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Effect of creatinine and specific gravity normalization on urinary biomarker 1,6-hexamethylene diamine

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Urine amine levels used as biomarkers of diisocyanate exposure have usually been normalized with creatinine concentration. The suitability of using creatinine concentration or specific gravity for these biomarkers in exposure assessment has not been established. We investigated the effect of creatinine concentration and specific gravity on urine 1,6-hexamethylene diamine (HDA) levels in multiple mixed linear regression models using quantitative dermal and inhalation exposure data derived from a survey of automotive spray painters occupationally exposed to 1,6-hexamethylene diisocyanate (HDI). Painters' dermal and breathing-zone HDI exposure were monitored for an entire workday for up to three workdays spaced approximately one month apart. One urine sample was collected before the start of work with HDI-containing paints, and multiple samples were collected throughout the workday. Both creatinine concentration and specific gravity were highly significant predictors ($p < 0.0001$) of urine HDA levels. When these two were used together in the same model, creatinine remained highly significant ($p < 0.0001$), but specific gravity decreased in significance (p-values 0.10–0.17). We used different individual factors to determine which affected creatinine and specific gravity. Urine collection time was a highly significant predictor of specific gravity ($p = 0.003$) and creatinine concentration ($p = 0.001$). Smoker status was significant ($p = 0.026$) in the creatinine model. The findings indicate that creatinine concentration is more appropriate to account for urine water content than specific gravity and that creatinine is best used as an independent variable in HDI exposure assessment models instead of traditional urine normalization with creatinine concentration.

Introduction

Urinary biomarkers are commonly used as indicators of xenobiotic exposure. However, an inherent problem with urinary biomarker analysis is the variability in the urine water content. Concentrations of both endogenous and exogenous compounds in urine can vary due to the person's hydration level that in turn is reflected in the water content of the collected urine sample. Therefore, urinary biomarker levels are generally normalized with urinary creatinine concentration, specific gravity, or urine volume.

Creatinine has been frequently used to normalize urinary biomarker levels to allow comparison between individuals, thus excluding variations in hydration levels. Creatinine, an organic cation, is an endogenous substance, which is formed from muscle creatine. The blood concentration of creatinine changes little during a 24 h period due to its release into the blood at a fairly constant rate.[1] In the kidneys, creatinine is mainly filtered through the glomerular capillaries with only a small portion secreted through the tubules. Thus, elimination of creatinine from the body resembles that of water, which is also filtered

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Environmental impact

Urinary biomarkers are commonly used as measures of xenobiotic exposures. However, the effect of the variable water content in urine samples poses difficulty in the determination of xenobiotic concentration, thus affecting exposure assessment. Our goal was to determine the most appropriate method to investigate urine amines as biomarkers of diisocyanate exposure. Our results indicate that amine concentration in a urine sample must be adjusted with creatinine concentration, and not with specific gravity. Furthermore, due to individual factors, creatinine concentration should be used as an independent variable, instead of dividing the amine level by creatinine concentration, in exposure assessment models. The presented information will aid occupational and environmental hygienist and researchers to built more accurate exposure assessment models using urine biomarkers.

through the glomerular capillaries. However, Greenberg and Levine[2] observed that creatinine elimination rate is not constant, but, in fact, depends on urine flow rate. They observed that spontaneously varying urinary flow rates explained 21% of the within-subject variation in creatinine excretion rates. They proposed that fluctuations in renal blood flow and individual variations in tubular excretion of creatinine were the most likely reasons for the variability in creatinine elimination rates. Considerable variation in creatinine excretion rate has also been observed in other studies.[3–6]

Newman *et al.*[7] observed greater variability in 24 h creatinine excretion rate than urine volume, which in turn showed greater variability than specific gravity. In addition, they observed that normalization for creatinine concentration corrected for exercise-induced proteinuria (*i.e.*, excess protein in the urine). Heavner *et al.*[8] observed that specific gravity and creatinine-normalized concentrations of urinary biomarkers for tobacco exposure exhibited lower variability than the unnormalized concentrations. They evaluated five normalization techniques for tobacco biomarkers: creatinine; specific gravity; combined specific gravity and Z, a statistic related to the change of creatinine and specific gravity with time; combined specific gravity, creatinine, and Z; and creatinine regression or the mean creatinine of the study population. Creatinine-normalized concentrations had the highest between-subject variability among the five normalization techniques that they evaluated. Thus, creatinine normalization may bias the estimated level of xenobiotic in the body due to potentially different excretion-rate and urine-flow relationships.

In contrast, Carrieri *et al.*[9] observed a good correlation (r = 0.82) between natural log-transformed creatinine and specific gravity and neither demonstrated significant intra- or inter-day variations with both reflecting the same extent of dilution of urine samples. They did find, however, that men showed higher values of creatinine and specific gravity than women and that creatinine, but not specific gravity, was significantly lower in subjects older than 50 years. Similarly, Suwazono *et al.*[10] observed that creatinine concentration was significantly influenced by age, gender, and body size, most likely reflecting the difference in muscle mass. They also found that meat intake influenced urinary creatinine excretion. Nermell *et al.*[11] also reported that creatinine concentration, but not specific gravity, was affected by age, gender, and body size. Since creatinine concentration is more affected by age and gender than specific gravity, adjustment with specific gravity may be more appropriate in the populations with large differences in muscle mass and meat intake.

Barr *et al.*[12] observed that age group, sex, race/ethnicity, body mass index (BMI), and fat-free mass are all significant predictors of urinary creatinine concentration. They also reported that the time of day that a urine sample was collected had a small but statistically significant effect (p = 0.001 for morning *versus* evening samples) on the urinary creatinine concentration. Thus, they proposed that exposure assessment models in which the level of external xenobiotic exposure is associated with urinary biomarker concentration include urinary creatinine concentration as an independent variable. This would allow the urinary chemical concentration to be distinguished from the urinary creatinine concentration, and the subject's hydration status to be accounted for along with the variation in creatinine concentration that is considered within the normal range. Further, because urinary chemical concentration and the covariates in the model would be independent of the urinary creatinine concentration, the statistical significance of other independent variables would not be due to confounding by urinary creatinine concentration.

Previously, we investigated the relationship between dermal and inhalation exposure to 1,6-hexamethylene diisocyanate (HDI) and urinary 1,6-hexamethylene diamine (HDA).[13] We observed that creatinine concentration was a significant independent covariate ($p < 0.0001$) along with dermal and inhalation exposure when investigating association between exposure and urinary HDA levels. This observation confirmed that the water content of a urine sample must be accounted for when biological monitoring is performed in HDI-exposed workers. However, a question remains whether creatinine concentration in a spot urine sample best describes the variability in the hydration levels of our study population. Since considerable variability in creatinine elimination has been documented in published studies,[2–4,6–8] we hypothesized that the specific gravity of the spot urine sample can explain more than creatinine concentration of the variability in the urinary HDA levels measured in the spray painters occupationally exposed to HDI.

Materials and methods

Study population

The study population has been described previously.[13,14] Briefly, spray painters in automotive repair shops who worked with HDI-containing paint were recruited for the study in the Raleigh-Durham area of North Carolina and the Puget Sound area of Washington State. Eleven shops in North Carolina with a total of 15 workers and 25 shops in Washington with a total of 33 workers participated in the study. Each exposed worker was monitored for up to three separate days over a 9–12 month period. Due to attrition, six subjects were monitored once and 15 subjects were monitored twice. The subjects were all male, ranging in age from 21 to 59 years with an average age of 34 years. Thirty-one subjects identified themselves as white, nine as Hispanic, four as African-American, one as Asian, one as Native American, and two as mixed race. This study was approved by the Institutional Review Board in the Office of Human Research Ethics at the University of North Carolina at Chapel Hill and by the Washington State Institutional Review Board (WSIRB) at the Washington State Department of Social and Health Services.

Dermal and breathing-zone air sampling

Personal breathing-zone sampling was performed to estimate inhalation exposure during every spray-application of HDI-containing paints and coatings. Dermal exposure was assessed using the tape-strip method previously developed in our laboratory.[15] The collection and analysis of the tape-strip and air samples have been described.[14,16] Briefly, on each sampling visit, breathing-zone air samples were collected during each HDI-containing painting task, and dermal tape-strip samples were collected immediately following each task. The painter was observed during the paint tasks to note the duration of exposure and the type of respirator worn. The assigned protection factor

(APF) designated by the Occupational Safety and Health Administration[17] for the respirator worn by a worker (none, APF = 1; air-purifying half-face, APF = 10; air-purifying full-face, APF = 50; supplied air full-face or hood, APF = 1000; powered air-purifying respirator (PAPR), full-face or hood, APF = 1000) was used to adjust the measured breathing-zone concentrations (BZCs) in order to account for the respiratory protection in inhalation exposure levels used in the analyses.

Urine sampling

The urine sampling has been described previously.[13] Briefly, during each sampling visit, one urine sample was obtained from each painter before the start of work with HDI-containing paint. During the workday, urine samples were obtained from the worker each time he urinated. At a minimum, one pre-exposure sample and one end-of-day sample were collected. An average of 3.5 samples were obtained per worker per day. The maximum number of samples obtained from a worker on a single day was nine. Fifteen samples were obtained from five workers on a day when they were not painting. They were all workers at shops with more than one painter.

HDA analysis

The HDA analysis has been described previously.[13] Briefly, 1,7-diaminoheptane (HpDA), an internal standard, was added to the urine sample before hydrolysis at 100 °C with concentrated sulfuric acid. The samples were cooled, neutralized with saturated sodium hydroxide (NaOH), mixed with sodium chloride (NaCl), and then extracted three times with toluene. The samples were then derivatized with heptafluorobutyric anhydride (HFBA) at 55 °C, cooled, and potassium phosphate buffer added to remove excess derivatizing agent. The organic layer was retained, and sodium sulfate was added to dry the sample. The organic solution was moved to a clean vial and dried using a TurboVap® LV Evaporator (Zymark Center, Hopkinton, MA). The samples were then reconstituted with ethyl acetate, sonicated, and transferred to gas-chromatograph vial inserts. The samples were again dried to completion in a SpeedVac® (Savant Instruments Inc., Holbrook, New York) and reconstituted with ethyl acetate. The samples were analyzed by gas-chromatography mass-spectrometry (Thermo, Austin, TX) in negative chemical ionization mode with methane as the reagent gas. The HDA and HpDA were determined using selective ion monitoring at m/z 448 and m/z 462, respectively.

Standard curves were prepared by spiking pooled urine from four unexposed individuals with HDA. Each standard curve consisted of a reagent blank (no HDA or HpDA), a negative control (HpDA but no HDA), and nine different HDA concentrations (0.08 to 20 μg l^{-1}) with HpDA (1.5 μg l^{-1}). Weighted linear regression was used to construct a standard curve using the HDA–HpDA ratio.[18] Different weighting factors ($w = x^{-0.5}$, x^{-1}, x^{-2}, $y^{-0.5}$, y^{-1}, y^{-2}, $y^{-1.5}$; where x = HDA/HpDA instrument response ratio, y = HDA concentration) were evaluated for fitting standard curves. The weighting factor that gave the smallest sum of absolute relative error as a percentage of the nominal concentration was used for fitting the standard curve.[18] The standard curve was linear from 0 to 20 μg l^{-1} ($w = y^{-2}$, $R^2 = 0.98$). The method detection limit (MDL) of 0.04 μg l^{-1} was calculated using the MDL procedure established by US EPA.[19]

Creatinine analysis

The creatinine concentration in the urine was analyzed using the Creatinine Companion assay kit (Exocell, Inc., Philadelphia, PA)[20,21] as described previously.[13] Briefly, samples were diluted in distilled water and then aliquoted, in duplicate, into a 96-well microtiter plate along with creatinine standards, in duplicate. NaOH was added to alkaline picrate reagent, and this solution was added to each well. The plate was incubated at room temperature for 10 min, and the absorbance determined at 500 nm (Emax, Molecular Devices, Sunnyvale, CA). The acid reagent provided with the kit was then added to each well, and the absorbance at 500 nm determined after a 5 min incubation at room temperature. The difference between the two absorbance values was recorded for each well. A standard curve was calculated based on the standards and their responses. Unknown samples were evaluated by comparing their responses to the standard curve.

Specific gravity analysis

The urine specific gravity was measured using a handheld refractometer (Schuco Clinical Refractometer model 5711-2021, Japan). On this instrument, either the refractive index or the specific gravity can be read directly. Since the refractive index scale is more sensitive than the specific gravity scale, a standard curve was constructed from the two scales to relate the refractive index to the specific gravity. Distilled water was used to calibrate the refractometer before each use and then after every twenty samples to check calibration.[22] While the instrument was in a horizontal position, a small drop of urine was placed on the prism, and the cover plate was placed over it. The scale seen in the eyepiece was focused, and the refractive index was read. The specific gravity was determined from the standard curve.

Statistical analysis

The data were analyzed using SAS statistical software (SAS 9.1; SAS Institute, Cary, NC). BZC and dermal HDI levels and urine HDA levels were natural log-transformed to satisfy normality assumptions (Shapiro–Wilks: $W > 0.85$) prior to statistical analysis. Creatinine concentrations were approximately normally distributed ($W = 0.89$). However, natural log-transformation of the creatinine concentrations improved the normality ($W = 0.96$).

Normalization of urine analyte level with specific gravity is performed with the following equation:

$$\mathrm{HDA}_{\mathrm{normalized}} = \mathrm{HDA}_{\mathrm{measured}} \frac{(1.02 - 1)}{(\mathrm{SG} - 1)} \quad (1)$$

where $\mathrm{HDA}_{\mathrm{normalized}}$ is the normalized HDA level, $\mathrm{HDA}_{\mathrm{measured}}$ is the measured HDA level, 1.02 is the average specific gravity of human urine, and SG is the measured specific gravity.[23] The use of this ratio increases the sensitivity of the analysis due to the small variation in the levels of specific gravity in human urine. Therefore, when investigating the significance of the specific

gravity in the regression models, we used the specific gravity ratio as given by the equation:

$$\text{Specific gravity ratio} = \frac{(\text{SG} - 1)}{(1.02 - 1)} \quad (2)$$

instead of the measured specific gravity itself. The specific gravity ratio was normally distributed ($W = 0.98$).

Factors affecting creatinine and specific gravity measurements. Mixed effects multiple linear regression modeling (PROC MIXED) was used to investigate the relative influence of factors that affected creatinine or specific gravity levels in HDI-exposed workers as well as to account for the repeated measurement strategy and to obtain both inter- and intra-person variance. All collected urine samples were used in these analyses including the samples taken from workers on days when they were not painting as well as those taken before the worker started painting. Each urine sample was used as a unique observation in the modeling. The candidate covariates included continuous variables such as urine collection time, subject's age, and BMI; and classification variables included smoking status and race. These covariates were previously observed to significantly affect creatinine concentration in urine.[5,9–12,24] The multiple linear mixed effects model took the form:

$$Y_{ij} = \beta_0 + \beta_1 X_{1ij} + \beta_2 X_{2ij} + \beta_3 X_{3ij} + \beta_4 X_{4ij} + \beta_5 X_{5ij} + \alpha_i + \varepsilon_{ij} \quad (3)$$

where Y_{ij} represents the natural logarithm of the creatinine concentration or the specific gravity ratio of the urine sample (the j^{th} measurement obtained for the i^{th} worker), X_{1ij} represents the collection time of the urine sample, X_{2ij}, X_{3ij}, X_{4ij}, and X_{5ij} represent the subject's age, BMI, smoking status, and race, respectively, while α_i and ε_{ij} represent the random effects associated with worker (α_i for $i = 1, 2,\ldots, 48$ workers) and an error term (ε_{ij} for $j = 1, 2,\ldots, 19$ measurements per worker). Models were constructed using standard regression techniques, and model fit was examined with regression diagnostics such as residual analysis. Final models were built using a backwards elimination procedure in which the least significant variables ($p > 0.10$) were eliminated one at a time.

Using this model, we 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 $\sigma^2_Y = \sigma^2_{\text{B}} + \sigma^2_{\text{W}}$. It is also assumed that Y_{ij} is normally distributed with mean $\mu_y = \beta_0 + \beta_1 X_{1ij} + \beta_2 X_{2ij} + \beta_3 X_{3ij} + \beta_4 X_{4ij} + \beta_5 X_{5ij}$ and variance σ^2_Y. Compound symmetry was used for the covariance structure.

Effect of creatinine or specific gravity on urine HDA levels. We investigated the effect of urine creatinine concentration or specific gravity on the relationship between urine HDA levels and HDI exposure (inhalation and dermal) by including creatinine or specific gravity as an independent variable in the mixed effects multiple linear regression models (PROC MIXED). Only those urine samples obtained after at least one spray-painting task had occurred were used in these analyses. Thus, the urine samples taken from workers on days when they were not painting or samples taken before the worker started painting were not included in these analyses. Each post-exposure urine sample was used as a unique observation in the exposure modeling.

Due to the relatively high percentage of non-detectable levels of HDA in the urine samples (38%) as well as HDI in the breathing-zone air (9%) and dermal tape-strip (63%) samples, multiple imputation consisting of 10 datasets was used to impute data below the detection limits. Methods for performing multiple imputation of exposure data are previously described.[13,14,16] In brief, a lower bound of zero was set for the imputations. In order to account for correlations in the multivariate exposure data, we imputed from truncated multivariate normal distributions, with truncation at the limit of detection for HDI and MDL for HDA. PROC MIANALYZE was used to combine the results of the analyses (PROC MIXED) carried out on the 10 imputed datasets and to obtain valid estimates and statistical inferences. Averages were computed where PROC MIANALYZE could not be used (*i.e.* fit statistics).

Dermal and breathing-zone exposure levels were used in the analyses separately or jointly to investigate whether both exposure routes contributed to urine HDA levels. Since all workers in the study wore respirators, the measured BZCs likely overestimated the inhalation exposure. Previously, we reported that adjustment of inhalation exposure level with the assigned protection factor for the respirator worn by the worker provided a better model fit when investigating the effect of dermal and inhalation exposure on urine HDA levels with or without creatinine normalization.[13] We also observed that BZC can be considered as an estimate for inhalation exposure only if a worker has no respiratory protection. Here, we again used both unadjusted and adjusted BZCs to determine the best-fit model.

The mixed effects multiple linear regression model to investigate the relative influence of natural log-transformed creatinine concentration (model A; Table 3), specific gravity ratio (model B; Table 3), or both natural log-transformed creatinine concentration and specific gravity ratio (model C; Table 3) as an independent variable to the relationship between unnormalized urine HDA levels and dermal and inhalation exposures are provided below:

$$Y_{ij} = \beta_0 + \beta_1 X_{1ij} + \beta_2 X_{2ij} + \beta_3 X_{3ij} + \alpha_i + \varepsilon_{ij} \quad (4)$$

where Y_{ij} represents the natural logarithm of the urinary HDA concentration (the j^{th} measurement obtained for the i^{th} worker), X_{1ij} represents the natural logarithm of the measured BZC unadjusted or adjusted for the APF based on the respirator worn, X_{2ij} represents the natural logarithm of the measured dermal exposure, X_{3ij} represents the natural logarithm of the creatinine concentration or the specific gravity ratio of the urine sample, and α_i and ε_{ij} represent the random effects associated with worker (α_i for $i = 1, 2,\ldots, 48$ workers) and an error term (ε_{ij} for $j = 1, 2,\ldots, 16$ measurements per worker). The same basic assumptions for eqn (3) apply to eqn (4). Models were constructed using standard regression techniques, and model fit was examined with regression diagnostics such as residual analysis. Compound symmetry was used for the covariance structure. The statistical significance was evaluated at α level of 0.10.

Cumulative exposure measures for BZC, APF adjusted BZC (BZC-APF), and dermal exposure were used in the statistical analyses. Cumulative exposure was calculated by summing all

the respective exposure levels that occurred before a urine sample was obtained, as provided by the following equation:

$$X_{ij} = \ln\left(\sum_{0}^{T} C_t(\text{if } t < T)\right) \quad (5)$$

where C_t is the concentration at time t of the measured dermal, BZC, or BZC-APF exposure, T is the time of the urine sample, and t is the exposure period. The exposure period is the time between when the air-sampling pump was turned on and off, and thus, the approximate period of time when the worker was exposed.

Since creatinine and specific gravity are closely related, models were also generated with standardized forms of natural log-transformed creatinine concentration and specific gravity ratio to test for co-linearity issue when they are included in the same model. The variables were standardized using the formula:

$$x^* = \frac{x - \bar{x}}{\text{stdev}(x)} \quad (6)$$

where x^* is the standardized value of x (natural log-transformed creatinine concentration or specific gravity ratio), $\bar{x}$ is the mean of x, and stdev(x) is the standard deviation of x.

In summary, a total of three exposure models (Table 3) were generated where (i) natural log-transformed creatinine concentration was used as an independent variable (model A), (ii) specific gravity ratio was used as an independent variable (model B), and (iii) both natural log-transformed creatinine concentration and specific gravity ratio were used as independent variables (model C). Within these three models, five submodels were built: (1) BZC, (2) BZC-APF, or (3) dermal levels as single exposure variables in a model, and (4) dermal and BZC levels, or (5) dermal and BZC-APF levels as two exposure variables in a model to determine the significance of these predictors in any given model. In addition, model sets A, B, and C were generated with a standardized forms of natural log-transformed creatinine concentration and specific gravity ratio to test for co-linearity (eqn (6)) (data not shown).

Results

Factors affecting creatinine and specific gravity measurements

A total of 417 urine samples obtained from 48 spray painters were analyzed for HDA, creatinine, and specific gravity. Creatinine concentrations ranged from 0.094 to 8.32 g l^{-1} with a mean and standard deviation (SD) of 1.55 g l^{-1} and 0.97, respectively, and a geometric mean and geometric SD of 1.26 g l^{-1} and 2.01, respectively. Specific gravity levels ranged from 1.00 to 1.04 with a mean of 1.02 (SD = 0.01). Urine is considered too dilute for biomarker analysis if the specific gravity is less than 1.010 or the creatinine concentration is less than 0.5 g l^{-1}.[23] Conversely, urine is too concentrated if the specific gravity is greater than 1.030 or the creatinine concentration is greater than 3 g l^{-1}.[23] The number of urine samples outside these normal ranges are presented in Table 1. Based on creatinine concentration and specific gravity, 43 and 63 urine samples, respectively, were too dilute. Only 40 were too dilute based on both creatinine concentration and specific gravity. Conversely, 29 urine samples were too concentrated based on creatinine concentration, but only nine were too concentrated based on specific gravity. All nine of the samples with too high specific gravity were also too concentrated based on their creatinine concentration. Of the urine samples considered too dilute based on creatinine concentration, 34 had non-detectable levels of HDA while 45 of those considered too dilute based on specific gravity had non-detectable levels of HDA. However, for the modeling conducted in this study, we did not exclude any of the samples even if, by convention, they were considered too dilute or too concentrated.

Table 1 Summary of measured creatinine and specific gravity levels and the number of urine samples (N) considered to be outside the normal range necessary for determinate analysis[23]

	Mean ± standard deviation	N
Creatinine	1.55 g l^{-1} ± 0.97	417
Specific gravity	1.02 ± 0.01	417
Below normal range	Creatinine < 0.5 g l^{-1}	43
	Specific gravity < 1.010	63
	Creatinine < 0.5 g l^{-1} and specific gravity < 1.010	40
Above normal range	Creatinine > 3 g l^{-1}	29
	Specific gravity > 1.030	9
	Creatinine > 3 g l^{-1} and specific gravity > 1.030	9

We observed an exponential ($R^2 = 0.80$) rather than linear ($R^2 = 0.69$) relationship between creatinine concentration and specific gravity (Fig. 1). The relationship between natural log-transformed creatinine concentration and the specific gravity ratio, the variables used in the models, exhibited a somewhat closer linear relationship ($R^2 = 0.76$) than that between creatinine and specific gravity (data not shown).

The factors that significantly affected natural log-transformed creatinine concentrations and specific gravity ratios in workers exposed to HDI are presented in Table 2. Urine collection time was a highly significant predictor of both specific gravity ratio ($p = 0.003$) and creatinine concentration ($p = 0.001$). Smoker status (current non-smoker or smoker) was only a significant predictor of creatinine concentration ($p = 0.026$). No other

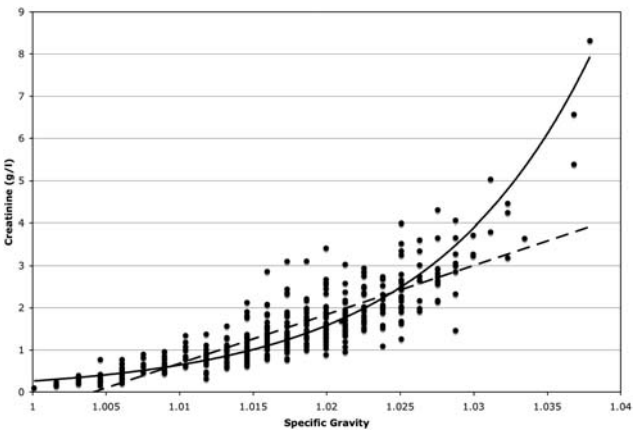

Fig. 1 The correlation between specific gravity and creatinine concentration in urine samples collected from 48 spray painters exposed to HDI in North Carolina and Washington State. The solid line indicates that this correlation is explained better with an exponential function (R^2 = 0.80) rather than a linear function (dashed line, $R^2 = 0.69$).

Table 2 Summary of the significant predictors for natural log-transformed creatinine concentration or specific gravity ratio in workers exposed to HDI[a]

Variable	Predictor	Estimate	Standard error	*p*-value	AIC
ln(creatinine concentration)	Urine collection time	8.71×10^{-6}	2.62×10^{-6}	0.001	764
	Smoker	0.296	0.131	0.026	
	Worker var.	0.201			
	Residual var.	0.272			
Specific gravity ratio	Urine collection time	3.87×10^{-6}	1.28×10^{-6}	0.003	171
	Worker var.	0.053			
	Residual var.	0.065			

[a] AIC = Akaike's information criterion; var. = variance.

covariates were observed to significantly affect either specific gravity ratio or creatinine concentration.

Effect of creatinine or specific gravity on urine HDA levels

The results of the three exposure models are provided in Table 3. When used alone with one or two exposure variables in the model (models A1–A5 and B1–B5), both natural log-transformed creatinine concentration and specific gravity ratio were always highly significant ($p < 0.0001$). Further, the significance levels of the exposure variables were very similar between the corresponding submodels. When the natural log-transformed creatinine concentration and specific gravity ratio were placed into the same model (models C1–C5), creatinine remained highly significant ($p < 0.0001$) while the *p*-value for specific gravity ratio ranged from 0.10 to 0.17. When these two were used together, the corresponding submodels gave similar results for the significance of the exposure variables (*i.e.* A1, B1, and C1 had similar *p*-values for the log-transformed BZC exposure). The Akaike's information criterion (AIC) is also provided for each model; a smaller AIC indicates a better model fit. The models that included both natural log-transformed creatinine concentration and specific gravity ratio (models C1–C5) had the smallest AIC indicating the best model fits. However, the specific gravity ratio only slightly improved the model fit (AIC = 1031; model C5) compared to using only natural log-transformed creatinine concentration (AIC = 1035; model A5).

All models were also investigated using the standardized forms of natural log-transformed creatinine concentration and specific gravity ratio (eqn (6); results not shown). No difference was observed between standardized and non-standardized forms with regard to the significance levels of any of the exposure variables, creatinine concentration, or specific gravity ratio. The non-standardized models had AICs about 3 points smaller than the standardized models, thus providing a slightly better model fit. No co-linearity issues between specific gravity ratio and log-transformed creatinine concentration were observed.

Discussion

Determination of systemic exposure to a xenobiotic in both environmental and occupational setting is commonly conducted by measuring a parent compound or its metabolite level in a spot urine sample. Due to fluctuations in the water content of urine, the urinary biomarker concentration is generally adjusted (or corrected) relative to the creatinine concentration or specific gravity in the urine sample. However, much controversy remains over the use of the methodologies for adjusting the urine biomarker levels due to inherent individual variability in physiological functions of the human body. For diisocyanate exposures, a common practice has been to normalize the amine urinary biomarker levels with creatinine concentration.[25–28] Previously, we reported that creatinine concentration was a significant covariate when used as an independent variable along with dermal and respirator-adjusted inhalation exposure to HDI.[13] We observed that including urinary creatinine concentration as a separate independent variable in the models provided better model fit than the traditional normalization method of dividing the urinary HDA level by the creatinine concentration. The independent variable method provided better model fit because the parameter estimate for creatinine was allowed to fluctuate as opposed to the parameter estimate being set as 1 with the traditional method.[13] Here, we report our findings on factors that affect creatinine concentration, specific gravity, and HDA levels in urine samples collected from spray painters occupationally exposed to HDI by using multiple linear regression analysis.

The urine collection time was a highly significant predictor for both creatinine concentration and specific gravity. Barr *et al.*[12] also observed that the time of day that a urine sample was collected had a small but statistically significant effect on the urinary creatinine concentration. Similarly, others have reported urinary creatinine concentrations[3–6] and specific gravity[3,4,6] fluctuate markedly. In contrast, Carrieri *et al.*[9] observed that spot urine samples did not demonstrate significant intra- or inter-day variations for either creatinine or specific gravity. However, in that study, only one morning and one afternoon urine samples were collected every day for five days from 14 men. Thus, the small number of intra-day samples per person (2) most likely limited the statistical power of intra-day variation observation.

Previously, age, gender, race/ethnicity, BMI, and fat-free mass have been observed to affect creatinine but not specific gravity levels.[5,9–12,24] These predictors did not affect creatinine concentration or specific gravity ratio in our study population. While the workers' demographic characteristics (age, BMI, and race/ethnicity) varied somewhat, they were all men sharing similar job characteristics, including frequent physical activity. This population homogeneity likely explains why our observations differed from the other published studies.

We observed that smokers had significantly increased creatinine concentrations ($p = 0.026$). Smoking has been associated with reduced serum creatinine concentrations.[29,30] As a group,

Table 3 Summary of the one (models 1–3) or two (models 4 and 5) cumulative exposure variable models for predicting natural log-transformed urinary HDA concentrations in the automotive spray painters using: (A) natural log-transformed creatinine concentration (lncreatinine), (B) specific gravity ratio (SG ratio), or (C) both lncreatinine and SG ratio as independent variables[a]

	A. lncreatinine					B. SG ratio					C. lncreatinine and SG ratio				
Model	Exposure variable	Estimate	Standard error	*p*-value	AIC	Exposure variable	Estimate	Standard error	*p*-value	AIC	Exposure variable	Estimate	Standard error	*p*-value	AIC
1	lnBZC	0.157	0.069	0.022	1037	lnBZC	0.166	0.071	0.019	1058	lnBZC	0.153	0.068	0.026	1033
	lncreatinine	1.254	0.168	<0.0001		SG ratio	2.062	0.349	<0.0001		lncreatinine	1.807	0.363	<0.0001	
											SG ratio	−1.217	0.738	0.100	
2	lnBZC-APF	0.179	0.055	0.001	1032	lnBZC-APF	0.190	0.058	0.001	1053	lnBZC-APF	0.170	0.056	0.002	1029
	lncreatinine	1.290	0.167	<0.0001		SG ratio	2.171	0.348	<0.0001		lncreatinine	1.749	0.360	<0.0001	
											SG ratio	−1.014	0.735	0.168	
3	lndermal	0.151	0.058	0.010	1036	lndermal	0.160	0.060	0.008	1057	lndermal	0.147	0.058	0.012	1032
	lncreatinine	1.256	0.168	<0.0001		SG ratio	2.071	0.351	<0.0001		lncreatinine	1.797	0.362	<0.0001	
											SG ratio	−1.190	0.740	0.108	
4	lnBZC	0.076	0.086	0.381	1038	lnBZC	0.081	0.089	0.365	1059	lnBZC	0.074	0.086	0.392	1034
	lndermal	0.113	0.074	0.124		lndermal	0.119	0.076	0.118		lndermal	0.110	0.073	0.135	
	lncreatinine	1.253	0.168	<0.0001		SG ratio	2.067	0.350	<0.0001		lncreatinine	1.791	0.362	<0.0001	
											SG ratio	−1.183	0.738	0.109	
5	lnBZC-APF	0.139	0.065	0.032	1035	lnBZC-APF	0.148	0.067	0.028	1055	lnBZC-APF	0.129	0.066	0.049	1031
	lndermal	0.079	0.068	0.244		lndermal	0.083	0.070	0.234		lndermal	0.080	0.068	0.238	
	lncreatinine	1.281	0.167	<0.0001		SG ratio	2.151	0.349	<0.0001		lncreatinine	1.744	0.360	<0.0001	
											SG ratio	−1.025	0.736	0.164	
	Worker var.	0.930				Worker var.	0.973				Worker var.	0.981			
	Residual var.	1.762				Residual var.	1.912				Residual var.	1.741			

[a] lnBZC = natural log-transformed breathing-zone concentration; lnBZC-APF = natural log-transformed respirator-adjusted breathing-zone concentration; lndermal = natural log-transformed dermal exposure; lncreatinine = natural log-transformed creatinine concentration; SG ratio = specific gravity ratio; AIC = Akaike's information criterion; var. = variance.

the smokers in our study were significantly younger in age than non-smokers (mean age for smokers and non-smokers 32.1 and 34.8, respectively; $p = 0.0017$), and they had a significantly higher BMI (mean for smokers and non-smokers 31.3 and 27.3, respectively; $p < 0.0001$). However, BMI and age were not significant predictors of creatinine concentration when used separately in the models. Thus, the relatively small number of subjects and the combined effect of smokers being younger and having a larger BMI, both of which have been associated with increased urinary creatinine concentrations, may have magnified this association in our study. However, it is possible that smoking causes reduced serum creatinine concentrations, as observed by Savdie *et al.*[29] and Pedersen,[30] which may have lead to increased urinary creatinine concentrations in our study population.

Natural log-transformed creatinine concentration and specific gravity ratio were always highly significant ($p < 0.0001$) when used alone with one or two exposure variables in the model (models A1–A5 and B1–B5). Thus, both natural log-transformed creatinine concentration and specific gravity ratio predicted the urine water content and thus the dilution level of HDA. Further, the significance levels of the exposure variables were very similar between the corresponding submodels. When creatinine concentration and specific gravity ratio were placed into the same model (models C1–C5), creatinine remained highly significant ($p < 0.0001$) while the p-value for specific gravity ratio ranged from 0.10 to 0.17.

All models using either natural log-transformed creatinine concentration or specific gravity ratio alone (models A1–A5 and B1–B5) indicated no predictive differences with regard to the HDA levels and exposure variables (all p-values < 0.0001). As reported previously,[13] BZC-APF ($p = 0.001$, model A1) was a more significant predictor of urine HDA levels than BZC ($p = 0.022$, model A2). Dermal exposure was also a significant predictor ($p = 0.010$, model A3) when used as a single exposure variable along with natural log-transformed creatinine concentration in the multiple linear regression analysis. Similarly, models in which specific gravity ratio was used as an independent variable indicated that both dermal and inhalation exposures were significant predictors of urinary HDA levels (models B1–B3). As with creatinine concentration, the specific gravity ratio models also showed that BZC-APF was a more significant predictor ($p = 0.001$; model B2) of HDA levels than BZC ($p = 0.019$; model B1).

The workers in our study were physically active during the entire workday. This constant activity may be one of the reasons that creatinine provides better fitting exposure models than specific gravity. Newman *et al.*[7] found that normalization for creatinine concentration in urine samples corrected for exercise-induced proteinuria. Exercise has also been observed to increase serum protein levels in urine.[31,32] Possible exercise-induced proteinuria due to work activities requiring constant physical straining during the workday may explain why 29 urine samples were too concentrated based on creatinine concentration, but only nine of those were too concentrated based on the specific gravity. Based on the specific gravity and creatinine concentration of the urine samples, a total of 92 samples should have been excluded for being too dilute or concentrated. Rosenberg *et al.*[23] stated that biomarker levels in urine samples outside the normal concentration range may not be reliable because the excretion mechanism may be altered. We chose not to exclude any of the urine samples from our analyses because the excretion mechanism for HDA is not completely understood.

Since we observed previously that the traditional normalization of urine HDA level by dividing it with the creatinine concentration of the sample was not an optimal method,[13] we sought to test if the same was true for normalization with specific gravity ratio. To accomplish this, we modeled the natural log-transformed specific gravity ratio as an independent variable along with the dermal and inhalation exposure variables to test if its parameter estimate was close to 1. The analyses of the models, conducted as shown in Table 3 for specific gravity ratio without log-transformation (model B), showed that the natural log-transformed specific gravity ratio had parameter estimates ranging from 1.14 to 1.19 (data not shown). Therefore, using specific gravity ratio as an independent variable provided only a minor improvement over the traditional normalization method. Natural log-transformed specific gravity ratio was not normally distributed, but specific gravity ratio was normally distributed. Consequently, specific gravity ratio was used in exposure model development.

As a limitation of the study, we were not able to collect all urine from each void during our study, nor were we able to collect urine samples either before the workers arrived or after they departed the work place. Thus, we could not investigate the use of urinary volume along with creatinine and specific gravity as a method to account for the hydration status of the worker. The inherent difficulty of obtaining the entire urine void throughout the 24 h period often precludes the use of urinary volume for monitoring of biomarkers in occupational and environmental setting. However, future studies should be conducted to investigate whether urinary volume may indeed be more appropriate to use for monitoring of urinary amines as biomarkers for diisocyanate exposure.

In summary, the data indicate that urinary HDA concentration should be adjusted for the water content of urine in this exposed population. Further, the models demonstrate that normalization with creatinine concentration provides a better model fit than normalization with specific gravity ratio when monitoring urine HDA levels in HDI-exposed workers. The observation that smoking status along with urine collection time affected the creatinine concentrations emphasizes a problem with traditional normalization. This observation provides another reason to use creatinine concentration as an independent variable (as proposed by Barr *et al.*[12]) instead of traditional normalization by dividing the HDA level with creatinine concentration in the urine sample when biological monitoring of HDI exposure is conducted. If other variables are tested in the model, using creatinine as an independent variable will assure that the relationship between a variable and HDA level, and not creatinine concentration, is discovered. Our results are supported by previous findings indicating that creatinine concentration can be affected by variables specific to that individual.[5,9–12,24]

Conclusion

To our knowledge, this is the first study to report on the suitability of using creatinine concentration or specific gravity for normalization of urinary amine levels after diisocyanate

exposure. We demonstrate that urinary creatinine concentration is more appropriate than normalization with specific gravity ratio when monitoring urine HDA levels in HDI-exposed workers. Further, our findings confirm that traditional normalization method of dividing the urinary HDA level by the creatinine concentration should not be used due to individual factors (*e.g.*, smoking) that affect the creatinine concentration in urine.

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