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Quantitative Plasma Biomarker Analysis in HDI Exposure Assessment

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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 1,6-hexamethylene diisocyanate (HDI)-containing paint in vehicle repair shops collected during repeated visits to their workplace and their relationship with dermal and inhalation exposure to 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^{-1}. 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 ∼20 to 60 days before blood collection ($N = 29$, $r = 0.57$, $P = 0.0014$). The correlation between plasma HDA levels and HDI dermal exposure measured on the same workday, although statistically significant, was low ($N = 108$, $r = 0.22$, $P = 0.040$) while the correlation between HDA and dermal exposure occurring ∼20 to 60 days before blood collection was slightly improved ($N = 29$, $r = 0.36$, $P = 0.053$). We evaluated various workplace factors and controls (i.e. location, personal protective equipment use and 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 and dermal exposure to HDI and for investigating the effectiveness of exposure controls in the workplace.

Keywords: biomarker; dermal exposure; 1,6-hexamethylene diamine (HDA); 1,6-hexamethylene diisocyanate (HDI); inhalation exposure; plasma

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 (Cockcroft and Mink, 1979; Vandenplas *et al.*, 1993; Zissu *et al.*, 1998; Pronk *et al.*, 2007). 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

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assessment. 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. 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 (Herrick *et al.*, 2002). Therefore, efficiency of personal protective equipment (PPE) (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 (Wisnewski *et al.*, 2000). Therefore, 1,6-hexamethylene diamine (HDA) blood biomarkers may provide a link between exposure and the relevant pathways contributing to the development of biological effects. 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 (Cocker, 2007). Albumin or hemoglobin (Hb) adducts in the blood resulting from diisocyanate exposure have been identified (Sepai *et al.*, 1995a; Lind *et al.*, 1997a; Pauluhn and Lewalter, 2002) and may result from direct interaction with blood proteins or through rapid hydrolysis to the corresponding diamine in the blood, catalyzed by bicarbonate (Berode *et al.*, 1991), 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 (Wikman *et al.*, 2002). The latter pathway is supported by Sepai *et al.* (1995a) who quantified *N*-acetylated Hb adducts resulting from methylene bisphenyl isocyanate (MDI) exposure indicating that the isocyanate group may be first hydrolyzed to the amine *in vivo*, followed by N-acetylation to monoacetylated MDA, N-hydroxylation to the *N*-hydroxyarylamine and subsequently to the nitroso compound, which can then form a sulfinamide adduct. Therefore, HDA measured from hydrolyzed plasma samples may represent a combination of unconjugated HDA and monoacetylated HDA (AcHDA) or diacetylated HDA (diAcHDA), as well as conjugated protein adducts of HDI and HDA or AcHDA 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 (Sepai *et al.*, 1995a). 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 (Sennbro *et al.*, 2004). 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 and isocyanurate), with the latter comprising the majority of HDI in the clear coat. While a test chamber exposure study by Liu *et al.* (2004) measured HDA in urine of persons exposed to biuret aerosol, the correlation between inhalation exposure to biuret and HDA was weak, indicating that HDA levels 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^{-1}) in two previous human exposure studies (Brorson *et al.*, 1990a; Tinnerberg *et al.*, 1995), a few volunteer and occupational exposure studies have demonstrated a positive association between HDI inhalation exposure and measured HDA in hydrolyzed urine (Tinnerberg *et al.*, 1995; Maitre *et al.*, 1996). However, due to the large interperson and intraperson 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 (Pronk *et al.*, 2006). 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 (Lind *et al.*, 1997b; Sennbro *et al.*, 2004), whereas the association between airborne MDI levels and MDA concentrations in hydrolyzed plasma or urine were much weaker (Kaaria *et al.*, 2001; Sennbro *et al.*, 2006), indicating the possible contribution of dermal uptake for MDI. Because quantitative plasma HDA levels among exposed workers are 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 (Pauluhn and Mohr, 1994; Rattray *et al.*, 1994). 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 (Rosenberg and Savolainen, 1986; Maitre *et al.*, 1993; Pauluhn and Lewalter, 2002; Creely *et al.*, 2006; Pronk *et al.*, 2006; Sennbro *et al.*, 2006; Austin, 2007). For example, urinary TDA among a group of workers in direct skin contact with uncured TDI-based foam occurred at higher levels compared to nonhandlers, even though both groups were exposed to similar airborne levels of TDI (Austin, 2007). 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 and plasma HDA levels.

Quantification of amines in biological samples requires highly sensitive and selective methods. Such methods for HDA (Tinnerberg *et al.*, 1995; Rosenberg *et al.*, 2002; Creely *et al.*, 2006), TDA (Lind *et al.*, 1997b) and MDA (Sepai *et al.*, 1995b) typically involve acid hydrolysis of samples and derivatization of the liberated amines with subsequent gas chromatography–mass spectrometry (GC–MS) analysis. Therefore, the total HDA concentration measured in hydrolyzed plasma represents the sum of covalently bound HDI monomer and HDA/AcHDA oxidation products, as well as non-covalently bound HDA and its metabolites (e.g. AcHDA and diAcHDA), 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^{-1} (Tinnerberg *et al.*, 1995) to 3 μg l^{-1} (Pronk *et al.*, 2006). Two test chamber human exposure studies were unable to detect HDA in hydrolyzed plasma of HDI-exposed subjects (Brorson *et al.*, 1990a; Tinnerberg *et al.*, 1995).

The objectives of this study were to (i) quantify HDA in hydrolyzed plasma of 46 auto-body shop workers applying HDI-containing clear coat through GC–MS analysis, (ii) investigate the relationship between plasma HDA levels and external exposure to HDI monomer (dermal and inhalation), and (iii) investigate PPE use (i.e. coverall and glove use and respirator type) and work environment (i.e. paint booth type and location) as modifiers of internal dose.

MATERIALS AND METHODS

Chemicals

High-performance liquid chromatography (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, USA). HDA (98%), 1,7-heptanediamine (HpDA), and derivatization grade heptafluorobutyric anhydride (HFBA) were obtained from Sigma-Aldrich (St Louis, MO, USA). Human plasma from whole blood [ethylenediaminetetraacetic acid

(EDTA) and potassium] was obtained from Biological Specialty Corporation (Colmar, PA, USA) and stored at −40°C in 5 ml aliquots.

Preparation of standard solutions

Stock solutions of HDA and HpDA (1 mg ml^{-1}) were prepared by dissolving 25 mg of the amine in 25 ml 1 M H_2SO_4 and diluted 1:1000 to a final concentration of 1 μg ml^{-1}. The solutions were stored at −40°C for up to 1 month. An internal standard solution (IS) was prepared by further dilution (1:100) of the HpDA solution in 1 M H_2SO_4 to 10 ng ml^{-1}. An HDA standard solution was prepared by further dilution (1:2) of the HDA solution in 1 M H_2SO_4 to 0.33 μg ml^{-1}. Just prior to the sample work-up procedures, HDA calibration standards ($N = 8$), in the range of 0–2 μg l^{-1} plasma, were prepared in duplicate by making 1:1 serial dilutions from the HDA standard solution in 1 M 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^{-1} plasma.

Instrumental 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, USA). Injections (1 μl) were made under splitless mode of 30 s with injector temperature of 220°C. Separation of the samples was carried out with a GC capillary column (DB5-MS, 30-m × 0.25-mm internal diameter, 0.1-μm film thickness; Agilent Technologies, Palo Alto, CA, USA). The ion source and GC transfer line temperatures were maintained at 150 and 260°C, respectively. Helium was used as the carrier gas with a constant flow of 1 ml min^{-1}. The GC oven temperature program was 50°C (1.0 min) to 155°C at 10°C min^{-1}, 155–185°C at 2°C min^{-1}, and 185–300°C at 25°C min^{-1} (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^{-1}). 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 three fluorine and three hydrogen groups ($[M - H]^- \rightarrow [M - H - 60]^-$), yielding m/z 448 (HDA) and 462 (HpDA) (Fig. 1).

Work-up procedures

General procedure for plasma samples. The sample work-up procedure was adapted from a previously published method describing the analysis of HDA in urine (Rosenberg *et al.*, 2002). Duplicate glass vials containing 1 ml plasma (±HDA) and 12 μl of IS (0.12 μg l^{-1} plasma) were hydrolyzed with 100 μl of concentrated H_2SO_4 at 100°C 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 was added to derivatize the amines. The vials were vortexed for several seconds and heated at 55°C for 1 h. The excess reagent was removed by extraction with phosphate buffer solution (4 ml of 1 M 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, USA). 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, NY, USA). 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.

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 100°C with 100 μl concentrated H_2SO_4 was measured. Stock solutions of HDA and HpDA were prepared in 1 M H_2SO_4, as described above, and spiked into 1 ml blank plasma for a final HDA concentration of 2 μg l^{-1} and IS concentration of 0.12 μg l^{-1}. 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 100°C). 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.

Method detection limit determination. The USEPA (1984) describes the method detection limit (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 *et al.* (1981). To calculate the MDL, a minimum of seven replicate (N) spikes are prepared at an appropriately low concentration (generally one to five times the expected MDL) and processed through the entire analytical method. Therefore, we spiked nine plasma samples with 0.12 µg l^{-1} HDA and 0.12 µg l^{-1} IS, and the work-up procedure was performed as previously described for HDA analysis. The MDL was calculated using the following general formula:

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

where

N = number of replicate spike determinations at one to five 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 $1 - \alpha$ (99%) confidence level. At $N = 9$ and $\alpha = 0.1$, then $t = 2.896$, and
α = level of significance.

Thus, based on values in our study: $s = 7$ ng l^{-1}, $N = 9$, and $t = 2.896$ at $\alpha = 0.1$, and the MDL was calculated at 0.02 µg l^{-1}.

Biomarker and exposure measurements among workers

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

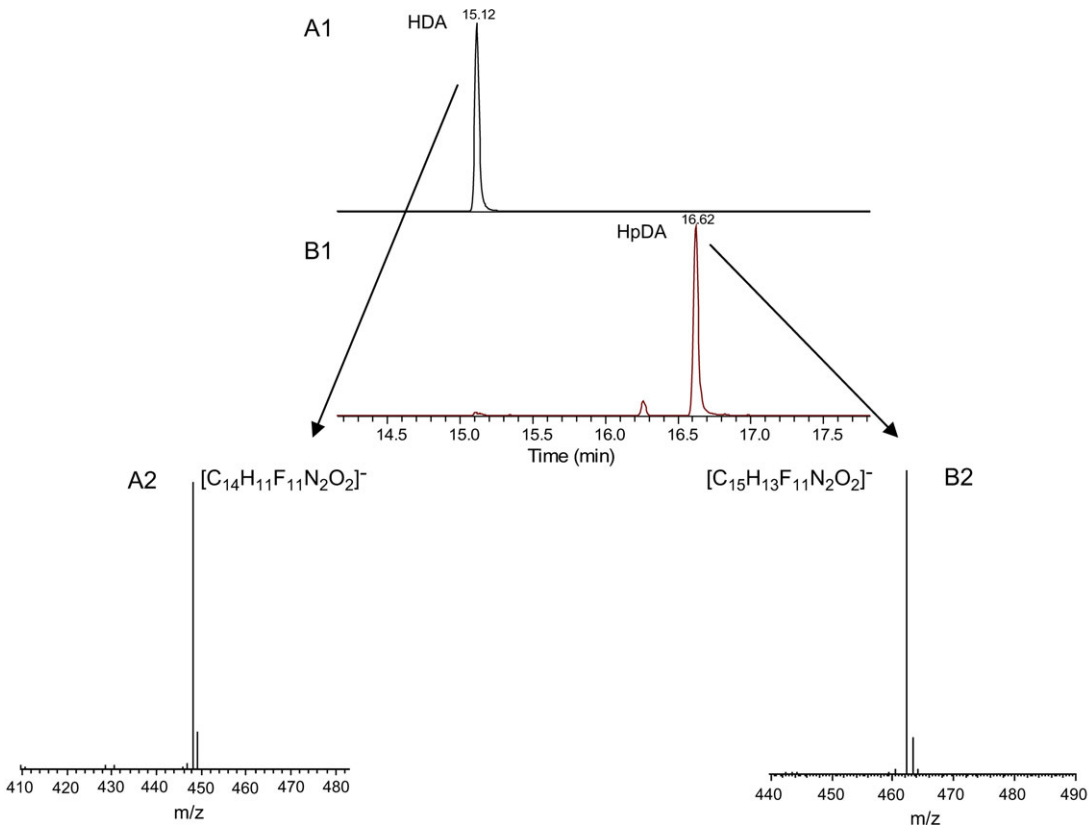

Fig. 1. Gas chromatogram and mass spectra of heptafluorobutyryl derivatives of 0.50 µg l^{-1} HDA (A1) and 0.12 µg l^{-1} HpDA (B1) in a plasma sample. A1 and B1 were recorded at *m/z* 448 and 462, corresponding to the heptafluorobutyryl derivative of HDA (A2) and the heptafluorobutyryl derivative of HpDA (B2).

in North Carolina (NC) ($n = 14$) and in Washington (WA) ($n = 32$). Blood samples were collected during each sampling visit when workers consented (112 total blood samples). No more than three 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 −40°C until analyzed. Samples (1 ml) were prepared in duplicate and the work-up procedure was performed as outlined above. Urine was also collected from the workers throughout the workday and stored at −40°C until analyzed. Results for HDA in hydrolyzed urine are published elsewhere (L. G. T. Gaines, K. W. Fent, S. L. Flack, J. M. Thomasen, L. M. Ball, D. B. Richardson, K. Ding, S. G. Whittaker, L. A. Nylander-French, in preparation).

Breathing-zone and dermal exposure measurements among these occupationally exposed spray painters have been published elsewhere (Fent *et al.*, 2009a,b). In brief, a personal air sample in the worker's breathing zone was collected during each spray-painting operation of clear coat using a one-stage or two-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^{-1} (SKC Inc.). The two-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 eight sampling visits (three workers), air sampling using one- and two-stage samplers was performed side by side. HDI on the skin was collected using three consecutive tape strips (10 cm^2) applied to six different sites on the worker's body (e.g. right and left forearms, hands, and neck) after each paint task. Air and skin samples were analyzed by liquid chromatography–MS as previously described by Fent *et al.* (2009a,b). An algorithm described by Fent *et al.* (2009b) was used to calculate the whole-body concentration of HDI in the skin (nanograms per cubic millimeter) for each worker and paint task.

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

Variable construction

The breathing-zone concentration (BZC) of HDI monomer for each paint task (micrograms per cubic millimeter) was multiplied by the total paint time (minutes) 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^{-1}) to estimate the HDI inhalation exposure (micrograms) (Adams, 1993). 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 (OSHA, 2006): none, APF = 1; air purifying (half face), APF = 10; air purifying (full facepiece), APF = 50; supplied air (full facepiece or hood), APF = 1000; and powered air-purifying respirator (PAPR) (full facepiece or hood), APF = 1000. Among visits where one- and two-stage air samplers were used side by side, we selected one-stage air data for regression and statistical analyses based on findings by Fent *et al.* (2009a). The HDI monomer dermal exposure concentrations (micrograms per square meter) for each paint task were multiplied by body surface area of the worker (square meters) and summed together to estimate the cumulative daily dermal exposure (micrograms). The internal HDA dose (micrograms) was calculated by multiplying the HDA concentration (micrograms per liter) in plasma with the individual plasma volume (liters), which was determined using individual's body weight (Svirbely and Iyengar, 1997).

Statistical analysis

Differences in HDA concentration in spiked plasma at hydrolysis times of 0, 4, and 16 h were 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 (intragroup 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 (intergroup variability) was investigated by comparing the %CV among calibration standards and field samples.

Due to the relatively high percentage of nondetectable 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^{-1}). Methods for performing multiple imputation of exposure data are previously described by Fent *et al.* (2009a,b) and were also applied to plasma HDA. In brief, 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 (GM) and geometric standard deviation (GSD) of dose and exposure data were calculated from the log-transformed data (PROC MEANS procedure in SAS, version 9.1; SAS Institute, Cary, NC, USA). Statistical analysis of the stratified data, according to different workplace covariates, was performed using Tukey's studentized range 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 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 across models with competing covariance structures (Keselman *et al.*, 1998). The intraclass correlation coefficient, 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–Wilks $W > 0.80$).

RESULTS

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 *et al.* (2004). The detection limit (MDL) of the analysis, from spiked plasma samples, was 0.02 μg l^{-1}. The repeatability among standards and field samples was assessed using the %CV. The %CV among replicate calibration standards (range of 0–2 μg l^{-1}) 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^{-1}) 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 in various biological media (Marand *et al.*, 2004). The lower quantitative precision among plasma samples compared with spiked standards may be attributed to the release of amines from biological matrices affecting the variation in recovered HDA from protein conjugates.

HDA plasma levels in occupationally exposed workers

The range of HDA concentrations in hydrolyzed plasma samples was $\leq$0.02–0.92 μg l^{-1}. After imputing values for samples <MDL (24%) and adjusting for individual plasma volume, the GM and 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 two repeated visits for 10 workers and three repeated visits for 28 workers. In addition, four of the 112 blood samples did not have corresponding dermal and breathing-zone samples 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 ~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 ~90% of paint tasks had detectable levels of HDI in the breathing zone of workers.

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 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 unadjusted HDI inhalation exposure ($N = 108$, $r = 0.22$, $P = 0.026$) (Fig. 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.

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 (Sepai *et al.*, 1995a) 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 to 60 days before blood collection, the association improved ($N = 29$, $r = 0.57$, $P = 0.0014$) (Table 2 and Fig. 3). In addition, the correlation improved between plasma HDA levels and APF-adjusted inhalation exposure to HDI occurring ~20 to 60 days (~1 to 3 albumin half-lives) before blood collection ($N = 29$, $r = 0.44$, $P = 0.018$) (Table 2).

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 APF-adjusted inhalation exposure was very low (<MDL/5 ≈ 0.005 μg) ($N = 17$, $r = 0.58$, $P = 0.031$) (Table 1). In addition, the correlation between plasma HDA levels and HDI dermal exposure occurring ~20 to 60 days before blood collection improved slightly compared to same-day exposure ($N = 29$, $r = 0.36$, $P = 0.053$) (Table 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 and uptake and elimination kinetics of HDI through the skin) may be obscuring the association between internal dose and dermal exposure.

Statistical models

We also investigated whether differences in PPE use (coveralls, gloves and 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 3. After implementing Tukey–Kramer multiple comparisons testing 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 GM values corresponding to 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 Tukey–Kramer multiple comparisons testing in SAS. However, we noted that PAPR users had a slightly higher GM for plasma HDA level (0.44 μg) compared to the other respirator types (Table 3), which may be related to maintenance and/or proper fit of respirator. Even though most workers (98%) wore

Table 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-values
HDI unadjusted inhalation (μg)	46	108	0.22	0.03–0.40	0.026
HDI adjusted inhalation (μg)	46	108	0.10	−0.10 to 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.

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 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 Tukey–Kramer multiple comparisons testing in SAS. The GM for plasma HDA levels was significantly lower among workers painting in a downdraft-ventilated booth compared to a semi-downdraft booth ($P = 0.0108$). The GM values for HDI dermal and inhalation exposure (unadjusted for APF) 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 demonstrates 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 (Woskie *et al.*, 2004).

The linear mixed model results for predicting plasma HDA level are summarized in Table 5. A large proportion (95%) of the total variance in plasma HDA level is attributed to the within-worker variance (σ_w^2), whereas ~5% of the total variance is explained by the between-worker variance (σ_b^2) (Table 5). The within-worker variance represented a majority of the total variance even when stratifying for location (0 = NC and 1 = WA), coverall or glove use (0 = no and 1 = yes), and respirator type or booth type (0 = no and 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-

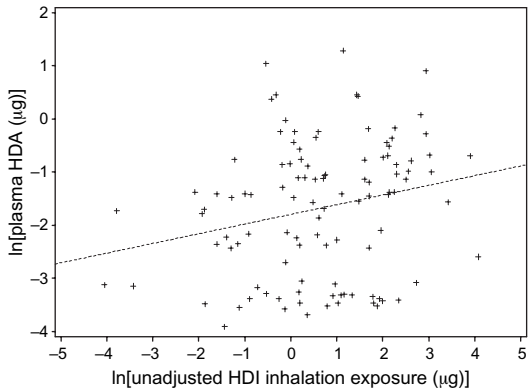

Fig. 2. Linear regression of the log-transformed plasma HDA levels (μg) versus the log-transformed unadjusted HDI inhalation exposure (μg) measured on the same workday ($N = 108$; $r = 0.22$; $P = 0.026$) for one to three visits.

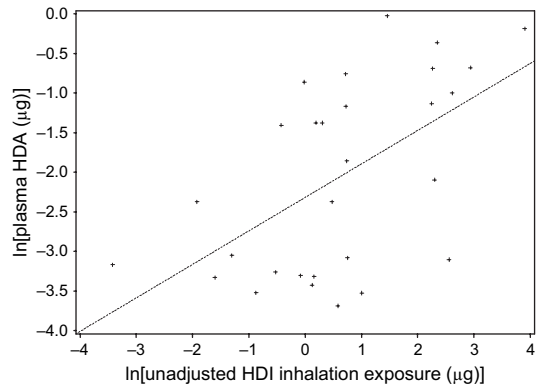

Fig. 3. Linear regression of the log-transformed plasma HDA levels (μg) versus the log-transformed unadjusted HDI inhalation exposure (μg) occurring ~20 to 60 days prior to blood collection ($N = 29$; $r = 0.57$; $P = 0.0014$).

Table 2. Correlation between log-transformed plasma HDA levels (μg) and inhalation or dermal HDI exposure (μg) ~20 to 60 days prior to blood collection

Predictor variables	n[a]	N[b]	Pearson's r	95% CI[c]	P-values
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 to 0.65	0.053

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

Table 3. Summary of plasma HDA levels (μg)[a] in 46 spray painters stratified by workplace covariates

Covariate	n[b]	N[c]	Summary statistics			P-values[d]
			GM	GSD	Range	
Location						
NC	13	30	0.20	3.83	<0.02–1.58	0.778
WA	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	

[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-values determined using Tukey–Kramer multiple comparisons testing.

Table 4. Summary of measurements in 46 spray painters[a] stratified by paint booth type

Variable	Booth type	n[b]	N[c]	Summary statistics			P-values[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	4.80	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	<0.10–1023	<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	<0.10–1023	

[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 Tukey–Kramer multiple comparisons testing.

downdraft ($P = 0.012$) booth types were significant predictors of plasma HDA levels.

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, uretdione, 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 (Fent *et al.*, 2009a,b). 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, AcHDA or diAcHDA 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 *et al.* (1990b) for urinary HDA levels, would have been masked by the acid treatment. Individuals who are fast acetylators would favor the metabolic pathway leading to the formation of diAcHDA, which is excreted directly into the urine, rather than the formation of protein adducts via the oxidation pathway. Also, Pauluhn and Lewalter (2002) observed differences in ratio of acetylated MDA/MDA Hb adducts between inhalation and dermal exposure routes in exposed rats, and, thus,

Table 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		ICC	P-values[f]
				σ^2_w	σ^2_b		
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 (dependent 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) and 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-values are based on F-tests of fixed effects.

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 AcHDA 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 BZCs 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), which implement filtration for reducing inhalation exposure, may not sufficiently remove diisocyanate aerosols, particularly if workers do not follow routine change-out of filters (Poovey and Rando, 2002). Consequently, adjusting BZC by APF may underestimate 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 or dermal exposure improved when HDA levels were correlated with exposures measured ~20 to 60 days prior to blood collection compared to same-day exposures. When we investigated the relationship between plasma HDA levels and HDI inhalation or dermal exposure measurements from >60 to 200 days before blood collection, the associations were weaker ($N = 37$, $r = 0.07$, $P > 0.05$). 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. On the other hand, urinary 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 h) (Brorson *et al.*, 1990a; Tinnerberg *et al.*, 1995).

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 five 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 to 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 and uretdione) 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 Hb 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.

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 and dermal exposure to HDI. 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 plasma HDA and same-day HDI inhalation or 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.

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