

## Hexamethylene diisocyanate (HDI) vapor reactivity with glutathione and subsequent transfer to human albumin

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### ABSTRACT

**Introduction:** Airway fluid glutathione (GSH) reactivity with inhaled vapors of diisocyanate, a common occupational allergen, is postulated to be a key step in exposure-induced asthma pathogenesis.

**Methods:** A mixed (vapor/liquid) phase exposure system was used to model the *in vivo* reactivity of inhaled HDI vapor with GSH in the airway fluid. HDI–GSH reaction products, and their capacity to transfer HDI to human albumin, were characterized through mass spectrometry and serologic assays, using HDI-specific polyclonal rabbit serum.

**Results:** HDI vapor exposure of 10 mM GSH solutions resulted in primarily S-linked, bis(GSH)–HDI reaction products. In contrast, lower GSH concentrations (100 μM) resulted in mainly mono(GSH)–HDI conjugates, with varying degrees of HDI hydrolysis, dimerization and/or intra-molecular cyclization, depending upon the presence/absence of  $H_2PO_4^-/HPO_4^{2-}$  and  $Na^+/Cl^-$  ions. The ion composition and GSH concentration of the fluid phase, during HDI vapor exposure, strongly influenced the transfer of HDI from GSH to albumin, as did the pH and duration of the carbamoylating reaction. When carbamoylation was performed overnight at pH 7, 25 of albumin's lysines were identified as potential sites of conjugation with partially hydrolyzed HDI. When carbamoylation was performed at pH 9, more rapid (within 3 h) and extensive modification was observed, including additional lysine sites, intra-molecular cross-linkage with HDI, and novel HDI–GSH conjugation.

**Conclusions:** The data define potential mechanisms by which the levels of GSH,  $H_2PO_4^-/HPO_4^{2-}$ , and/or other ions (e.g.  $H^+/OH^-$ ,  $Na^+$ ,  $Cl^-$ ) affect the reactivity of HDI vapor with self-molecules in solution (e.g. airway fluid), and thus, might influence the clinical response to HDI respiratory tract exposure.

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### 1. Introduction

Isocyanate (N=C=O) chemicals used to make polyurethane are a well-recognized cause of occupational asthma (Bernstein, 1999; Redlich and Karol, 2002; Tarlo and Liss, 2002). Two classes of isocyanates, aromatic and aliphatic, possess different chemical properties and are typically used for distinct applications (<http://www.cdc.gov/niosh/topics/isocyanates/>; Klees and Ott, 1999; Ulrich, 1996). Aromatic isocyanates, such as toluene diisocyanate (TDI) and methylene diphenyl diisocyanate (MDI) are excellent for foam production, elastomers and durable coatings (e.g. truck-bed liners), but are sensitive to photo-oxidation (Allport et al., 2003; Davis and Sims, 1983; Ulrich, 1996). Aliphatic isocyanates,

such as HDI, possess better UV light resistance, and are typically used for exterior coatings, such as protective finishes on automobiles and aircrafts (Davis and Sims, 1983; <http://www.alipa.org/>; Ulrich, 1996).

The pathogenic mechanisms through which isocyanates cause asthma remain uncertain but, are believed to depend upon N=C=O reactivity with "self" molecules at exposure sites (Day et al., 1996, 1997; Lange et al., 1999; Lantz et al., 2001; Wisnewski and Jones, 2010). The fluid lining the human lower respiratory tract contains a relatively high level (>100 μM) of an essential tri-peptide, GSH, postulated to be a primary reactant for inhaled isocyanates (Cantin et al., 1987; Day et al., 1997; Lange et al., 1999; Lantz et al., 2001). Airway fluid GSH levels are normally maintained within a limited range, by complex genetically-defined homeostatic feedback mechanisms (Day et al., 2004; Rahman and MacNee, 2000). However, substantial individual variability may occur secondary to environmental exposures (e.g. smoking, infection), or genetic mutations/polymorphisms, and has been suggested to influence the pathogenesis of several different diseases (Day, 2005, 2009; Pacht et al., 1991; Rahman and MacNee, 1999, 2002; Roum et al.,

**Abbreviations:** GSH, reduced glutathione; GSSG, oxidized glutathione; HDI, hexamethylene diisocyanate; TDI, toluene diisocyanate.

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1999; Smith et al., 1993; van der Vliet et al., 1999). In animal studies, inhalation of isocyanate vapors acutely alters airway fluid GSH levels, while GSH depletion exacerbates MDI's respiratory tract toxicity (Pauluhn, 2000a,b). Among occupationally exposed workers, the development of isocyanate asthma, and rate of chemical excretion have been associated with genetic polymorphisms in GSH-dependent enzymes (Broberg et al., 2010; Littorin et al., 2008; Mapp et al., 2002; Piirila et al., 2001; Wikman et al., 2002).

*In vitro* studies have begun to dissect possible mechanisms through which isocyanate reactivity with GSH might influence immunologic and/or other biological responses to exposure. For the aromatic diisocyanate, TDI, it has been demonstrated that chemical vapors readily cross a liquid phase barrier to react with GSH, and cause acute depletion of intracellular GSH in human airway epithelial cells (Lantz et al., 2001; Wisnewski et al., 2011). Furthermore, thiol-linked vapor TDI–GSH reaction products are capable of carbamoylating (transferring TDI to) peptide/protein molecules, including human albumin, the major “carrier” protein for TDI *in vivo*, and essential element of allergenic (IgE) recognition by the human immune system (Day et al., 1997; Lange et al., 1999; Wass and Belin, 1989; Wisnewski et al., 2011).

The reactivity of GSH with HDI, and other aliphatic isocyanates used in polyurethane manufacturing, remains less clear and may differ from aromatic isocyanates, given inherent differences in their chemical reactivity. The hydrolysis of aliphatic isocyanates is much slower than aromatic isocyanates, which may provide greater opportunity to react with GSH in solution (Brown et al., 1987). Thiol-linked (e.g. GSH) reaction products with aliphatic isocyanates are likely more stable than reaction products with aromatic isocyanates, based on prior studies with cysteine-methyl-ester (Chipinda et al., 2006). In short-term (1 h) vapor exposure studies *in vitro*, GSH has been reported to prevent HDI protein conjugation, rather than mediate transcarbamoylation as observed with TDI (Day et al., 1997; Wisnewski et al., 2005, 2011). A better understanding of the GSH reactivity with HDI vapor should provide insight into the basic mechanisms underlying the biological effects of HDI exposure.

The present study used a mixed (vapor/liquid) phase exposure system to model the biophysics of HDI reactivity in the airway microenvironment, where vapor phase chemical contacts the airway lining fluid, which contains high levels of GSH (Wisnewski et al., 2004, 2011). A variety of physiologically relevant, inter-dependent exposure variables (pH, [GSH], and ion/buffer content of the solution) were evaluated for their potential influence on HDI vapor phase reactivity with GSH, and subsequent carbamoylation of human albumin, the major carrier protein for isocyanates *in vivo*. The potential biological relevance of the *in vitro* data is discussed.

## 2. Experimental procedures

### 2.1. HDI vapor exposure

Solutions (100  $\mu$ M–10 mM) of reduced or oxidized glutathione (GSH or GSSG respectively) from Sigma (St. Louis, MO) were prepared in 20 mM phosphate buffered saline (PBS, pH 7.4) from Gibco (Grand Island, NY), 140 mM NaCl, or de-ionized water (Sigma). All GSH solutions and reaction products were 0.2  $\mu$ m filtered (Millipore; Billerica, MA) to ensure sterility. Solutions were exposed as previously described, to room air or HDI vapor, for 18 h, in 35  $\times$  10 mm Petri dishes obtained through VWR International from Bridgeport, NJ (Wisnewski et al., 2004, 2011). HDI vapors were obtained by passive diffusion from puriss grade HDI (PubChem Substance ID: 24874557, CAS Number: 822-06-0) solution (Sigma-Aldrich),  $\geq 99\%$  purity by gas chromatography,

with a refractive index of  $n_{20}/D$  1.453, and a density of 1.047 g/mL at 20 °C. HDI vapor concentration was monitored with an Auto-step toxic gas monitor (GMD Systems, Pittsburgh, PA, USA), and maintained at  $180 \pm 20$  ppb, by adjusting the intake air flow rate. The mixed phase exposure conditions were empirically developed in prior studies, based on specific recognition of (albumin) reaction products by serum IgG from HDI exposed workers. Each exposure condition was replicated in four independent experiments to determine biological variability of the experimental results.

### 2.2. LC–MS

Experiments were performed on a Waters (Milford, MA) nanoACQUITY ultra-performance liquid chromatography (UPLC) system. Aliquots (1  $\mu$ L) of each GSH–HDI mixture were injected and trapped/desalted on a 5  $\mu$ m SymmetryC<sub>18</sub> (180  $\mu$ m  $\times$  20 mm) trapping column with 99.5/0.5 A/B (A:0.1% formic acid; B:0.1% formic acid in acetonitrile) at a flow rate of 15  $\mu$ L/min for 1 min. Separation was performed on a 1.7  $\mu$ m BEH130 C<sub>18</sub> (100  $\mu$ m  $\times$  100 mm) analytical column utilizing gradient elution at a flow rate of 400 nL/min and a gradient of 99/1 to 60/40 A/B over 60 min. The eluent from the UPLC system was directed to the nanoelectrospray source of a Waters SYNAPT MS quadrupole time-of-flight (qTOF) mass spectrometer. Positive ion nanoelectrospray was performed utilizing 10  $\mu$ m PicoTip (Waters) emitters held at a potential of +3.5 kV. The cone voltage was held constant at +40 V for all experiments. Dry N<sub>2</sub> desolvation gas was supplied to the instrument via a nitrogen generator (NitroFlowLab, Parker Hannifin Corp., Haverhill, MA). [Glu]<sup>1</sup>-Fibrinopeptide B (100 fmol/ $\mu$ L in 75/25 A/B) was supplied to an orthogonal reference probe and the [M + 2H]<sup>2+</sup> ion ( $m/z$  = 785.84265 u) measured as an external calibrant at 30 s intervals. Ultra-high purity (UHP) argon was used as collision gas. Data were analyzed with MassLynx v. 4.1 (Waters). Samples from four independent experiments were analyzed to assess reproducibility of the experimental findings.

### 2.3. GSH–HDI mediated carbamoylation of human albumin

Solutions of HDI vapor exposed GSH were co-incubated 1:2 (v/v) with a 5 mg/mL solution of human albumin (Sigma) at 37 °C. Initial studies, including those analyzed by ELISA and Western blot (Fig. 3A and B), were performed overnight in 0.1 M carbonate, pH 9.0. Subsequent carbamoylation reactions were performed for varying time periods (1 h–3 days) in carbonate buffer, or at pH 7.0 using 0.1 M (mono/dibasic) phosphate buffer. Four different experiments were performed in triplicate using GSH–HDI reaction products from four independent experiments.

### 2.4. Anti-HDI ELISA

Maxisorp® microtiter plates from Nunc (VWR International) were incubated overnight at 4 °C with 5  $\mu$ g/well of human albumin that had been co-incubated with solutions of HDI vapor-exposed GSH. Plates were coated in 0.1 M carbonate buffer, pH 9.5, washed, and “blocked” with 3% (w/v) dry milk, before addition of anti-HDI rabbit serum, diluted 1:200 (v/v). As previously described, the HDI-specific rabbit polyclonal antiserum was raised against HDI–KLH, depleted of KLH binding activity by affinity chromatography, and specifically recognizes HDI when bound to a larger carrier protein, such as human albumin (Wisnewski et al., 1999, 2000, 2004, 2008, 2010). ELISA plates were developed with peroxidase-conjugated anti-rabbit IgG (Pharmingen; San Diego, CA), diluted 1:2000 (v/v), and TMB substrate. Optical density (OD) measurements (absorbance of light at 450 nm, minus absorbance at a reference wavelength), reflecting detection of HDI, were obtained on a Benchmark microtiter plate reader from Bio-Rad. Samples from

four independent experiments, described above, were analyzed in triplicate to obtain mean and standard error values. Statistical differences in IgG binding data were calculated using the Wilcoxon rank-sum test.

### 2.5. Anti-HDI Western blot

Human albumin carbamoylation reactions (1  $\mu$ L) or control samples from three independent experiments were electrophoresed under reducing conditions (without heating to avoid protein aggregation) on precast 4–15% gradient gels and transferred to nitrocellulose membrane using a trans-blot system from BioRad (Hercules, CA). Nitrocellulose membranes were blocked with 3% dry milk in PBS, and probed with rabbit anti-HDI polyclonal antiserum (anti-KLH depleted), diluted 1:200 as previously described (Wisnewski et al., 1999, 2000, 2004, 2008, 2010). Following incubation with 1:1000 peroxidase-conjugated anti-rabbit IgG (Pharmingen; San Diego, CA), blots were developed with enhanced luminescence reagent from Thermo Fisher Scientific (Rochester, NY).

### 2.6. Quantitative measurements of hexamethylene diamine (HDA)

Samples were treated with equal volume of 3 M  $\text{H}_2\text{SO}_4$  at 100 °C for 16 h, and then neutralized with 2.5 volumes of saturated sodium hydroxide, vortexed and cooled in an ice bath to cool for 10 min. Samples were extracted 2× with dichloromethane (DCM), evaporated at 40 °C under  $\text{N}_2$ , to 1 mL. Five hundred microliters of 0.5%  $\text{H}_2\text{SO}_4$  was then added to the extract, of which 250  $\mu$ L was finally used for derivatization. An equal volume of saturated borate buffer (pH 8.5) and 450  $\mu$ L of acetonitrile were added and vortexed for 1 min before adding 50  $\mu$ L of 15 mg/mL fluorescamine in acetonitrile. Samples were separated on a 250 × 4.6 mm, 5  $\mu$ m particle size Discovery C18 column run using a Shimadzu Prominence HPLC system (Columbia, MD, USA) consisting of an online vacuum degasser (model DGU-20A5), a quaternary pump (model LC-20AT), autosampler (model SIL-10AD-VP), and fluorescence detector (model RF-10AXL). HDA-fluorescamine derivatives were eluted from the column into a fluorometer at 1 mL/min using a linear solvent gradient of 0–30% ACN/water. Analytes were excited at 410 nm and emission was measured at 510 nm. The concentration of HDA in albumin samples carbamoylated by GSH–HDI was based on calibration standards containing 10–1600 ng/mL HDA in 1.65 mg/ml albumin solution in PBS, which were acid hydrolyzed at the same time. Analysis of samples from three independent experiments is provided in *Supplemental material* (on-line Fig. E6).

### 2.7. MS/MS analysis of GSH–HDI reaction products

For FT-ICR MS analysis, total GSH–HDI reaction products were desalting using a C18 ZipTip, eluted into 60% acetonitrile/0.1% formic acid and infused, via Triversa NanoMate, into a Bruker 9.4T FT-ICR MS (Bruker Daltonics; Billerica, MA), as previously described (Stone et al., 2007; Wisnewski et al., 2011).

### 2.8. Preparative reverse phase HPLC purification of GSH–HDI reaction products

GSH–HDI reaction products from three independent experiments were fractionated by the Yale Keck Center on a Hewlett-Packard 1090 HPLC system equipped with an Isco Model 2150 Peak Separator and a 1 mm × 25 cm Vydac C-18 (5  $\mu$ m particle size, 300 Å pore size) reverse phase column (Williams and Stone, 1997). Following equilibration with 98% buffer A (0.06% TFA) and 2% buffer B (0.052% TFA, 80% acetonitrile), mono(GSH)–HDI\* and bis(GSH)–HDI reaction products eluted with distinct retention times (~12 and ~20 min, respectively), when buffer B was increased from 2% to 37% over the course of 1 h. Elution was tracked by  $A_{210}$ , and purity was verified by MS/MS. For carbamoylation reactions, fractions containing equimolar amounts of mono (GSH)–HDI\* or bis(GSH)–HDI, based on HDA released by acid hydrolysis, were speed-vacuumed to dryness and resuspended with 100  $\mu$ L of 3.3 mg/mL human albumin in 0.1 M carbonate, pH 9.0 and incubated overnight at 37 °C.

### 2.9. MS/MS identification of albumin carbamoylation sites

Samples of albumin carbamoylated by GSH–HDI, were reduced, acetylated, and trypsin digested, prior to LC–MS/MS at the Yale University Keck Center, as previously described (Stone et al., 2007; Williams and Stone, 1997). Samples from three independent experiments were analyzed on an LTQ Orbitrap Elite mass spectrometer and all MS/MS spectra were searched using the automated Mascot algorithm against the NCBInr database. A 95% confidence level was set within the MASCOT search engine for protein hits based on randomness search. In addition, two or more MS/MS spectra must have matched the same protein entry and been derived from trypsin digestion. Peptide scores >20 are likely correct based on past experience, and the higher the score the better the match. In addition to oxidation of methionine and acetylation of cysteine (during workup), the data were further queried for anticipated mass modifications due to carbamoylation by GSH–HDI reaction product(s). *Supplemental material* Fig. E1 provides chemical structures of the predicted  $\Delta$  mass modifications shown in Table 1.

## 3. Results

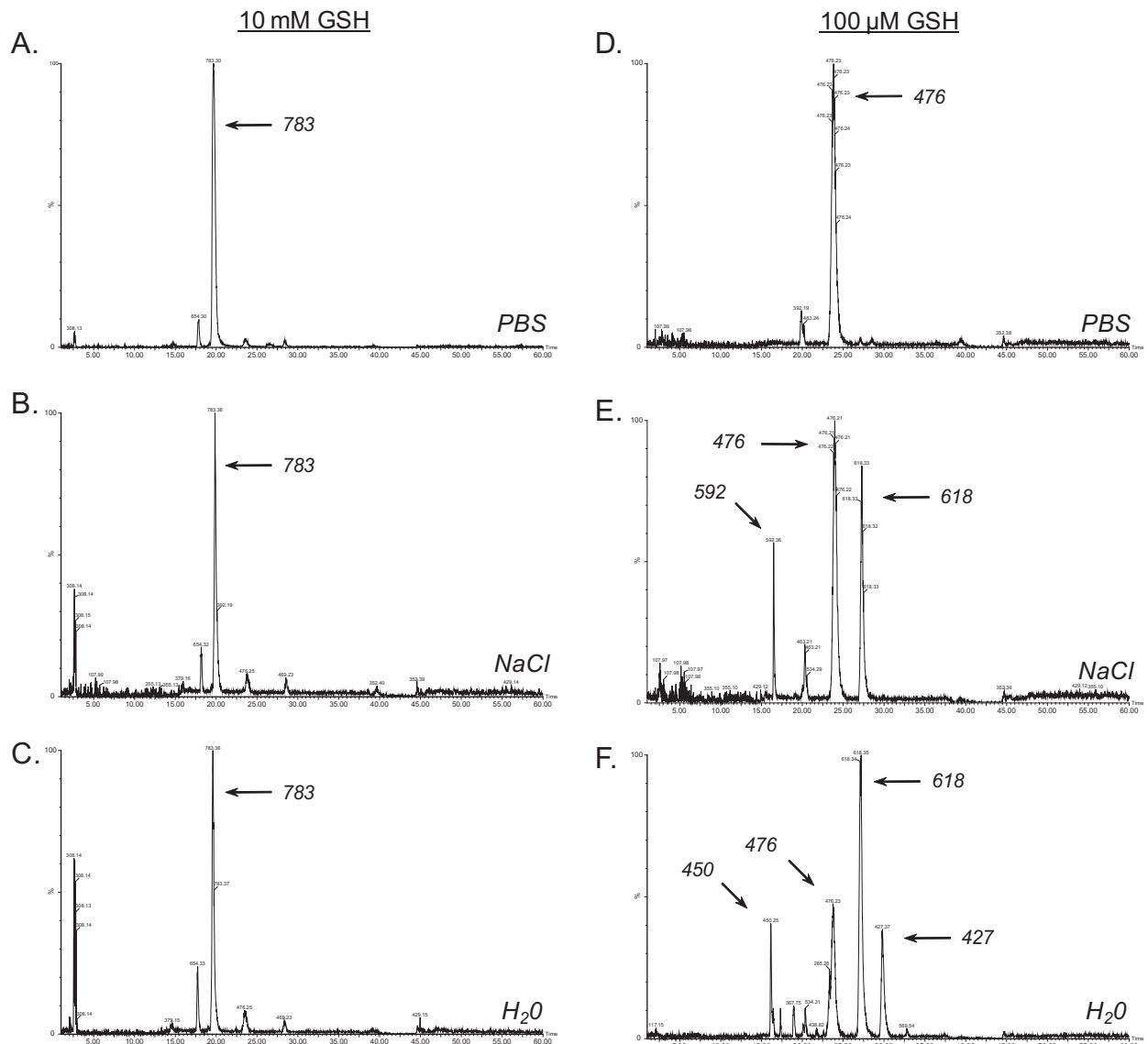
### 3.1. HDI–GSH reaction products resulting from mixed phase exposure

Reaction products of HDI vapor with reduced glutathione (GSH) in fluid phase, were analyzed by LC–MS methods. Initial experiments were performed with solutions of GSH at two different concentrations, 10 mM and 100  $\mu$ M, to mimic approximate human intra- and extra-cellular (e.g. airway fluid) concentrations. When the aqueous phase contained 10 mM GSH, HDI vapor exposure consistently resulted in a single major product, with an LC-column retention time around 20 min, and an  $m/z$  of 783.3 (Fig. 1). Further MS/MS analysis (see *Supplemental materials*, Fig. E2) identified the product as bis(GSH)–HDI, in which HDI's two NCO groups were conjugated via thiocarbamate linkages to GSH's cysteine side

**Table 1**

Predicted mass modifications due to carbamoylation with HDI via GSH.

$\Delta$ Mass	Chemical formula	Description
142.11	$\text{C}_7\text{H}_{14}\text{N}_2\text{O}$	HDI*, *2nd NCO hydrolyzed
168.09	$\text{C}_8\text{H}_{12}\text{N}_2\text{O}_2$	HDI, cross-linked
532.19	$\text{C}_{20}\text{H}_{32}\text{N}_6\text{O}_5\text{S}$	HDI–GSH (2nd NCO conjugated to $\gamma$ -glu of GSH, acetylation of GSH's thiol)
674.30	$\text{C}_{27}\text{H}_{46}\text{N}_8\text{O}_{10}\text{S}$	HDI*–HDI–GSH (two HDI molecules linked via one hydrolyzed NCO, conjugated to GSH's $\gamma$ -glu, acetylation of GSH's thiol)



**Fig. 1.** LC-MS analysis of GSH-HDI reaction products formed under different conditions. LC-MS base peak intensity chromatograms of the reaction products formed in the presence of 10 mM (left) or 100  $\mu$ M GSH, dissolved in PBS, or  $\text{H}_2\text{O}$  as labeled. The  $m/z$  of the major products are highlighted. Data shown are representative of  $N = 4$  independent experiments.

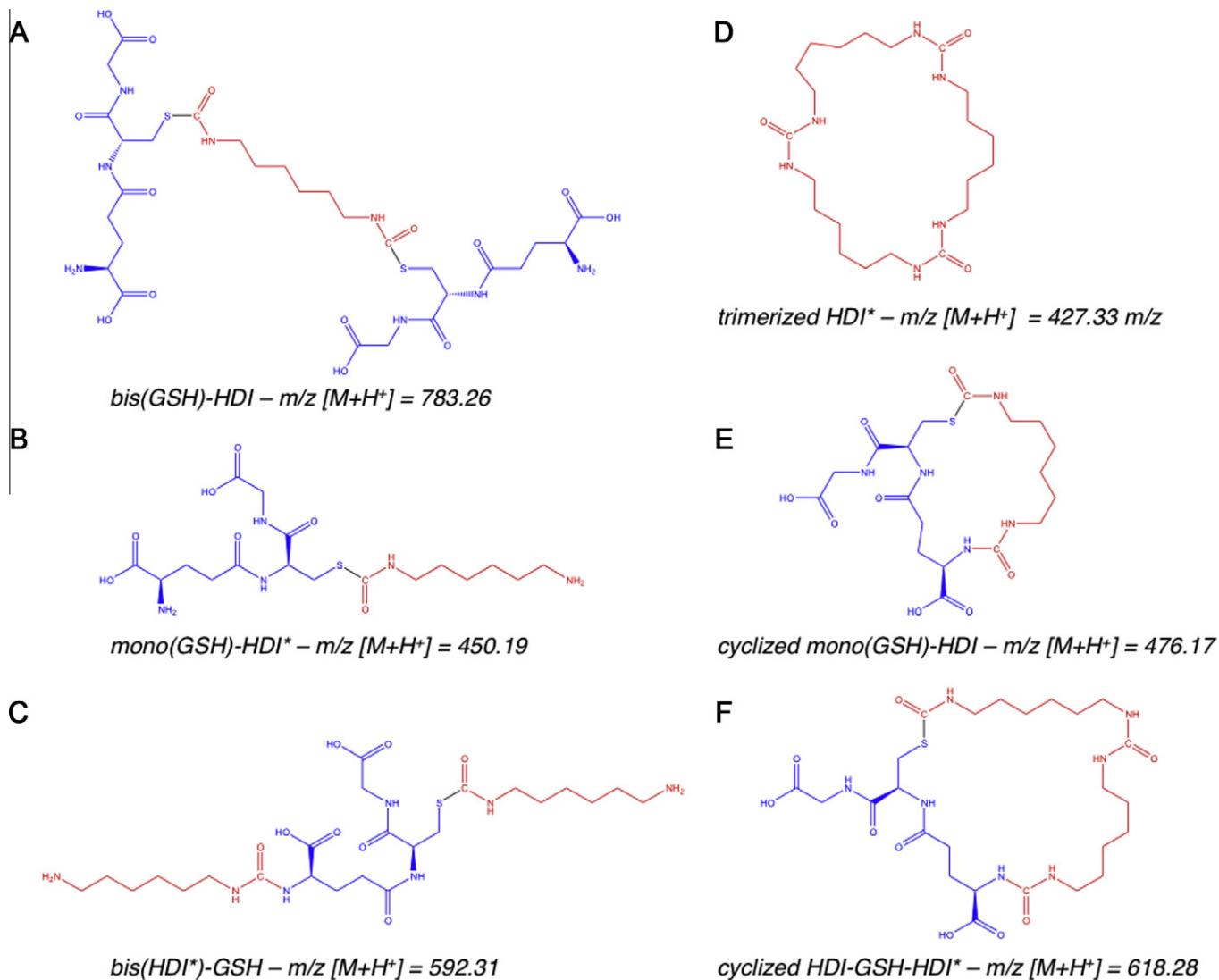
chains (rather than via carbamide linkages to the primary amine of  $\gamma$ -glutamate) as shown in Fig. 2A. Formation of bis(GSH)-HDI was similarly observed when 10 mM GSH was dissolved in PBS, 140 mM NaCl, or deionized H<sub>2</sub>O (Fig. 1A–C).

When HDI vapor exposed solutions contained 100-fold lower concentrations of GSH (e.g. 100  $\mu$ M), LC-MS analysis revealed different reaction products, which further varied depending upon the ionic composition of the fluid phase (Fig. 1D-F). In PBS, a single major product, with a *m/z* of 476.2 was observed. In saline solution (140 mM NaCl), the 476.2 *m/z* product was similarly present; however, two other major products were also observed, with *m/z* of 618.3 and 592.3. In deionized H<sub>2</sub>O, four major GSH-HDI reaction products were observed; the 618.3 *m/z* and 476.23 *m/z* products described above, as well as unique products with *m/z* of 450.2 and 427.4. The structures of the 100  $\mu$ M GSH reaction products with HDI vapor were further characterized by MS/MS (see *Supplemental materials* Figs. E3-E5), which confirmed molecular assignments based on the exact mass (Fig. 2). The data identify four different GSH-HDI reaction products, each containing a single

GSH molecule, in contrast to the major bis(GSH) product formed at higher (10 mM) GSH concentrations. Furthermore, lower (100  $\mu$ M) GSH concentration resulted in more reaction with GSH's "amino" terminus, intramolecular cyclization, and/or reaction products with two HDI molecules. Together, the data describe the formation of markedly different fluid phase reaction products, despite identical HDI vapor exposure, depending upon the GSH concentration and ionic composition.

### 3.2. Carbamoylation of human albumin by GSH-HDI reaction products

The potential influence of the fluid phase starting composition (GSH concentration, buffer/ion content) on the subsequent carbamoylation capacity of different HDI vapor exposed solutions was initially investigated through ELISAs and Western blots with HDI-specific polyclonal rabbit serum. Under the conditions tested, HDI vapor exposed-solutions possessed the greatest capacity to "antigenically" transfer HDI to human albumin, when GSH was present at 1 mM (Fig. 3A). However, the relationship between



**Fig. 2.** Chemical structures of GSH-HDI reaction products. The chemical structures of the major reaction products of HDI vapor with fluid phase GSH are based on the exact mass and MS/MS data included in the *Supplemental materials*.

starting GSH concentration and carbamoylating capacity appeared to be non-linear, and significantly ( $p < 0.002$ ) influenced by the presence of  $\text{H}_2\text{PO}_4^-/\text{HPO}_4^{2-}$  ions (Fig. 3A and B). Further studies suggested that the pH level during the albumin carbamoylation reaction (after HDI vapor exposure) also had substantial affects on HDI transfer kinetics. As shown in Fig. 3C, “antigenic” HDI transfer occurred rapidly (within 1 h), and reached near maximal levels within 3 h at pH 9. In contrast, modification occurred significantly ( $p < 0.001$ ) slower at pH 7, requiring 3 days to reach maximal levels.

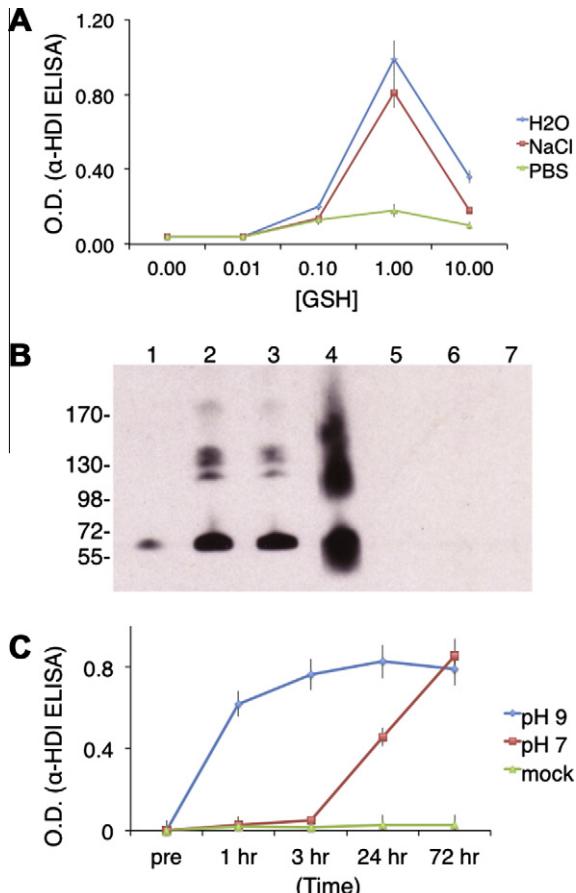
Quantitation of the amount of HDI transferred to human albumin by GSH-HDI was subsequently evaluated by measuring the amount of hexamethylene diamine (HDA) released from selected samples upon acid hydrolysis (e.g. mol hydrolyzed HDA/mol albumin). The maximum amount of HDI/albumin (e.g. substitution ratio) mediated by GSH transfer was 0.47 ( $\pm 0.18$ ), while albumin directly exposed to HDI vapor contained 1.99 ( $\pm 0.4$ ) mol HDI/mol albumin. Overall, the levels of HDA released by acid hydrolysis correlated well with anti-HDI ELISA absorbance measurements of individual samples ( $r^2 = 0.91$ ) (Supplemental Fig. E6, and data not shown).

### 3.3. Evidence for mono(GSH)-HDI\* as the major carbamoylating reaction product of HDI vapor with GSH

Additional comparative LC-MS was performed to characterize HDI exposed GSH solutions with potent vs. weak carbamoylating capacity, as defined immunochemically. As shown (Fig. 4), mono(GSH)-HDI\* is uniquely present in HDI vapor exposed solutions with potent carbamoylating capacity (1 mM GSH in deionized  $\text{H}_2\text{O}$ ), and consistently absent from HDI exposed solutions (1 mM GSH in PBS) with weak carbamoylating activity. Further (anti-HDI) Western blot analysis (Fig. 5), with different HPLC purified GSH-HDI products, similarly implicate mono (GSH)-HDI as the major carbamoylating species formed upon HDI vapor exposure of fluid phase GSH.

### 3.4. Loci of albumin carbamoylation by GSH-HDI

The loci of albumin modification by HDI vapor exposed-GSH reaction products were assessed by LC-MS/MS. Data from three independent experiments consistently identify GSH-mediated HDI conjugation of up to 25 of albumin's lysines (Fig. 6, and



**Fig. 3.** Carbamoylation of human albumin by GSH-HDI based on immunochemical analyses. Panel A-HDI vapor exposed solutions containing different concentrations of GSH (X-axis = mmoles/L) in PBS, NaCl, or H<sub>2</sub>O, were tested for their ability to transfer HDI to human albumin, based on HDI-specific polyclonal rabbit serum IgG binding. \*p < 0.002 comparing PBS vs. H<sub>2</sub>O or NaCl when [GSH] is 1 mM. Panel B-Western blot with HDI-specific antisera of human albumin co-incubated with HDI vapor exposed solutions of 1 mM GSH in PBS (lane 1), H<sub>2</sub>O (lane 2), or NaCl (lane 3). For comparison, albumin directly conjugated by HDI vapors (lane 4), or incubated with control (room air exposed) GSH solutions prepared in PBS (lane 5), H<sub>2</sub>O (lane 6), or NaCl (lane 7). Panel C-solutions of HDI vapor exposed 1 mM GSH in H<sub>2</sub>O were tested for their ability to transfer HDI to albumin over time (X-axis) at pH 7.0 or pH 9.0 as labeled. \*p < 0.001 comparing pH 7 vs. pH 9 at 1, 3 and 24 h. Note: ELISA O.D. values (Y-axis) in Panels A and C reflect “antigenic” HDI transfer (mean and standard error) observed in N = 4 independent experiments, while Western blot in Panel B is representative of N = 3 independent experiments.

Supplemental materials, Table E2) when carbamoylation was performed overnight at pH 7, with the total reaction products of 1 mM GSH in deionized H<sub>2</sub>O (which exhibited maximal “antigenic” HDI transfer). Ion fragmentation patterns upon CID further suggested hydrolysis of the unbound NCO group of HDI, to a free amine (see Supplemental materials Fig. E1a and Table E1) (Hettick et al., 2011; Wisnewski et al., 2004). The potential sites of albumin modification by GSH-HDI were numerous compared with those directly conjugated by HDI vapors in previous reports, and overlapped with, but were qualitatively distinct from those conjugated by GSH-TDI, or direct exposure to TDI or MDI (Hettick and Siegel, 2012; Hettick et al., 2011; Wisnewski et al., 2004, 2010, 2011). Among the sites carbamoylated by GSH-HDI were all (four) of albumin’s di-lysine site motifs, and Lys<sup>351</sup>, recently suggested to be most susceptible to direct TDI vapor conjugation (Hettick et al., 2011). It should be noted that although the number of potential HDI conjugation sites (via GSH-HDI) was numerous, the absolute amount of conjugation/albumin molecule was relatively low,

based on HDA analysis as described above, suggesting conjugation to different lysine groups in different albumin molecules.

Consistent with the immunochemical assessment of HDI transfer, LC-MS/MS analysis demonstrated more extensive and rapid albumin carbamoylation by GSH-HDI at pH 9 vs. pH 7. When albumin carbamoylation was performed for three hours at pH 9.0, an additional eight lysines were modified by HDI, in which the unbound NCO was hydrolyzed as described above (Fig. 6 and Supplemental materials Table E2). Also observed were internal cross-linking of an individual peptide with HDI (see Supplemental materials Fig. 1b), and a previously undescribed carbamoylation product apparently resulting from reaction of albumin’s K<sup>389</sup> with intra-molecularly cyclized GSH-HDI, as shown (Fig. 7 and Supplemental material Figs. E1c, E7, E8 and Table E2). Together, the mass spectrometry data identify specific loci of structural modification, and describe novel HDI-mediated cross-linking of GSH to human albumin.

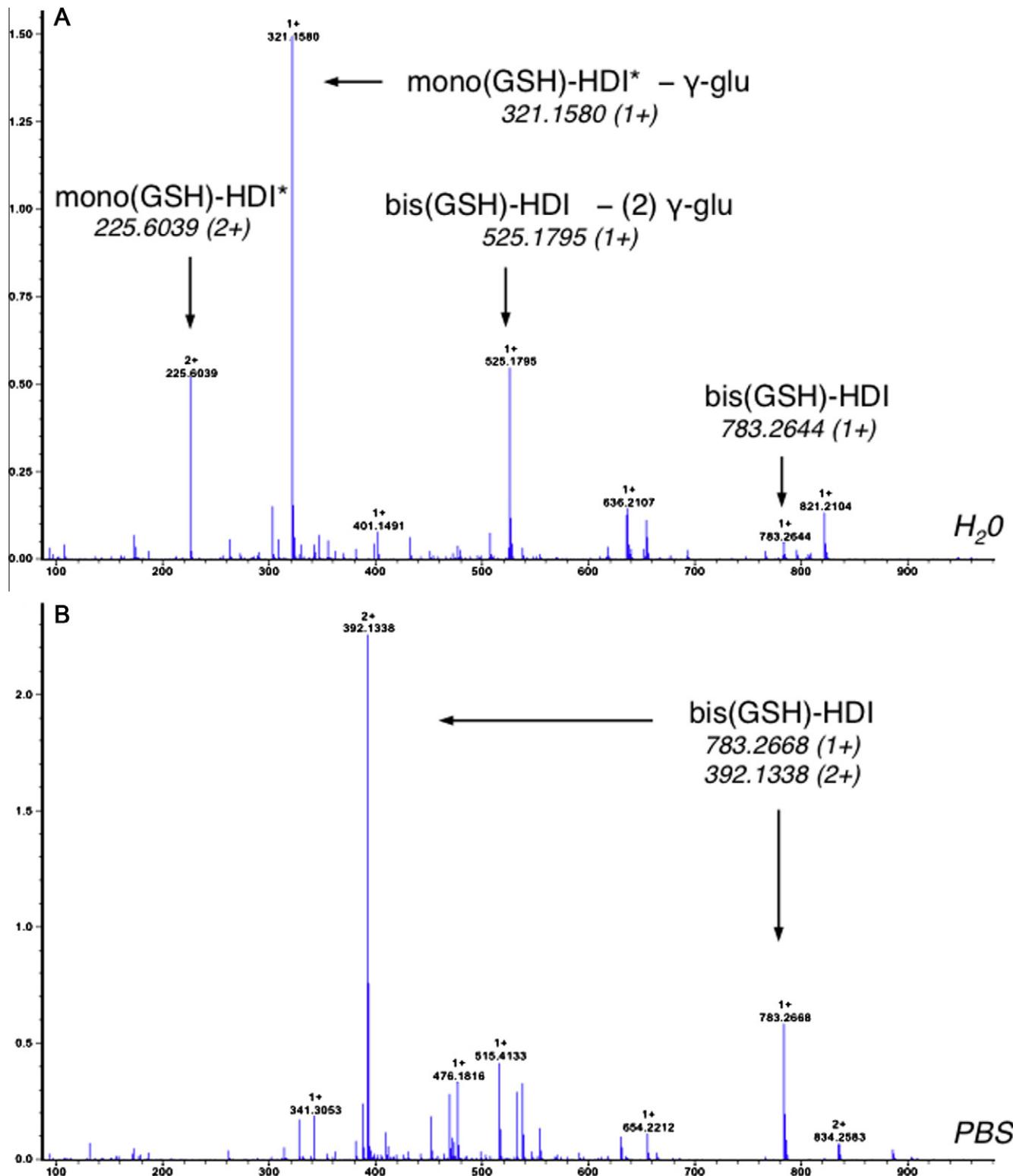
#### 4. Discussion

This report demonstrates the ability of the aliphatic diisocyanate, HDI, to react with glutathione across a vapor/liquid phase boundary, and describes unique reaction products formed in the presence of physiologic GSH concentrations. Notably, different reaction products formed under identical exposure conditions, depending upon the GSH concentration and the ionic composition of the fluid phase. Among the different vapor HDI-GSH reaction products, mono(GSH)-HDI\* exhibited particularly potent carbamoylating activity, based on immunochemical analyses, and was a major product present in HDI-vapor exposed GSH solutions proven to transfer HDI to albumin by LC-MS/MS. Together, the data support a hypothetical mechanism by which GSH in the airway fluid may mediate HDI vapor uptake from the respiratory tract, as reversible reaction products. The data further suggest that individual differences in airway fluid composition could modify this process.

The present study extends recent investigations modeling mixed (vapor/liquid) phase reactivity between GSH and asthma-causing diisocyanate chemicals used for commercial polyurethane production. The formation of bis(GSH)-diisocyanate conjugates, as observed here upon HDI vapor exposure of 10 mM GSH, is analogous to that previously reported with the aromatic diisocyanate, TDI (Day et al., 1997; Wisnewski et al., 2011). However, the mono(GSH)-HDI reaction products observed at lower GSH concentrations are distinct and include partially hydrolyzed HDI dimers and/or unique cyclized structures. Importantly, mono rather than bis(GSH)-HDI conjugates were the major reaction products resulting from HDI vapor exposure of 100 μM GSH, the approximate concentration of the lower airway fluid *in vivo* in humans.

The influence of the fluid phase ionic composition and [GSH] on the formation of GSH-HDI reaction products highlights the potential complexity of HDI reactivity *in vivo* upon inhalational exposure. Inorganic ions such as H<sub>2</sub>PO<sub>4</sub><sup>-</sup>/HPO<sub>4</sub><sup>2-</sup>, which affect pH levels, also alter reactivity of GSH’s thiol toward NCO, and may participate in hydrolysis of NCO and solvolysis of GSH-NCO thiocarbamates (Ng et al., 2004). Inter-individual differences in airway fluid GSH levels, due to genetic and/or other environmental exposures (e.g. smoking), might further result in different HDI reaction products (Day et al., 2004; Duan et al., 1993; Rahman and MacNee, 1999). Thus, variability in airway fluid GSH and ion composition might alter the clinical response to HDI vapor exposure, and could help explain individual differences in HDI asthma susceptibility.

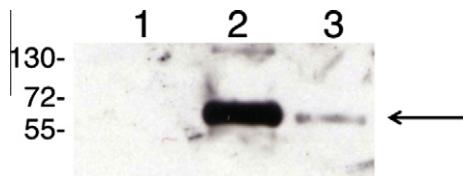
The carbamoylating activity of the presently described (aliphatic) HDI-GSH conjugates differs from that of (aromatic) TDI-GSH conjugates (Day et al., 1997; Wisnewski et al., 2011). While



**Fig. 4.** Effect of buffer on GSH-HDI products formed at 1 mM GSH. FT-ICR MS analysis of GSH-HDI reaction products formed in (HDI vapor exposed) solutions of 1 mM GSH, prepared in  $H_2O$  (A) or PBS (B). The major species were identified based on exact mass and MS/MS (see Supplemental materials, Figs. E2 and E3). Data shown are representative of  $N = 3$  independent experiments.

bis(GSH)-TDI is more potent than mono(GSH)-TDI\* at transferring TDI to albumin, the opposite appears to be true for the corresponding GSH-HDI conjugates, at least based on immunochemical (anti-HDI IgG) analysis. Perhaps more importantly, the kinetics of GSH-HDI mediated albumin carbamoylation, at neutral pH, are

substantially delayed compared with those of GSH-TDI. Such differences may reflect inherent differences in stability of thiocarbonate linkages between aromatic vs. aliphatic isocyanate (e.g. TDI vs. HDI) as mentioned earlier, and may be especially relevant to “detoxification” and elimination *in vivo* (Chipinda et al., 2006). As



**Fig. 5.** Immunochemical identification of mono(GSH)-HDI as the major carbamoylating reaction product of GSH with vapor phase HDI. HDI-specific Western blot was performed on albumin that had been co-incubated overnight at pH 9, with reverse phase HPLC purified GSH (lane 1), or equimolar amounts of mono(GSH)-HDI\* (lane 2), or bis(GSH)-HDI (lanes 3), based on quantitation of HDA released by acid hydrolysis. Data shown are representative of  $N = 3$  independent experiments.

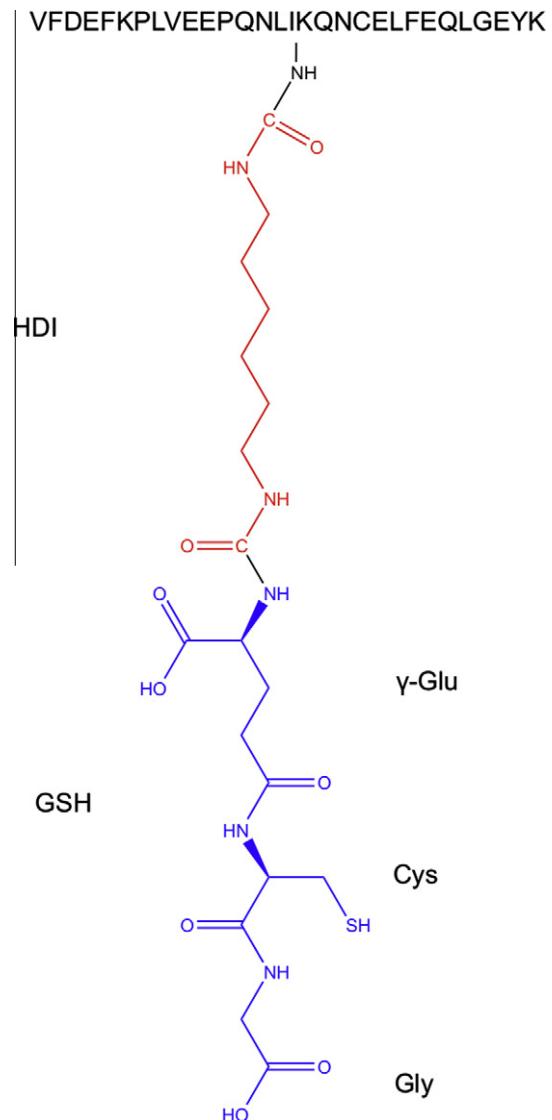
DAHKSEVAHRFKDLGENFKALVLIAFAQYLQQCPFEDHVKLVNEVTEFAKTCVADESA  
ENCKSLSHTLFGDKLCTVATLRETYGEMADCCAKQEPERNECFLQHKDDNPNNPLRIVRP  
EVDMCATAFHNEETFLKLYELARRHYFYAPELLFAAKRYKAAFTCCQAAKAAC  
LLPKLDELDRGKASSAKQLRKCASLQKGGERAKAWAVARLSQRFPKAEFAEVSKLVT  
DLTKVHTECCHGDLLECADRDLAKVICENQDSISSLKECCEKPLLESHClAEVEN  
DEMPADPLSLAADFVESKDVCKNYAEAKDVFLGMLFYAYARRHPDYSVVVLLRLAKTYE  
TTLKCCAAADPHECYAKVDFEKPLVEEPQNLIKQNCELFEQLGEYKFQNALLVRYTK  
KVPQVSTPLVEVSRNLGVGSCKCKHPEAKRMPCAEDYLSVVLNQLCVLHKTPVSDR  
VTKCCTESLVNRRPCFSALEDETYVPKEFNAETFTFHADICTLSEKERQIKKQTA  
LVKHKPKATKEQIKAVMDFAAFVEKCCKADDKETCFAEEGKLVAAQALGL

**Fig. 6.** Loci of HDI conjugation to human albumin, resulting from carbamoylation by GSH-HDI. Sites of carbamoylation at pH 7 and/or pH 9, identified by MS/MS, are highlighted. K-conjugated w/HDI\* (\*=2nd NCO hydrolyzed) resulting from pH 7 carbamoylation reaction overnight. Additional loci of conjugation resulting from carbamoylation reaction at pH 9 for 3 h are also highlighted, including K (no underline)-conjugated w/HDI\* (\*=2nd NCO hydrolyzed), K (double underline)-conjugated w/ HDI-GSH (GSH bound via  $\gamma$ -glu), or K (underline and strikethrough)-peptide internally conjugated with HDI.

HDI is excreted rapidly (within hours) from exposed workers, the relative stability of GSH-HDI conjugation (vs. GSH conjugates with aromatic isocyanates) may serve an overall protective, rather than pathogenic role, consistent with previous short-term *in vitro* HDI vapor studies (Liu et al., 2004; Wisnewski et al., 2005).

Differences between albumin carbamoylated by GSH-HDI, and albumin directly conjugated by HDI, are further suggested by the present data. Under the current experimental conditions, the amount of HDI per albumin molecule, resulting from transfer by GSH-HDI, was four times lower than that resulting from direct HDI vapor exposure. However, while over 25 of albumin's lysines could be modified by GSH-HDI at neutral pH, vapor exposure appears to target a limited number of sites (Wisnewski et al., 2004). By native gel electrophoretic analysis, albumin conjugated by GSH-HDI was virtually unaffected, while the migration of albumin directly conjugated by HDI vapor (and liquid, not shown) was dose-dependently increased, suggesting differences in conformation and/or charge. Such data are consistent with the identification of partially hydrolyzed HDI, vs. cross-linked HDI, as the major modification resulting from carbamoylation via GSH-HDI. Further studies will be needed to better understand the difference(s) between albumin directly reacted with HDI vapor vs. GSH-HDI, including, structural/conformational changes and preferential loci of HDI conjugation *in vivo*.

Particular strengths and weaknesses of the present study are important considerations in the data interpretation. The immunology-based assessment of HDI conjugation, using polyclonal HDI-specific rabbit serum, facilitated high-throughput analysis of albumin carbamoylation in a cost and time-effective manner. However, while the approach recognizes "antigenic" HDI, it is possible that HDI carbamoylation may alter albumin's structure in a way that affects antiserum binding in an HDI dose-independent manner. Although this possibility cannot be ruled out entirely, quantitation of hydrolyzable HDA and the LC/MS-MS data from studies performed at different pH levels are



**Fig. 7.** Novel type of albumin conjugation by HDI-GSH. Proposed structure of novel HDI-GSH conjugation to albumin based on MS/MS data, where one NCO of the HDI is linked to  $K^{389}$  side chain, and the 2nd NCO is linked to the "N-terminus" of GSH (e.g.  $\gamma$ -glutamate side chain). Structure is based on identical MS/MS data from  $N = 3$  independent experiments.

consistent with anti-HDI serology, and positively identify numerous sites of HDI conjugation. It should be noted that the workup (reduction/acetylation) of protein samples for LC/MS/MS, prevented potential identification of albumin carbamoylation on cys-34, the only free thiol in albumin, which could have occurred by a thiol-exchange mechanism. Similar S-linked HDI-albumin conjugates, if they occur *in vivo*, are likely to be relatively unstable, susceptible to further thiol-exchange as they are *in vitro*.

Inherent limitations of the *in vitro* mixed-phase exposure system, in modeling the airway microenvironment, should be also recognized. In particular, the exposure system lacks many components essential to airway fluid's functional activity (surfactant, protein, alveolar macrophages, etc.). Furthermore, while the system recapitulates a mixed (vapor/liquid) phase exposure, the HDI vapor concentration is difficult to compare with that *in vivo*, given differences in the volume:surface area, and lack of fluid phase mixing. As a starting point, we relied upon a fixed HDI vapor concentration established in previous studies, and evaluated the potential influence of variability in (airway) fluid composition. Future studies

with titrated levels of HDI vapor, and other structural/functional components of the airway lining fluid mentioned above, should better reflect GSH–HDI interactions *in vivo* under “normal” working conditions. Ultimately however, *in vivo* studies will be necessary to confirm the biological relevance of the present *in vitro* findings.

In summary, the ability of HDI to react with glutathione across a vapor/liquid phase, as exists in the airway microenvironment *in vivo*, was documented using a mixed-phase exposure system. The concentration of GSH, across a physiologic range, and the ionic composition of the fluid phase markedly influenced the reaction, with five distinct reaction products observed depending upon the experimental conditions. Notably, HDI vapor exposure of liquid solutions with GSH concentrations similar to normal human airway fluid (100  $\mu$ M) resulted in predominately mono(GSH)–HDI conjugates and the capacity to carbamoylate human albumin in a pH-dependent manner. Together, the data support the hypothesis that GSH serves as a primary reaction target for HDI *in vivo*, with a potentially important role in the clinical response to respiratory tract exposure.

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## Conflict of interest

All authors declare no conflict of interest.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.tiv.2012.11.013>.

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