BIOLOGICAL MONITORING OF OCCUPATIONAL EXPOSURE TO MONOMERIC 1,6-HEXAMETHYLENE DIISOCYANATE

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ABSTRACT

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Biological Monitoring of Occupational Exposure to
Monomeric 1,6-Hexamethylene Diisocyanate
(Under the direction of Leena A. Nylander-French)

1,6-hexamethylene diisocyanate (HDI) is used throughout the automotive repair industry and is a commonly reported cause of occupational asthma in industrialized populations. However, the exact pathological mechanism remains uncertain. Biomarkers from HDI exposure can fill important knowledge gaps between exposure, susceptibility, and the rise of immunological reactions and sensitization leading to asthma. Use of biomarkers in HDI exposure assessment has been limited due to the lack of specific and sensitive analytical methods for their measurement. The objective of this research was to develop and apply quantitative methods for the analysis of novel blood and urinary biomarkers stemming from exposure to HDI monomer to be utilized in HDI exposure assessment studies. To achieve this, we monitored dermal and inhalation exposure to HDI monomer and collected blood and urine from 46 automotive painters. Analytical methods were developed and utilized to quantify 1,6-hexamethylene diamine (HDA) in plasma and hemoglobin and to quantify *N*-acetyl-1,6-hexamethylene diamine (monoacetyl-HDA) and *N*,*N*'-diacetyl-1,6-hexamethylene diamine (diacetyl-HDA) in urine and hemoglobin. Strong associations between cumulative exposure to HDI monomer (dermal or inhalation) and plasma HDA or HDA-hemoglobin adducts were observed ($p \leq 0.10$). The significant workplace determinants of plasma HDA levels were paint booth type and coveralls ($p \leq 0.10$). These biomarkers may be used in HDI exposure assessment to evaluate workplace controls for reducing exposures and, thus, prevent adverse health effects among workers.

To my husband, Bob

and to my parents, John and Thelma Flack.

ACKNOWLEDGEMENTS

Man cannot discover new oceans unless he has the courage to lose sight of the shore.

Andre Gide

There are a number of individuals who have been an integral part of this journey and to whom I owe enormous thanks. I am especially grateful to my advisor, Leena Nylander-French, for her guidance, vision, and support in making all this possible. She has challenged me to think deeper and more critically while always encouraging the development of new ideas in an enjoyable academic environment. I thank all of my colleagues Kenny, Linda, and Jen at UNC and Steve, Rob, and Diana in Washington State who were instrumental in the sample collection. I also thank Connie, David, Rong, Vandy, and Evelyn for their assistance, advice, and friendship during my time at UNC. I am also indebted to my committee members: Louise Ball for providing help with any chemistry-related questions; Mike Flynn for shedding light on exposure-biomarker relationships; Joachim Pleil for discussions involving new and enlightening perspectives; and David Richardson for providing epidemiological insights. I thank them for always being available whenever I needed help or advice. Lastly, but most importantly, I thank my husband, Bob, for his encouragement, patience, and care during those stressful times and long hours in the lab.

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LIST OF ABBREVIATIONS

ADME	absorption, distribution, metabolism, elimination
APF	assigned protection factor
BZC	breathing-zone concentration
CYP450	cytochrome P-450
diacetyl-HDA	*N,N'*-diacetyl-1,6-hexamethylene diamine
FAD	flavine adenine dinucleotide
GC	gas chromatography
GFF	glass fiber filter
GM	geometric mean
GSD	geometric standard deviation
GSH	glutathione
GST	glutathione-*S*-transferase
Hb	hemoglobin
HDI	1,6-hexamethylene diiocyanate
HDA	1,6-hexamethylene diamine
HFBA	heptafluorobutyric anhydride
HpDA	1,7-heptanediamine
IS	internal standard
m^3	cubic meter
µg	microgram
µl	microliter
mg	milligram
ml	milliliter
ng	nanogram

L	liter
LC	liquid chromatography
LOD	limit of detection
LOQ	limit of quantification
MDA	4,4’-methylenedianiline
MDI	methylene diphenyl diisocyanate
MDL	method detection limit
monoacetyl-HDA	*N*-acetyl-1,6-hexamethylene diamine
monoacetyl-MDA	*N*’-acetyl-4,4’-methylenedianiline
MS	mass spectrometry
m/z	mass-to-charge ratio
NAT	*N*-acetyltransferase
OSHA	Occupational Safety and Health Administration
PAPR	powered-air purifying respirator
PFPA	pentafluoropropionic anhydride
PPE	personal protective equipment
PTFE	polytetrafluoroethylene
TDA	2,6-toluene diamine
TDI	toluene diisocyanate
TFECF	2’,2’,2-trifluoroethyl chloroformate
USEPA	United States Environmental Protection Agency

CHAPTER 1

BACKGROUND AND SIGNIFICANCE

The high reactivity of 1,6-hexamethylene diisocyanate (HDI) serves as a basis for producing polyurethane-based coatings and is widely used in the automotive refinishing industry. However, exposure to these compounds can present health risks in the workplace. Measurement of HDI in the air alone is insufficient for exposure assessment as individual differences in breathing rate, deposition, absorption, distribution, and metabolism are not taken into account. Furthermore, the mechanisms of toxic reactions and information on exposure routes and their contribution to internal dose levels have not yet been adequately addressed in HDI exposure assessment. Utilizing biomarkers in HDI exposure assessment would improve our understanding of mechanisms and susceptibility factors involved in diisocyanate-induced asthma and help establish exposure mitigation controls in the workplace. In this chapter, I describe the current knowledge of HDI metabolism, proposed mechanisms involved in diisocyanate-induced asthma, methods used in biomonitoring of HDI exposure, and the specific aims of this project.

1.1 Health effects of exposure to HDI

HDI (see Figure 1.1: **1**[a]) is the most commonly used aliphatic diisocyanate for the production of polyurethane foams and coatings[1]. Its highly reactive isocyanato (NCO) moieties react readily with the hydroxyl (OH) group of polyols to form polyurethanes, and hence have been utilized in the automotive repair industry as a component of polyurethane paints and coatings. Painting air levels

[a] Numbers and letters in bold (e.g., **1, 1A**) refer to the specific chemical reaction product and protein adducts, respectively, in Figure 1.

typically consist of trace amounts of HDI monomer and larger amounts of HDI oligomers (e.g., uretdione, biuret, and isocyanurate)[2, 3]. However, skin sensitization produced at these trace levels to HDI monomer (0.1%) is sufficient to induce HDI-induced asthma following inhalation exposure[4]. Therefore, characterizing and quantifying biomarkers of HDI monomer exposure continues to be critical in evaluating workplace exposure, developing strategies for mitigating exposure, and preventing adverse health effects. In this section, we review our current understanding of health effects associated with HDI exposure, metabolism, mechanisms, and risk factors involved in diisocyanate-induced asthma.

1.1.1 Overview of health effects

Isocyanate-induced asthma is the major health problem associated with exposure to HDI, and has been documented among occupationally exposed workers[5-8]. Other effects resulting from HDI exposure include allergic rhinitis, hypersensitivity pneumonitis, and contact dermatitis[9, 10]. A diisocyanate-exposed worker may become sensitized after a single, acute exposure, but in most cases, sensitization takes months to several years of exposure[11]. Additionally, once a worker is sensitized, s/he can experience an asthmatic response when exposed to inhalation levels below the occupational exposure limit[12]. This response is characterized by an early asthmatic reaction and rapid recovery period, followed by a prolonged decline in lung function[5]. There is evidence that inducing HDI skin sensitization at trace levels of HDI monomer (0.1%) with subsequent inhalation challenge may result in lung and airway inflammation, characterized by a mixed Th1/Th2 immune response[4]. The low prevalence (5 – 10%) of diisocyanate-induced asthma among exposed individuals and HDI-specific IgE among asthma cases[13-15] has generated interest in understanding the role of genetic factors driving HDI metabolism as modifiers of individual susceptibility to asthma[16].

1.1.2 Metabolism of HDI

Several non-enzymatic and enzymatic-directed metabolic pathways have been identified following HDI exposure, which are summarized Figure 1.1. Once absorbed into the body, the NCO moieties of HDI react with protein functional groups such as amines, hydroxyls, and thiols, with the last of these reactions favored under physiologic conditions[17]. HDI reacts spontaneously, or through catalysis by glutathione-*S*-transferase (GST), with thiol groups in cysteine residues of glutathione (GSH) and other proteins to form mono- and bis-dithiocarbamate adducts (see Figure 1.1: **2**)[17, 18]. *In vitro* studies indicated that HDI-protein conjugates are also created via the base-catalyzed elimination reaction of the bis-dithiocarbamate adducts (**2**) to form an intermediate *S*-glutathionyl adduct (**3**), which can undergo carbamoylation reactions with proteins or undergo solvolysis to regenerate HDI (**1**), resulting in potential carbamoylations with other nucleophilic sites on albumin or hemoglobin far from the initial site of contact (**2A**). This reaction pathway competes with the base-catalyzed biomolecular substitution reaction, leading to the formation of the very labile carbamic acid group (**4**), which decomposes to HDA (**5**)[18]. Because these reaction pathways were demonstrated *in vitro*, it is still unknown whether these metabolic products are formed in humans following occupational exposure to HDI.

Sepai and colleagues (1995) proposed an alternative pathway for diisocyanate-adduct formation based on measurements of 4,4'-methylenedianiline (MDA) and *N*'-acetyl-4,4'-methylenedianiline (monoacetyl-MDA) in blood and urine samples[19]. This proposed metabolic pathway for HDI is based on the *N*-hydroxylation of the amines (**5, 6**), catalyzed by cytochrome P-450 isoforms (CYP450) or flavine-adenine dinucleotide (FAD) dependent enzyme system (e.g., flavine-containing monooxygenase)[20], resulting in the formation of *N*-hydroxyamine (**8**) and the nitroso compound (**9**), which can react with thiols on albumin or hemoglobin (**3A**). This pathway competes with *N*-acetylation of the amine, catalyzed by *N*-acetyltransferase (NAT) enzymes, to form monoacetylated (**6**) and diacetylated amines (**7**), which are excreted in urine. The identification of *N*-acetyl-1,6-hexamethylene diamine (monoacetyl-HDA) in individuals receiving HDA by oral administration also

indicates the potential for this metabolic pathway[21]. However, *N*-acetylation following occupational exposure to HDI, where dermal and inhalation are the major exposure routes, has not been established. How HDI is transformed *in vivo* may have important implications in exposure assessment, the biological availability of different reactive intermediates, as well as the development of adverse health effects.

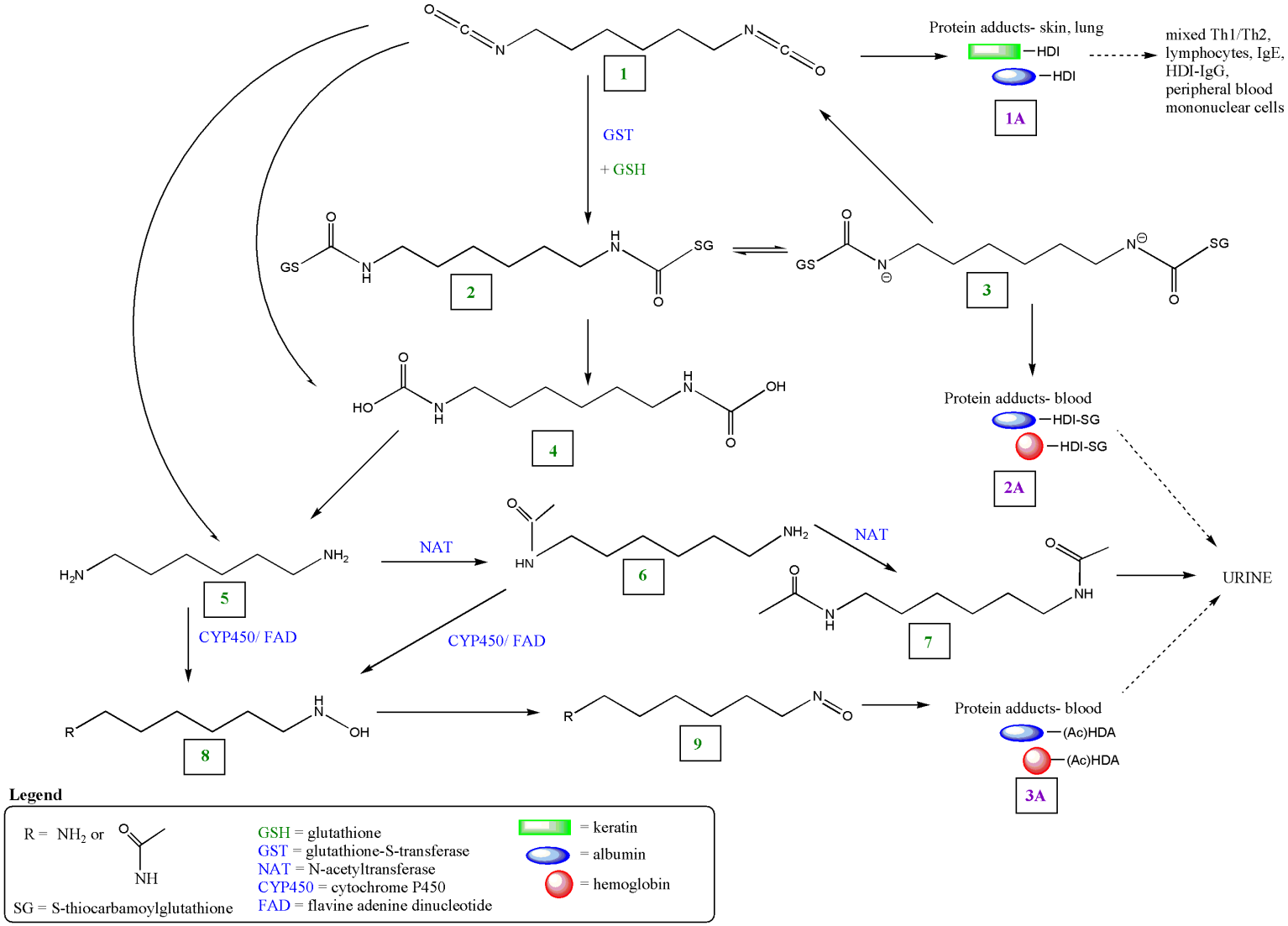

Figure 1.1 Proposed enzymatic (GST, NAT, CYP450, FAD) and non-enzymatic directed HDI metabolic pathways leading to formation of HDI-protein adducts, potential immune response, and elimination.

1.1.3 Mechanisms of diisocyanate-induced asthma

While the biological mechanisms by which diisocyanates cause asthma remain poorly understood, the low proportion of diisocyanate-induced asthmatics with detectable specific IgE[7, 14] has generated theories concerning disease mechanisms and genetic susceptibility. Unlike high molecular weight allergens, diisocyanates may react with amino, hydroxyl, and sulfhydryl groups of various proteins and peptides, including albumin and keratin, to form hapten-protein complexes[22]. Cell-mediated mechanisms would enable these proteins to act as hapten-carriers (i.e., antigen presenting cells) in presenting HDI to the immune system (see Figure 1.1: **1A**), resulting in induction of a mixed Th1/Th2 type response, lymphocyte production[4, 23-25], and peripheral blood mononuclear cell responses, which has been demonstrated in human subjects receiving inhalation exposure to HDI-albumin conjugate[26] (Figure 1.1). Analogously, allergen-induced delayed dermatitis (or type IV hypersensitivity) occurs when sensitizing chemicals penetrate the viable epidermis and stimulate IgG/IgE production and T-cell response. Langerhan's cells can transport HDI as a hapten-carrier complex from the skin to the lymph nodes, where it is presented to naïve T-cells with subsequent transformation into memory T-cells (i.e., sensitization phase). With subsequent exposure, memory T-cells would travel to the site of exposure and produce inflammation and systemic immune response (i.e., elicitation phase). A similar class of hapten-carrier complexes (e.g., HDI-conjugated keratins) formed following skin and airway exposure to HDI may explain cases of asthmatic response elicited by inhalation challenge in guinea pigs or mice sensitized previously by dermal exposure to diisocyanates[4, 23, 27].

Hereditary factors may also work in conjunction with cell-mediated mechanisms to modify individual susceptibility to diisocyanate-induced asthma. Cysteinyl leukotrienes are potent mediators of airway narrowing and have been implicated as possible mediators of hyperresponsiveness to histamine, which is a key feature in bronchial asthma[28]. GSTs can utilize a variety of pro-inflammatory leukotrienes as substrates, and exhibit polymorphic expression due to their allelic variants in the respective genes[16]. Genetic polymorphisms of GSTs may modify asthmatic responses to diisocyanate exposure through detoxification of diisocyanates or their metabolites, acting as pro-

inflammatory mediators in airway reaction to diisocyanates, or toxicity via GSH-protein conjugates which could lead to the formation of hapten complexes giving rise to immunological reactions. Similarly, large inter-individual variability in NAT enzyme activities exists due to polymorphisms in NAT1 and NAT2 genes. Therefore, NATs are another group of enzymes whose genetic polymorphisms may modify the development of adverse effects due to diisocyanate exposure. The *N*-acetylation pathway competes with *N*-hydroxylation, which is a metabolic activation route leading to the formation of *N*-hydroxyamine and the nitroso compound capable of binding covalently to molecules. Thus, hapten formation, and consequently asthma risk, would be predicted to be enhanced in humans with slow acetylator phenotype compared to fast acetylator phenotype. In support of this hypothesis, Wikman and colleagues (2002) demonstrated that the largest increase in asthma risk was observed with the combined GSTM1 null genotype and the NAT1 slow acetylator phenotype (OR = 4.5)[16]. Additionally, Brorson and colleagues (1990) found that individuals with the NAT slow acetylator phenotype cleared lower amounts of HDA following oral administration of HDA compared to individuals with fast acetylator phenotype, indicating modification of hapten formation with acetylator status[21]. Further investigation into factors driving HDI metabolism and clearance following occupational exposure to HDI would contribute to our understanding of mechanisms and susceptibility factors involved in diisocyanate-induced asthma.

1.2 Automotive refinishing industry

Auto body shop painters and repair technicians use polyurethane paints containing HDI for spray and refinishing work. The auto body industry in the U.S. employs >205,000 workers in ~35,500 auto body shops (U.S. Bureau of Census, 2002). Spray painters have been reported to be the highest risk group for developing occupational asthma compared to other occupations[29]. Therefore, characterizing occupational exposure to HDI is important for establishing and improving protection controls and mitigating disease. In this section, I describe the painting process in the automotive refinishing industry and methods involved in exposure monitoring.

1.2.1 Painting process and HDI exposure

Paints used in the automotive refinishing industry contain aliphatic diisocyanates, consisting of trace amounts of HDI monomer (< 1%) and much higher amounts of HDI oligomers[30, 31], including the dimer, uretidone, and trimers, biuret and isocyanurate. Paints are applied using a two-stage system in which the first stage is the base coat, and the second stage is the clearcoat to which hardener, containing monomeric and polymeric HDI, is added. Once mixed, the polyisocyanates in the hardener react with the polyols in the clearcoat solution to form a polyurethane coating. Automotive painting is performed using a compressed-air spray gun inside a ventilated paint booth (i.e., downdraft, crossdraft, or semi-downdraft). Some of the paint droplets may be captured in the airflow around the surface of the vehicle, forming a mist or overspray that is likely to contain unreacted polyisocyanates[32] and come into the workers' personal space. Therefore, dermal and inhalation exposure to HDI are potential exposure routes in this occupation. To reduce exposure risk, the most common types of personal protective equipment (PPE) used during spray painting include respirators (e.g., half-face or full-face air purifying, powered-air purifying (PAPR), and supplied air), coveralls, and gloves.

1.2.2 Methods for dermal and inhalation exposure monitoring

Inhalation exposure to HDI most likely occurs during spray-painting when the paint is aerosolized and enters the breathing-zone of the worker. Dermal exposure to HDI may occur during spray-painting as the aerosol is deposited on the skin or the vapor is absorbed through the skin, contact with contaminated or freshly painted surfaces, or directly with the paint containing HDI. The extent of inhalation or dermal exposure depends on the use and efficiency of PPE. While spray painters typically wear a respirator, coveralls and gloves are worn less frequently[33]. Even when PPE is worn, HDI may break through latex gloves or respirator cartridges with repeated use[34]. Air-purifying respirators that implement filtration for reducing inhalation exposure may not sufficiently remove diisocyanate aerosols, particularly if workers do not follow routine change-out of filters[35]. Improper maintenance of coveralls or inadequate changing of gloves could also result in dermal exposure to HDI.

Because HDI oligomers likely exist as aerosols in the overspray while HDI monomer, with its higher vapor pressure (0.05 mmHg at 25°C) may partially exist as a vapor, methods to accurately measure these compounds present challenges. Due to their reactivity with water, alcohols, and amines, most air sampling methods require immediate derivatization of collected diisocyanates. Filter sampling, using single-stage or dual-stage cassettes, are the most commonly used methods for collecting diisocyanates in the air. In a dual-stage system, the first stage consists of a polytetrafluoroethylene (PTFE) filter designed to collect aerosols and the second stage contains an impregnated glass fiber filter (GFF) designed to collect and derivatize vapor (ISO-CHECK®). After sampling, the PTFE filter is placed into derivatizing solution. The single-stage system lacks the PTFE filter, and consequently, the impregnated GFF collects and derivatizes all phases of the diisocyanates (OSHA 1983)[36].

HDI dermal exposure assessment is much less developed than that of inhalation exposure [22], although the role of dermal exposure in sensitization and diisocyanate-induced asthma has been demonstrated[27, 37]. Several different techniques have been used to detect and/or quantify these levels

in the workplace including SWYPE pads[34], wipes[38], glove washes[39], and tape-strips[40, 41]. The lack of a standardized and fully validated method to assess HDI dermal exposure has added to the difficulty in characterizing dermal exposure. Evidence that dermal exposure occurs among automotive spray painters stresses the need to validate methods to measure skin exposure[34, 38, 39, 41]. Post-exposure tape-stripping of the skin has the advantage of quantifying dermal penetration of HDI monomer and oligomers within the same sample [41] and the potential to be adapted to collect and measure HDI-keratin adducts that have been identified *in vivo*[23].

1.3 Biological monitoring of exposure to HDI

Measures of exposure levels alone, through tape-stripping of the skin or air monitoring, are insufficient to fully understand mechanisms involved in sensitization. Information on HDI metabolism and protein adduct formation following diisocyanate exposure has contributed to our understanding of the possible mechanisms involved in sensitization and development of diisocyanate-induced asthma[23, 24, 42]. Biological monitoring, through measurement of one or more internal markers in the blood or urine, can be combined with HDI exposure and disease monitoring to understand workplace and personal factors including susceptibility that modify exposure and asthma risk, respectively. The integration of biological monitoring (i.e., internal dose of a chemical or its metabolites) within the source-disease framework for HDI is summarized in Figure 1.2. The internal dose resulting from HDI exposure can be related with both HDI exposure and the target organ dose, which may continue towards disease progression and produce HDI-induced asthma. In this section, I describe the methodology used in biological monitoring of exposure to HDI, including blood and urine analysis, and their relationship with HDI exposure levels.

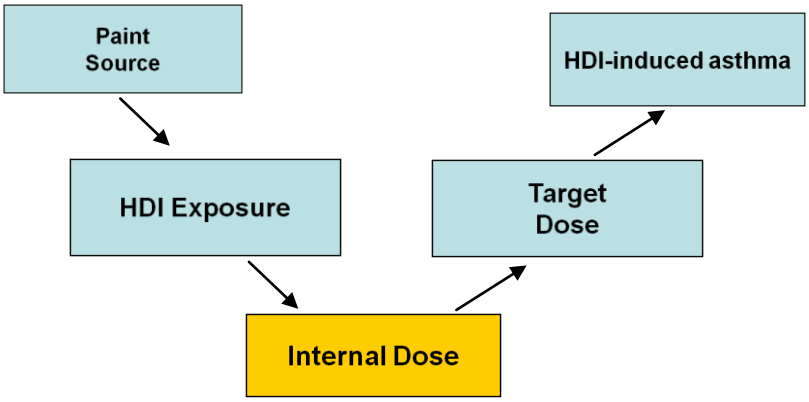

Figure 1.2 Source to disease framework for HDI exposure.

1.3.1 Analytical methods for biomarker quantification (sensitivity and specificity)

Biological monitoring of HDI exposure has primarily involved analysis of HDA, the hydrolysis product of HDI, in urine or plasma as a marker of exposure to HDI. Typically, 1-2 ml of urine or plasma is hydrolyzed by acid or base, and the liberated diamines are extracted into toluene followed by derivatization with heptafluorobutyric anhydride (HFBA) or pentafluoropropionic anhydride (PFPA) and analyzed by gas chromatography-mass spectrometry (GC-MS) in negative chemical ionization mode[43, 44] or by liquid chromatography-mass spectrometry (LC-MS)[45]. The former has been the most commonly used method for HDA analysis and implemented primarily when HFBA was used as the derivatization agent. The detection limits for HDA in plasma or urine as reported in various publications are in the range of 0.02 – 3.0 µg/l (summarized in Table 1.1). The high detection limit of HDA in samples, resulting in low analytical sensitivity, has hindered quantification of plasma HDA in several studies[43, 46].

Table 1.1 Summary of analytical method and HDA detection limits (µg/l) in biological media.

Reference	Biological media	Derivatization agent[a]	Analytical method[b]	Detection limit (µg/l)
Brorson et al. 1990[43]	plasma & urine	HFBA	GC-MS	0.5
Dalene et al. 1994[44]	urine	TFECF	GC-MS	0.5
Skarping et al. 1994[45]	urine	PFPA	LC-MS	0.1
Tinnerberg et al. 1995[46]	plasma & urine	PFPA	GC-MS	≤0.1
Maitre et al. 1996[47]	urine	HFBA	GC-MS	0.35
Rosenberg et al. 2002[48]	urine	HFBA	GC-MS	0.4[c]
Liu et al. 2004[49]	urine	HFBA	GC-MS	0.2
Pronk et al. 2006[39]	urine	HFBA	GC-MS	3.0
Gaines et al. 2009[50]	urine	HFBA	GC-MS	0.04
Flack et al. 2009[51]	plasma	HFBA	GC-MS	0.02

a. HFBA = heptafluorobutyric anhydride; TFECF = 2',2',2-trifluoroethyl chloroformate; PFPA = pentafluoropropionic anhydride.
b. GC-MS = gas chromatography-mass spectrometry; LC-MS = liquid chromatography-mass spectrometry.
c. Reported as limit of quantification (LOQ) = 5 nmol/l = limit of detection (LOD) × 1.5 [48], and LOD (µg/l) calculated as follows: 5 nmol/l ÷ 1.5 × 1 mol/10^9 nmol × 116.21 g/mol × 10^6 µg/g = 0.4 µg/l.

Acid hydrolysis releases HDA-protein conjugates from biological matrixes and converts the acetyl functional groups on monoacetyl-HDA and *N*,*N*'-diacetyl-1,6-hexamethylene diamine (diacetyl-HDA) back to HDA. Thus, the HDA detected following these treatment conditions may be derived from a number of other metabolites and adducts, and any individual variability in specific metabolite formation would be masked by acid hydrolysis. Therefore, analytical specificity for HDI exposure biomarkers must be further developed to determine individual differences in specific metabolite formation related to HDI monomer and oligomer exposure. This information would be used to understand individual differences in metabolism of HDI and susceptibility factors related to disease development.

1.3.2 Urine analysis for HDA

Positive associations between HDI inhalation exposure and urinary HDA levels have been observed in several studies[39, 43, 46, 47, 49]. Automotive repair workers may be exposed to HDI monomer and oligomers simultaneously, complicating methods for biological monitoring of HDI exposure. However, Liu and colleagues (2004) demonstrated that the correlation between inhalation exposure to biuret and urinary HDA was weak, indicating that HDA levels better reflect HDI monomer exposure rather than oligomers[49]. In addition, knowledge on oligomer metabolism and their association with external exposures is lacking, thereby making HDA the only valid biomarker for HDI monomer to date.

Unlike inhalation exposure, the impact of dermal exposure on urine HDA levels has been quantitatively investigated in only one study[50]. Investigation of these associations between exposure and biomarker levels has been hindered not only by the sensitivity of analytical methods, but also by the large inter- and intra-individual variability in HDA urine biomarker levels[39]. In urine, this is partially attributed to the biphasic elimination pattern of HDA, characterized by a rapid elimination phase ($t_{1/2}$ = 1.2 – 2.9 h) and a slow elimination phase, which may be attributed to the breakdown of HDI-protein conjugates in the blood[43, 46, 49]. Furthermore, analyses of HDA in acid hydrolyzed urine may represent a combination of various metabolites, including free and protein conjugates of HDA and acetylated HDA. Therefore, the quantification of specific HDI metabolites, such as monoacetyl-HDA or diacetyl-HDA, would improve biological monitoring methods for HDI exposure by reducing the inter- and intra-individual variability in biomarker measures and providing further information on HDI metabolism. The association between exposure and internal dose may be investigated to evaluate the effectiveness of PPE and other workplace factors in mitigating HDI exposure. Modifiers of HDI metabolism, such as hereditary factors, may also be integrated with these internal dose measures to understand individual susceptibility for asthma development.

1.3.3 Blood analysis for HDA

Similar to urine HDA, measurement of HDA in blood may also be used in evaluating the effectiveness of PPE for mitigating exposure, as well as investigating individual susceptibility in disease development. As mentioned previously, if hapten-carrier complexes are important in immune response, then measures of blood protein adducts resulting from HDI exposure may be directly related with asthma risk. There are no publications reporting the amount of free or protein-conjugated HDA in the blood after exposure to HDI monomer. However, for other diisocyanates it has been reported that the majority of MDA measured in the plasma of workers exposed to methylene diphenyl diisocyanate (MDI) is covalently bound to albumin, and these levels are up to 450-fold higher than hemoglobin (Hb) adduct levels[19]. Assuming that modified proteins are stable, albumin or Hb adducts accumulating in blood may be indicative of cumulative diisocyanate exposure and the biological availability of different reactive intermediates over the half-life of albumin (19 days) and the life-span of Hb (120 days). The association between 2,6-toluene diamine (TDA) in hydrolyzed urine and airborne levels of toluene diisocyanate (TDI) were more strongly correlated compared to plasma TDA levels, perhaps due to the presence of these longer-lived TDA-albumin adducts[52]. Thus, HDA blood biomarkers (e.g., HDA-albumin or HDA-Hb adducts) may also be used to evaluate HDI exposures in retrospective studies, where actual exposure measurements are missing.

1.4 Modeling the exposure-biomarker relationship

Predictive models describing the relationship between HDI exposure and biomarkers can be used to identify the major routes of exposure, as well as workplace and individual-level covariates that may modify these relationships, such as PPE and engineering controls in the workplace. The inter-individual variability in HDI metabolism may also be investigated to understand susceptibility factors, such as NAT and GST gene polymorphisms, associated with disease risk. In this section, we describe how HDI exposure and biomarker levels, modified by PPE and workplace factors, may be associated using statistical modeling.

1.4.1 Internal dose modeling

Linear regression models have demonstrated good associations ($r \geq 0.49$) between HDI air exposure and post-shift urinary HDA[47, 49] indicating that urine HDA is a useful marker of short-term HDI exposure. Logistic regression has also been used to investigate the association between urine HDA levels, external dermal exposure, and glove use, with odds ratios (ORs) adjusted for correlation among repeated measures[39]. The limitation in the latter study was implementing glove washes to estimate dermal exposure rather than direct quantitative measures of the skin exposure, as glove type (e.g., nitrile *vs.* latex) may affect the extent of dermal exposure to the hands. Additionally, differences in respiration rate, HDI uptake, absorption, metabolism (e.g., acetylator status), and HDI-conjugate clearance rates would contribute to the large inter-individual variability in urinary HDA levels observed in these studies.

Mixed modeling has been implemented for identifying the main determinants of dermal and breathing-zone HDI monomer and oligomers[41, 53]. Such models could also be implemented to identify the main determinants of biomarker concentration resulting from HDI exposure using repeated measures. Mixed models are statistical models that incorporate both fixed and random effects in making predictions of the dependant variable (e.g., biomarker concentration). They are useful in settings where repeated measures are made on the same statistical units (e.g., worker). Due to the likelihood of serial correlation with repeated measures, the mixed model approach would be an appropriate method for biological samples.

The general form of the linear mixed model investigating the association of exposure concentration and personal or workplace covariates on biomarker concentration is:

$$Y_{ij} = \beta_0 + \beta_1 X_{1ij} + \beta_2 X_{2ij} + \alpha_i + \varepsilon_{ij},$$

where Y_{ij} represents the log-transformed biomarker concentration (the *j*th measurement obtained for the *i*th worker), X_{1ij} represents the log-transformed exposure concentration (e.g., inhalation exposure),

X_{2ij} represents the covariate being tested (e.g., coverall use), β_1 and β_2 represent regression coefficients of the explanatory variables, β_0 represents the intercept, and α_i and ε_{ij} represent the random effects associated with worker (α_i for i = 1, 2,..., n workers) and an error term (ε_{ij} for j = 1, 2,. . ., N measurements for the ith worker). It is assumed that α_i and ε_{ij} are mutually independent and normally distributed with means of zero and respective variances σ^2_b and σ^2_w representing the between and within-worker variance components, where total variance $\sigma^2_Y = \sigma^2_w + \sigma^2_b$. It is also assumed that Y_{ij} is normally distributed with mean $\mu_y = \beta_0 + \beta_1 X_{1ij} + \beta_2 X_{2ij}$ and variance σ^2_Y.

These statistical models may be used to evaluate the association between exposure and biomarker concentrations, and how personal and workplace covariates, such as PPE use, modify these associations and biomarker concentration. Identifying sources of the between and within-worker variability in biomarker measurements would help establish workplace and personal protection strategies in mitigating exposure and disease.

Another useful statistical analysis is the Tukey-Kramer multiple comparison test (Tukey's test) which is used to identify which means, among groups of measurements, are different from each other. Thus, it compares all possible pairs of means, and is based on a studentized range distribution q. Tukey's test is essentially a t-test, except that it corrects for experiment-wise error rate; when there are multiple comparisons being made, the probability of making a type I error increases. Tukey's test corrects for type I error, and is thus more suitable for multiple comparisons than perfoming a number of t-tests. Additionally, for unequal sample sizes, the confidence coefficient is greater than $1 - \alpha$. Therefore, Tukey's test is more conservative when dealing with unequal sample sizes.

The general formula for Tukey's test is: $$q_s = \frac{Y_A - Y_B}{SE},$$

where Y_A is the larger of the two means being compared, and Y_B is the smaller of the two means being compared and SE is the standard error of the data in question. This q_s value can then be compared to a q value from the studentized range distribution ($q_{critical}$). If the q_s value is larger than the

$q_{critical}$ value obtained from the distribution, the two means are significantly different. In exposure assessment, this test may be used to compare the means in biomarker levels (e.g., plasma HDA, HDA-Hb) when workers are grouped by PPE use (e.g., coverall use *vs.* non-coverall use).

1.4.2 Retrospective HDI exposure assessment

Several studies have indicated both fast and slow transformation and elimination of HDI in the human body, with the possibility of the latter phase comprised of the breakdown and release of protein adducts, such as HDA-albumin and HDA-Hb adducts[43, 46, 49]. Therefore, exposure-biomarker associations should also aim to investigate the relationship between biomarker and retrospective HDI exposure occurring over a range of time spanning the life-span of Hb (120 days) and, at minimum, the half-life of albumin (19 days). The strong association between urinary HDA and the same day HDI exposure was attributed to the rapid elimination of HDI[47, 49]. However, the measurement of blood-protein adducts may be applied in retrospective HDI exposure assessment studies where exposure measures are missing. How long these adducts remain in circulation may reflect the biological availability of these products over this time period and their potential contribution to sensitization and asthma. Thus, HDA-albumin and HDA-Hb adducts and urine HDA levels may be utilized as biomarkers of HDI exposure occurring at various times (i.e., same day, 0.5 – 2 months, 0.5 – 4 months) relative to the collection of the biological sample.

1.5 Specific aims of this project

The primary objective of this project was to develop and apply quantitative methods for the analysis of novel blood and urinary biomarkers stemming from exposure to HDI monomer. This investigation was conducted to identify the most significant personal and workplace determinants for estimating the received internal dose and evaluate the effectiveness of PPE and other workplace controls in mitigating exposure. To achieve our primary objective we identified the following specific aims:

Specific Aim 1: Quantify HDA in plasma of workers exposed to HDI monomer, investigate their relationship with HDI dermal and inhalation exposure levels, and identify the major determinants for plasma HDA levels among the various workplace covariates (e.g., coveralls, respirator type, booth type).

Specific Aim 2: Develop and apply quantitative methods for the analysis of monoacetyl-HDA and diacetyl-HDA in the urine of workers exposed to HDI monomer and investigate their relationship with HDI dermal and inhalation exposure levels.

Specific Aim 3: Quantify hemoglobin adducts of HDA, monoacetyl-HDA, and diacetyl-HDA in workers exposed to HDI monomer and investigate their relationship with HDI dermal and inhalation exposure levels, urine HDA concentration, and various workplace covariates.

CHAPTER 2

QUANTITATIVE PLASMA BIOMARKER ANALYSIS IN HDI EXPOSURE ASSESSMENT

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2.1 Abstract

Quantification of amines in biological samples is important for evaluating occupational exposure to diisocyanates. In this study, we describe the quantification of 1,6-hexamethylene diamine (HDA) levels in hydrolyzed plasma of 46 spray painters applying HDI-containing paint in vehicle repair shops collected during repeated visits to their workplace and their relationship with dermal and inhalation exposure to 1,6-hexamethylene diisocyanate (HDI) monomer. HDA was detected in 76% of plasma samples, as heptafluorobutyryl derivatives, and the range of HDA concentrations was ≤0.02 – 0.92 μg/l. After log-transformation of the data, the correlation between plasma HDA levels and HDI inhalation exposure measured on the same workday was low (N = 108, r = 0.22, p = 0.026) compared with the correlation between plasma HDA levels and inhalation exposure occurring approximately 20 – 60 days before blood collection (N = 29, r = 0.57, p = 0.0014). In addition, the correlation between plasma HDA levels and HDI dermal exposure measured on the same workday was also low (N = 108, r = 0.22, p = 0.040). We evaluated various workplace factors and controls (*i.e*., location, personal protective equipment use, paint booth type) as modifiers of plasma HDA

levels. Workers using a downdraft-ventilated booth had significantly lower plasma HDA levels relative to semi-downdraft and crossdraft booth types ($p = 0.0108$); this trend was comparable to HDI inhalation and dermal exposure levels stratified by booth type. These findings indicate that HDA concentration in hydrolyzed plasma may be used as a biomarker of cumulative inhalation exposure to HDI and for investigating the effectiveness of exposure controls in the workplace.

2.2 Introduction

The high reactivity of 1,6-hexamethylene diisocyanate (HDI) serves as a basis for producing polyurethane-based coatings and is widely used in the auto-repair industry. The reactive nature of HDI has also resulted in cases of skin and respiratory sensitization, and occupational asthma with repeated or prolonged exposure[5-7, 54]. However, the mechanisms of toxic reactions and information on exposure routes and their contribution to internal dose levels have not yet been adequately addressed in HDI exposure assessment. Evidence that inducing HDI skin sensitization, with subsequent inhalation challenge, can result in HDI-induced asthma stresses the importance of investigating dermal exposure to HDI and its relationship with internal dose levels[4]. Therefore, efficiency of personal protective equipment (*e.g.*, respirators and coveralls) and routes of exposure (*e.g.*, dermal and inhalation) are important considerations in exposure assessment.

Biomonitoring may supplement traditional air monitoring methods, or used in combination with dermal exposure methods, by integrating previous exposures across multiple exposure routes. Thus, biomarker analysis may be used to evaluate the effectiveness of protection controls in the workplace through internal dose estimation of a particular compound. In addition, HDI-conjugated proteins in the blood may contribute to the pathogenesis of diisocyanate-induced asthma by acting as protein carriers that present HDI to the immune system as hapten-protein complexes[23]. Therefore, HDA blood biomarkers may provide a link between exposure and the relevant pathways contributing to the development of biological effect(s). Not only may blood biomarkers of HDI exposure be used to evaluate the effectiveness of workplace protection controls, but also to investigate biological factors

contributing to disease development. Investigating how internal dose varies with external exposure to HDI monomer may be performed by utilizing a sensitive method for the measurement of HDA in the hydrolyzed plasma, and correlating these levels with HDI monomer measured in a worker's breathing-zone and skin.

The high reactivity and toxicity of diisocyanates has generated interest in measuring the internal dose of these compounds through analysis of one or more biomarkers. Diisocyanate protein adducts form as a result of conjugation of diisocyanates to various macromolecules at the site of contact or after systemic absorption[9]. Albumin or hemoglobin (Hb) adducts in the blood resulting from diisocyanate exposure have been identified[19, 55, 56] and may result from direct interaction with blood proteins, or through rapid hydrolysis to the corresponding diamine in the blood, catalyzed by bicarbonate[57], subsequent conversion to the N-acetylated diamine by N-acetyltransferase, and/or further hydroxylation catalyzed by cytochrome P450 1A2 leading to the formation of protein adducts[16]. The latter pathway is supported by Sepai and colleagues[19] who quantified N-acetylated hemoglobin adducts resulting from MDI exposure indicating that the isocyanate group may be first hydrolyzed to the amine *in vivo*, followed by N-acetylation to monacetylated MDA, N-hydroxylation to the N-hydroxyarylamine and subsequently to the nitroso compound, which can then form a sulphinamide adduct. Therefore, HDA measured from hydrolyzed plasma samples may represent a combination of unconjugated HDA and monoacetyl-HDA or diacetyl-HDA, as well as conjugated protein adducts of HDI and HDA or monoacetyl-HDA hydroxylation products.

There are no publications reporting the amount of free or protein-conjugated HDA in the blood after exposure to HDI monomer. However, for other diisocyanates it has been reported that the majority of MDA measured in the plasma of workers exposed to MDI is covalently bound to albumin and these levels are up to 450-fold higher than Hb adduct levels[19]. Assuming that modified proteins are stable, albumin or Hb adducts accumulating in blood may be indicative of cumulative diisocyanate exposure and biological availability of different reactive intermediates over the half-life of albumin (3 weeks) and life span of Hb (17 weeks). The association between 2,6-toluene diamine (TDA) in hydrolyzed

urine and airborne levels of toluene diisocyanate (TDI) were more strongly correlated compared to plasma TDA levels, perhaps due to the longer half-life of TDA in plasma (21 days) resulting from the formation of albumin adducts[52]. Thus, HDA blood biomarkers may also be used to evaluate exposures in retrospective studies, where actual exposure measurements are missing.

Blood or urinary biomarkers may be utilized in exposure assessment by investigating how these measures relate to external exposure levels. Automotive repair workers may be exposed to HDI monomer and/or oligomers (*e.g.*, biuret, isocyanurate), with the latter comprising the majority of HDI in the clear coat. While a test chamber exposure study by Liu and colleagues[49] measured HDA in urine of persons exposed to biuret aerosol, the correlation between inhalation exposure to biuret and HDA was weak, indicating that HDA may better reflect HDI monomer exposure rather than oligomers. In addition, knowledge on oligomer metabolism and their association with external exposures is lacking, thereby making HDA the only valid biomarker for HDI monomer to date.

While HDA blood levels were observed to be below the limit of detection (<0.1 µg/l) in two previous human exposure studies[43, 46], a few volunteer and occupational exposure studies have demonstrated a positive association between HDI inhalation exposure and measured HDA in hydrolyzed urine[46, 47]. However, due to the large inter- and intra-person variability in urinary HDA levels among automotive repair workers and its biphasic elimination pattern, these measures are not easily interpretable as markers of HDI exposure[39]. For other diisocyanates, associations between biomarker levels in plasma or urine and diisocyanate air exposure levels have been investigated, although the relationship between dermal exposure and internal dose has not been investigated adequately. Strong, positive correlations were found between airborne TDI levels and TDA concentrations in hydrolyzed plasma and urine[52, 58], whereas the association between airborne MDI levels and MDA concentrations in hydrolyzed plasma or urine were much weaker[59, 60], indicating the possible contribution of dermal uptake for MDI. Because quantitative plasma HDA levels among exposed workers is lacking, we present a sensitive method for HDA analysis and investigate the association between plasma HDA and HDI inhalation and dermal exposure levels.

The contribution of dermal exposure to diisocyanates in sensitization and development of diisocyanate-induced asthma has been demonstrated in several animal studies[27, 37]. The issue of dermal absorption of diisocyanates in the occupational setting has been raised by several authors who have speculated that dermal exposure may contribute significantly to internal dose[39, 56, 60-64]. For example, urine TDA among a group of workers in direct skin contact with uncured TDI-based foam occurred at higher levels compared to non-handlers, even though both groups were exposed to similar airborne levels of TDI[64]. The author suggested that dermal absorption accounted for this large difference in TDA levels between the two groups. However, one limitation in this study was not demonstrating whether urinary levels were attributed to skin exposure to the hydrolyzed diisocyanate (*i.e.*, diamine) from contaminated surfaces or to the diisocyanate itself. Because quantitative information relating dermal exposure to diisocyanates (e.g., HDI monomer) and biomarker levels is lacking, one of our goals was to investigate the relationship between dermal exposure to HDI monomer on the skin and plasma HDA levels.

Quantification of amines in biological samples requires highly sensitive and selective methods. Such methods have been described previously for HDA[46, 48, 63], TDA[58], and MDA[65], and typically involve acid hydrolysis of samples and derivatization of the liberated amines with subsequent GC-MS analysis. Therefore, the total HDA concentration measured in hydrolyzed plasma represents the sum of covalently bound HDI monomer and HDA/monoacetyl-HDA oxidation products, as well as non-covalently bound HDA and its metabolites (*e.g.*, monoacetyl-HDA, diacetyl-HDA), and these levels may be directly correlated with levels of HDI monomer in the air and/or on the skin. Detection limits of HDA in hydrolyzed urine reported in previous occupational exposure studies ranged from <0.1 µg/l[46] to 3 µg/l[39]. Two test chamber human exposure studies were unable to detect HDA in hydrolyzed plasma of HDI-exposed subjects[43, 46].

The objectives of this study were to (i) quantify HDA in hydrolyzed plasma of 46 auto-body shop workers applying HDI-containing clearcoat through GC-MS analysis, (ii) investigate the relationship between plasma HDA levels and external exposure to HDI monomer (dermal and inhalation), and (iii)

investigate personal protective equipment (PPE) use (*i.e.*, coverall and glove use, respirator type) and work environment (*i.e.*, paint booth type, location) as modifiers of internal dose.

2.3 Methods

2.3.1 Chemicals

HPLC grade toluene (≥ 99.9%), HPLC grade ethyl acetate, certified American Chemical Society (ACS) grade sulfuric acid (H_2SO_4), sodium sulfate anhydrous, laboratory grade potassium phosphate monobasic (KH_2PO_4), ACS grade sodium chloride (NaCl), and certified ACS grade sodium hydroxide (NaOH) were obtained from Fisher Scientific (Hampton, NH). HDA (98%), 1,7-heptanediamine (HpDA) and derivatization grade heptafluorobutyric anhydride (HFBA) were obtained from Sigma-Aldrich (St. Louis, MO). Human plasma from whole blood (EDTA, potassium) was obtained from Biological Specialty Corporation (Colmar, PA) and stored at –40ºC in 5 ml aliquots.

2.3.2 Preparation of standard solutions

Stock solutions of HDA and HpDA (1 mg/ml) were prepared by dissolving 25 mg of the amine in 25 ml 1M H_2SO_4 and diluted 1:1000 to a final concentration of 1 μg/ml. The solutions were stored at –40ºC for up to one month. An internal standard solution (IS) was prepared by further dilution (1:100) of the HpDA solution in 1M H_2SO_4 to 10 ng/ml. An HDA standard solution was prepared by further dilution (1:2) of the HDA solution in 1M H_2SO_4 to 0.33 μg/ml. Just prior to the sample work-up procedures HDA calibration standards (n = 8), in the range of 0 – 2 μg/l plasma, were prepared in duplicate by making 1:1 serial dilutions from the HDA standard solution in 1M H_2SO_4 and spiking 12 μl of each diluted standard to 1 ml control plasma. To each HDA calibration standard, 12 μl of IS was added for a final concentration of 0.12 μg/l plasma.

2.3.3 Instrumental analysis

Samples were analyzed by gas chromatography-mass spectrometry (GC-MS; Thermo Trace GC Ultra interfaced with a PolarisQ ion trap mass spectrometer and AI/AS 3000 injector, and Xcalibur 1.4 SR1 software, Thermo Electron Corporation, Austin, TX). Injections (1 µl) were made under splitless mode of 30 s with injector temperature of 220ºC. Separation of the samples was carried out with a GC capillary column (DB5-MS, 30 m × 0.25 mm ID, 0.1 µm film thickness; Agilent Technologies, Palo Alto, CA). The ion source and GC transfer line temperatures were maintained at 150ºC and 260ºC, respectively. Helium was used as the carrier gas with a constant flow of 1 ml/min. The GC oven temperature program was 50ºC (1.0 min) to 155ºC at 10ºC/min, 155ºC to 185ºC at 2ºC/min, and 185ºC to 300ºC at 25ºC/min (final temperature held for 10 min). Ions were monitored in negative ion chemical ionization mode using methane as the reagent gas (1.8 ml/min). Mass spectra were acquired in the mass-to-charge ratio (*m/z*) range 400 – 500. Quantification of HDA derivatives in plasma samples was performed using a linear calibration graph of peak-area response of HDA to peak-area response of IS ratio versus concentration of HDA in calibration standards (r^2 = 0.998). The retention times of derivatized HDA and HpDA were 15.1 and 16.6 min, respectively. The most abundant fragment ions were produced from losses of 3 fluorine and 3 hydrogen groups ($[M - H]^- \rightarrow [M - H - 60]^-$), yielding *m/z* 448 (HDA) and 462 (HpDA) (Figure 2.1).

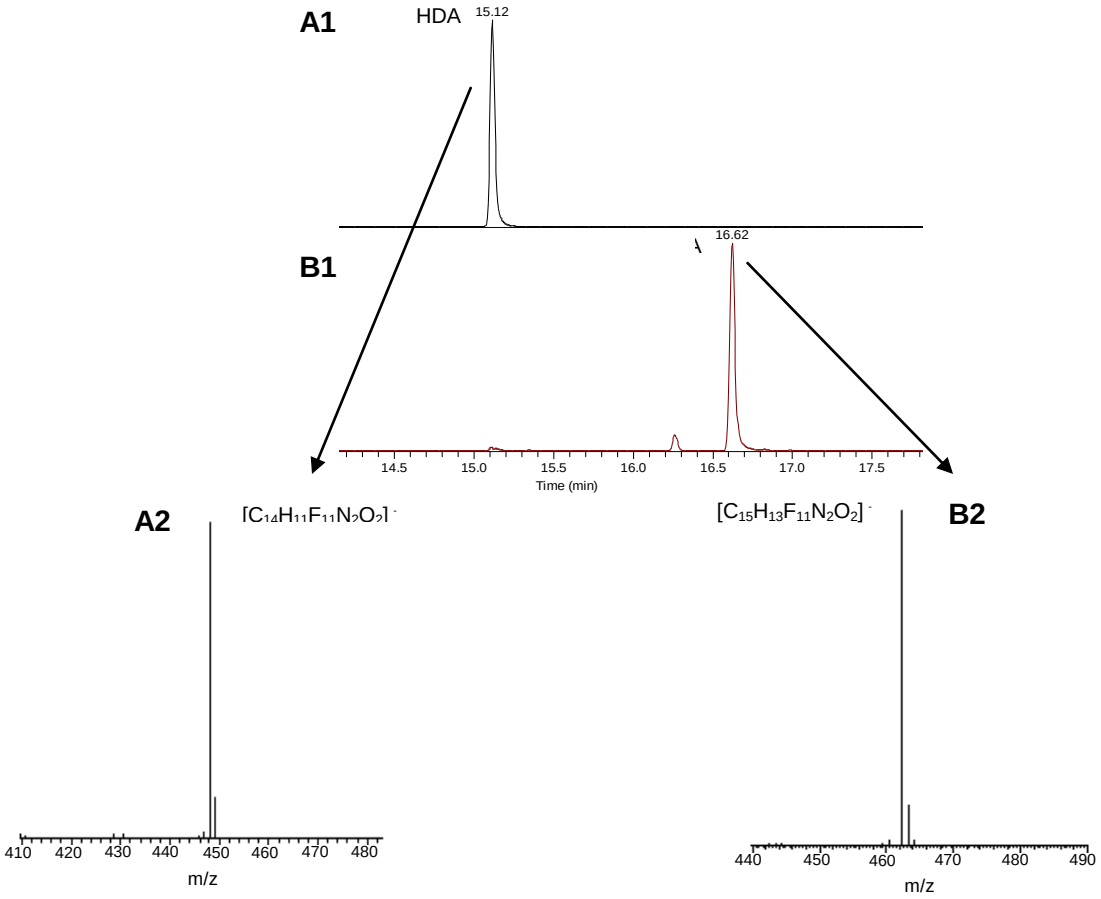

Figure 2.1 Gas chromatogram and mass spectra of heptafluorobutyryl derivatives of 0.50 µg/l HDA (A1) and 0.12 µg/l HpDA (B1) in a plasma sample. A1 and B1 were recorded at *m/z* 448 and 462, corresponding to the heptafluorobutyryl derivative of HDA (A2) and the heptafluorobutyryl derivative of HpDA (B2).

2.3.4 Work-up procedures

2.3.4.1 General procedure for plasma samples. The sample work-up procedure was adapted from a previously published method describing the analysis of HDA in urine [48]. Duplicate glass vials containing 1 ml plasma (± HDA) and 12 µl of IS (0.12 µg/l plasma) were hydrolyzed with 100 µl of concentrated H_2SO_4 at 100ºC for 16 h. The sample vials were cooled to room temperature and 0.5 g NaCl was weighed and added to the vials. A solution of saturated NaOH (4 ml) was added and the liberated amines extracted, with vortexing between extractions, into toluene (3 × 2 ml). The toluene layers were transferred to new tubes and 20 µl of HFBA added to derivatize the amines. The vials were vortexed for several seconds and heated at 55ºC for 1 h. The excess reagent was removed by extraction with phosphate buffer solution (4 ml of 1M KH_2PO_4, pH 7). The toluene layers were transferred to new vials and dried over sodium sulfate. The samples were dried under nitrogen using a TurboVap® LV Evaporator (Zymark Center; Hopkinton, MA). The dried residues were dissolved in 200 µl ethyl acetate and placed in an ultrasonic bath for several minutes. The sample solutions were transferred to GC vial inserts and evaporated to dryness using a SpeedVac® (Savant Instruments Inc., Holbrook, New York). The dried residues were dissolved in 60 µl ethyl acetate. One reagent blank and two calibration standards were prepared with every set of plasma samples collected from the workers. The samples were analyzed by GC-MS as described above.

2.3.4.2 Stability of HDA in plasma during hydrolysis. Because the stability of amine metabolites in biological samples may be related to the hydrolysis conditions, we compared changes of hydrolysis time on the concentration of HDA in plasma. The concentration of HDA in plasma after 0, 4, and 16 h hydrolysis times at 100ºC with 100 µl concentrated H_2SO_4 was measured. Stock solutions of HDA and HpDA were prepared in 1M H_2SO_4, as described above, and spiked into 1 ml blank plasma for a final HDA concentration of 2 µg/l and IS concentration of 0.12 µg/l. To one set of HDA spiked plasma (N = 5), the work-up procedure was performed without hydrolysis (*i.e.*, no addition of

concentrated H_2SO_4 and heating at 100ºC). A second set of HDA spiked plasma solutions (N = 5) was prepared and the work-up procedure was performed involving hydrolysis for 4 h in concentrated H_2SO_4 and IS added after hydrolysis. A third set of HDA spiked plasma solutions (N = 5) was prepared and the work-up procedure was performed involving hydrolysis for 16 h in concentrated H_2SO_4 and IS added after hydrolysis. The amine residues for all sample sets were dissolved in 60 µl ethyl acetate and analyzed by GC-MS.

2.3.4.3 Method detection limit (MDL) determination. The USEPA[66] describes the MDL as the minimum concentration of a substance that can be measured and reported with 99% confidence that the analyte concentration is greater than zero, and is based on the approach of Glaser and colleagues[67]. To calculate the MDL, a minimum of seven replicate (n) spikes are prepared at an appropriately low concentration (generally 1 to 5 times the expected MDL) and processed through the entire analytical method. Therefore, we spiked 9 plasma samples with 0.12 µg/l HDA and 0.12 µg/l IS, and the work-up procedure performed as previously described for HDA analysis. The MDL was calculated using the following general formula:

$$\text{MDL} = s \times t_{(n-1,\ 1-\alpha = 0.99)}$$

where

n = number of replicate spike determinations at 1 to 5 times the estimated MDL.

s = standard deviation of measured concentration of *n* spike determinations.

t = Student's *t* value at *n*-1 degrees of freedom and 1-α (99%) confidence level. At n = 9 and α = 0.1, then *t* = 2.896, and

α = level of significance

Thus, based on values in our study: s = 7 ng/l, n = 9, and t = 2.896 at α = 0.1, the MDL was calculated at 0.02 µg/l.

2.3.5 Biomarker and exposure measurements

One blood sample (10 ml) was obtained at the end of the work-shift from each of the 46 spray painters applying HDI-containing paint in auto-repair shops in North Carolina (n = 14) and in Washington (n = 32). Blood samples were collected during each sampling visit when workers consented (112 total blood samples). No more than 3 sampling visits (at least 3 weeks apart) were made for each worker. Blood was collected in heparin and EDTA tubes, and the plasma separated within 24 h of collection. After the plasma was isolated, samples were stored at –40ºC until analyzed. Samples (1 ml) were prepared in duplicate and the work-up procedure performed as outlined above. Urine was also collected from the workers throughout the workday, and stored at –40ºC until analyzed. Results for HDA in hydrolyzed urine are published elsewhere[50].

Breathing-zone and dermal exposure measurements among these occupationally exposed spray painters have been published elsewhere[41, 53]. Briefly, a personal air sample in the worker's breathing-zone was collected during each spray-painting operation of clear-coat using a 1-stage or 2-stage filter sampling system housed in a 37-mm polystyrene cassette (SKC Inc., Eighty Four, PA, USA) attached to a high-flow air pump operating at 1.0 l/min (SKC). The 2-stage sampler used in this study contained an untreated polytetrafluoroethylene pre-filter (designed to collect diisocyanate aerosols) and a glass-fiber filter impregnated with derivatizing agent (designed to collect and derivatize diisocyanate vapors). The one-stage sampler was identical to the two-stage samplers except that the pre-filter was not included in the cassette. For 8 sampling visits (3 workers), air sampling using 1- and 2-stage samplers was performed side-by-side. HDI on the skin was collected using 3 consecutive tape-strips (10 cm^2) applied to 6 different sites on the worker's body (*e.g.*, right and left forearms, hands, and neck) after each paint task. Skin and air samples were analyzed by LC-MS as previously described by Fent and colleagues[41, 53]. An algorithm described by Fent and colleagues was used to calculate the whole-body concentration of HDI in the skin (ng/mm^3) for each worker and paint task[41].

Work diaries and questionnaires about workers' physical characteristics (*e.g.*, age, height, weight), type and frequency of PPE use (*e.g.*, coveralls, respirator, gloves), work environment (*e.g.*, paint booth, number of paint tasks per week), and duration of paint tasks were completed during each visit.

2.3.6 Variable construction

The breathing-zone concentration (BZC) of HDI monomer for each paint task (μg/m^3) was multiplied by the total paint time (min) for each task and summed together to obtain a daily cumulative air exposure (DCAE). Each DCAE value was multiplied by the average male breathing rate (0.023 m^3/min) to estimate the HDI inhalation exposure (μg)[68]. We also estimated the respirator-adjusted HDI inhalation exposure after dividing each DCAE value by the OSHA Assigned Protection Factor (APF) based on respirator type[69]: none, APF = 1; air purifying (half face), APF = 10; air purifying (full facepiece), APF = 50; supplied air (full facepiece or hood), APF = 1000; PAPR (full facepiece or hood), APF = 1000. Among visits where 1- and 2-stage air samplers were used side-by-side, we selected 1-stage air data for regression and statistical analyses based on findings by Fent and colleagues[53]. The HDI monomer dermal exposure concentrations (μg/m^2) for each paint task were multiplied by body surface area of the worker (m^2) and summed together to estimate the cumulative daily dermal exposure (μg). The internal HDA dose (μg) was calculated by multiplying the HDA concentration (μg/l) in plasma with the individual plasma volume (l), which was determined using individual's body weight[70].

2.3.7 Statistical analysis

Differences in HDA concentration in spiked plasma at hydrolysis times of 0, 4, and 16 h was analyzed using single factor analysis of variance in Excel (Microsoft Office, 2004) at α-level of 0.05. The effect of sample treatment on repeatability (intra-group variability) was investigated by calculating the percent coefficient of variation (%CV), which was derived as the ratio of the standard deviation of replicate samples to the mean and multiplied by 100, among calibration standards and

field samples. The effect of sample treatment on reproducibility (inter-group variability) was investigated by comparing the %CV among calibration standards and field samples.

Due to the relatively high percentage of non-detectable levels of HDA in hydrolyzed plasma samples (24%), multiple imputation (n = 10 imputed datasets) was used to impute plasma HDA data below detection limits (*i.e.*, <0.02 µg/l). Methods for performing multiple imputation of exposure data are previously described by Fent and colleagues[41, 53], and were also applied to plasma HDA. Briefly, a lower bound of zero was set for the imputations, and we imputed from truncated multivariate normal distributions, with truncation at the MDL. The geometric mean and geometric standard deviation of dose and exposure data were calculated from the log-transformed data (PROC MEANS procedure in SAS, version 9.1; SAS Institute, Cary, NC). Statistical analysis of the stratified data, according to different workplace covariates, was performed using analysis of variance and Tukey-Kramer multiple comparison test (Tukey's test, PROC GLM procedure) at α-level 0.05 across each imputed data set and p-values averaged.

SAS PROC MIANALYZE was used to combine the results of the analyses on 10 imputed datasets in order to obtain valid estimates and statistical inferences across repeated visits. Linear mixed models for predicting plasma HDA levels were constructed and parameter estimates, as well as the restricted maximum likelihood (REML) estimates for the within- and between-worker variance of the logged values were obtained using SAS PROC MIXED of the fixed effects, which included location, coverall use, glove use, respirator type, and booth type. Each fixed effect was evaluated one at a time with the log-transformed plasma HDA level as the dependent variable, and the *p*-value of the prediction determined. A significance level of 0.05 was used to investigate these relationships. The variance components covariance structure type was selected based on comparisons of the Akaike's Information Criteria (AIC) across models with competing covariance structures[71]. The intraclass correlation coefficient (ICC), defined as the proportion of total variance explained by the between-worker variability, and calculated by dividing the between-worker variance by the sum of the between- and within-worker variance, was calculated for plasma HDA.

The correlation between biomarker and exposure data was investigated using linear regression analysis (PROC CORR procedure in SAS). PROC MIANALYZE was used to obtain a Pearson's correlation coefficient (r) and p-value from the set of 10 imputed datasets. We computed Fisher's z transformation of r to determine the 95% confidence intervals on Pearson's r. For regression and statistical analyses, all continuous values were log-transformed to meet the assumption of normality (Shapiro-Wilk $W > 0.80$).

2.4 Results

2.4.1 Stability of HDA with sample treatment

Hydrolysis of HDA spiked plasma samples for up to 16 h did not significantly affect the concentration of HDA ($p = 0.31$). This result is similar to HDA stability in water and urine under similar hydrolysis conditions described by Marand and colleagues[72]. The detection limit (MDL) of the analysis, from spiked plasma samples, was 0.02 µg/l. The repeatability among standards and field samples was assessed using the %CV. The %CV among replicate calibration standards (range of 0 – 2 µg/l) was in the range of 0.3 – 5%, demonstrating good repeatability. The %CV among replicate field samples (range of <0.02 – 0.92 µg/l) was in the range of 0.5 – 20%, demonstrating lower repeatability compared to calibration standards with spiked HDA. Thus, sample treatment affected the reproducibility of the method between calibration standards (spiked standards) compared with field samples. The higher variability of amines in samples compared to spiked standards has been observed previously using different biological matrices[72]. This may be a due to the efficiency of the hydrolysis step in cleavage of HDA – protein covalent bonds among field samples yielding greater variability in recovered HDA, whereas spiked plasma does not require bond cleavage to extract HDA.

2.4.2 HDA plasma levels in occupationally exposed workers

The range of HDA concentrations in hydrolyzed plasma samples was ≤0.02 – 0.92 µg/l. After imputing values for samples <MDL (24%) and adjusting for individual plasma volume, the geometric mean (GM) and geometric standard deviation (GSD) for the plasma HDA dose was 0.29 µg and 3.53, respectively. Of the 46 workers participating in the study, we collected blood on exactly 2 repeated visits for 10 workers, and 3 repeated visits for 28 workers. Dermal and breathing-zone samples were not collected from 4 workers during 4 of the 112 sampling visits because no paint tasks were performed on those days. Therefore, we analyzed 108 plasma samples from 46 workers with corresponding dermal and breathing-zone measurements that were used in regression and statistical

analyses. The GM and GSD for dermal HDI monomer exposure was 4.9 µg and 11.2, respectively, and approximately 35% of paint tasks had detectable levels of HDI on the skin. The GM and GSD for unadjusted inhalation exposure was 1.9 µg and 4.8, respectively, and approximately 90% of paint tasks had detectable levels of HDI in the breathing-zone of workers.

2.4.3 Linear regression

The correlation coefficients and significance of the associations between plasma HDA levels and HDI exposure (dermal and inhalation) measured on the same workday are summarized in Table 2.1. The correlation between plasma HDA levels and HDI inhalation exposure, adjusted by APF, was weak (N = 108, r = 0.10, $p = 0.336$) compared with that unadjusted by APF (N = 108, r = 0.22, $p = 0.026$) (Figure 2.2). Individual differences in respirator fit and maintenance (*e.g.*, change-out of respirator filters), the uptake and elimination kinetics of HDI via the inhalation route, and/or the contribution of HDI dermal exposure on plasma HDA level may have obscured the association between HDI inhalation exposure and dose.

Table 2.1 Correlation between log-transformed plasma HDA levels (µg) and inhalation or dermal HDI exposure (µg) measured during the same workday.

Predictor Variables	n[a]	N[b]	Pearson's r	95% CI[c]	p-value
HDI Unadjusted Inhalation (µg)	46	108	0.22	0.03 – 0.40	0.026
HDI Adjusted Inhalation (µg)	46	108	0.10	-0.10 – 0.29	0.336
HDI Dermal (µg)	46	108	0.22	0.01 – 0.41	0.040
HDI Dermal (µg)[d]	10	17	0.58	0.06 – 0.85	0.031

a. Number of workers.
b. Number of samples.
c. 95% confidence interval for Pearson's r.
d. Inhalation exposure to HDI <0.005 µg.

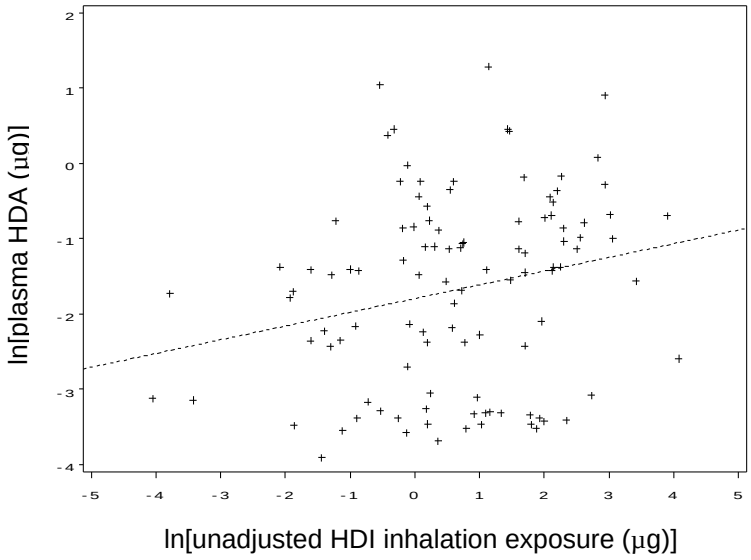

Figure 2.2 Linear regression of the log-transformed plasma HDA levels (µg) versus the log-transformed unadjusted HDI inhalation exposure (µg) measured on the same workday (N = 108; r = 0.22; *p* = 0.026) for visits 1-3.

HDA concentration in hydrolyzed plasma may be partially derived from the cleavage of long-lived albumin conjugates during hydrolysis, as was demonstrated for MDI exposures [19], and may, thus, be correlated with previous HDI inhalation exposure. Consequently, when plasma HDA levels were correlated with unadjusted inhalation exposure to HDI occurring ~20 – 60 days before blood

collection, the association improved (N = 29, r = 0.57, p = 0.0014) (Table 2.2 and Figure 2.3). In addition, the correlation improved between plasma HDA levels and adjusted inhalation exposure to HDI occurring ~20 – 60 days before blood collection (N = 29, r = 0.44, p = 0.018).

Table 2.2 Correlation between log-transformed plasma HDA levels (µg) and inhalation or dermal HDI exposure (µg) ~20 – 60 days prior to blood collection.

Predictor Variables	n[a]	N[b]	Pearson's r	95% CI[c]	p-value
HDI Unadjusted Inhalation (µg)	26	29	0.57	0.14 – 0.78	0.0014
HDI Adjusted Inhalation (µg)	26	29	0.44	0.06 – 0.69	0.018
HDI Dermal (µg)	26	29	0.36	-0.08 – 0.65	0.053

a. Number of workers.
b. Number of plasma samples.
c. 95% confidence interval for Pearson's r.

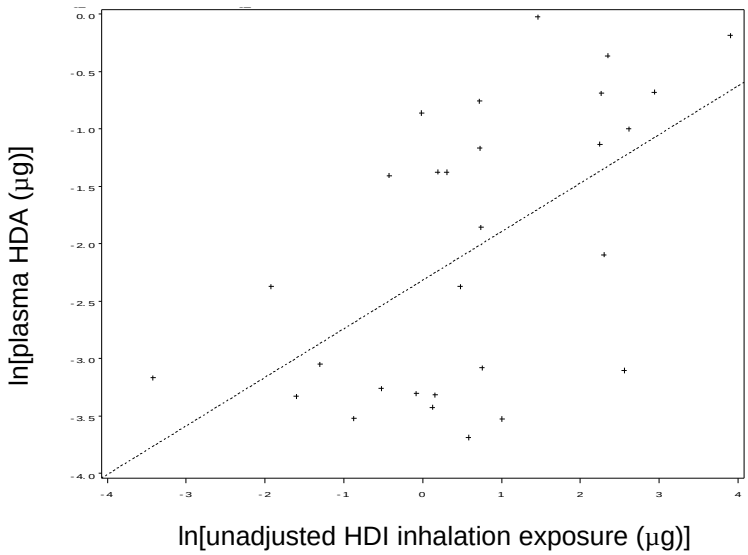

Figure 2.3 Linear regression of the log-transformed plasma HDA levels (µg) versus the log-transformed unadjusted HDI inhalation exposure (µg) occurring ~20 – 60 days prior to blood collection (N = 29; r = 0.57; p = 0.014).

Due to the potential issue of high collinearity between HDI inhalation and dermal exposure (N = 108, r = 0.77, p <0.0001), we investigated whether plasma HDA levels may be better correlated with

dermal exposure levels measured on the same workday when workers' exposure through the inhalation route was minimized (*i.e*., measured air levels <MDL). The correlation between plasma HDA levels and HDI dermal exposure was low (N = 108, r = 0.22, $p = 0.040$) compared with that among workers where adjusted inhalation exposure was very low (<MDL/5 ≈ 0.005 µg) (N = 17, r = 0.58, $p = 0.031$) (Table 2.1). In addition, the correlation between plasma HDA levels and HDI dermal exposure occurring ~20 – 60 days before blood collection, did not improve the association (N = 29, r = 0.36, $p = 0.053$) (Table 2.2). The large percentage of paint tasks with non-detectable HDI measures on the skin (65%), exposure through the inhalation route, as well as other workplace and individual factors (*e.g*., PPE use, uptake and elimination kinetics of HDI through the skin) may be obscuring the association between internal dose and dermal exposure.

2.4.4 Statistical models

We also investigated whether differences in PPE use (coveralls, gloves, respirator type) and sampling location (NC versus WA) would significantly modify plasma HDA levels. The GM and GSD values, as well as ranges for plasma HDA levels stratified by these various workplace covariates are summarized in Table 2.3. After implementing analysis of variance in SAS, insignificant differences in the GM of plasma HDA levels were observed between location NC and WA (mean difference = 0.02 µg, $p = 0.778$), coverall non-users and users (mean difference = 0.09 µg, $p = 0.097$), and glove non-users and users (mean difference = 0.03 µg, $p = 0.629$).

Insignificant differences among the different respirator types were also observed for plasma HDA levels ($p = 0.305$), unadjusted HDI inhalation exposure ($p = 0.460$), and HDI dermal exposure ($p = 0.250$), using analysis of variance and Tukey's test in SAS. However, we noted that PAPR users had a slightly higher GM for plasma HDA level (0.44 µg) compared to the other booth types (Table 2.3), which may be related to maintenance and/or proper fit of respirator. Even though most workers (98%) wore a respirator during spraying, there is the potential for inhalation exposure through

respirator filters and mask resulting from inadequate change-out of filters and/or loose-fitting mask due to improper fit, which was not factored into the adjusted inhalation exposure calculations.

Table 2.3 Summary of plasma HDA levels (µg) in 46 spray painters stratified by workplace covariates.

Covariate	n[b]	N[c]	Summary Statistics			p-value[d]
			GM	GSD	Range	
Location						
North Carolina	13	30	0.20	3.83	<0.02-1.58	0.778
Washington	33	78	0.18	3.46	<0.02-3.61	
Coveralls						
no	18	35	0.25	3.57	<0.02-1.58	0.097
yes	32	73	0.16	3.48	<0.02-3.61	
Gloves						
no	13	22	0.21	3.51	<0.02-1.54	0.629
yes	39	86	0.18	3.57	<0.02-3.61	
Respirator						
Air purifying (half)	35	80	0.17	3.24	<0.02-2.85	0.305
Air purifying (full)	1	3	0.20	4.5	0.03-0.64	
Supplied air (full or hood)	8	17	0.18	5.31	<0.02-3.61	
PAPR (full or hood)	4	7	0.44	2.48	0.16-2.47	
none	1	1	0.79			
Overall	46	108	0.29	3.53	0.02-3.61	

GM, geometric mean; GSD, geometric standard deviation; PAPR, powered-air purifying respirator

[a.] Among workers performing paint tasks on day of sample collection (N = 108). Multiple imputation (*n* = 10 imputed datasets) was used to impute plasma data below MDL.

[b.] Number of workers.

[c.] Number of samples

[d.] *p*-value determined using analysis of variance.

Table 2.4 summarizes plasma HDA levels as well as HDI inhalation (adjusted and unadjusted) and dermal exposures stratified by paint booth type with *p*-values determined using analysis of variance testing in SAS. The plasma HDA levels was significantly lower among workers painting in a downdraft ventilated booth compared to a semi-downdraft booth ($p = 0.0108$) using Tukey's test. HDI dermal and inhalation (unadjusted for APF) exposures were also significantly lower among downdraft booth users compared to the other booth types ($p < 0.0001$). The similar trend for HDI exposure levels (dermal and inhalation) and plasma HDA levels demonstrate that paint booth type may be an important modifier of the relationship between exposure and internal dose. These findings also reaffirm previous evidence of differential HDI exposure levels by booth type indicating that the downdraft booth may produce lower particulate exposures relative to other booth types[31]. Additionally, among workers painting in a downdraft booth (n = 31), HDA plasma levels were significantly lower among workers wearing coveralls compared to those not wearing coveralls ($p = 0.010$), indicating a protective effect from coverall use and that dermal exposure is contributing to internal dose.

Table 2.4 Summary of measurements in 46 spray painters[a] stratified by paint booth type.

Variable	Booth Type	n[b]	N[c]	Summary Statistics			p-value[d]
				GM	GSD	Range	
Plasma HDA (µg)	Downdraft	31	72	0.15	3.48	<0.02-2.85	0.0108
	Semi-downdraft	10	21	0.35	3.53	<0.02-3.61	
	Crossdraft	6	15	0.26	2.79	0.03-0.83	
	Overall	46	108	0.29	3.53	<0.02-3.61	
HDI Adjusted Inhalation (µg)	Downdraft	31	72	0.05	9.35	<0.0002-5.91	0.0051
	Semi-downdraft	10	21	0.05	10.5	0.001-1.70	
	Crossdraft	6	15	0.39	5.04	0.013-4.94	
	Overall	46	108	0.07	9.76	<0.0002-5.91	
HDI Unadjusted Inhalation (µg)	Downdraft	31	72	1.18	4.46	<0.02-59.1	<0.0001
	Semi-downdraft	10	21	6.88	2.49	0.84-21.4	
	Crossdraft	6	15	3.33	4.8	0.13-49.4	
	Overall	46	108	1.92	4.84	<0.02-59.1	
HDI Dermal (µg)	Downdraft	31	72	1.94	7.31	<0.10-1,023	<0.0001
	Semi-downdraft	10	21	28.8	8.82	0.24-440	
	Crossdraft	6	15	33.3	8.91	1.14-538	
	Overall	46	108	4.86	11.2	<0.10-1,023	

GM, geometric mean; GSD, geometric standard deviation

[a.] Among workers performing paint tasks on day of sample collection (N = 108). Multiple imputation (n = 10 imputed datasets) was used to impute plasma data below MDL.

[b.] Number of workers.

[c.] Number of samples.

[d.] p-value determined using analysis of variance.

The linear mixed model results for predicting plasma HDA level are summarized in Table 2.5. A large proportion (95%) of the total variance in plasma HDA level is attributed to the within-worker

variance (σ^2_w), whereas ~5% of the total variance is explained by the between-worker variance (σ^2_b) (Table 2.5). The within-worker variance represented a majority of the total variance even when stratifying for location (0 = NC, 1 = WA), coverall or glove use (0 = no, 1 = yes), and respirator type or booth type (0 = no, 1 = yes for each type) as a fixed-effect covariate in the prediction of plasma HDA level in our mixed model. In addition, location ($p = 0.806$), coverall use ($p = 0.114$), glove use ($p = 0.758$), and use of various respirator types ($p = 0.068 – 0.942$) were not-significant predictors of plasma HDA levels. On the other hand, downdraft ($p = 0.004$) and semi-downdraft ($p = 0.012$) booth types were significant predictors of plasma HDA levels.

Table 2.5 Linear mixed models for predicting log-transformed plasma HDA (μg)[a] in 46 automotive spray painters.

Covariates[b]	n[c]	N[d]	Parameter Estimates	REML[e] Estimates σ^2_w	REML[e] Estimates σ^2_b	ICC	p-value[f]
Intercept	46	108	-1.676	1.77	0.10	0.05	<0.001
Location	33	78	-0.071	1.44	0.34	0.19	0.806
Coveralls	32	73	-0.423	1.55	0.26	0.14	0.114
Gloves	39	86	-0.096	1.80	0.09	0.05	0.758
Respirator Type							
Air purifying (half)	35	80	-0.407	1.48	0.12	0.08	0.137
Air purifying (full)	1	3	0.058	2.40	NA	NA	0.942
Supplied Air (full or hood)	8	17	-0.034	3.25	0.11	0.03	0.934
PAPR (full or hood)	4	7	0.931	0.66	0.19	0.22	0.068
none	1	1	1.452	NA	NA	NA	0.258
Booth Type							
Downdraft	31	72	-0.745	1.80	0.02	0.01	0.004
Semi-downdraft	10	21	0.774	1.86	0.03	0.02	0.012
Crossdraft	6	15	0.377	1.04	0.18	0.15	0.310

REML, restricted maximum likelihood; ICC, intraclass correlation coefficient; NA, not applicable

[a.] Multiple imputation (n=10 imputed datasets) was used to impute air-sampling data below MDL.

[b.] Each fixed-effect dichotomous covariate tested in the linear mixed model for log-transformed plasma HDA level (dependant variable); location (NC=0, WA=1), coveralls (no=0, yes=1), gloves (no=0, yes=1) respirator type (no=0, yes=1 for each type), booth type (no=0, yes=1 for each type).

[c.] Number of workers.

[d.] Number of samples.

[e.] Restricted maximum likelihood estimates of the log-transformed data for the within-worker (σ^2_w) and between-worker variance (σ^2_b).

[f.] p-value is based on F-tests of fixed effects.

2.5 Discussion

We collected blood samples from 46 automotive spray painters at the end of the workday, which yielded internal dose estimations of HDI exposure. Using linear regression, as well as multiple comparisons testing of workplace covariates and linear mixed models to predict plasma HDA level, we investigated relevant exposure pathways and identified significant predictors of internal dose. However, there were several limitations that warranted consideration in the interpretation of our results.

In this study, we investigated HDA concentration in hydrolyzed plasma as a biomarker of exposure to HDI monomer. However, HDI oligomers, isocyanurate, uretidone, and biuret, were also present in the workers' breathing-zone and on the skin, with the highest levels for both air and skin occurring for isocyanurate. Because current knowledge about oligomer absorption, metabolism, and the corresponding biomarkers that best reflect exposure to these compounds is lacking, we treated HDA as the only validated biomarker of exposure to HDI monomer. Consequently, the relationship between plasma HDA level and exposure to HDI oligomers was not investigated, leaving future exposure assessment studies to consider how best to estimate dose of these compounds.

Because we investigated HDA concentration in hydrolyzed plasma, we were unable to differentiate the proportion of unconjugated to protein-conjugated HDI/HDA or metabolic products, monoacetyl-HDA or diacetyl-HDA in plasma. Consequently, individual differences in HDA acetylation (fast versus slow), which may affect HDI metabolism rates and extent of protein adduct formation, as was demonstrated by Brorson and colleagues for urinary HDA levels[21], would have been masked by the acid treatment. Individuals who are fast acetylators would favor the metabolic pathway leading to the formation of diacetyl-HDA, which is excreted directly into the urine, rather than the formation of protein adducts via the oxidation pathway. Also, Pauluhn and colleagues[56] observed differences in ratio of acetylated MDA/MDA hemoglobin adducts between inhalation and dermal exposure routes in exposed rats, and, thus, exposure route may influence metabolic pathways and elimination kinetics of these compounds. Methods for distinguishing between these pathways in monitoring for HDA

biomarkers may aid to identify relevant mechanisms contributing to biological effects. Further investigation into the proportion of unconjugated versus conjugated HDA and acetylated HDA protein adducts would also provide insights into the half-life of these compounds in the blood and their possible use as indicators of cumulative exposure to HDI.

Estimating inhalation exposure from breathing-zone concentrations is challenging due to the presence of exposure modifiers, such as respirator use. Adjusting air concentrations by the APF, according to respirator type, in order to estimate inhaled concentrations has several limitations including the inability to account for improper fit and/or maintenance. Air-purifying respirators (*e.g.*, half-face air purifying), that implement filtration for reducing inhalation exposure, may not sufficiently remove diisocyanate aerosols, particularly if workers do not follow routine change-out of filters[35]. Consequently, adjusting breathing-zone concentration by APF may under-estimate workers' true exposure if proper fit-testing and scheduled maintenance are not employed. Therefore, the correlation between plasma HDA level and inhalation exposure to HDI, adjusted by APF, needs to be interpreted with caution due to these limitations in estimating inhaled dose.

One of the main advantages in measuring blood biomarkers of HDI exposure would be to obtain internal dose measures of past or cumulative exposures from the formation of protein adducts. Assuming the modified proteins are stable, these markers may be indicative of cumulative diisocyanate exposure over the half-life of these proteins, such as albumin (~3 weeks). The presence of protein adducts may explain why the association between plasma HDA level and HDI inhalation exposure improved when HDA levels were correlated with exposures measured approximately 20–60 days prior to blood collection compared to same day exposures. When we investigated the relationship between plasma HDA levels and HDI inhalation exposure measurements from >60–200 days before blood collection, the association was weaker ($N = 37$, $r = 0.07$, $p = 0.66$). Assuming that the majority of plasma HDA was in the form of albumin adducts, most of these products would have been cleared over this time period. Our relationships with inhalation exposure should be interpreted with caution due to the limitations in estimating workers' inhaled dose through respirators. Unlike

plasma, urine HDA concentration has been demonstrated to be a good indicator of recent (short-term) inhalation exposure to HDI, due to the evidence for a fast elimination phase (1.2 – 2.5 hours) [46, 49].

The correlation between plasma HDA level and dermal HDI exposure should be interpreted with caution due to the large percentage of paints tasks (65%) with non-detectable levels of HDI on the skin. Although tape-strips of the skin were collected after each paint task, the possibility of rapid formation of HDI-keratin adducts on the skin and/or rapid absorption through the stratum corneum would contribute to the large number of non-detects of HDI monomer on the skin. An interesting observation was the slightly higher plasma HDA levels between non-coverall users compared with coverall users ($p = 0.097$), which may suggest an association between plasma HDA level and dermal HDI exposure modified by skin protection. Further research on the dermal penetration of HDI monomer and oligomers may provide information on the uptake and elimination rates from this route of exposure and the extent of its impact on internal dose levels.

Another important point to consider is the high collinearity between dermal and HDI inhalation exposure ($r = 0.77$, $p < 0.0001$), and the impact of that association with attempts at interpreting causation with respect to plasma HDA concentration. Therefore, establishing relative significance of either route on internal dose levels based on the strength of the correlations between plasma HDA levels and exposure measures may be limited in this study. The influence of multiple exposure routes and the possible differences in metabolism, uptake, and elimination rates for each of these exposure routes, may contribute to the large unexplained within-worker variability in plasma HDA levels among the workers in this study. Although significant correlation ($p < 0.05$) between plasma HDA level and dermal or inhalation exposure to HDI was observed, the large unexplained variance in plasma HDA levels prevents drawing strong conclusions about the dominant exposure pathway.

Of the four plasma samples collected from four workers who did not paint on collection day, two plasma samples had detectable concentrations of HDA (0.08 and 0.09 µg/l). These workers did not enter the paint booth during spray-painting tasks nor were they involved in mixing of the paint. However, the workers reported painting within the past 5 working days. This implies for the presence

of HDA adducts in plasma that could be related to cumulative HDI exposure. Another possible explanation is a potential for dermal exposure to trace amounts of HDA (resulting from HDI hydrolysis) from contaminated surfaces. This could result in "false-positive" readings of HDI exposure and contribute to the large variability in internal dose. Thus, biomarker measures integrating dosimeters of exposure should also be accompanied by adequate air and dermal monitoring across repeated visits.

The large variability in plasma HDA levels when correlated with dermal or inhalation exposure to HDI, as well as the large unexplained within-worker variance, reflect the limitations of using plasma HDA measurements as markers of short-term exposure to HDI. The improvement in the association between plasma HDA level and past HDI inhalation exposure, occurring ~20–60 days before blood collection, indicates the presence of plasma protein adducts with longer half-lives. Thus, the detection and measurement of HDA in the plasma may provide important clues linking cumulative HDI exposure, the evaluation of workplace protection controls, and the relevant metabolic pathways leading to sensitization.

The presence of HDI oligomers (*i.e.,* isocyanurate, biuret, uretidone) in the breathing-zone and in the skin of these workers at higher levels relative to HDI monomer, stresses the importance of investigating additional biomarkers related to HDI oligomer exposures. Future studies investigating individual differences in HDI albumin and hemoglobin adduct levels and half-lives in the blood will provide further knowledge of how long these biomarkers may remain in systemic circulation and, thus, how exposure patterns may contribute to immune response, sensitization, and development of diisocyanate-induced asthma.

2.6 Conclusions

We have applied a sensitive method for quantifying HDA in hydrolyzed plasma samples collected in the occupational exposure setting. This study reveals the potential use of plasma HDA as a marker of cumulative inhalation exposure to HDI, as well as the possible contribution of dermal exposure to

internal dose levels. Users of the downdraft paint booth had significantly lower plasma HDA levels, as well as inhalation and dermal HDI exposure levels compared to the other booth types, and booth type was a significant predictor of plasma HDA levels in the linear mixed model. However, the investigation of workplace covariates did not adequately explain the within-worker variability in plasma HDA levels, and the low correlations between HDI inhalation and dermal exposure reveals the limitation of using plasma HDA as a marker of short-term exposure to HDI. Other environmental factors, as well as the uptake and elimination kinetics of HDI via dermal and inhalation exposure routes, and the formation of protein adducts need to be further investigated.

CHAPTER 3

OCCUPATIONAL EXPOSURE TO HDI: PROGRESS AND CHALLENGES IN BIOMARKER ANALYSIS

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3.1 Abstract

1,6-hexamethylene diisocyanate (HDI) is extensively used in the automotive repair industry and is a commonly reported cause of occupational asthma in industrialized populations. However, the exact pathological mechanism remains uncertain. Characterization and quantification of biomarkers resulting from HDI exposure can fill important knowledge gaps between exposure, susceptibility, and the rise of immunological reactions and sensitization leading to asthma. Here, we discuss existing challenges in HDI biomarker analysis including the quantification of *N*-acetyl-1,6-hexamethylene diamine (monoacetyl-HDA) and *N*,*N*'-diacetyl-1,6-hexamethylene diamine (diacetyl-HDA) in urine samples based on previously established methods for HDA analysis. In addition, we describe the optimization of reaction conditions for the synthesis of monoacetyl-HDA and diacetyl-HDA, and utilize these standards for the quantification of these metabolites in the urine of three occupationally exposed workers. Diacetyl-HDA was present in untreated urine at 0.015 – 0.060 µg/l. Using base hydrolysis, the concentration range of monoacetyl-HDA in urine was 0.19 – 2.2 µg/l, 60-fold higher than in the untreated samples on average. HDA was detected only in one sample after base hydrolysis (0.026 µg/l). In contrast, acid hydrolysis yielded HDA concentrations ranging from 0.36

to 10.1 µg/l in these three samples. These findings demonstrate HDI metabolism via *N*-acetylation metabolic pathway and protein adduct formation resulting from occupational exposure to HDI.

3.2 Introduction

Biological monitoring of diisocyanate exposure through the analysis of diisocyanate-derived diamines in the urine of exposed individuals has been performed for a few decades (Rosenberg et al., 1986). However, the relationship of these diamines with dermal and inhalation exposure measures has been more difficult to discern due to the lack of a standardized method to assess dermal exposure, as well as the low sensitivity and poor specificity of analytical methods for biomarker quantification. In addition, workers receive dermal and inhalation exposure to different forms of diisocyanates (e.g., monomers, oligomers, and polymers) simultaneously, further complicating attempts to investigate the relationship between exposure and dose. Nevertheless, several volunteer and occupational studies have demonstrated a good association between 1,6-hexamethylene diamine (HDA), in urine or plasma, and inhalation exposure to HDI monomer[39, 43, 46, 47, 49].

HDI dermal exposure assessment is much less developed than that of inhalation exposure[22], although the role of dermal exposure in sensitization and diisocyanate-induced asthma has been demonstrated[27, 37]. Several different techniques have been used to detect and/or quantify these levels in the workplace including SWYPE pads[34], wipes[38], glove washes[39], and tape-strips[40]. The lack of a standardized and fully validated method to assess HDI dermal exposure has added to the difficulty in investigating associations between dermal exposure and internal dose. Evidence that dermal exposure occurs among automotive spray painters stresses the need to validate methods to measure skin exposure[41]. Post-exposure tape-stripping of the skin has the advantage of quantifying dermal penetration of HDI, and has the potential to be adapted to collect and measure HDI-keratin adducts that have been identified *in vivo*[23].

Automotive repair shop workers are becoming increasingly exposed to HDI oligomers (i.e., isocyanurate, biuret, uretidone) relative to HDI monomer during spray-painting operations, and analytical methods for quantifying specific HDI monomer and oligomers in the breathing-zone and skin of exposed workers are available[40, 41, 53]. Since these compounds differ in size and volatility, absorption and metabolism may differ by type of HDI oligomer and between monomer and oligomers. Because exposure to these compounds can now be quantified by LC-MS analysis[73], analytical methods to identify and validate specific biomarkers stemming from HDI monomer and oligomers need to be developed in order to help characterize exposure patterns to these compounds, as well as to understand their potential role in sensitization and asthma.

Measures of exposure levels alone, through tape-stripping of the skin or air monitoring, are insufficient to fully understand mechanisms involved in sensitization. Information on HDI metabolism and protein adduct formation has contributed to our understanding of the possible mechanisms involved in sensitization and development of diisocyanate-induced asthma[23, 24, 42]. As only 5-10% of diisocyanate-exposed individuals develop asthma[13], attention has been focused on the role of genetic factors in modifying individual susceptibility to asthma. Polymorphisms in glutathione-*S*-transferase (GST) or *N*-acetyltransferase (NAT) genes may result in inter-individual variation in GST or NAT enzyme activities that are involved in HDI metabolism, and could lead to increased risk of disease development[16]. Thus, highly sensitive and specific analytical methods for the quantification of HDI metabolites would improve our understanding of genetic susceptibility factors contributing to exposure-disease relationships.

Analytical methods for HDA in biological media are hindered by extensive sample processing involving lengthy acid or base hydrolysis (4 – 16 h), multiple extraction steps with toluene, derivatization and drying of samples[39, 45, 46, 48], and relatively large volume of plasma required to conduct replicate analyses (2 – 4 ml). Acid hydrolysis releases HDA-protein conjugates from biological matrixes and converts the acetyl functional groups on *N*-acetyl-1,6-hexamethylene diamine (monoacetyl-HDA) and *N*,*N*'-diacetyl-1,6-hexamethylene diamine (diacetyl-HDA) back to HDA.

Thus, the HDA detected following these treatment conditions may be derived from a number of other metabolites and adducts, and any individual variability in specific metabolite formation would be masked by acid hydrolysis. Base hydrolysis of hemoglobin has resulted in identification of 4,4'-methylenedianiline (MDA) and *N*'-acetyl-4,4'-methylenedianiline (monoacetyl-MDA) hemoglobin adducts[19, 65]. Whether base hydrolysis could liberate albumin and hemoglobin adducts of HDA and monoacetyl-HDA or adducts of HDI oligomers, has yet to be determined. HDA was non-detectable in untreated urine while base hydrolysis produced detectable levels of HDA[45], indicating that HDA may be covalently bound to proteins, and/or metabolized to other products such as diacetyl-HDA. Therefore, analytical specificity for HDI exposure biomarkers must be further developed to determine individual differences in specific metabolite formation related to HDI monomer and oligomer exposure.

Because no studies have been conducted to quantify monoacetyl-HDA or diacetyl-HDA in biological samples, and analytical standards are not commercially available, optimized conditions for their synthesis and detection are not well established, leading to variable analytical conditions being reported in a few studies[21, 74, 75]. Brorson and colleagues (1990) reported conditions for the synthesis of monoacetyl-HDA using a 1:1 molar ratio of acetic anhydride to HDA in acetonitrile and reacting for 2 h[21]. However, the final results for monoacetyl-HDA in urine samples were only "semi-quantitative" because monoacetyl-HDA standards were not purified or fully characterized. In addition, the individuals in their study were orally exposed to HDA, not HDI. Thus, monoacetyl-HDA and diacetyl-HDA metabolites among HDI exposed workers, and whose exposure occurs primarily via dermal and inhalation routes, need to be further investigated. Therefore, our intent was to (i) modify and improve on existing protocols for monoacetyl-HDA and diacetyl-HDA synthesis and quantification, (ii) use these methods to quantify monoacetyl-HDA and diacetyl-HDA in urine from three occupationally exposed workers, using GC-MS analysis, and (iii) discuss our current understanding of HDI metabolism by integrating these findings with additional reaction pathways.

3.3 Materials and Methods

Solvents and chemicals were purchased from Thermo Fisher (Hampton, NH): HPLC grade (99.9%) dichloromethane (#AC61005-0040), ethyl acetate (#AC61006-0040) and acetonitrile (#AC61001-0040), sodium sulfate anhydrous (certified ACS, #S421-500), acetic anhydride (>99%, #AC14949-0010), and sodium hydroxide (certified ACS, #S318-500); Sigma Aldrich (St. Louis, MO): 1,6-hexamethylene diamine (≥99%, #33000), 1,7-diaminoheptane (98%, #D17408), and heptafluorobutyric anhydride (HFBA, derivatization grade, #394912).

3.3.1 Reaction with acetic anhydride. Acetylation of HDA in acetonitrile for synthesis of monoacetyl-HDA has been published previously (Brorson et al., 1990a). Because ethyl acetate has been used previously in the GC-MS analysis of HDA in urine and plasma (Rosenberg et al., 2002), we were interested in comparing acetylation of HDA carried out in ethyl acetate or acetonitrile. HDA was dissolved in acetonitrile according to Brorson and colleagues (1990)[21], and in ethyl acetate (2 mg/ml, 100 ml total volume). Acetic anhydride was added dropwise with constant stirring at seven molar ratios of acetic anhydride to HDA increasing from 0.7:1 to 4:1, (S1A – S7A and S1E – S7E, respectively) during which time a precipitate formed. Solutions were allowed to react at room temperature for 2 h with continuous stirring then filtered under vacuum using a 0.22 µm, 25 mm nylon filter. The precipitate collected on filters was dissolved in ethyl acetate (1 mg/ml). The filtrates were dried under N_2 and dissolved in ethyl acetate at 1 mg/ml.

1,7-Heptanediamine (HpDA), the internal standard (IS) for HDA, was dissolved in ethyl acetate (2 mg/ml, 100 ml total volume). Acetic anhydride was added dropwise with constant stirring at 0.9:1 molar ratio of acetic anhydride to HpDA. The solution was allowed to react at room temperature for 2 h with continuous stirring, filtered, and filtrates dried under N_2. The dried products were dissolved in ethyl acetate to make a 1 mg/ml IS mixture.

Each 1 mg/ml sample, synthesized in acetonitrile or ethyl acetate, was diluted 1:1000 in ethyl acetate (final concentration = 1 µg/ml). A solution containing monoacetyl-HDA mixture (S3E) and

IS mixture (final concentrations = 1 µg/ml) was also prepared in ethyl acetate. To 1 ml of these diluted samples, 10 µl HFBA was added and reacted for 1 h at 55°C with periodic shaking.

3.3.2 GC-MS analysis. Samples were immediately analyzed by GC-MS (Thermo Trace GC Ultra interfaced with a PolarisQ ion trap mass spectrometer and AI/AS 3000 injector, and Xcalibur 1.4 SR1 software, Thermo Electron Corporation, Austin, TX) in negative chemical ionization mode, according to Flack and colleagues (2009), in scan mode (*m/z* 200-600) using methane as the reagent gas. Injections (1 µl) were made under splitless mode of 30 s with injector temperature of 220ºC. Samples were separated on a GC capillary column (DB5-MS, 30 m × 0.25 mm ID, 0.1 µm film thickness; Agilent Technologies, Palo Alto, CA). The ion source and GC transfer line temperatures were maintained at 150ºC and 260ºC, respectively. Helium was used as the carrier gas with a constant flow of 1 ml/min. The GC oven temperature program was 50ºC (1.0 min) to 155ºC at 10ºC/min, 155ºC to 185ºC at 2ºC/min, and 185ºC to 300ºC at 25ºC/min (final temperature held for 10 min). Ions were monitored in negative ion chemical ionization mode using methane as the reagent gas (1.8 ml/min). Major ions corresponding to HDA, monoacetyl-HDA, and diacetyl-HDA, as well as internal standards, HpDA, *N*-acetyl-1,7-heptanediamine (monoacetyl-HpDA), and *N*,*N*'-diacetyl-1,7-heptanediamine (diacetyl-HpDA), were identified. The relative percent abundance of each compound (i.e., peak intensity of diamine/sum of peak intensities × 100) was calculated for each molar ratio by solvent type (acetonitrile or ethyl acetate).

3.3.3 Reaction time. HDA was dissolved in ethyl acetate (2 mg/ml, 100 ml total volume) to which acetic anhydride (0.9:1 molar ratio acetic anhydride to HDA) was dropwise added with constant stirring at room temperature. At each respective time point (1, 2, 3, 4, and 21 h) 10 ml aliquot of the mixture was filtered and the filtrate diluted 1:1000 in ethyl acetate (5 ml final volume). Solutions were reacted with 10 µl HFBA for 1 h at 55ºC and immediately analyzed by GC-MS. The percent abundance of each compound was calculated for each reaction time point.

3.3.4 Standard curves (diacetyl-HDA and HDA). A portion of the synthesized diacetyl-HDA solution (S7E) was dried under N_2, dissolved in ethyl acetate (1 mg/ml), and diluted to a concentration range (0 – 4 µg/ml) in ethyl acetate. A concentration range of HDA was also prepared in ethyl acetate (0 – 1 µg/ml). To 1 ml diluted samples, 5 µl of 1 mg/ml IS mixture was added. Each solution was derivatized with 10 µl HFBA and allowed to react for 1 h at 55ºC. Samples were immediately analyzed by GC-MS.

3.3.5 Treatment of spiked urine (base hydrolysis vs. no treatment). To two control (unexposed) urine samples (1 ml aliquots), 10 µl of the 1 µg/ml optimized monoacetyl-HDA mixture synthesized in ethyl acetate (S3E) was added and shaken (set A). To two control urine samples (1 ml aliquots), 10 µl of the 1 µg/ml optimized diacetyl-HDA mixture synthesized in ethyl acetate (S7E) was added and shaken (set B). A reagent blank (1 ml urine) was also prepared, which was not spiked with monoacetyl-HDA or diacetyl-HDA mixture. Aqueous NaOH (32%, 50 µl) was added to one sample in set A and B, as well as the reagent blank, shaken for several seconds, and allowed to react at room temperature for 20 min, according to Sepai and colleagues (1995)[19], while the other samples were left untreated. Samples were extracted into 6 ml dichloromethane and dried over sodium sulfate. Dried extracts were transferred to fresh vials and reacted with 10 µl HFBA for 30 min at room temperature. The derivatized extracts were dried under nitrogen, reconstituted in 200 µl ethyl acetate, sonicated for several minutes, and analyzed by GC-MS.

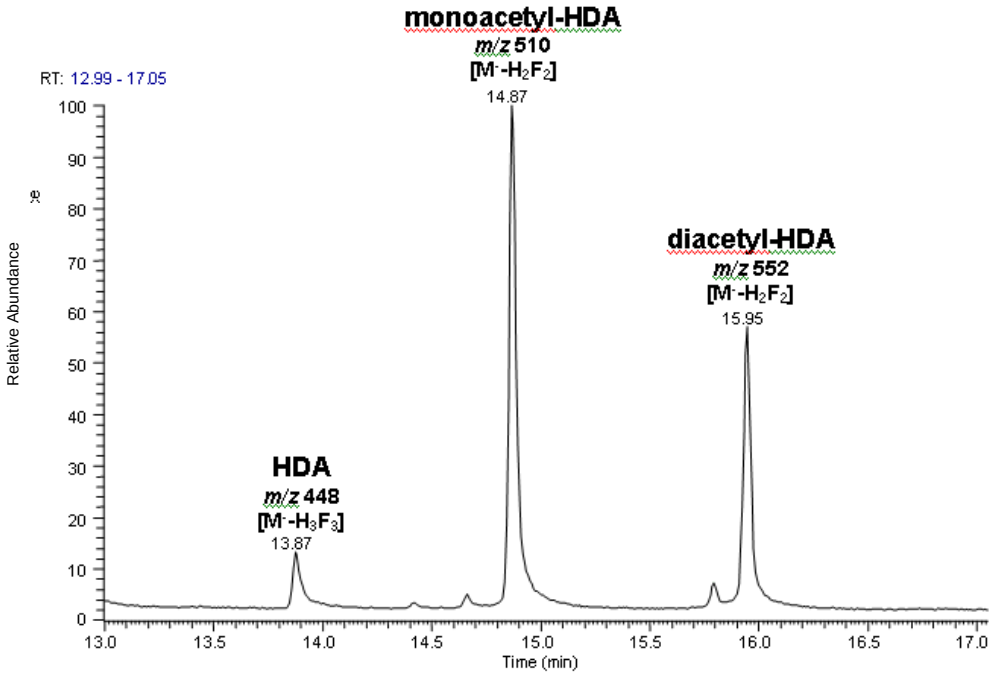
3.3.6 Analysis of urine samples. Urine samples were collected from three automotive spray painters at the end of the workday who applied HDI-containing paint. These samples were previously analyzed for total HDA using acid hydrolysis (0.36 – 10.1 µg/l) [50], and were analyzed for acetylated HDA using base hydrolysis or no treatment (section 3.3.5) and GC-MS (section 3.3.2). The acetylated HDA levels were quantified in duplicate samples against a standard curve of urine spiked

with synthesized 1 mg/ml (S3E) amine mixture (final urine concentrations: 0 – 0.09 µg HDA/l, 0 – 2.1 µg monoacetyl-HDA/l, 0 – 2.8 µg diacetyl-HDA/l). These standards were subjected to both base hydrolysis or no treatment and analyzed by GC-MS. In addition, breathing-zone and dermal tape-strip samples were collected during and after each paint task, respectively, and previously analyzed by LC-MS[41, 53]. These data for total urinary HDA in acid-hydrolyzed samples and HDI in breathing-zone and dermal tape-strip samples have been published[41, 50, 53].

3.4 Results

3.4.1 Acetylation of HDA. GC-MS analysis of the synthesized mixtures in ethyl acetate or acetonitrile, as well as untreated urine spiked with the mixtures, revealed that the major ions corresponding to derivatized HDA, monoacetyl-HDA, and diacetyl-HDA was *m/z* 448 [M^--H_3F_3] eluting at 13.9 min, 510 [M^--H_2F_2] eluting at 14.9 min, and 552 [M^--H_2F_2] eluting at 16.0 min, respectively (Figure 3.1A). The major ion for HpDA, monoacetyl-HpDA, and diacetyl-HpDA in the IS mixture was *m/z* 462 [M^--H_3F_3] eluting at 15.6 min, 524 [M^--H_2F_2] eluting at 16.6 min, and 566 [M^--H_2F_2] eluting at 17.8 min, respectively (Figure 3.2). The mass spectra for each corresponding standard peak reflected successive losses of hydrogen (H) and fluorine (F) atoms from derivatized parent amines (HDA=508, monoacetyl-HDA=550, diacetyl-HDA=592 g/mol) and IS amines (HpDA=522, monoacetyl-HpDA=564, diacetyl-HpDA=606 g/mol). Ion selection used in quantification of each amine was based on the most abundant ion fragment in the mass spectra (Figure 3.3).

A. Standard mixture synthesized in ethyl acetate.



B. Urine spiked with mixture and base-hydrolyzed.

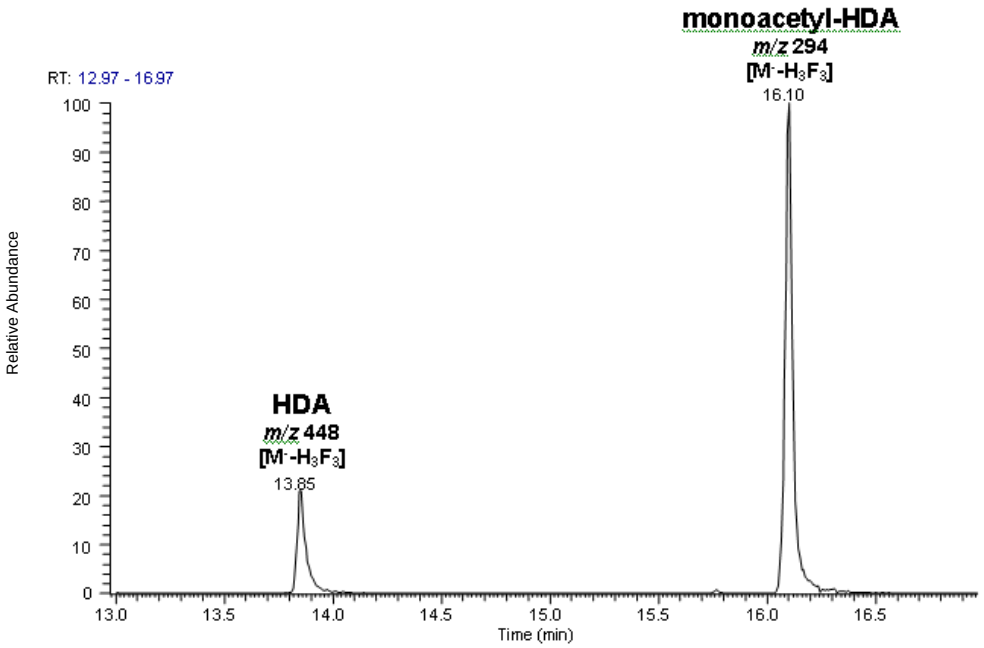


Figure 3.1 GC-MS chromatograms (*m/z* 200-600) of (A) a filtered solution containing HDA, monoacetyl-HDA, and diacetyl-HDA synthesized with 0.9:1 molar ratio (acetic anhydride to HDA) in ethyl acetate using 2 h reaction time and derivatization with HFBA and (B) derivatized extract from control urine spiked with the 0.9:1 molar ratio mixture and subjected to base hydrolysis.

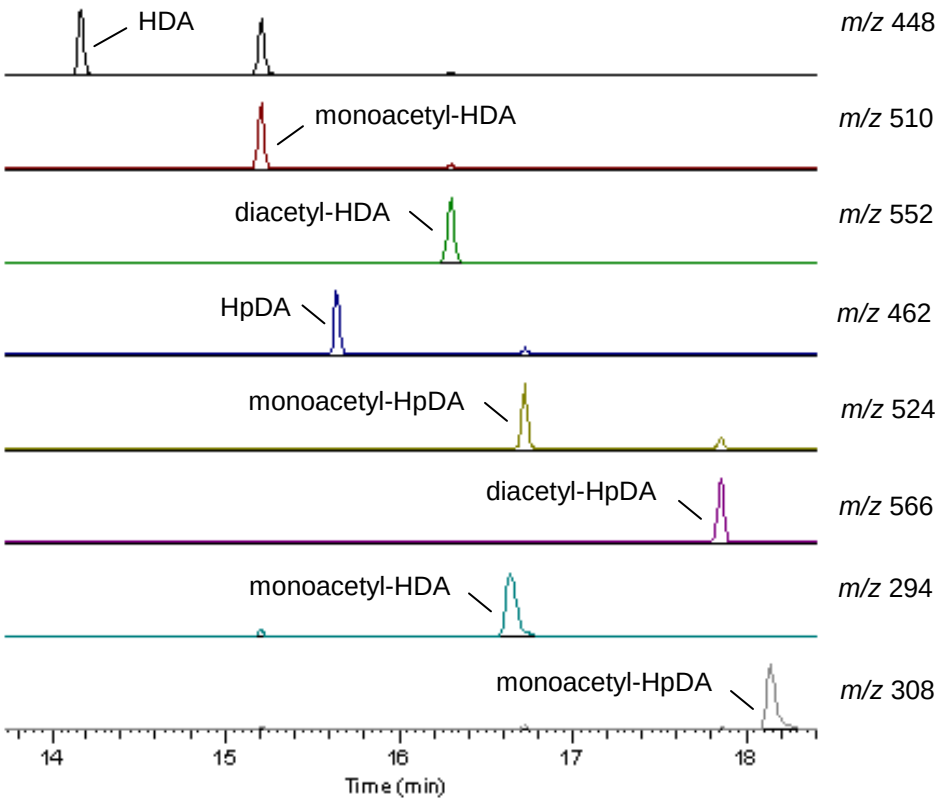
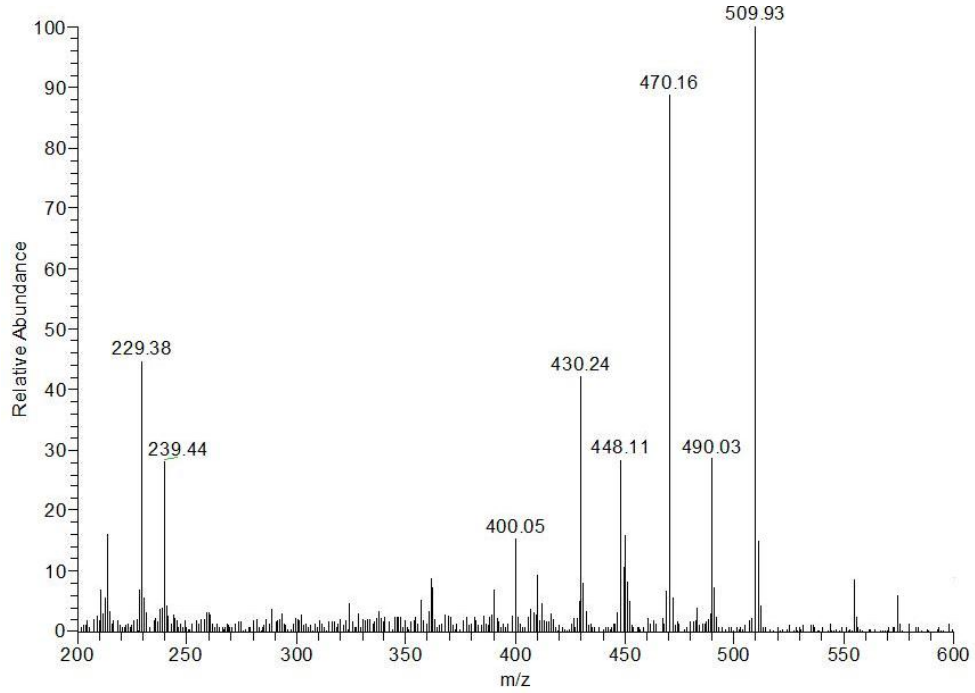


Figure 3.2 GC-MS chromatogram and most abundant mass fragment ion (*m/z*) for each amine (HDA, monoacetyl-HDA, diacetyl-HDA) and the internal standards (HpDA, monoacetyl-HpDA, diacetyl-HpDA) in derivatized extract from base-hydrolyzed control urine sample spiked with 0.9:1 molar ratio (acetic anhydride to HDA or HpDA) mixtures.

A. Monoacetyl-HDA in spiked urine.



B. Diacetyl-HDA in spiked urine.

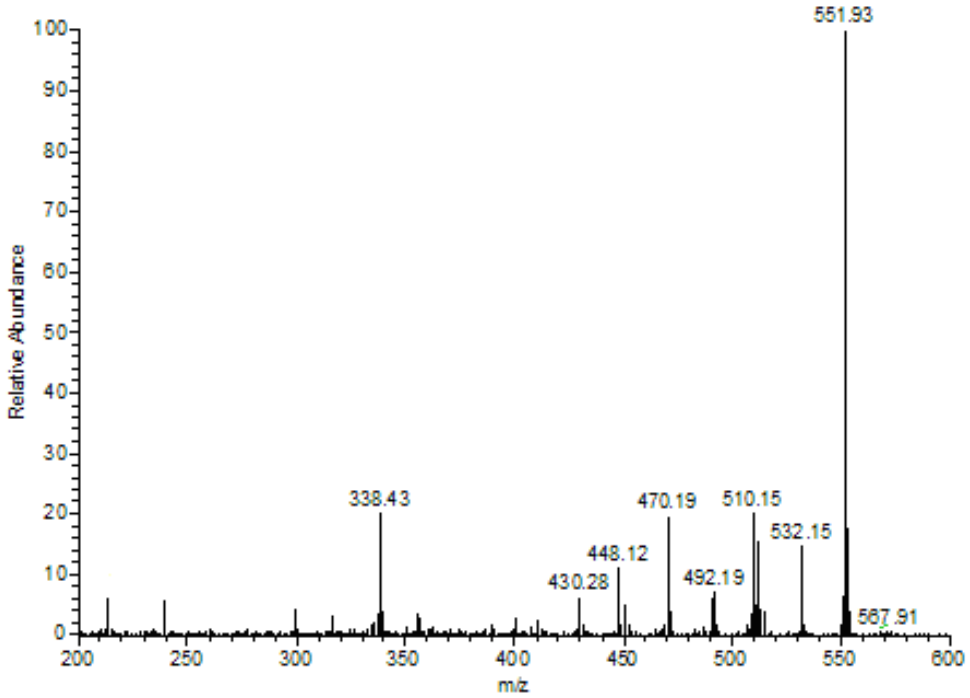
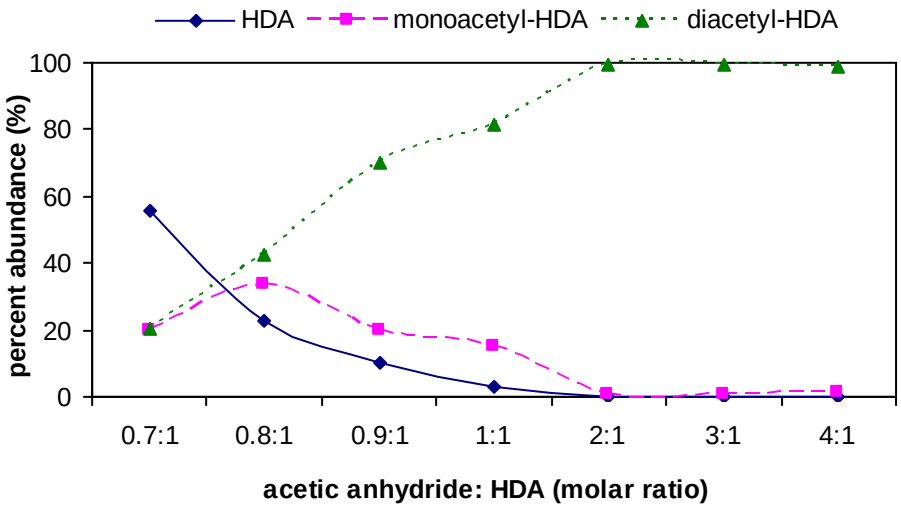


Figure 3.3 GC mass spectra (*m/z* 200 – 600) of monoacetyl-HDA (A) and diacetyl-HDA (B) in derivatized extract from untreated control urine sample spiked with 0.9:1 molar ratio (acetic anhydride to HDA or HpDA) mixtures.

Comparison of various molar ratios of acetic anhydride to HDA synthesized in acetonitrile or ethyl acetate is summarized in Figure 3.4. The reaction conditions yielding >99% diacetyl-HDA was a 2:1 molar ratio in acetonitrile (S5A) or a 4:1 molar ratio in ethyl acetate (S7E). Reaction in ethyl acetate at molar ratio 0.9:1 (acetic anhydride to HDA) gave the maximum yield of monoacetyl-HDA (55%). Monoacetyl-HDA was quantified in the product by ascertaining the concentration of HDA and diacetyl-HDA in a known concentration of mixture against pure (≥99%) HDA and diacetyl-HDA standards. Monoacetyl-HDA was quantified in these mixtures by subtracting out the known concentrations of HDA and diacetyl-HDA in the 1 mg/ml mixture quantified against pure (≥99%) HDA (0 – 1 µg/ml) and diacetyl-HDA standards (S7E; 0 – 4 µg/ml) prepared in ethyl acetate (R^2 = 0.999). The limit of detection (LOD) of diacetyl-HDA in ethyl acetate, defined as a signal-to-noise ratio of 3, was 1 ng/ml, corresponding to 1 pg on-column injection. The optimal reaction time resulting in both the maximum percent yield of monoacetyl-HDA and minimal percent of unreacted HDA was 2 h, which was also the reaction time used by Brorson and colleagues (1990)[21]. These reaction conditions may also be used for the synthesis of monoacetyl-HDA and diacetyl-HpDA, used as the internal standard for monoacetyl-HDA and diacetyl-HDA quantification in biological samples.

A. Acetonitrile



B. Ethyl acetate

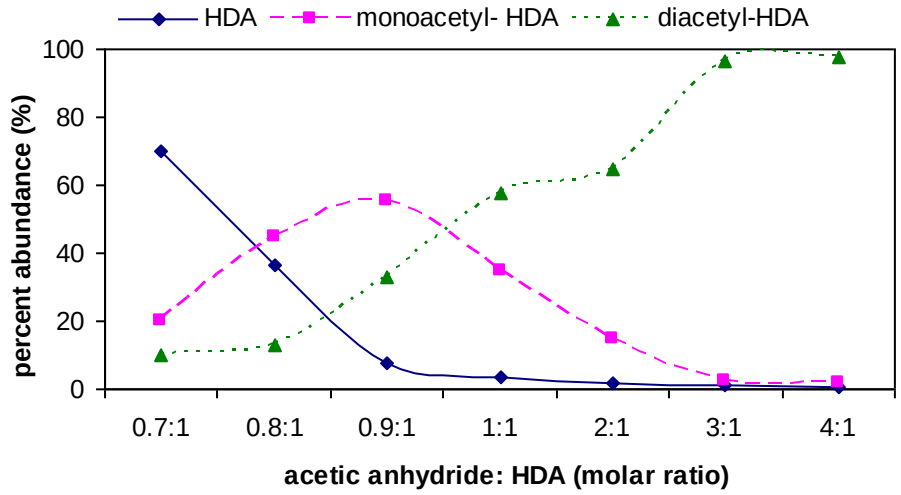


Figure 3.4 Relative percent abundance of HDA (♦),monoacetyl-HDA (■), and diacetyl-HDA (▲) in derivatized mixtures synthesized by varying the molar ratios of acetic anhydride to HDA (0.7:1 to 4:1) in acetonitrile (A) or ethyl acetate (B), reacting for 2 h, drying filtrates under N_2, and preparing 1 µg/ml solutions in ethyl acetate.

3.4.2 Analysis of spiked urine. GC-MS analysis of the spiked base-hydrolyzed urine revealed a chromatographic profile with a major peak at retention time of 16.1 min and containing an ion corresponding to monoderivatized monoacetyl-HDA, *m/z* 294 [M^--H_3F_3] eluting at 16.10 min (Figure

3.1B). This major ion fragment stemmed from derivatized monoacetyl-HDA and whose acetyl group did not react with HFBA. Furthermore, diacetyl-HDA in the base-hydrolyzed urine (spiked with S3E or S7E) was approximately 5 to 20-fold lower in sensitivity, revealing a limitation of base hydrolysis and subsequent HFBA derivatization for detection of both monoacetyl-HDA and diacetyl-HDA in biological samples. The limit of detection (LOD; signal-to-noise of 3:1) and limit of quantification (LOQ; signal-to-noise of 10:1) for HDA, monoacetyl-HDA, and diacetyl-HDA in spiked urine subjected to no treatment or base hydrolysis are summarized in Table 3.1. We postulated that base hydrolysis of urine modified the derivatization reaction of HFBA with the acetyl groups on monoacetyl-HDA and diacetyl-HDA. It is unlikely that base hydrolysis converted diacetyl-HDA to monoacetyl-HDA or HDA since the chromatographic profiles of base-hydrolyzed urine samples spiked with pure diacetyl-HDA solution (S7E) did not show peaks corresponding to HDA or monoacetyl-HDA. The lower sensitivity and different extraction method may explain why Brorson and colleagues (1990) did not detect diacetyl-HDA in base-hydrolyzed urine samples, while monoacetyl-HDA was detectable[21]. Therefore, no hydrolysis treatment could be implemented to improve detection of diacetyl-HDA.

Table 3.1 Summary of the LOD and LOQ values for HDA, monoacetyl-HDA, and diacetyl-HDA in spiked urine under base hydrolysis or no treatment conditions.

Analyte	LOD (µg/l)		LOQ (µg/l)	
	Base hydrolysis	**No treatment**	**Base hydrolysis**	**No treatment**
HDA	0.035	0.030	0.12	0.10
Monoacetyl-HDA	0.022	0.020	0.080	0.070
Diacetyl-HDA	0.050	0.015	0.20	0.050

LOD = limit of detection; LOQ = limit of quantification.

3.4.3 Analysis of urine samples. Urine samples collected from three HDI exposed workers at the end of the workday were analyzed for HDA, monoacetyl-HDA, and diacetyl-HDA using base hydrolysis or no treatment. The results are summarized in Table 3.2 along with HDI breathing-zone and dermal exposure levels as well as HDA concentrations analyzed in these same samples using acid-hydrolysis and published previously[41, 50, 53]. All three untreated urine samples had detectable levels of diacetyl-HDA (0.015 – 0.062 µg/l) while one sample had a detectable level after base hydrolysis (0.065 µg/l). Additionally, the signal-to-noise ratio of diacetyl-HDA in base-hydrolyzed samples was 2 to 10-fold lower compared to untreated samples. Therefore, the sensitivity of diacetyl-HDA analysis may be improved by not hydrolyzing the samples. All three urine samples subjected to base hydrolysis had detectable levels of monoacetyl-HDA (0.19 – 2.2 µg/l) whereas monoacetyl-HDA was detectable in two untreated samples (0.020 and 0.061 µg/l). In addition, base-hydrolyzed samples had an average of 60-fold higher concentrations compared with the untreated samples. These differences between untreated and base-hydrolyzed samples indicate the presence of monoacetyl-HDA-protein adducts that are released after base-hydrolysis. One urine sample had a detectable level of HDA after base hydrolysis (0.026 µg/l) while HDA was non-detectable in all untreated samples.

Table 3.2 Summary of the inhalation and dermal HDI levels and HDA and acetylated HDA concentrations in the end-of-day urine samples collected from three workers occupationally exposed to HDI using different treatment conditions.

Worker	Breathing-zone HDI[a] ($\mu g/m^3$)	Dermal HDI[b] (ng/mm^3)	**HDA (µg/l)**			**Monoacetyl-HDA (µg/l)**		**Diacetyl-HDA (µg/l)**	
			Acid[c]	Base	None	Base	None	Base	None
1	0.94	15.8	4.1	ND	ND	1.7	0.020	ND	0.048
2	15.0	730	10.1	0.026	ND	2.2	0.061	0.065	0.060
3	4.3	20.3	0.36	ND	ND	0.19	ND	ND	0.015

ND = non-detectable.
[a.] Average HDI breathing-zone level across paint tasks ($\mu g/m^3$) determined using LC-MS.
[b.] Total dermal HDI exposure level across paint tasks (ng/mm^3) determined using LC-MS.
[c.] Urinary HDA concentration determined using acid hydrolysis and GC-MS[50].

3.5 Discussion

Our analysis of monoacetyl-HDA and diacetyl-HDA in the urine of three HDI exposed workers adds to our understanding of an important metabolic pathway for HDI involving *N*-acetylation of HDA. The difference in monoacetyl-HDA levels between untreated and base-hydrolyzed urine also demonstrates the presence of monoacetyl-HDA-protein adducts that are secreted into urine, and subsequently released with base hydrolysis. The absence of HDA in untreated or base-hydrolyzed urine samples indicates that HDA is covalently bound to proteins, which was also demonstrated by Skarping and colleagues (1994)[45]. Moreover, the concentration of bound monoacetyl-HDA (i.e., base-hydrolyzed minus untreated) in the urine samples represented 21 – 52% of the total HDA concentration (acid hydrolysis) while diacetyl-HDA represented 0.6 – 4% of the total HDA concentration indicating that monoacetyl-HDA adducts comprise a large portion of the total HDA with unbound diacetyl-HDA representing a minor component. The concentration of total HDA in urine and HDI exposure levels followed a similar trend with monoacetyl-HDA and diacetyl-HDA (Table 3.2). Further work analyzing these metabolites among a larger population of HDI exposed workers is needed to understand the significance of this metabolic pathway in the occupational setting. We may integrate these findings for HDI metabolism with additional reaction pathways identified in other publications in our overview of HDI metabolism (Figure 1.1).

The identification of monoacetyl-HDA in individuals receiving HDA by oral administration also indicates the potential for *N*-acetylation metabolic pathway[21]. However, prior to our study reported here, monoacetyl-HDA had not yet been identified in individuals exposed to HDI in the occupational setting where dermal and inhalation exposures are the major exposure routes. Therefore, the identification and quantification of monoacetyl-HDA and diacetyl-HDA in urine of occupationally exposed workers demonstrates the relevance of the *N*-acetylation pathway in the metabolism of HDA. In addition, the difference in monoacetyl-HDA levels between base-hydrolyzed and untreated urine demonstrates the presence of monoacetyl-HDA-protein adducts that are secreted into urine.

The association between HDI exposure and biomarker levels has been evaluated in several HDI exposure assessment studies and a good correlation between HDI inhalation exposure and urinary HDA levels has been observed[39, 43, 46, 47, 49]. However, the impact of dermal exposure on urinary HDA levels has been quantitatively investigated in only one study[50]. Investigation of these associations between exposure and biomarker levels has been hindered not only by the sensitivity of analytical methods, but also by the large inter- and intra-individual variability in plasma and urinary biomarker levels of HDA[39]. In urine, this is partially attributed to the biphasic elimination pattern of HDA, characterized by a rapid elimination phase ($t_{1/2}$ = 1.2 – 2.9 h) and a slow elimination phase, which may be attributed to the breakdown of HDI-protein conjugates in the blood[43, 46, 49]. Furthermore, analyses of HDA in acid hydrolyzed urine may represent a combination of various metabolites, including free and protein conjugates of HDA and acetylated HDA. Therefore, the identification of specific HDI metabolites, such as monoacetyl-HDA or diacetyl-HDA, would improve biological monitoring methods for HDI exposure by reducing the inter- and intra-individual variability in biomarker measures and providing further information on HDI metabolism.

The low proportion of diisocyanate-induced asthmatics with detectable specific IgE[7, 14] has generated theories concerning disease mechanisms and genetic susceptibility. Lung epithelial cells, such as keratin and serum albumin, can act as carriers (antigen presenting cells) that present HDI to the immune system, resulting in induction of mixed Th1/Th2 type response and lymphocyte production[4, 23, 25]. Therefore, biomarker type and yield may be applied further to investigate metabolic pathways contributing to increased risk of diisocyanate-induced asthma. Not only is the large inter-individual variability in HDA levels partially attributed to workplace exposure factors, but also to individual differences in GST or acetylator status resulting from variable NAT enzyme activity that influence metabolism and susceptibility for disease[16]. We have demonstrated the elimination of monoacetyl-HDA and diacetyl-HDA in the urine of occupationally exposed workers. Factors driving these pathways may be further investigated using improved genotyping technologies for identifying individual single nucleotide polymorphisms and copy number variants affecting variable enzyme

activities. Thus, analytical specificity for HDI biomarkers needs to be developed in order to investigate factors driving metabolic activation versus detoxification of HDI and susceptibility of diisocyanate-induced asthma.

Our analysis of monoacetyl-HDA and diacetyl-HDA in the urine samples collected from occupationally exposed workers demonstrates the relevance of the *N*-acetylation metabolic pathway for HDI following exposure. Our proposed analytical methods for the quantification of monoacetyl-HDA and diacetyl-HDA in untreated or base-hydrolyzed urine may be utilized and applied to further investigate individual differences in HDI metabolism and relate these measurements to HDI exposure levels and susceptibility factors for HDI-associated diseases.

3.6 Conclusions

Highly sensitive and specific analytical methods for HDI biomarkers would allow for improved characterization of HDI exposure patterns and predicting systemic exposure and dose, as well as enhance our understanding of exposure-disease relationships. Progress has been made in identifying HDI metabolic pathways and intermediates leading to the formation of various protein adducts that may play a role in disease development. However, further research is needed to improve the sensitivity and specificity of analytical methods for the detection of HDA and other metabolites to better explain the sources of intra- and inter-individual variability in metabolite formation related to HDI exposure. The lack of commercially available analytical standards for monoacetyl-HDA and diacetyl-HDA has also hindered quantification of these metabolic products in biological samples. Therefore, we have optimized the reaction conditions for monoacetyl-HDA and diacetyl-HDA synthesis and presented analytical methods for their analysis in order to improve quantification of these metabolites in biological samples. In addition, we have demonstrated that monoacetyl-HDA and diacetyl-HDA may be quantified in the urine of exposed workers, thereby further contributing to our understanding of HDI metabolism.

CHAPTER 4

HEMOGLOBIN ADDUCTS IN WORKERS EXPOSED TO 1,6-HEXAMETHYLENE DIISOCYANATE

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(Manuscript)

4.1 Abstract

We investigated the utility of 1,6-hexamethylene diamine (HDA) hemoglobin adducts as biomarkers of exposure to 1,6-hexamethylene diisocyanate (HDI) monomer. Here, we describe the hemoglobin (Hb) analysis of HDA (HDA-Hb), *N*-acetyl-1,6-hexamethylene diamine (monoacetyl-HDA-Hb), and *N*,*N*'-diacetyl-1,6-hexamethylene diamine (diacetyl-HDA-Hb) in 15 spray painters (N = 35) applying HDI-containing paint in vehicle repair shops. These biomarkers were detected by GC-MS as heptafluorobutyryl derivatives in base-treated Hb. HDA-Hb was quantified in all workers ($\leq$1.2 – 37 ng/g Hb). Monoacetyl-HDA-Hb was detected in one worker (0.06 ng/g Hb) while diacetyl-HDA-Hb was not detected in any of the workers. There was a stronger, positive association between HDA-Hb adduct concentration and cumulative HDI dermal (r^2 = 0.32, p = 0.058), inhalation (r^2 = 0.35, p = 0.042), and air (r^2 = 0.34, p = 0.048) exposure than the same day exposure levels ($p \geq$ 0.13). In addition, there was a strong, inverse association between HDA concentration in acid-hydrolyzed urine and cumulative HDA-Hb adduct (r^2 = 0.28 – 0.32, p = 0.06-0.08) or total plasma HDA (r^2 = 0.21, p = 0.13) concentration. The association between HDA-Hb adduct and plasma HDA concentration within the same blood sample was weak (r^2 = 0.039, p = 0.38). These findings indicate long-term elimination kinetics for HDA-Hb adducts and, potentially, competing HDI metabolic pathways. These findings on the type and yield of different HDA blood biomarkers, and their

association with urine HDA concentration and HDI dermal and inhalation exposure, indicate that these biomarkers are suitable for further validation as biomarkers of exposure and for characterization of HDI-specific metabolic pathways that may contribute to disease susceptibility.

4.2 Environmental Impact Statement

While exposure to HDI monomer has been linked with occupational asthma, few studies have quantitatively investigated the association between HDI exposure and blood biomarker levels in the occupational setting. These biomarkers may be important indicators of cumulative exposure as a result of the formation of long-lived protein adducts during HDI metabolism. We were able to measure HDI-specific hemoglobin adducts in workers exposed to HDI and demonstrate their potential use as biomarkers of indicated cumulative exposure to HDI.

4.3 Introduction

1,6-hexamethylene diamine (HDA) in biological media (e.g., urine, plasma) has been utilized as a biological marker for 1,6-hexamethylene diisocyanate (HDI) exposure[39, 43, 45-51]. During automotive spray-painting, workers are exposed to HDI present in paint formulations via skin or inhalation routes. In biological samples these markers represent the integration of HDI exposure across multiple exposure routes. Repeated or prolonged exposure to HDI may also result in skin or respiratory sensitization[7, 54] and development of occupational asthma[5, 6]. Trace amounts of HDI monomer and larger amounts of HDI oligomer (e.g., uretdione, biuret, and isocyanurate) as vapors and/or aerosols are produced during painting with isocyanate containing paints[2, 3, 39, 76]. Repeated skin exposure at these trace levels to HDI monomer (0.1%) may cause skin sensitization, which can develop into asthma following inhalation exposure to HDI monomer[4]. Therefore, characterizing and quantifying biomarkers of HDI monomer exposure continues to be critical in evaluating workplace exposure, developing strategies for mitigating exposure, and preventing adverse health effects.

HDA in acid-hydrolyzed urine and plasma are the only validated biomarkers to date for short-term[46, 47, 50] and cumulative exposure to HDI monomer[51]. Once in the body, the isocyanato moiety (NCO) may be hydrolyzed to an amine (e.g. HDA), catalyzed by bicarbonate in the blood[57], or react with proteins, such as albumin and hemoglobin (Hb), to form protein adducts. HDA could be *N*-acetylated by *N*-acetyltransferase (NAT) to form monoacetyl-HDA[21] and diacetyl-HDA and/or undergo *N*-oxidation by cytochrome P450 (CYP450) or a flavine adenine dinucleotide (FAD)-dependent enzyme system[20] and further oxidized to form a nitroso compound[19]. The oxidation pathway would eventually result in the formation of amine-derived protein adducts, persisting over the half-life of albumin ($t_{1/2}$ = 19 days)[77] or the life-span of Hb (120 days)[78]. Individual differences in acetylator genotype (fast *vs*. slow) that drive these dominant metabolic pathways have been linked with asthma risk[16]. Protein adducts are also formed via hydrolysis of bis-thiocarbamate intermediates resulting from the reaction of NCO with thiols, such as cysteine[18]. Different protein adducts or metabolites formed from these various non-enzymatic- or enzymatic-directed pathways following HDI monomer exposure have not been fully characterized in exposed individuals. Such information would help to identify important metabolic intermediates that are indicators of individual susceptibility for asthma and other health effects.

The main advantage in measuring blood biomarkers of HDI exposure is to obtain internal dose measures of cumulative exposures due to the formation of long-lived protein adducts. The stronger correlation between HDA in acid-hydrolyzed plasma and dermal or inhalation exposure to HDI monomer occurring ~0.5 – 2 months before blood collection compared with the same-day exposure among 46 spray painters demonstrated the presence of longer-lived albumin adducts[51]. Moreover, a biphasic elimination pattern has been described for diisocyanate-derived amines, thought to result from the rapid urinary excretion of short-term metabolites within a few hours[43, 46], followed by the excretion of long-lived protein adducts days later[58]. The presence of HDA in the majority of first-day, pre-exposure urine samples among HDI exposed spray painters, also indicated the slow elimination of protein conjugates accumulated during the work-week[50]. In addition to being an indicator of past

exposure to diisocyanates, protein adducts may contribute to the pathogenesis of diisocyanate-induced asthma by their action as protein carriers in presenting HDI to the immune system[23]. Therefore, the correlation between HDA-protein adducts in blood and HDI exposure levels or urine biomarkers would provide additional information on the uptake and elimination kinetics of HDI metabolites and, thus, the biological availability of these reaction products in contributing to disease development.

The presence of Hb adducts may indicate cumulative exposure across a more extensive period of time (≤120 days) compared with albumin adducts (≤60 days) or free HDA (several hours). A benefit of Hb adduct analysis is the enhanced specificity compared with HDA analysis using acid-hydrolysis. Measured HDA from acid-hydrolyzed plasma (i.e., plasma HDA) or urine (i.e., urine HDA) may be derived from both unbound and protein-conjugated HDA, monoacetyl-HDA, and diacetyl-HDA[79]. Acid treatment of plasma or urine masks individual variability in the types and yield of various metabolites which, in turn, could provide information about susceptibility factors and mechanisms related to disease development. Hb isolation followed by mild base treatment, has resulted in the identification of 4,4'-methylene dianiline (MDA) and monoacetyl-MDA adducts[19], and these methods could be applied to blood samples for identifying HDA-Hb and monoacetyl-HDA-Hb adducts.

The mechanism by which reactive diisocyanates are transported across the epithelial layer of the respiratory tract, into the blood, and through the erythrocyte membrane to react with Hb is unknown, but may involve the formation of mono- and bis-thiocarbamate intermediates[18]. Such products formed by the reaction of diisocyanate with cysteine would allow transport across cellular membranes. Chipinda and colleagues postulated that the bis-thiocarbamate product could be carried from the lung to a distal site, whereupon hydrolysis would occur, releasing a free diisocyanate that could react with another nucleophile[18]. Because the rates of hydrolysis among the different diisocyanate intermediates vary widely, the biological availability of reactive intermediates may also vary. Thus, investigation of HDA-Hb adducts would provide important information on potential metabolic pathways and reaction products related to diisocyanate-mediated hypersensitivity diseases.

There are no publications reporting the proportion of Hb to albumin adducts resulting from HDI exposure and how these protein adduct concentrations vary with urine HDA concentration. Sepai and colleagues reported that MDA was detected as Hb adducts in all 20 exposed workers to 4,4'-methylene diphenyl diisocyanate (MDI), and monoacetyl-MDA was detected as a Hb adduct in 1 worker[19]. However, the albumin adduct levels were 450-fold higher than the Hb adduct levels. Whether albumin adducts are also the dominant products formed from HDI exposure has yet to be investigated. Pauluhn and colleagues observed different levels of Hb adducts and urinary MDA corresponding to MDI or MDA exposure and varying MDA-Hb levels by exposure route (i.e., dermal or inhalation)[56]. In our previous study, plasma HDA levels were modified by coverall use and type of ventilated paint booth that the workers used during spray painting[51]. Therefore, exposure route, stability of metabolic intermediates, and exposure to the diisocyanate versus the diamine (e.g., HDI *vs.* HDA) may drive specific metabolic pathways of HDI and, consequently, the types and yields of products formed.

The objectives of this study were to: (i) quantify Hb adducts of HDA, monoacetyl-HDA, and diacetyl-HDA resulting from HDI exposure, using mild base treatment, in 15 exposed spray painters, (ii) investigate these measures as biomarkers of short- or long-term HDI exposure by correlating plasma HDA or Hb adduct concentrations with same day or cumulative HDI monomer air, dermal, or inhalation exposures, and (iii) compare elimination kinetics of Hb adducts and plasma HDA by correlating these concentrations with urine HDA concentration in acid-treated samples (pre-shift, post-shift, daily average).

4.4 Materials and Methods

4.4.1 Chemicals

Solvents and chemicals purchased from Thermo Fisher (Hampton, NH): HPLC grade (99.9%) dichloromethane (#AC61005–0040), ethyl acetate (#AC61006–0040), acetonitrile (#AC61001–0040), sodium sulfate anhydrous (certified ACS, #S421–500), acetic anhydride (>99%, #AC14949–0010),

sodium hydroxide (certified ACS, #S318–500), and hemoglobin (#ICN10071425); and from Sigma Aldrich (St. Louis, MO): 1,6–hexamethylene diamine (≥99%, #33000), 1,7–diaminoheptane (98%, #D17408), and heptafluorobutyric anhydride (derivatization grade, #394912).

4.4.2 Synthesis of standards

4.4.2.1 Preparation of HDA and acetylated HDA (standards). A standard mixture of HDA, monoacetyl-HDA and diacetyl-HDA was prepared by making a 2 mg/ml solution of HDA in ethyl acetate (50 ml total volume). Acetic anhydride was added dropwise at 0.9:1 molar ratio (acetic anhydride to HDA) with constant stirring at room temperature. The solution was allowed to react for 2 h and was subsequently filtered under vacuum using a 0.22 µm, 25 mm nylon filter. A portion of the filtrate was dried under nitrogen and a 1 mg/ml solution of the dried product prepared in ethyl acetate, and stored at 4ºC.

4.4.2.2 Synthesis of diacetyl-HDA (≥99%). A solution of diacetyl-HDA (≥ 99%) was synthesized by preparing a 2 mg/ml solution of HDA in ethyl acetate (50 ml total volume). Acetic anhydride was added dropwise at 4:1 molar ratio (acetic anhydride to HDA) with constant stirring at room temperature. The solution was allowed to react for 2 h and was subsequently filtered under vacuum using a 0.22 µm, 25 mm nylon filter. A portion of the filtrate was dried under nitrogen and a 1 mg/ml solution of the dried product prepared in ethyl acetate and stored at 4ºC.

4.4.2.3 Preparation of HpDA and acetylated HpDA (internal standards). An internal standard (IS) mixture of 1,7-diaminoheptane (HpDA), monoacetyl-HpDA and diacetyl-HpDA was prepared by making a 2 mg/ml solution of HpDA in ethyl acetate (50 ml total volume). Acetic anhydride was added dropwise at 0.9:1 molar ratio (acetic anhydride to HpDA) with constant stirring at room temperature. The solution was allowed to react for 2 h and was subsequently filtered under vacuum

using a 0.22 µm, 25 mm nylon filter. A portion of the filtrate was dried under nitrogen and a 1 mg/ml solution of the dried product prepared in ethyl acetate, and stored at 4ºC.

4.4.2.4 Preparation of HDA and diacetyl-HDA standard curves. To quantify HDA, monoacetyl-HDA, and diacetyl-HDA in the standard mixture, HDA and diacetyl-HDA standard curves were prepared in ethyl acetate. First, HDA and IS solutions (1 µg/ml) were prepared. A 5 µg/ml solution of diacetyl-HDA was prepared in ethyl acetate. A 1:1 serial dilution (1 ml total volume) of HDA and diacetyl-HDA solutions (in duplicate) was performed in ethyl acetate to obtain a concentration range: HDA (0 – 1 µg/ml) and diacetyl-HDA (0 – 5 µg/ml). Each diluted sample of HDA or diacetyl-HDA was spiked with 100 µl of 1 µg/ml IS mixture (final concentration = 0.1 µg/ml). Diluted standard mixture solutions (5 µg/ml) were prepared in ethyl acetate (1 ml total volume) and spiked with 100 µl of 1 µg/ml IS mixture. Solutions were reacted with 10 µl HFBA for 1 h at 55ºC and immediately analyzed by gas chromatography-mass spectrometry (GC–MS).

4.4.3 Analysis and work-up procedures

4.4.3.1 GC-MS analysis. Samples were analyzed by GC-MS (Thermo Trace GC Ultra interfaced with a PolarisQ ion trap mass spectrometer and AI/AS 3000 injector, and Xcalibur 1.4 SR1 software, Thermo Electron Corporation, Austin, TX). Injections (1 µl) were made under splitless mode of 30 s with injector temperature of 220ºC. Separation of the samples was carried out with a GC capillary column (DB5-MS, 30 m × 0.25 mm ID, 0.1 µm film thickness; Agilent Technologies, Palo Alto, CA). The ion source and GC transfer line temperatures were maintained at 150ºC and 260ºC, respectively. Helium was used as the carrier gas with a constant flow of 1 ml/min. The GC oven temperature program was 50ºC (1.0 min) to 155ºC at 10ºC/min, 155ºC to 185ºC at 2ºC/min, and 185ºC to 300ºC at 25ºC/min (final temperature held for 10 min). Ions were monitored in negative ion chemical ionization mode using methane as the reagent gas (1.8 ml/min). Mass spectra were acquired in the mass-to-charge ratio (*m/z*) range 200 – 600.

GC-MS analysis of the standard curves and standard mixture revealed that the major ions corresponding to derivatized HDA, monoacetyl-HDA, and diacetyl-HDA were *m/z* 448 $[M–H_3F_3]^-$ eluting at 14.1 min, 510 $[M–H_2F_2]^-$ eluting at 15.1 min, and 552 $[M–H_2F_2]^-$ eluting at 16.2 min, respectively (Figure 1). The major ion for HpDA, monoacetyl-HpDA, and diacetyl-HpDA in IS mixture was *m/z* 462 $[M–H_3F_3]^-$ eluting at 15.6 min, 524 $[M^–H_2F_2]^-$ eluting at 16.6 min, and 566 $[M–H_2F_2]^-$ eluting at 17.7 min, respectively. The mass spectra for each corresponding standard peak reflected successive losses of hydrogen (H) and fluorine (F) atoms from derivatized parent amines (HDA = 508, monoacetyl-HDA = 550, diacetyl-HDA = 592 g/mol) and internal standard amines (HpDA = 522, monoacetyl-HpDA = 564, diacetyl-HpDA = 606 g/mol). Ion selection used in quantification of each amine was based on the most abundant ion fragment in mass spectra.

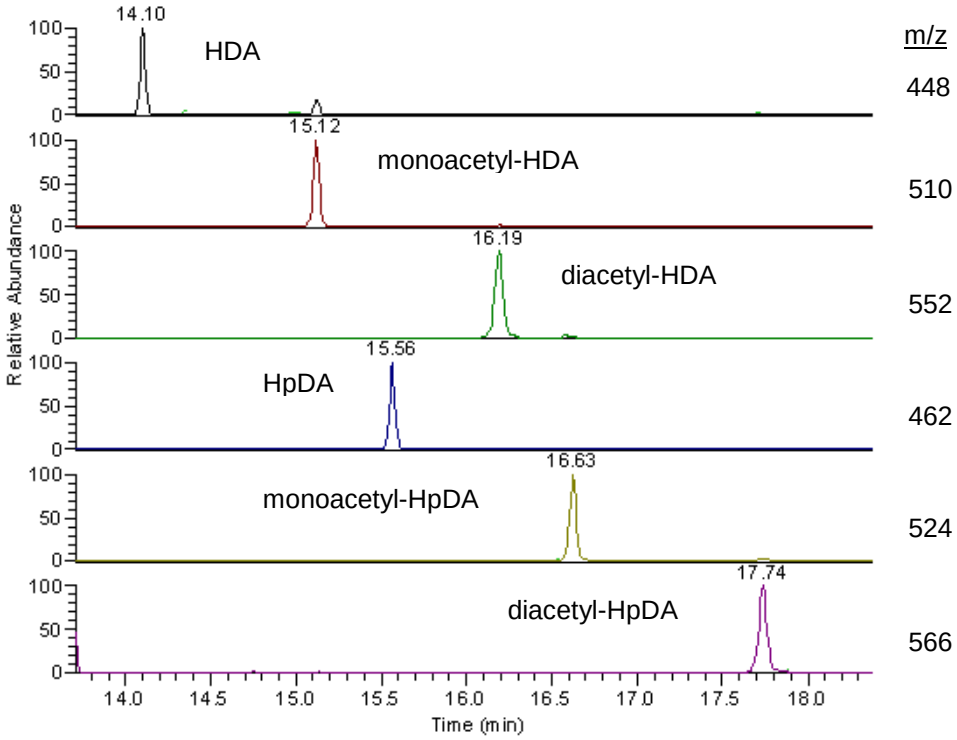


Figure 4.1 GC-MS chromatogram of various amines extracted from base-treated Hb solution (50 mg/ml) spiked with standard mixture (per mg Hb: 0.010 µg HDA, 0.11 µg monoacetyl-HDA, 0.14 µg diacetyl-HDA) and IS mixture (0.50 µg/mg Hb) with the corresponding major fragment ion (m/z).

4.4.3.2 Quantification of HDA and acetylated HDA in mixture. Peaks corresponding to HDA and acetylated HDA, as well as HpDA and acetylated HpDA were integrated. Quantification of HDA and

diacetyl-HDA in the standard mixture was performed using a linear calibration graph of peak-area response of HDA to peak-area response of HpDA in calibration standards (r^2 = 0.999) and a graph of peak-area response of diacetyl-HDA to peak-area response of diacetyl-HpDA in calibration standards (r^2 = 0.998), respectively. The concentration of monoacetyl-HDA was calculated by subtracting the known concentration of HDA and diacetyl-HDA from the total concentration (5 µg/ml). The standard mixture yielded 0.09 µg/ml HDA (1.8%), 2.11 µg/ml monoacetyl-HDA (55.7%), and 2.80 µg/ml diacetyl-HDA (42.5%).

4.4.3.3 Biomarker and exposure measurements. Fifteen spray painters (4 from North Carolina and 11 from Washington State) were randomly selected from our larger HDI study population (n = 46) reported previously[51]. Blood samples were collected during each sampling visit when workers consented (N = 35). Six workers were sampled twice, and eight workers were sampled three times. Blood was collected in EDTA and heparin tubes at the end of the workday and plasma separated within 48 h of collection. After the plasma and red blood cells were isolated, samples were stored at –35ºC until analyzed. Urine was collected throughout the workday, and stored at –35°C until analyzed. Analyses for urine and plasma HDA in acid-hydrolyzed samples have been published elsewhere[50, 51]. Briefly, 1 ml of urine or plasma (in duplicate), containing 100 µl sulfuric acid and HpDA internal standard, was baked at 100°C for 16 h. HDA was extracted 3 times into 2 ml toluene, pooled extracts derivatized with 20 µl HFBA at 55°C for 1 h, and dried under nitrogen. Samples were reconstituted into 60 µl ethyl acetate and analyzed by GC-MS.

Breathing-zone and dermal exposure measurements among these occupationally exposed spray painters have been published elsewhere[41, 53]. Briefly, a personal air sample in the worker's breathing-zone was collected during each spray-painting operation of clear-coat using a 1-stage or 2-stage filter sampling system housed in a 37-mm polystyrene cassette (SKC Inc., Eighty Four, PA, USA) attached to a high-flow air pump operating at 1.0 l/min (SKC). The 2-stage sampler used in this study contained an untreated polytetrafluoroethylene pre-filter (designed to collect diisocyanate aerosols)

and a glass-fiber filter impregnated with derivatizing agent (designed to collect and derivatize diisocyanate vapors). The 1-stage sampler was identical to the 2-stage sampler except that the pre-filter was not included in the cassette. Among visits where 1- and 2-stage air samplers were used side-by-side (n = 1), we selected 1-stage air data for regression and statistical analyses based on findings by Fent and colleagues[53]. Air and skin samples were analyzed by LC-MS as previously described by Fent and colleagues[41, 53]. Work diaries and questionnaires about workers' physical characteristics (*e.g.*, age, height, weight), type and frequency of PPE use (*e.g.*, coveralls, respirator, gloves), and work environment (*e.g.*, paint booth type, duration of paint tasks) were completed during each visit.

4.4.3.4 Work-up procedure for Hb adducts. The sample work-up procedure was adapted from a previously published method describing the analysis of Hb adducts of MDA and monoacetylated MDA[19]. The separated red blood cells (4 ml) were removed from –35ºC storage and sat at room temperature until thawed (~30 min). The Hb was precipitated from the lysate with 20 ml ethanol and centrifuged (4000 *g*, 10 min). The precipitate was washed with a series of organic solvents (20 ml each of 1:1 ethanol: water, ethanol, 4:1 diethyl ether: ethanol, and diethyl ether), with centrifuging between each wash. The Hb was dried in a dessicator at 4°C overnight.

Portions (200 mg) of Hb were dissolved in 4 ml sodium hydroxide (0.1 M) in the presence of IS mixture (final concentration 5 ng/g Hb). After 60 min at room temperature, the amines were extracted (2x) from basic solution into 6 ml dicholoromethane, vortexed for 2 min, and then centrifuged for 10 min at 1000 *g*. To achieve optimal separation, samples were frozen at –35ºC and then allowed to thaw at room temperature. The pooled extracts were dried over sodium sulfate and derivatized with 10 µl HFBA. After 30 min at room temperature, the samples were dried under nitrogen using a TurboVap® LV Evaporator (Zymark Center; Hopkinton, MA). The dried residues were dissolved in 200 µl ethyl acetate and placed in an ultrasonic bath for several minutes. The

sample solutions were transferred to GC vial inserts and evaporated to dryness using a SpeedVac® (Savant Instruments Inc., Holbrook, New York). The dried residues were dissolved in 60 µl ethyl acetate. One reagent blank and two calibration standards were prepared with every set of Hb samples collected from the workers. The samples were analyzed by GC-MS as described above.

4.4.3.5 Standard curve preparation for Hb adduct analysis. A 50 mg/ml solution of Hb was prepared in 0.1 M sodium hydroxide (100 ml total volume). Portions (4 ml, in duplicate) of Hb solution were spiked with HDA and acetylated HDA mixture to make concentration range (N = 7, in duplicate): HDA (0 – 0.045 ng/mg Hb), monoacetyl-HDA (0–1.05 ng/mg Hb), and diacetyl-HDA (0 – 1.40 ng/mg Hb). Each sample was spiked with internal standard mixture (final concentration 5 pg/mg Hb). Each calibration sample was treated according to procedure for Hb-adduct analysis. Samples were analyzed by GC-MS according to the above procedure. The limit of detection (LOD) for HDA, monoacetyl-HDA, and diacetyl-HDA was 1.2 ng/g Hb, 0.03 ng/g Hb, and 0.05 ng/g Hb.

4.4.4 Variable construction.

The breathing-zone concentration (BZC) of HDI monomer for each paint task (µg/m^3) was multiplied by the total paint time (min) for each task and summed together to obtain a daily air exposure level (µg/m^3 × min). To obtain a daily inhalation exposure level (µg/m^3 × min) the BZC was divided by the OSHA Assigned Protection Factor (APF) based on respirator type[69]: none, APF = 1; air purifying (half face), APF = 10; air purifying (full facepiece), APF = 50; supplied air (full facepiece or hood), APF = 1000; PAPR (full facepiece or hood), APF = 1000. The dermal concentration from tape-strips (ng/mm^3) were summed together for each task, and the task-based dermal values were summed together to obtain a daily dermal exposure level (ng/mm^3). HDA concentration in the pre-shift (i.e., beginning of day) urine sample, post-shift (i.e., end of day) urine sample, as well as daily total urine HDA concentration (ng/ml) was used in the data analysis. The concentration of Hb adducts (ng/g Hb), and plasma HDA (ng/ml) were used in the data analysis.

4.4.5 Statistical analysis.

The percent coefficient of variation (CV) was calculated for Hb adduct analysis among replicate samples. The correlation between Hb adducts and plasma or urine HDA and between Hb adducts and HDI air exposure was investigated using linear regression analysis (PROC REG procedure in SAS, version 9.1; SAS Institute, Cary, NC), with a significance level of 0.10. For regression and summary statistics all variables were natural log-transformed to meet the assumption of normality (Shapiro-Wilk $W > 0.80$). For all biomarker or exposure concentrations below the LOD, values of LOD/√2 were imputed.

Because plasma albumin has a circulating serum half-life of 19 days and hemoglobin has a lifespan of 120 days we selected a time frame of 0 to 4 months to investigate the associations between the blood biomarker concentration in plasma or Hb and cumulative HDI air or dermal exposure. This 4-month time frame would correspond to ~98% loss of HDA-albumin adducts and ~100% loss of HDA-Hb adducts resulting from HDI exposure. This time frame was also used to investigate the association between cumulative blood biomarkers and HDA concentration in urine. These cumulative exposure estimates were obtained by summing the daily HDI air or dermal concentrations across all repeated sampling visits for each worker. The cumulative blood biomarker estimates were obtained by summing the plasma HDA or HDA-Hb adduct concentrations across all repeated sampling visits for each worker. We investigated the associations between HDI exposure or urine HDA and blood HDA biomarkers among workers sampled on two or three repeated visits.

4.5 Results

4.5.1 Biomarker and exposure concentrations in occupationally exposed workers.

The range of HDA-Hb adduct concentrations among NC workers (n = 4) was 1.3 – 6.6 ng/g Hb and among WA workers (n = 11) was ≤1.2 – 37 ng/g Hb. The majority of samples were above the LOD (95%) for HDA-Hb concentration. Monoacetyl-HDA-Hb was detected in one worker (0.06 ng/g Hb), and diacetyl-HDA was not detected in any of the workers. The CV among replicate Hb samples was

in the range of 1 – 20%, which is similar to those reported for plasma HDA, while spiked standards were in the range of 1 – 10%. The lower quantitative precision among Hb samples compared with spiked standards may be attributed to the release of amines from biological matrices affecting the variation in recovered amines from Hb conjugates.

The range of HDA in base-treated Hb (N = 35) was ≤1.2 – 37, and in acid-hydrolyzed plasma (N = 35) was ≤0.02 – 0.71 ng/ml (Table 4.1). The majority of plasma samples (~90%) were ≥LOD for HDA. The average and standard deviation for HDA-Hb adduct and plasma HDA concentrations in each worker are provided in Figure 4.2, which depicts the large within-worker variability in biomarker levels and the weak association between these biomarker types. The within-worker variability (>90%) comprised a greater portion of the total variability in both HDA-Hb adduct and plasma HDA concentration. The range of HDA concentrations in acid-hydrolyzed urine was ≤0.03 – 66 µg/l (N = 116; Table 4.1). All breathing-zone concentrations were greater than the LOD while 40% of dermal exposure concentrations by paint task were ≥LOD. The HDI monomer dermal exposure range was 2.0 – 38,100 ng/mm^3, inhalation exposure range was 0.034 – 864 µg/m^3 × min, and air exposure range was 0.25 – 2,570 µg/m^3 × min (N = 35; Table 4.1).

Table 4.1 Summary of the measured HDA-Hb adduct (ng/g Hb), plasma HDA (ng/ml), and urine HDA (ng/ml) concentrations as well as HDI dermal (ng/mm^3), inhalation ($\mu g/m^3 \times min$), and air ($\mu g/m^3 \times min$) exposure among 15 automotive spray painters.

Sample	n	N	GM	GSD	Range
HDA-Hb	15	35	3.0	2.0	1.3 – 37
Plasma HDA	15	35	0.061	2.7	0.012 – 0.71
Urine HDA					
pre-shift	15	35	0.18	5.0	0.021 – 7.8
post-shift	15	35	0.25	5.6	0.021 – 66
all samples	15	116	0.22	4.5	0.021 – 66
HDI dermal exposure[a]	15	35	131	7.1	2.0 – 38,100
HDI inhalation exposure[b]	15	35	7.8	11.0	0.034 – 864
HDI air exposure[c]	15	35	72	6.1	0.25 – 2,570

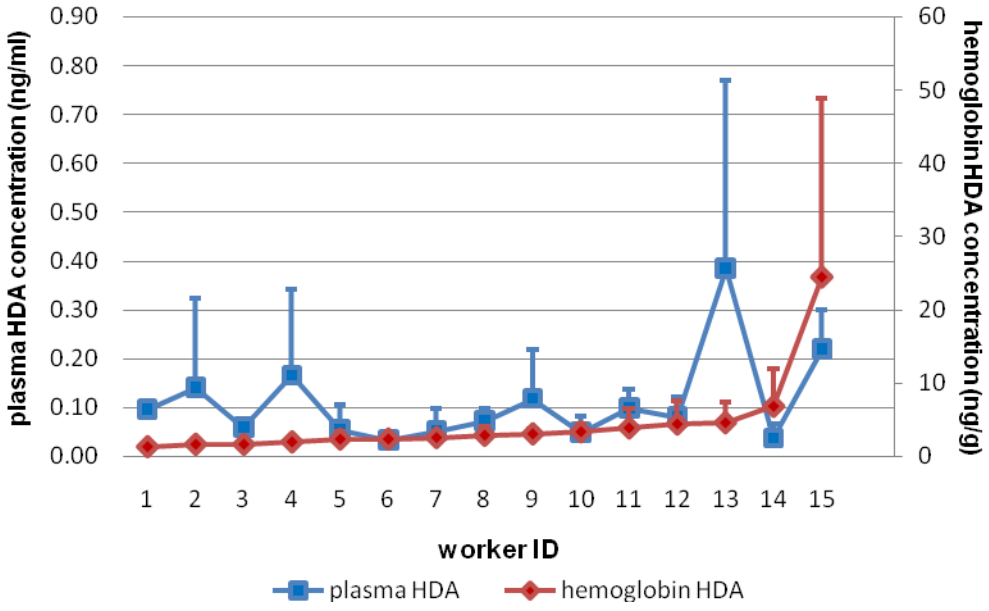
GM, geometric mean; GSD, geometric standard deviation; n, number of workers; N, number of samples

a. $\sum_{j=1}^{n}$ (HDI dermal exposure)$_j$; Sum of HDI dermal concentration across all daily tasks (j) was obtained by calculating the sum of the dermal concentration measured for each task and then summing the task-based dermal values.

b. $\sum_{j=1}^{n}$ (HDI inhalation exposure)$_j$; Sum of HDI inhalation concentration across all daily tasks (j) was obtained by dividing the measured breathing-zone concentration by the OSHA Assigned Protection Factor (APF) based on respirator type for each task and then summing the task-based inhalation values.

c. $\sum_{j=1}^{n}$ (HDI air exposure)$_j$; Sum of HDI air concentration across all daily tasks (j) was obtained by multiplying the measured breathing-zone concentration for each paint task ($\mu g/m^3$) by the paint time (min) for each task and then summing the task-based air values.

Figure 4.2 Average HDA-Hb adduct (ng/g) and plasma HDA levels (ng/ml) in each worker (n = 15).



4.5.1.1 Blood biomarker concentration vs. HDI exposure. Table 4.2 summarizes the regression analysis of Hb-adduct or plasma HDA concentration versus same day or cumulative HDI dermal, inhalation, or air exposure. The positive association between HDA-Hb adduct concentration and HDI exposure was strongest with cumulative dermal (N = 12, r^2 = 0.32, p = 0.058), cumulative inhalation (N = 12, r^2 = 0.35, p = 0.042), or cumulative air exposure (N = 12, r^2 = 0.34, p = 0.048). The association between plasma HDA concentration and HDI exposure was strongest with cumulative inhalation (N = 12, r^2 = 0.32, p = 0.023) or cumulative air exposure (N = 12, r^2 = 0.37, p = 0.036), similar to previous findings reported for the entire study population (n = 46) which reported stronger associations with past HDI exposure levels[9]. However, plasma HDA was negatively associated with the same day or cumulative HDI exposures among these workers. These different time-dependent associations (i.e., same day or cumulative) between HDI air or dermal exposure and plasma HDA or HDA-Hb concentration may be attributed to the half-life of albumin ($t_{1/2}$ = 19 days) and Hb lifespan (120 days), respectively.

Table 4.2 Log-transformed HDA-Hb adduct (ng/g Hb) or plasma HDA (ng/ml) concentration on last sampling visit versus the log-transformed same day or cumulative HDI dermal (ng/mm^3), inhalation ($\mu g/m^3 \times min$), or air ($\mu g/m^3 \times min$) exposure among workers sampled two (n = 4) or three times (n = 8).

Exposure Route	Time of HDI exposure[a]	Blood HDA	n	N	r^2	p-value[b]	Association (+/-)[c]
Dermal	same day	Hb	12	12	0.15	0.206	+
		Plasma	12	12	0.033	0.571	-
	cumulative	Hb	12	12	**0.32**	**0.058**	+
		Plasma	12	12	0.13	0.255	-
Inhalation	same day	Hb	12	12	0.22	0.126	+
		Plasma	12	12	**0.36**	**0.041**	-
	cumulative	Hb	12	12	**0.35**	**0.042**	+
		Plasma	12	12	**0.42**	**0.023**	-
Air	same day	Hb	12	12	0.18	0.173	+
		Plasma	12	12	0.20	0.150	-
	cumulative	Hb	12	12	**0.34**	**0.048**	+
		Plasma	12	12	**0.37**	**0.036**	-

n, number of workers; N, number of values
[a.] Same day or cumulative HDI exposure.
[b.] *p*-value determined using linear regression analysis; significant associations ($p \leq 0.10$) are bolded.
[c.] Positive (+) or negative (-) association between HDI exposure and blood HDA biomarkers.

4.5.1.2 Urine HDA concentration vs. blood biomarker concentration. The association between HDA-Hb adduct and plasma HDA concentrations in the same blood sample was weak (N=35, r^2 = 0.039, p = 0.38). Table 4.3 summarizes the correlations between HDA concentration in pre-shift, post-shift, or daily average urine sample versus HDA-Hb adduct or plasma HDA concentration in blood samples collected on the same day or cumulative blood HDA concentration. Urine HDA concentration was more strongly correlated with cumulative HDA-Hb adduct (r^2 = 0.28-0.32, p = 0.055-0.080) or plasma HDA concentration (r^2 = 0.21, p = 0.13) compared to the same day blood concentrations (Table 3). There was a positive association between HDA concentration in urine sample (pre-shift, post-shift, or total HDA) and plasma HDA measured on the same day, while there was a negative association between urine HDA concentration and cumulative plasma HDA concentration. Of the different urine samples collected during the workday (i.e., pre-shift, post-shift,

total HDA), the total daily urine HDA concentration was most strongly correlated with cumulative plasma HDA (N = 12, $r^2 = 0.21$, $p = 0.13$). There was no association between urine HDA and the same day HDA-Hb adduct concentration, while there was a strong, negative association between urine HDA (pre-shift, post-shift, and total HDA) and cumulative HDA-Hb adduct concentration.

Table 4.3 Log-transformed HDA concentration (ng/ml) in urine (pre-shift, post-shift, or daily total HDA) from last sampling visit versus the log-transformed same day or cumulative HDA-Hb adduct (ng/g Hb) or plasma HDA (ng/ml) concentration among workers sampled two (n = 4) or three times (n = 8).

Blood samples[a]	**HDA in urine sample(s)**[b]	**Blood HDA**[c]	**n**	**N**	***r²***	**p-value**[d]	**Association (+/-)**[e]
same day	pre-shift	Hb	12	12	0.003	0.859	NA
		Plasma	12	12	0.16	0.191	+
	post-shift	Hb	12	12	0.001	0.938	NA
		Plasma	12	12	0.13	0.249	+
	total	Hb	12	12	0.005	0.831	NA
		Plasma	12	12	0.075	0.389	+
cumulative	pre-shift	Hb	12	12	**0.32**	**0.056**	-
		Plasma	12	12	0.19	0.152	-
	post-shift	Hb	12	12	**0.32**	**0.055**	-
		Plasma	12	12	0.11	0.285	-
	total	Hb	12	12	**0.28**	**0.080**	-
		Plasma	12	12	0.21	0.133	-

n, number of workers; N, number of values; NA, no association

[a.] Same day or cumulative (i.e., 0 – 4 months) HDI exposure.

[b.] Dependent variable

[c.] Independent variable.

[d.] *p*–value determined using linear regression analysis; significant associations ($p \leq 0.10$) are bolded.

[e.] Positive (+) or negative (-) association between urine HDA and blood HDA biomarkers.

4.6 Discussion

We developed a method to measure HDA-Hb, monoacetyl-HDA-Hb, and diacetyl-HDA-Hb adducts as biomarkers of HDI exposure. Because diacetyl-HDA-Hb concentrations were not detectable and monoacetyl-HDA-Hb was only detected in one worker, further statistical investigation of these compounds was not feasible. Using linear regression, we were able to demonstrate the utility of HDA-Hb adduct as a biomarker of cumulative HDI dermal or inhalation exposure. The detection of HDA-Hb adducts in the majority of blood samples indicate that HDA-Hb adducts are sufficiently sensitive and specific as biomarker of cumulative exposure to HDI.

We observed ~10-fold higher total circulating amounts of HDA-Hb adducts (GM = 2.5 µg, GSD = 2.1) relative to plasma HDA (GM = 0.21 µg, GSD = 2.9), after adjusting for plasma volume or blood volume[70] and average male Hb concentration (16 g/dl), which is contrary to observations for other diamine adducts (e.g., plasma MDA > MDA-Hb)[19]. This may be related to differences in the stability of metabolic intermediates, which could influence the yields of various protein adducts (albumin *vs.* Hb). The bis-thiocarbamate intermediate of HDI, which can be easily transported into RBCs, is more stable compared to TDI or MDI[18]. Therefore, the stability of this metabolic intermediate would allow greater opportunity for cellular uptake and subsequent Hb-adduct formation following further transformation. Another interesting point to consider is the involvement of different enzyme systems in the metabolism of primary aliphatic amines (e.g., HDA, monoacetyl-HDA) versus primary aromatic amines (e.g., MDA), which may be oxidized by cytochrome P450[80]. The possible involvement of both an FAD-dependent enzyme system, which is involved in oxidation of basic aliphatic amines (e.g., HDA), and cytochrome P450-dependent system[20] may explain these differences in the types and yields of various plasma and Hb adducts.

The time-dependent associations between plasma HDA or HDA-Hb and urine HDA concentration indicate long-term elimination kinetics among blood biomarkers. The association between cumulative plasma HDA and urine HDA concentration was stronger compared to the same day plasma HDA concentration, indicating the breakdown and excretion of longer-term plasma protein

adducts. The association between cumulative HDA-Hb adducts and urine HDA concentration was stronger than the same day HDA-Hb adduct concentration, indicating the breakdown of Hb upon death of the red blood cell (120 day lifespan) and subsequent excretion of HDA-Hb adducts. The long-term elimination kinetics of these metabolites (HDA-Hb adducts and plasma HDA) may have important implications in HDI exposure monitoring and biological availability of these adducts for disease development.

The time-dependent associations between plasma HDA or HDA-Hb and HDI dermal, inhalation, or air exposure indicated the utilization of plasma HDA and HDA-Hb adducts as biomarkers of cumulative HDI exposure. The inverse association between plasma HDA and HDI exposure may be partially attributed to the non-specificity of plasma HDA measures and indicate competing metabolic pathways. The benefit of HDA-Hb or monoacetyl-HDA-Hb adduct analyses is obtaining measures of specific biomarkers that may be related to different exposure routes and metabolic pathways contributing to disease development. Protein adducts may be formed via enzymatic-directed pathways, such as conjugation of metabolites to cysteine residues of glutathione by GST or oxidation of HDA or monoacetyl-HDA by FAD or cytochrome P450 systems. Polymorphisms in GST have been suggested to play an important role in the inception of ill effects related to diisocyanate exposure[81]. Therefore, individual differences in how diisocyanates are metabolized and the types of products formed may be important factors related to individual susceptibility for disease.

The inverse association between cumulative HDA-Hb adducts or plasma HDA and urine HDA concentration may be attributed to competing metabolic pathways leading to the formation of long-lived protein adducts. HDA in acid-hydrolyzed urine and plasma (i.e., non-specific) may be partially derived from albumin adducts, and HDA in base-treated Hb (i.e., specific) is completely derived from Hb adducts. The variable specificity of plasma HDA and HDA-Hb measures may also explain the poor association between the same day HDA-Hb adduct and plasma HDA concentrations. Therefore, exposure or metabolism that favors albumin or Hb adduct formation (e.g., slow acetylators) would result in less acetylated-HDA metabolites (*N*-acetylation pathway) and, consequently, less urine

HDA. Brorson and colleagues observed lower HDA amounts in acid-treated urine among slow acetylators receiving oral exposure to HDA compared with rapid acetylators[21], supporting this hypothesis of competing metabolic pathways. The lack of detectable diacetyl-HDA-Hb and monoacetyl-HDA-Hb adducts also supports the presence of alternative metabolic pathways (e.g., oxidation of HDA) resulting in Hb adduct formation. These findings for plasma HDA and HDA-Hb adducts may indicate long residence-time characteristics, which may be utilized in HDI exposure assessment.

There were several limitations in our estimates of HDI exposure through the inhalation route. All 15 workers wore a respirator during spray painting (83% wore half-face air purifying, 8% wore full-face air purifying, and 8% wore supplied air) which would reduce exposure through the inhalation route. However, inhalation exposure may occur due to improper fit and maintenance of respirators, particularly among workers wearing air-purifying respirators, and would contribute to the within- and between-worker variability in biomarker levels. Because breathing-zone measures were taken outside of the respirator, we were unable to adjust for these various factors in our HDI inhalation exposure estimations. Therefore, adjusting by the APF in estimating inhalation exposure may underestimate the workers' true exposure if these issues were a concern. However, the associations between blood biomarker concentrations and inhalation exposure estimates were stronger than associations with air exposure estimates indicating that adjusting for respirator use better reflects exposure through the inhalation route.

There were also several limitations in our estimates of HDI exposure through the dermal route. The high percentage of non-detectable dermal exposure measures (60% of paint tasks) for HDI monomer using tape-stripping of the skin limited our ability in making strong conclusions about the dermal exposure route. However, several workers did not wear gloves (13%) or coveralls (27%) and, thus, dermal exposure was a potential route for uptake of HDI. Analytical methods to measure skin protein adducts from HDI exposure and secondary exposure to HDA, the hydrolysis product of HDI, may result in better estimates of dermal exposure. Additionally, there was a strong, positive association

between HDI dermal and inhalation exposure (N = 35, $r^2 = 0.27$, $p = 0.0015$). Therefore, the issue of high collinearity between these exposure routes prevents drawing strong conclusions about the relative significance of either route on internal dose. However, we demonstrated a strong, positive association between cumulative HDI dermal exposure and HDA-Hb adduct concentration, indicating the potential importance of dermal exposure among spray painters. Whether exposure route (i.e., dermal *vs.* inhalation) or exposure type (HDI *vs.* HDA) has an impact on the type and yield of protein adducts formed requires further investigation.

Another source of the within-worker variability in plasma HDA or HDA-Hb concentrations may be the aging of PPE (e.g., coveralls, respirator filters) over long periods of use, which would lead to eventual breakthrough of the material. Additionally, differences in exposure route (e.g., dermal *vs.* inhalation) or exposure type (e.g., HDI *vs.* HDA) would also contribute to both the within- and between-worker variability. Other sources of the between-worker variability in biomarker concentrations may be related to genetic polymorphisms affecting enzymes regulating HDI metabolism (e.g., GST, NAT). Future studies should investigate whether differences in exposure route, PPE use, and genetic factors play a role in predicting these biomarker levels. Such information could be used to understand the relative importance of dermal and inhalation exposure, workplace covariates, and genetic factors on susceptibility for developing adverse health effects.

4.7 Conclusions

We presented a method for quantifying HDA, monoacetyl-HDA, and diacetyl-HDA in Hb from HDI exposed individuals and demonstrated the utility of HDA-Hb adducts as biomarkers of exposure to HDI monomer. The time-dependent associations between HDI dermal, inhalation, or air exposure and HDA-Hb adduct or plasma HDA concentrations demonstrate their utilization as biomarkers of cumulative HDI exposure. The time-dependent associations between urine HDA and HDA-Hb or plasma HDA concentrations demonstrate long-term elimination kinetics among these biomarker types. Monoacetyl-HDA and diacetyl-HDA hemoglobin adducts may have limited use as markers of

HDI exposure due to the low levels observed in this study population. We demonstrated that HDA-Hb adducts are sensitive and specific biomarkers of HDI monomer exposure. Further characterization of urine and blood biomarkers resulting from HDI exposure may reveal important metabolic pathways contributing to individual susceptibility for disease.

CHAPTER 5

DISCUSSION AND CONCLUSIONS

In this dissertation, I have presented new methodologies for the identification and quantification of novel biomarkers related to HDI exposure and applied these methods to biological samples collected in the occupational setting (Chapters 2 – 4). In Chapter 2, I presented a method that was developed and applied to quantify plasma HDA in a cohort of 46 workers occupationally exposed to HDI monomer and discussed how these measures were associated with HDI dermal and inhalation exposure and modified by personal and workplace covariates. In Chapter 3, I presented a method that was developed and applied to quantify novel urine biomarkers (i.e., monoacetyl-HDA and diacetyl-HDA) in three workers exposed to HDI monomer, as well as integrated currently proposed HDI metabolic pathways based on these measures and previously published metabolic pathways. In Chapter 4, I presented a method that was developed and applied to quantify hemoglobin adducts in a cohort of 15 workers occupationally exposed to HDI monomer and discussed how these measures were associated with other biomarkers (i.e., urine and plasma HDA), as well as cumulative dermal and inhalation exposure to HDI monomer. In these chapters I provided important information on the significant determinants of blood biomarker concentrations related to HDI exposure and demonstrated the importance of integrating HDI exposure measures, workplace covariates, and urine HDA concentration with blood biomarker levels to further understand the exposure – dose relationship.

5.1 Biomarker analysis

Variable reaction conditions using acid or base hydrolysis were implemented prior to GC-MS analysis in the identification and quantification of HDI biomarkers in urine, plasma, or hemoglobin. Acid hydrolysis, while improving the analytical sensitivity of HDA analysis in urine and plasma, also prevents identifying the specific adducts comprising HDA (e.g., HDA *vs.* monoacetyl-HDA). Base hydrolysis or no hydrolysis of urine allowed the identification and quantification of both monoacetyl-HDA and diacetyl-HDA, respectively. Base hydrolysis of hemoglobin also allowed the detection and quantification of HDA-Hb and monoacetyl-HDA-Hb adducts. Using these different treatment conditions, various acetylated-HDA and HDA-proteins adducts were quantified and associated with HDI exposure levels. While base hydrolysis or no hydrolysis resulted in improved analytical detection of specific biomarkers compared to acid hydrolysis, the overall sensitivity of the analysis is diminished; HDA detection in acid hydrolyzed urine may not necessarily yield detectable monoacetyl-HDA or diacetyl-HDA. However, these specific biomarkers may be used to investigate inter-individual differences in HDI metabolism, which may be associated with enhanced risk for diisocyanate-induced asthma[16]. These various biomarker concentrations may be modified by exposure route[56] due to differences in absorption, distribution, metabolism and elimination (ADME) via skin *vs.* lungs, timing of urine or blood sample collection relative to exposure, and personal or workplace modifiers (e.g., PPE use). Thus, factors related to ADME of HDI and biological availability of various metabolic intermediates may be relevant in asthma development.

5.2 Metabolism of HDI

The identification of monoacetyl-HDA and diacetyl-HDA in base-hydrolyzed urine demonstrated, for the first time, the occurrence of *N*-acetylation pathway catalyzed by NAT enzymes among workers occupationally exposure to HDI monomer (Chapter 2). The quantification of HDA-Hb and monoacetyl-HDA-Hb adducts also indicated the presence of *N*-oxidation pathway, which may be catalyzed by CYP450 isoforms, an FAD-dependent enzyme system[20], and/or GST enzymes. The

presence of HDA in the plasma of workers exposed to HDI monomer also indicates the possible formation of HDA albumin adducts, which may be formed via similar pathways as HDA-Hb adducts. The inter-individual variability in protein adduct formation may be associated with enhanced risk of developing occupational asthma. Polymorphisms in GST and NAT genes have been suggested to play an important role in the inception of ill effects related to diisocyanate exposure by altering GST and NAT enzyme activities involved in HDI metabolism. The lower urine HDA amounts among slow acetylators receiving oral exposure to HDA compared with fast acetylators also indicates variability in the type and yield of metabolites based on individual phenotype. Therefore, personal and workplace factors modifying HDI exposure as well as inter-individual differences in gene polymorphisms affecting enzyme activity involved in HDI metabolism may be factors contributing to the enhanced risk of developing occupational asthma.

5.3 Blood HDA *vs.* HDI exposure

Time-dependent associations between plasma HDA, HDA-Hb, or urine HDA concentrations with HDI dermal or air exposure (i.e., unadjusted for respirator type) are summarized in Table 5.1, which highlights (in red) the strongest associations between the correlated variables. These time-dependent associations between plasma HDA and dermal or air exposure to HDI monomer was observed in our cohort of 46 workers (Chapter 2). The association between plasma HDA and dermal or air exposure to HDI occurring in the previous 0.5 – 2 months (i.e., 1 – 3 albumin half-lives) was stronger than with the same day HDI exposure. Because albumin has a half-life of 19 days, these findings indicate the presence of HDA-albumin adducts which may persist, at least, over the albumin half-life. There was also a strong association between HDA-Hb and cumulative dermal, inhalation, or air exposure to HDI, occurring within 4 months prior to blood collection, in our cohort of 15 workers (Chapter 4). This time-frame corresponds to the lifespan of Hb (120 days) at which time the RBC, and Hb adducts contained within, are broken down. The time-dependent associations between plasma HDA or HDA-Hb concentration and HDI exposure, which are summarized in Table 5.1, demonstrate their potential

use as indicators of past HDI exposure. In addition, the advantage of HDA-Hb or monoacetyl-HDA-Hb adduct analyses is obtaining measures of specific biomarkers that may be related to different exposure routes (dermal *vs.* inhalation), biological availability, and inter-individual differences in HDI metabolism associated with enhanced risk for asthma development.

Table 5.1 Time-dependent associations between log-transformed same day or previous HDI monomer exposure (air or dermal) with HDA biomarker concentration in plasma (ng/ml), hemoglobin (ng/g Hb), and urine (ng/ml) and correlations between same day or previous blood (plasma and hemoglobin) and urine HDA biomarker concentrations.

Association	(Bio)marker[a]	n	N	Same day[c]			0.5 – 2 months[d]			0.5 – 4 months[e]		
				r^2	p-value	Sample range	r^2	*p*-value	Sample range	r^2	p-value	Sample range
Air: Dermal[f]	Dermal HDI	48	278	**0.40**	**<0.001**	≤0.01 – 179 µg/mm^3	NA	NA	NA	NA	NA	NA
Plasma: Hb	HDA	46	108	0.039	0.38	≤1.2 – 37.2 ng/g	NA	NA	NA	NA	NA	NA
Air:												
Plasma	Total HDA	46	108	0.058	0.013	≤0.02 – 0.90 µg/l	**0.33**	**0.0014**	≤0.02 – 0.14 µg/l	0.14	0.061	≤0.02–0.71 µg/l
Hb	HDA[b]	15	35	0.085	0.10	≤1.2 – 37.2 ng/g	0.15	0.12	≤1.2 – 37.2 ng/g	**0.34**	**0.008**	≤1.2–7.6 ng/g
Urine	Total HDA[b]	15	35	0.018	0.45	≤0.03 – 66 µg/l	0.11	0.44	≤0.03 – 0.80 µg/l	0.007	0.97	≤0.03–4.0 µg/l
Dermal:												
Plasma	Total HDA	46	108	0.063	0.012	≤0.02 – 0.90 µg/l	**0.13**	**0.053**	≤0.02 – 0.14 µg/l	0.086	0.15	≤0.02–0.71 µg/l
Hb	HDA[b]	15	35	0.10	0.071	≤1.2 – 37.2 ng/g	0.20	0.38	≤1.2 – 37.2 ng/g	**0.13**	**0.10**	≤1.2–7.6 ng/g
Urine	Total HDA[b]	15	35	0.024	0.51	≤0.03 – 66 µg/l	0.19	0.33	≤0.03 – 0.80 µg/l	0.021	0.58	≤0.03–4.0 µg/l
Plasma:												
Hb	HDA[b]	15	35	0.039	0.38	≤0.02 – 0.90 µg/l	0.27	0.26	≤1.2 – 37.2 ng/g	0.096	0.33	≤1.2–7.6 ng/g
Urine	Total HDA[b]	15	35	0.30	0.001	≤0.03 – 25.9 µg/l	**0.50**	**0.007**	≤0.03 – 0.80 µg/l	0.26	0.017	≤0.03–4.0 µg/l
Hb: Urine	Total HDA[b]	15	35	0.015	0.49	≤0.03 – 25.9 µg/l	0.016	0.69	≤0.03 – 0.80 µg/l	**0.21**	**0.065**	≤0.03–4.0 µg/l

Air = Σ_{task}(breathing-zone concentration × paint time) = µg/m^3 × min; Dermal = Σ_{task} (skin concentration) = µg/mm^3; Hb = hemoglobin; NA = not applicable; LOD = limit of detection; n = number of workers; N = number of samples

[a.] Exposure or biomarker variable analyzed; total HDA derived from acid-hydrolyzed urine.
[b.] Random subset of workers from 46 total workers used in regression analysis; post-exposure urine samples used in regression analysis.
[c.] HDI exposure (BZ, dermal), urine HDA, plasma HDA, or HDA-Hb measured on the same day. Strongest association with dermal HDI (bolded).
[d.] HDI exposure (BZ, dermal), plasma HDA, or HDA-Hb measured in the previous 0.5 – 2 months. Strongest association with Hb (red) or plasma (blue).
[e.] HDI exposure (BZ, dermal), plasma HDA, or HDA-Hb measured in the previous 0.5 – 4 months. Strongest association with Hb (red) or plasma (blue).
[f.] Reported in previous publication (Fent et al., 2009)[41].

5.4 Blood HDA *vs.* urine HDA

Time-dependent associations between plasma HDA or HDA-Hb and urine HDA concentration were observed in our cohort of 15 workers and are summarized in Table 5.1. The strongest association between plasma HDA and total urine HDA occurred using urine collected 0.5 – 2 months following blood collection, indicating the breakdown and excretion of long-term albumin adducts (Table 5.1). The strongest association between HDA-Hb and total urine HDA occurred using urine collected 0.5 – 4 months following blood collection, demonstrating the breakdown and excretion and longer-lived HDA-Hb adducts. The narrower excretion time-frame associating plasma and urine HDA indicates a more rapid turnover of albumin and elimination of HDA-albumin adducts compared with HDA-Hb adducts. A strong association between cumulative HDA-Hb and pre-shift, post-shift, or total urine HDA concentrations was observed (Chapter 4), indicating long-term elimination of these protein adducts. These time-dependent associations between plasma HDA or HDA-Hb and urine HDA may have important implications in HDI exposure monitoring and in the biological availability of these adducts in development of adverse health effects. The strong, inverse association in HDA-Hb and urine HDA concentration may also indicate the presence of competing metabolic pathways (e.g., *N*-acetylation *vs. N*-oxidation) involved in forming these various biomarkers. Inverse association between an administered oral HDA dose and urine HDA among slow acetylators (i.e., greater protein adduct formation) have been previously observed and attributed to the relative contribution of these competing metabolic pathways[21].

5.5 Workplace covariates

Workplace and personal covariates (e.g., coverall use, booth type) may affect biomarker levels by modifying the received HDI exposure dose through inhalation and dermal routes. From analysis of variance and mixed models of measured biomarker levels, the most significant covariates affecting total plasma HDA dose (after adjusting for plasma volume) were coverall use and booth type (Chapters 2). The various significant or non-significant trends in plasma HDA, HDA-Hb

concentrations, HDI air or HDI dermal exposure stratified by these workplace covariates among the cohort of 15 workers are summarized in Table 5.2. Each covariate (i.e., coverall use or downdraft booth) was evaluated by holding one variable constant (i.e., group) and observing the trend in exposure or biomarker levels from non-use (0) to use (1) of the other variable. Among workers grouped by coverall use (Table 5.2), HDI exposure (dermal and/or air) and HDA-Hb concentration varied significantly between downdraft booth users compared to non-users indicating that use of the downdraft booth type decreases both air and dermal exposures, which in turn decreases HDA-Hb concentration. Interestingly, coverall use was inversely associated with HDA-Hb concentration; coverall users had moderately higher HDA-Hb concentration compared to non-coveralls users. This may be attributed to the significantly higher HDI air exposure levels among coverall users ($p < 0.05$) due to longer exposure times and higher HDI paint concentration relative to non-coverall users. Thus, higher air exposure may have contributed to enhanced HDI inhalation exposure among coverall users, resulting in higher HDA-Hb concentration. Whether these observations indicate exposure-route specific biomarkers, which may have important applications in HDI exposure assessment, need to be further investigated. Other sources of variability in these levels, which were not analyzed in these studies, may include genetic differences in HDI metabolism that can affect the types and yields of different blood biomarkers and the proportion of biomarker concentration attributed to specific exposure routes (dermal *vs.* inhalation).

Table 5.2 Summary of blood biomarker and HDI monomer exposure trends stratified by workplace covariates among 15 workers.

Covariate		N	Biomarker[a]				Exposure[a]			
			Plasma HDA (ng/l)		HDA-Hb (ng/g Hb)		HDI Air (µg/m^3 × min)		HDI Dermal (ng/mm^3)	
Group	Comparison		GM	trend	GM	trend	GM	trend	GM	trend
Downdraft booth[b]	Coveralls									
yes	yes	17	39	↓	2.7	↑	79	↑	54	↓
	no	6	153		2.0		16		89	
Coveralls	Downdraft booth									
yes	yes	17	39	↓	2.7	↓	79	↓	54	↓
	no	6	71		5.5		210		260	
no	yes	6	153	↑	2.0	↓	16	↓	89	↓
	no	6	73		3.3		85		980	

N = number of samples; GM = geometric mean

a. Colored arrows (red or blue) indicate significant difference ($p \leq 0.10$) in HDA biomarker concentration or HDI exposure stratified by covariate (no = 0; yes = 1) using Tukey-Kramer multiple comparisons testing; significant difference stratified by covariate and similar directional trend between HDA-Hb and HDI exposure (red) or plasma HDA and HDI exposure (blue); black arrows indicate non-significant difference ($p > 0.10$); direction of arrow indicates whether association is increased (up arrow) or decreased (down arrow) within each comparison from 0 to 1.

b. No significant differences ($p > 0.10$) in biomarker or exposure concentrations stratified by coverall use among workers not using a downdraft booth.

5.6 Limitations and suggestions for future research

There were several limitations in these studies involving both plasma HDA and inhalation and dermal HDI exposure estimates (Chapter 2 and 4). Because HDA analysis in plasma involved acid hydrolysis, information on the specific constituents of HDA was masked (e.g., HDA *vs*. monoacetyl-HDA, adduct *vs*. unbound). Additionally, estimates of inhalation exposure adjusted or unadjusted for respirator type (Chapter 2 and 4) used breathing-zone concentrations measured outside of the respirator and did not take into account improper fit and maintenance of respirators, which may increase the potential for inhalation exposure. Therefore, adjusted inhalation exposure by respirator type would have underestimated the true inhalation exposure while the unadjusted values most likely overestimated inhalation exposure. Because the majority (65%) of task-based HDI dermal exposure

measurements was less than the LOD[41], the associations between biomarker levels and HDI dermal exposure must be interpreted with caution. These limitations in inhalation and dermal exposure measurements also prevent making strong conclusions about the major routes of exposure or the proportion of internal dose attributed to dermal or inhalation exposure routes.

Further work investigating the dermal uptake kinetics of HDI monomer and the formation of skin-protein adducts with HDI (e.g., keratin adducts) may help improve dermal exposure assessment strategies for future studies and better understand the contribution of dermal exposure route on internal dose levels. Based on our preliminary analysis of monoacetyl-HDA and diacetyl-HDA in urine from 3 exposed workers (Chapter 3), specific biomarkers related to *N*-acetyl metabolism may be investigated further in this study population. These biomarkers may be associated with individual differences in enzyme-directed metabolism (e.g., NAT, GST) resulting from gene associated single nucleotide polymorphisms (SNPs). Candidate SNPs may be selected and integrated with the significant HDI exposure variables and workplace covariates identified in these studies to improve predictive models for biomarker concentrations. This would not only help mitigate exposures in the workplace by advising optimum protection controls but also identify susceptibility factors (e.g., slow *vs.* fast acetylation) that may enhance risk for development of occupational asthma.

REFERENCES

1. H. Urlich, *Chemistry and technology of isocyanates*, Wiley, West Sussex, UK, 1996.
2. M. Janko, K. McCarthy, M. Fajer and J. van Raalte, *Am Ind Hyg Assoc J*, 1992, **53**, 331-338.
3. J. Sparer, M. H. Stowe, D. Bello, Y. Liu, R. J. Gore, F. Youngs, M. R. Cullen, C. A. Redlich and S. R. Woskie, *J Occup Environ Hyg*, 2004, **1**, 570-581.
4. C. A. Herrick, L. Xu, A. V. Wisnewski, J. Das, C. A. Redlich and K. Bottomly, *J Allergy Clin Immunol*, 2002, **109**, 873-878.
5. D. W. Cockcroft and J. T. Mink, *Can Med Assoc J*, 1979, **121**, 602-604.
6. O. Vandenplas, J. L. Malo, M. Saetta, C. E. Mapp and L. M. Fabbri, *Br J Ind Med*, 1993, **50**, 213-228.
7. A. Pronk, L. Preller, M. Raulf-Heimsoth, I. C. Jonkers, J. W. Lammers, I. M. Wouters, G. Doekes, A. V. Wisnewski and D. Heederik, *Am J Respir Crit Care Med*, 2007, **176**, 1090-1097.
8. J. L. Malo, G. Ouimet, A. Cartier, D. Levitz and C. R. Zeiss, *J Allergy Clin Immunol*, 1983, **72**, 413-419.
9. J. Cocker, *Occup Med (Lond)*, 2007, **57**, 391-393.
10. Q. Liu and A. Wisnewski, *Ann Allergy Asthma Immunol.*, 2003, **90 (5 Suppl 2)**, 35-41.
11. J. Weber, *A Summary of Health Hazard Evaluations: Issues Related to Occupational Exposure to Isocyanates, 1989 to 2002*, 2004.
12. NIOSH, *Criteria for a recommended standard: occupational exposure to diisocyanates*, 1978.
13. B. T. Butcher, R. N. Jones, C. E. O'Neil, H. W. Glindmeyer, J. E. Diem, V. Dharmarajan, H. Weill and J. E. Salvaggio, *Am Rev Respir Dis*, 1977, **116**, 411-421.
14. J. A. Bernstein, *Toxicology*, 1996, **111**, 181-189.
15. H. Keskinen, O. Tupasela, U. Tiikkainen and H. Nordman, *Clin Allergy*, 1988, **18**, 597-604.
16. H. Wikman, P. Piirila, C. Rosenberg, R. Luukkonen, K. Kaaria, H. Nordman, H. Norppa, H. Vainio and A. Hirvonen, *Pharmacogenetics*, 2002, **12**, 227-233.
17. B. W. Day, R. Jin, D. M. Basalyga, J. A. Kramarik and M. H. Karol, *Chem Res Toxicol*, 1997, **10**, 424-431.
18. I. Chipinda, S. J. Stetson, G. J. Depree, R. H. Simoyi and P. D. Siegel, *Chem Res Toxicol*, 2006, **19**, 341-350.
19. O. Sepai, D. Henschler and G. Sabbioni, *Carcinogenesis*, 1995, **16**, 2583-2587.
20. J. W. Gorrod, *Chem Biol Interact*, 1973, **289**, 289-303.
21. T. Brorson, G. Skarping, J. F. Sandstrom and M. Stenberg, *Int Arch Occup Environ Health*, 1990, **62**, 79-84.
22. D. Bello, C. A. Herrick, T. J. Smith, S. R. Woskie, R. P. Streicher, M. R. Cullen, Y. Liu and C. A. Redlich, *Environ Health Perspect*, 2007, **115**, 328-335.
23. A. V. Wisnewski, R. Srivastava, C. Herick, L. Xu, R. Lemus, H. Cain, N. M. Magoski, M. H. Karol, K. Bottomly and C. A. Redlich, *Am J Respir Crit Care Med*, 2000, **162**, 2330-2336.
24. A. V. Wisnewski, R. Lemus, M. H. Karol and C. A. Redlich, *J Allergy Clin Immunol*, 1999, **104**, 341-347.
25. C. A. Redlich, M. H. Karol, C. Graham, R. J. Homer, C. T. Holm, J. A. Wirth and M. R. Cullen, *Scand J Work Environ Health*, 1997, **23**, 227-231.
26. A. V. Wisnewski, Q. Liu, J. Liu and C. A. Redlich, *Clin Exp Allergy*, 2008, **38**, 957-967.
27. N. J. Rattray, P. A. Botham, P. M. Hext, D. R. Woodcock, I. Fielding, R. J. Dearman and I. Kimber, *Toxicology*, 1994, **88**, 15-30.
28. P. Devillier, N. Baccard and C. Advenier, *Pharmacol Res*, 1999, **40**, 3-13.
29. F. Di Stefano, S. Siriruttanapruk, J. McCoach, M. Di Gioacchino and P. S. Burge, *Eur Ann Allergy Clin Immunol*, 2004, **36**, 56-62.

30. D. Bello, S. R. Woskie, R. P. Streicher, Y. Liu, M. H. Stowe, E. A. Eisen, M. J. Ellenbecker, J. Sparer, F. Youngs, M. R. Cullen and C. A. Redlich, *Am J Ind Med*, 2004, **46**, 480-491.
31. S. R. Woskie, J. Sparer, R. J. Gore, M. Stowe, D. Bello, Y. Liu, F. Youngs, C. Redlich, E. Eisen and M. Cullen, *Ann Occup Hyg*, 2004, **48**, 393-403.
32. G. N. Carlton and E. C. England, *Appl Occup Environ Hyg*, 2000, **15**, 705-712.
33. S. G. Whittaker, R. Anderson and C. Whittaker, *Health and safety in Washington state's collision repair industry: a needs assessment. Techinical report 69-4-2005* 69-4-2005, Safety & Health Assessment & Research for Prevention, Washington State Department of Labor and Industries, Olympia, 2005.
34. Y. Liu, D. Bello, J. A. Sparer, M. H. Stowe, R. J. Gore, S. R. Woskie, M. R. Cullen and C. A. Redlich, *Ann Occup Hyg*, 2007, **51**, 429-439.
35. H. G. Poovey and R. J. Rando, in *Isocyanates: sampling, analysis, and health effects, ASTM STP 1408*, ed. D. I. Lesage J, Danchik RS, American Society for Testing and Materials, West Conshohocken, PA, Editon edn., 2002, pp. 110-122.
36. OSHA, *Diisocyanates: Method 42.* , U.S. Department of Labor, OSHA, Salt Lake CIty, UT, 1983.
37. J. Pauluhn and U. Mohr, *Toxicology*, 1994, **92**, 53-74.
38. D. Bello, C. A. Redlich, M. H. Stowe, J. Sparer, S. R. Woskie, R. P. Streicher, H. D. Hosgood and Y. Liu, *Ann Occup Hyg*, 2008, **52**, 117-124.
39. A. Pronk, F. Yu, J. Vlaanderen, E. Tielemans, L. Preller, I. Bobeldijk, J. A. Deddens, U. Latza, X. Baur and D. Heederik, *Occup Environ Med*, 2006, **63**, 624-631.
40. K. W. Fent, K. Jayaraj, A. Gold, L. M. Ball and L. A. Nylander-French, *Scand J Work Environ Health*, 2006, **32**, 225-240.
41. K. W. Fent, L. G. Trelles Gaines, J. M. Thomasen, S. L. Flack, K. Ding, A. H. Herring, S. G. Whittaker and L. A. Nylander-French, *Ann Occup Hyg*, 2009, **53**, 691-702.
42. R. Jin, B. W. Day and M. H. Karol, *Chem Res Toxicol*, 1993, **6**, 906-912.
43. T. Brorson, G. Skarping and J. Nielsen, *Int Arch Occup Environ Health*, 1990, **62**, 385-389.
44. M. Dalene, G. Skarping and H. Tinnerberg, *J Chromatogr B Biomed Appl*, 1994, **656**, 319-328.
45. G. Skarping, M. D. Dalene and H. Tinnerberg, *Analyst*, 1994, **119**, 2051-2055.
46. H. Tinnerberg, G. Skarping, M. Dalene and L. Hagmar, *Int Arch Occup Environ Health*, 1995, **67**, 367-374.
47. A. Maitre, M. Berode, A. Perdrix, M. Stoklov, J. M. Mallion and H. Savolainen, *Int Arch Occup Environ Health*, 1996, **69**, 65-68.
48. C. Rosenberg, K. Nikkila, M. L. Henriks-Eckerman, K. Peltonen and K. Engstrorm, *J Environ Monit*, 2002, **4**, 711-716.
49. Y. Liu, M. Berode, M. H. Stowe, C. T. Holm, F. X. Walsh, M. D. Slade, M. F. Boeniger and C. A. Redlich, *Int J Occup Environ Health*, 2004, **10**, 262-271.
50. L. G. T. Gaines, K. W. Fent, S. L. Flack, J. M. Thomasen, L. M. Ball, D. B. Richardson, K. Ding, S. G. Whittaker and L. A. Nylander-French, *Ann Occup Hyg*, submitted.
51. S. L. Flack, K. W. Fent, L. G. Trelles Gaines, J. M. Thomasen, S. Whittaker, L. M. Ball and L. A. Nylander-French, *Ann Occup Hyg*, 2009, **54**, 41-54.
52. C. J. Sennbro, C. H. Lindh, H. Tinnerberg, H. Welinder, M. Littorin and B. A. Jonsson, *Scand J Work Environ Health*, 2004, **30**, 371-378.
53. K. W. Fent, L. G. Gaines, J. M. Thomasen, S. L. Flack, K. Ding, A. H. Herring, S. G. Whittaker and L. A. Nylander-French, *Ann Occup Hyg*, 2009, **53**, 677-689.
54. D. Zissu, S. Binet and J. C. Limasset, *Contact Dermatitis*, 1998, **39**, 248-251.
55. P. Lind, M. Dalene, V. Lindstrom, A. Grubb and G. Skarping, *Analyst*, 1997, **122**, 151-154.
56. J. Pauluhn and J. Lewalter, *Exp Toxicol Pathol*, 2002, **54**, 135-146.
57. M. Berode, B. Testa and H. Savolainen, *Toxicol Lett*, 1991, **56**, 173-178.
58. P. Lind, M. Dalene, H. Tinnerberg and G. Skarping, *Analyst*, 1997, **122**, 51-56.

59. K. Kaaria, A. Hirvonen, H. Norppa, P. Piirila, H. Vainio and C. Rosenberg, *Analyst*, 2001, **126**, 476-479.
60. C. J. Sennbro, C. H. Lindh, C. Mattsson, B. A. Jonsson and H. Tinnerberg, *Int Arch Occup Environ Health*, 2006, **79**, 647-653.
61. C. Rosenberg and H. Savolainen, *J Chromatogr*, 1986, **367**, 385-392.
62. A. Maitre, M. Berode, A. Perdrix, S. Romazini and H. Savolainen, *Int Arch Occup Environ Health*, 1993, **65**, 97-100.
63. K. S. Creely, G. W. Hughson, J. Cocker and K. Jones, *Ann Occup Hyg*, 2006, **50**, 609-621.
64. S. Austin, *Occup Med (Lond)*, 2007, **57**, 444-448.
65. O. Sepai, D. Schutze, U. Heinrich, H. G. Hoymann, D. Henschler and G. Sabbioni, *Chem Biol Interact*, 1995, **97**, 185-198.
66. USEPA, *Definition and procedure for determination of the method detection limit. Title 40, Appendix B to Part 136 Revision 1.11*, http://ecfr.gpoaccess.gov, Accessed 2008 Aug.
67. J. A. Glaser, D. L. Foerst, G. D. McKee, S. A. Quane and W. L. Budde, *Environmental Science and Technology*, 1981, **15**, 1426-1435.
68. W. C. Adams, *Measurement of breathing rate and volume in routinely performed daily activities* A033-205, Human Performance Laboratory, University of California, Davis, CA, 1993.
69. OSHA, Respiratory protection - 1910.134 Regulation (Standards - 29 CFR) US Department of Labor, http://www.osha.gov, Accessed 2008 Aug.
70. J. R. Svirbely and S. Iyengar, *The Medical Algorithms Project, Release 24.0. Chapter 2: Body dimensions and blood volumes. Blood volume from height and weight*, http://www.medal.org, Accessed 2008 Aug.
71. H. J. Keselman, A. J., R. K. Kowalchuck and R. D. Wolfinger, *Communications in statistics: Simulation and computation*, 1998, **27**, 591-604.
72. A. Marand, D. Karlsson, M. Dalene and G. Skarping, *Analyst*, 2004, **129**, 522-528.
73. K. W. Fent, K. Jayaraj, L. M. Ball and L. A. Nylander-French, *J Environ Monit*, 2008, **10**, 500-507.
74. E. Meilhoc, M. J. Moutin and H. B. Osborne, *Biochem J*, 1986, **238**, 701-707.
75. S. Naik, G. Bhattacharjya, B. Talukdar and B. K. Patel, *Eur J Org Chem*, 2004, 1254-1260.
76. J. Lesage, N. Goyer, F. Desjardins, J. Y. Vincent and G. Perrault, *Am Ind Hyg Assoc J*, 1992, **53**, 146-153.
77. T. J. Peters, in *The plasma proteins. Structure, function, and genetic control*, ed. F. W. Putnam, Academic Press, New York, 1975, vol. 1, pp. 133-181.
78. S. A. Landaw, in *Hematology: basic principles and practice*, ed. B. E. Hoffman R, Shattil SJ, Furie B, Cohen HJ, Churchill Livingstone, New York, Editon edn., 1991, pp. 274-290.
79. S. L. Flack, L. M. Ball and L. A. Nylander-French, *J Chromatogr B Analyt Technol Biomed Life Sci*, 2010, doi: 10.1016/j.jchromb.2010.01.012.
80. F. P. Guengerich and T. Shimada, *Chem Res Toxicol*, 1991, **4**, 391-407.
81. P. Piirila, H. Wikman, R. Luukkonen, K. Kaaria, C. Rosenberg, H. Nordman, H. Norppa, H. Vainio and A. Hirvonen, *Pharmacogenetics*, 2001, **11**, 437-445.