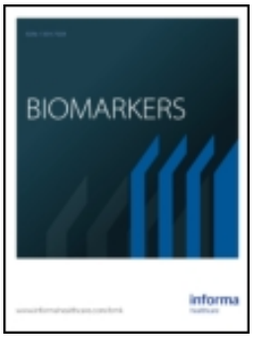

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RESEARCH ARTICLE

Determination of isocyanate specific albumin-adducts in workers exposed to toluene diisocyanates

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Abstract
Toluene diisocyanates (2,4-TDI and 2,6-TDI) are important intermediates in the chemical industry. Among the main damages after low levels of TDI exposure are lung sensitization and asthma. It is therefore necessary to have sensitive and specific methods to monitor isocyanate exposure of workers. Urinary metabolites or protein adducts have been used as biomarkers in workers exposed to TDI. However, with these methods it was not possible to determine if the biomarkers result from exposure to TDI or to the corresponding toluene diamines (TDA). This work presents a new procedure for the determination of isocyanate-specific albumin adducts. Isotope dilution mass spectrometry was used to measure the adducts in albumin present in workers exposed to TDI. 2,4-TDI and 2,6-TDI formed adducts with lysine: N^{ε}-[({3-amino-4-methylphenyl}amino)carbonyl]-lysine, N^{ε}-[({5-amino-2-methylphenyl}amino)carbonyl]-lysine, and N^{ε}- [({3-amino-2-methylphenyl}amino)carbonyl]-lysine. In future studies, this new method can be applied to measure TDI-exposures in workers.

Keywords: Mass spectroscopy, chemical carcinogenesis, environmental Pollution/Ecotoxicology

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Introduction

Toluene diisocyanate (TDI) (Brochhagen 1991) is the most important isocyanate product after methylene diphenyl diisocyanate (MDI) in industry. TDI is an intermediate in the manufacturing of polyurethanes, dyes, pigments, and adhesives. Higher concentrations of isocyanates cause respiratory irritation. Among, the main damages after low levels of isocyanate-exposures are lung sensitization and asthma (Raulf-Heimsoth and Baur 1998; Maestrelli et al. 2009; Redlich and Karol 2002; Ye et al. 2006; Ott, Diller, and Jolly 2003). The sensitization properties of TDI are well documented (Raulf-Heimsoth and Baur 1998; Maestrelli et al. 2009; Redlich and Karol 2002; Ye et al. 2006; Ott, Diller, and Jolly 2003). Polyurethane products might be a cause of asthma also in environmentally exposed people: for example, for people living close to isocyanate-manufacturing sites (Wilder et al. 2011) or for people exposed to polyurethane products in the daily life (Krone and Klingner 2005). Un-reacted isocyanate groups were found in polyurethane foam (Krone et al. 2003) and polyurethanes (Krone, Klingner, and Ely 2003; Damant, Jickells, and Castle 1995). Krone et al (Krone and Klingner 2005; Krone 2004) postulated that the increasing incidence of asthma in developing and developed nations is somehow, in part, linked with isocyanate/polyurethane exposure.

One of the corresponding aromatic amines of TDI, 2,4-toluenediamine (2,4-TDA), is carcinogenic in animal experiments (IARC 1978). Commercial grade TDI has been classified by the International Agency for Research on Cancer (IARC) as a carcinogen in animals, with"sufficient evidence" and as a potential occupational carcinogen for humans (IARC 1999). Isocyanates and arylamines can bind with proteins and/or DNA (Figure 1) and lead to cytotoxic and genotoxic effects (Jeong, Park, and Kim 1998; Bolognesi et al. 2001). Protein adducts of isocyanates might be involved in the etiology of sensitization reactions (Pauluhn and Mohr 1994; Raulf-Heimsoth and

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Abbreviations

3A2MP-Lys, N^{ε}-[({3-amino-2-methylphenyl}amino)carbonyl]-lysine;
3A4MP-Lys, N^{ε}-[({3-amino-4-methylphenyl}amino)carbonyl]-lysine;
3A4MP-[$^{13}C_6{}^{15}N_2$]Lys, N^{ε}-[({3-amino-4-methylphenyl}amino) carbonyl]-[$^{13}C_6{}^{15}N_2$]lysine;
5A2MP-Lys, N^{ε}-[({5-amino-2-methylphenyl}amino)carbonyl]-lysine;
AcMDI–Lys, N^{ε}-[({4-[4-(acetylamino)benzyl]phenyl}amino) carbonyl] lysine;
N_{α}-Boc-Lys, N_{α}-(*tert*-butoxycarbonyl)-L-lysine;
ESI, electrospray ionization;
MDI, methylenediphenyl diisocyanate;
MDI-Lys, N^{ε}-[({4-[4-aminobenzyl]phenyl}amino)carbonyl]-lysine,
MS, mass spectrometry;
TDA, toluenediamine;
TDI, toluene diisocyanate.

Baur 1998; Wisnewski and Jones 2010). To improve the risk assessment for workers exposed to TDI, it is important to develop dosimeters to establish if the toxic reactive intermediate is TDI or a metabolite of TDA (Figure 1). An established method to biomonitor exposed people is the determination of blood protein adducts (Skipper and Tannenbaum 1990; Sabbioni and Jones 2002; Tornqvist et al. 2002). Arylamine specific adducts with cysteine of hemoglobin are of the sulfinic acid amide type (Green et al. 1984). Mild base hydrolysis of such adducts releases the parent aromatic amine (Skipper et al. 2010; Sabbioni 1994). Hemoglobin adducts of TDA have been found in animals (Wilson, La, and Froines 1996) and humans (Jones et al. 2005). 2,4-TDA binds covalently with DNA (Wilson et al, 1996). TDI binds to DNA (Jeong, Park, and Kim 1998) but the chemical structure was not identified. The chemical structure of TDI adducts with plasma proteins found *in vivo* is unknown (Wilson, La, and Froines 1996; Sepai et al. 1995), since harsh hydrolyses conditions were used to determine the released parent aromatic amine (Lind,

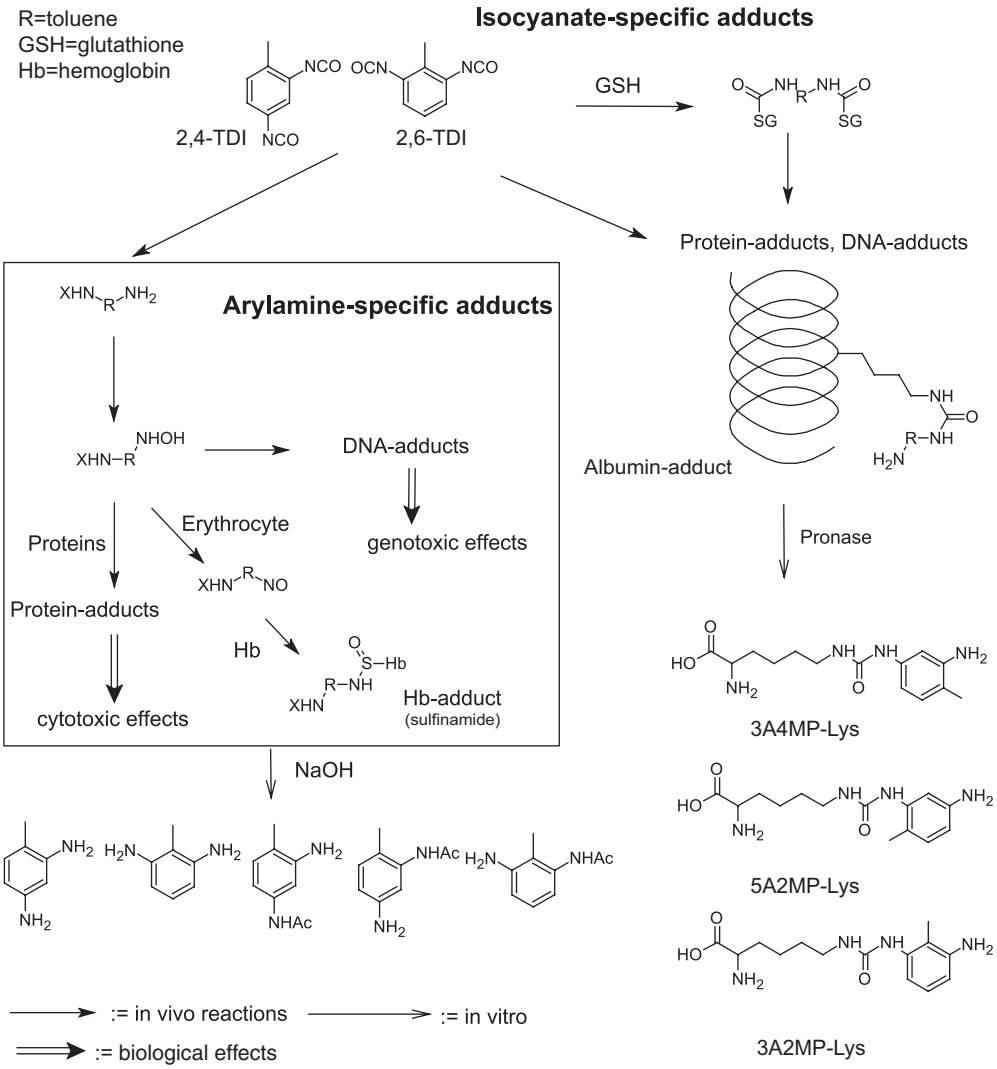

Figure 1. Metabolism and possible reaction products of TDI.

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Dalene, Lindstrom, et al. 1997; Lind, Dalene, Tinnerberg, et al. 1997; Sepai et al. 1995). The distribution of TDI after oral dosage or inhalation has been studied in rats with radiolabeled TDI (Timchalk, Smith, and Bartels 1994; Kennedy et al. 1994). Day et al. (Day, Jin, and Karol 1996) found carbamylation products of TDI with hemoglobin in guinea pigs exposed to 2,4-TDI. For all adducts, one of the two original isocyanato groups had been hydrolyzed to the amine. In addition an amine-nitroso adduct on the α chain of hemoglobin was found. In the bronchoalveolar lavage fluid (Jin, Day, and Karol 1993) of guinea pigs exposed to TDI, five proteins (10.5, 38, 45, 66, and 148 kDa) were found which were positive with a TDI-antiserum. A portion of the 66 kDa protein was identified as albumin (Jin, Day, and Karol 1993). *N*-Terminal valine adducts of TDI with hemoglobin were found in workers and in females with polyurethane covered breast implants (Sabbioni, Hartley, and Schneider 2001). The reaction products of TDI derivatives with single amino acids were obtained and characterized with ^{1}H-, ^{13}C-NMR and mass spectrometry (MS) in our laboratory (Sabbioni, Hartley, and Schneider 2001). Mraz and Bouskova described the reaction of TDI with single amino acids (Mraz and Bouskova 1999) using MS. *In vitro* modifications of albumin with TDI showed only adducts with lysine and the *N*-terminal amino acids according to the MS-analyses of tryptic digests (Hettick and Siegel 2011). At a low (1:2) TDI/albumin ratio, five binding sites were identified, whereas at a high (40:1) ratio, near-stoichiometric conjugation was observed with a maximum of 37 binding sites identified. Sabbioni et al. (Sabbioni et al. 2000) found a *N*-terminal valine adduct with hemoglobin after chronic exposure of rats to MDI and a lysine adduct of MDI in albumin (Kumar, Dongari, and Sabbioni 2009) of the same rats. The levels of the albumin adducts were ca. 10 times higher than the hemoglobin adduct levels. The same albumin adduct of MDI was found in workers exposed to MDI (Sabbioni, Dongari, and Kumar 2010). *In vitro* reactions of MDI with albumin yielded only products with lysine and asparagine according to the MS-analyses of the tryptic digests (Wisnewski, Liu, and Redlich 2010).

For the present work, we developed a method to determine isocyanate specific adducts of TDI with albumin formed *in vivo*. Lysine adducts of isocyanates present in albumin might be crucial for the immunological response of antibodies present in exposed workers (Wisnewski et al. 2010). Thus, a biomonitoring method is needed to determine isocyanate-specific adducts with albumin in order to a have an isocyanate-specific biomarker which is relevant to the immunogenic response observed in TDI exposed workers. For the present project we developed a method to find isocyanate-specific adducts with albumin, which can be applied to human samples.

Experimental procedures

Chemicals

Amicon ultra centrifugal filter tube (30K MWC; 4 mL) (#UFC803096), ammonium bicarbonate (A643-500), methanol (A454–4) for sample preparation, methanol (Optima, A456–4), acetonitrile (A998-4) and water (Optima, W6-4) for LC–MS/MS were obtained from Fisher Scientific (New Jersey, USA). Pronase E (protease, type XIV bacterial, from Streptomyces griseus) (#P5147), Acylase I from porcine kidney (#3010), ammonium acetate, tris(hydroxymethyl)amino–methane, Celite® 577 fine (#22142 from Fluka), deuterated DMSO (DMSO-d_6), deuterium oxide (D_2O), dry 1,4-dioxane (# 296309), N_α-(*tert*–butoxycarbonyl)–L–lysine, L–[$^{13}C_6{}^{15}N_2$]lysine hydrochloride, copper (II) carbonate basic (#207896), human Palladium 10% on carbon (Pd/C), serum albumin (# A1653), sodium phosphate monobasic monohydrate, sodium sulfide hydrate puriss. p.a. (32–38%) (#71975), sodium thiocyanate, trifluoroacetic acid, were purchased from Sigma–Aldrich (St.Louis, MO). 4-Methyl-3-nitrophenyl isocyanate, 2-methyl-5-nitrophenyl isocyanate, 2-methyl-3-nitrophenyl isocyanate were acquired from Lancaster (Muhlheim am Main, Germany). Coomassie plus protein assay reagent (#23236) for protein determination was ordered from Thermo Scientific (Rockford, IL). Strata–X–33u (8B–S100–FBJ) polymeric reversed phase columns (200 mg/3 mL) was obtained from Phenomenex Inc. (Torrance CA). N^{α}-acetyl-N^{ε}-[{(3-amino-4-methylphenyl}amino)carbonyl]-lysine was synthesized as described in the literature (Sabbioni, Hartley, and Schneider 2001).

Diagnostic samples

We obtained blood samples from 10 workers (age = 46.8 ± 8.7 years) accidentally exposed to TDI. The first set of samples were drawn 26 days after the accident and a second set of samples was obtained from 6 workers after an additional 28 days. Plasma samples were shipped on dry ice from Europe and were stored in the freezer at –25°C.

Instrumentation

A API 4000Q Trap (ABSciex, Foster City, CA) mass spectrometer interfaced to a HPLC (Shimadzu Prominance 20AD) was used for the LC–MS/MS analyses. A UV-1800 Spectrophotometer from Shimadzu was used for protein determination. Centrifugations were performed on a Beckman Coulter Allegra™ X–22R centrifuge equipped with a SX4250 swing out bucket rotor. HPLC analyses to determine the purity of the synthesized compounds were performed on a Hewlett–Packard 1100 system with a quaternary HPLC pump and a photodiode array detector: Lichrosphere RP18 (125 × 4.6 mm, 5 μm) column, with a 20 min 30–80% methanol and ammonium acetate (10 mM) gradient, flow rate of 1.0 mL/min, and λ = 225, 250 nm. NMR spectra were recorded on a Bruker AC 500 instrument with deuterium oxide and/or DMSO-d_6 as the solvent and as the internal standard. The raw NMR data were processed with the program ACD NMR Processor Academic Edition (Advanced Chemistry Development, Inc., Toronto, Ontario, Canada).

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N^{ε}-[({3-Amino-2-methylphenyl}amino) carbonyl]-lysine (3A2MP-Lys)

2-Methyl-3-nitrophenyl isocyanate (4.0 mmol) was added to a solution of N^{α}-Boc-protected lysine (2.0 mmol) in 0.25 M $NaHCO_3$ (40 mL). The reaction mixture was stirred at 80°C. After 3 h, the solution was cooled with ice, the precipitate was filtered off and washed with 0.25 M $NaHCO_3$ (5 mL). The filtrate was acidified to pH 1–2 with 2 M HCl, and extracted with ethyl acetate (3 × 50 mL). The water phase was discarded. The organic phase was extracted with 0.5 M $NaHCO_3$ (3 × 50 mL). The water phase was made acidic to pH 1–2 with 2 M HCl and re-extracted with ethyl acetate (3 × 50 mL). The organic extract was dried ($MgSO_4$), filtered, and evaporated. Trifluoroacetic acid (5 mL) was added to remove the remaining Boc group. After 1 h the mixture was evaporated under reduced pressure and the residue was dried using a high vacuum pump. N^{ε}-[({3-nitro-2-methylphenyl}amino)carbonyl]-lysine was obtained with 31.6% yield (205.2 mg).

Pd/C (50 mg) and ammonium formate (1109.6 mg, 17.6 mmol) were added under nitrogen at room temperature to a solution of N^{ε}-[({3-nitro-2-methylphenyl} amino)carbonyl]-lysine (200 mg, 617 µmol) in dry MeOH (10 mL). After 3 h the reaction mixture was filtered over Celite. The solvent was evaporated and the residue was dried using a high vacuum pump. To eliminate ammonium formate, the residue was dried overnight at 0.01 Torr and 30°C. *N*-[({3-Amino-2-methylphenyl}amino) carbonyl]-lysine was obtained with 34.5% (62.5 mg) yield. According to HPLC analyses on a Nucleodur 100-5 C18 EC column (125 × 2 mm) with a 21 min of 90% to 10% 10 mM ammonium formate buffer/acetonitrile gradient the purity of 3A2MP-Lys (t_R = 1.7 min) was 97% at λ = 225 nm. UV: λ(E[mAU]) = 214_{max} nm (495), $234_{shoulder}$ nm (194), 264_{min} (17.1), 284_{max} (35.3).

^{1}H-NMR (D_2O): 7.02 (1H, t = 7.8 Hz, H-C_6); 6.76 (1H, d, *J* = 7.8 Hz, H-C_5); 6.72 (1H, d, *J* = 7.8 Hz, H-C_4); 3.62 (1H, t = 6.1 Hz, NH_2-C*H*); 3.06 (2H, t = 6.8 Hz, NH-CH_2); 1.96 (s, 3H, Me-Ar); 1.76 (2H, m_c, NH_2-CH-CH_2-); 1.43 (2H, m_c, NH_2-CH-CH_2-CH_2-); 1.30 (2H, m_c, NH_2-CH-CH_2-CH_2-CH_2-).

ESI-MS: 295.2 $[M+H]^+$

N^{ε}-[({5-Amino-2-methylphenyl}amino) carbonyl]-lysine (5A2MP-Lys)

2-Methyl-5-nitrophenyl isocyanate (4.0 mmol) was added to a solution of N^{α}-Boc-protected lysine (2.0 mmol) in 0.25 M $NaHCO_3$ (40 mL). The solution was stirred at 80°C for 3 h. After cooling the solution with ice, the precipitate was filtered off and washed with 0.25 M $NaHCO_3$ (5 mL). The filtrate was acidified to pH 1–2 with 2 M HCl, extracted with ethyl acetate (3 × 50 mL). The water phase was discarded. The organic phase was extracted with 0.5 M $NaHCO_3$ (3 × 50 mL). The water phase was made acidic to pH 1–2 with 2 M HCl and re-extracted with ethyl acetate (3 × 50 mL). The organic extract was dried ($MgSO_4$), filtered, and evaporated. Trifluoroacetic acid (5 mL) was added to remove remaining Boc group from lysine. After 1 h stirring at room temperature, the residue was evaporated under reduced pressure and the residue was dried using a high vacuum pump. N^{ε}-[({5-nitro-2-methylphenyl}amino)carbonyl]-lysine was obtained with 28.4% (184.5 mg) yield.

Pd/C (50 mg) and ammonium formate (540 mg, 8.56 mmol) were added under nitrogen at room temperature to a solution of the nitro compound *N*-[({5-nitro-2-methylphenyl}amino)carbonyl]-lysine (97.2 mg 0.3 mmol) in dry MeOH (10 mL). After 3 h, the reaction mixture was filtered over Celite. The solvent was evaporated and the residue was dried using a high vacuum pump. To eliminate ammonium formate, the residue was dried overnight at 0.01 Torr and 30°C. 5A2MP-Lys was obtained with 21.5% (18.5 mg) yield. According to HPLC analyses on a Nucleodur 100-5 C18 EC column (125 × 2 mm) with a 21 min 90% to 10% 10 mM ammonium formate buffer/acetonitrile gradient the purity of 5A2MP-Lys (t_R = 2.2 min) was 98% at λ= 225 nm. UV: λ (E[mAU]) = 216_{max} nm (395), $238_{shoulder}$ nm (172), 266_{min} (12), 292_{max} (40.6).

^{1}H-NMR (D_2O): 7.01 (1H, *d* = 8.1 Hz, H-C_3); 6.65 (1H, *d*, *J* = 2.3 Hz, H-C_6); 6.58 (1H, *dd*, *J* = 2.3, 8.1 Hz, H-C_4); 3.63 (1H, *t* = 6.0 Hz, NH_2-C*H*); 3.06 (2H, broad *t* = 6.8 Hz, NH-CH_2); 2.01 (s, 3H, Me-Ar); 1.78 (2H, m_c, NH_2-CH-CH_2-); 1.45 (2H, m_c, NH_2-CH-CH_2-CH_2-); 1.32 (2H, m_c, NH_2-CH-CH_2-CH_2-CH_2-).

ESI-MS: 295.2 $[M+H]^+$

N^{ε}-[{(3-Amino-4-methylphenyl} amino)carbonyl]-lysine (3A4MP-Lys)

N^{α}-Acetyl-N^{ε}-[({3-amino-4-methylphenyl}amino) carbonyl]-lysine (Ac3A4MP-Lys) (100 mg, 0.3 mmol) in 0.1 M phosphate buffer (10 mL, pH 7.5) was incubated with Acylase I (50 mg) for 4 days at 37°C. The pH was monitored every 12 h. The reaction was monitored by HPLC on a Nucleodur 100-5 C18 EC column (125 × 2 mm) with a 21 min 90% to 10% 10 mM ammonium formate buffer/acetonitrile gradient: t_R = 1.7 min (Ac3A4MP-Lys) and t_R = 2.7 min (3A4MP-Lys) (λ= 225, 250 nm). After 4 days another 25 mg Acylase I was added. After an additional 2 days the reaction mixture was purified by solid phase extraction (Strata–X–33u, polymeric reversed phase columns, 200 mg). The columns were first activated with 3 mL of methanol and then equilibrated 3 mL of of 0.1 M phosphate buffer (pH 7.5). The samples were applied on the column and subsequently washed with 3 mL. One fifth of the reaction mixture (2 mL) was loaded on the column. The column was washed with 3 mL of water. 3A4MP-Lys, was eluted with 6 mL of 80% methanol in water. This step was repeated for the rest of the reaction mixture. The combined eluates were evaporated at the roto-evaporator to yield 18.8 mg (21.5%) of 3A4MP-Lys. According to HPLC analyses on a Nucleodur 100-5 C18 EC column (125 × 2 mm) with a 21 min 90% to 10% 10 mM ammonium formate buffer/acetonitrile gradient the purity of 3A4MP-Lys (t_R = 2.9 min) was 98.9% at λ = 225 nm. UV-Spectrum: λ(E[mAU]) = 218_{max} nm (420), $244_{shoulder}$ nm (140), 268_{min} (14.1), 292_{max} (36.1).

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^{1}H-NMR (DMSO-d_6 + drops of D_2O): 6.71 (1H, *d* = 8.3 Hz, H-C_5); 6.70 (1H, *d*, *J* = 2.1 Hz, H-C_2); 6.47 (1H, *dd*, *J* = 2.1, 8.3 Hz, H-C_6); 3.15 (1H, m_c, NH_2-C*H*); 3.02 (2H, '*t*' = 6.7 Hz, NH-CH_2); 1.95 (s, 3H, Me-Ar); 1.72 (1H, m_c, NH_2-CH-CH_2-); 1.61 (1H, m_c, NH_2-CH-CH_2-); 1.38 (4H, m_c, NH_2-CH-CH_2-CH_2-CH_2-).

^{1}H-NMR (D_2O): 7.12 (1H, *d* = 8.0 Hz, H-C_5); 6.91 (1H, *d*, *J* = 2.1 Hz, H-C_2); 6.77 (1H, *dd*, *J* = 2.1, 8.0 Hz, H-C_6); 3.74 (1H, *t* = 5.8 Hz, NH_2-C*H*); 3.19 (2H, *t* = 6.7 Hz, NH-CH_2); 2.16 (s, 3H, Me-Ar); 1.88 (2H, m_c, NH_2-CH-CH_2-); 1.56 (2H, m_c, NH_2-CH-CH_2-CH_2-); 1.42 (2H, m_c, NH_2-CH-CH_2-CH_2-CH_2-).

ESI-MS: 295.2 $[M+H]^+$

N^ε-[({3-Amino-4-methylphenyl}amino)carbonyl]-[$^{13}C_6{}^{15}N_2$] lysine (3A4MP-[$^{13}C_6{}^{15}N_2$]Lys)

A solution of L-[$^{13}C_6{}^{15}N_2$] lysine hydrochloride (20 mg, 104 μmol) in water (2 mL), 300 μl of NaOH (20 mg/mL) and copper(II) carbonate (23 mg, 104 μmol) was refluxed for 30 min. The reaction mixture was cooled to room temperature and used for further reaction. A solution of 4-methyl-3-nitrophenyl isocyanate (16 mg, 84 μmol) in 1,4-dioxane (0.5 mL) was added slowly. The reaction mixture was stirred for 4 h at room temperature. Na_2S (20 mg) was added and stirred at room temperature for about 10 min. The precipitated cuprous sulfide was eliminated after centrifugation and filtration. The filtrate was washed with ethyl acetate (3 × 3 mL), acidified to pH 4 with 2 M HCI, and extracted again with ethyl acetate (3 × 4 mL). The collected organic phases were dried on anhydrous Na_2SO_4, filtered, and evaporated under vacuum. The obtained white solid was treated with 100 μL of TFA for 25 min. After evaporation of TFA under reduced pressure, the residue was dried using a high vacuum pump to obtain 8 mg (28.7%) of N^ε-[({3-nitro-4-methylphenyl}amino)carbonyl]-[$^{13}C_6{}^{15}N_2$] lysine.

Pd/C (50 mg) and ammonium formate (43.1 mg, 684 μmol) were added under nitrogen at room temperature to a solution of N^ε-[({3-nitro-4-methylphenyl}amino) carbonyl]-[$^{13}C_6{}^{15}N_2$] lysine (8.0 mg, 24 μmol) in dry MeOH (10 mL). After 3 h the reaction mixture was filtered over Celite. The solvent was evaporated and the residue was dried using a high vacuum pump. To eliminate ammonium formate, the residue was dried overnight at 0.01 Torr and 30°C. 3A4MP-[$^{13}C_6{}^{15}N_2$]Lys was obtained with 20.6% (1.5 mg) yield. According to HPLC analyses on a Nucleodur 100-5 C18 EC column (125 × 2 mm) with a 21 min 90% to 10% 10 mM ammonium formate buffer/acetonitrile gradient, the purity of 3A4MP-[$^{13}C_6{}^{15}N_2$] Lys (t_R = 2.2 min) was 90% at λ = 225 nm. UV: $\lambda(E[mAU]) = 218_{max}$ nm (94.8), $244_{shoulder}$ nm (31.8), 268_{min} (3.8), 292_{max} (8.5)

ESI-MS: 303.2 $[M+H]^+$. ESI-MS/MS of m/z 303.2: m/z 181 N^ε-isocyanato [$^{13}C_6{}^{15}N_2$] lysine, m/z 149.

4-methyl-3-aminophenyl isocyanate, m/z 123 toluene diamine.

Isolation of albumin

Albumin was purified with HiTrap Blue HP column 1.0 mL (0.7 × 2.5 cm) from GE-Life sciences, Inc, USA. The HiTrap Blue HP column was equilibrated with 6 volumes of binding buffer (50 mM KH_2PO_4, pH 7.0). Human plasma (0.5 mL) was diluted with binding buffer (0.5 mL). After centrifugation the supernatant was loaded on the column. After eluting with 6 mL of binding buffer, albumin was eluted with 6 mL of elution buffer (20 mM KH_2PO_4+1.5 M KCl, pH 7.0). The columns were then rinsed with washing buffer (10 mL, 50 mM Tris buffer +0.2 M NaSCN, pH 7.5). Purified fractions were concentrated in Amicon ultra centrifugal filter tube (30k MWC; 4 mL) by centrifuging with 4000 rpm at 4°C for 40–60 min, and washed with water (3 × 4 mL). Samples were re-dissolved in 10 mM sodium phosphate buffer (pH 7.0). The concentrations of the isolated albumin solutions were determined with a Coomassie protein Assay kit for total protein quantitation from Pierce. For some samples albumin was also isolated from plasma using ammonium sulfate precipitation as described previously (Kumar and Sabbioni 2010).

Isolation of globin

A method published by Lewalter et al. (Lewalter, Leng, and Ellrich 2003) was followed. Blood samples were centrifuged for 5 min at 1200*g*. The plasma was drawn off using a pipette and stored separately. The erythrocytes were washed three times with an equal volume of 0.9% sodium chloride solution. After adding 1 equal volume of distilled water the erythrocyets were kept at <–20°C overnight in a freezer. The frozen lysate samples were thawed and then homogenized by shaking. Then 6 volumes of 0.05 M HCl in propanol was added. After vigorous shaking of the mixture, the cell debris were removed after centrifugation. Four volumes (1 volume = original lysate volume) of ethyl acetate were added to the supernatant and globin precipitated. The globin precipitate was washed with 2 volumes of ethyl acetate, centrifuged and decanted. This procedure was repeated three times. Globin was washed additionally with 2 volumes of hexane. The isolated globin was placed overnight in a desiccator.

Digestion of proteins

Protein (9 mg) in 50 mM ammonium bicarbonate, pH 8.9 (0.9 mL) was spiked with 3A4MP–[$^{13}C_6{}^{15}N_2$]Lys (10 pmol) and incubated with 500 μL of freshly prepared pronase E solution (6 mg/mL; 50 mM ammonium bicarbonate, pH 8.9) for 20 h at pH 8.9 and 37°C. The digest was purified with solid phase extraction (Strata–X–33u, polymeric reversed phase columns, 200 mg). The column was first activated with 3 mL of methanol and then equilibrated with 3 mL of 50 mM ammonium bicarbonate (pH 8.9). The samples were applied on the column and subsequently washed with 3 mL of water. 3A4MP-Lys, 3A4MP–[$^{13}C_6{}^{15}N_2$]Lys, 5A2MP-Lys and 3A2MP-Lys were eluted with 6 mL of methanol. The eluate was concentrated to approximately 0.3 mL in a speed evaporator.

Quantification of albumin adducts, 3A4MP-Lys, 5A2MP-Lys and 3A2MP-Lys, using LC–MS/MS

Shimadzu Prominance 20AD interfaced to a API 4000Q Trap LC–MS/MS (ABSciex, Foster City, CA) mass

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spectrometer system was used for all the quantitative analysis. The MS parameter were optimized in the electrospray ionization mode (ESI). Parameter optimizations were carried out with 100 pg/μL solution of analyte with the flow rate of 10 μL/min in the positive ionization mode. Quantitative optimization mode was used to maximize the molecular ion signal and to set the maximum suitable parameters for the compounds: $[M+1]^+ = m/z$ 295.1 for 3A4MP-Lys, 5A2MP-Lys and 3A2MP-Lys and $[M+1]^+ = m/z$ 303.1 for 3A4MP–[$^{13}C_6{}^{15}N_2$]Lys. For better resolution and sensitivity of the analytes, quadrupole mass analyzers (Q1 and Q3) were set on 0.7 ± 0.1 amu resolution window.

The mass spectrometer was operated in the positive ionization mode with an electrospray voltage 5500 V and a source temperature of 500°C. Nitrogen was used as ion spray (GS1), drying gas (GS2) and curtain gas at 40, 40 and 15 arbitrary unit, respectively. The entrance potential (EP) and the declustering potential (DP) were set for all compounds at 10 and 56 V respectively. The CXP (Collision Cell Exit Potential) and the and collision energy (CE) for lysine adducts and the internal standard were 6, 12 V and 22, 18 V for the quantification masses 295.1/123.1 and 303.1/123.1 respectively. For the qualification ion m/z = 295.1/173.1, the CXP and CE were set at 22 and 10 V respectively. All data were processed using Analyst software 1.4.2 (ABSciex, Foster City, CA).

The lysine adducts of TDI (3A4MP-Lys, 5A2MP-Lys, and 3A2MP-Lys) and the internal standard (3A4MP–[$^{13}C_6{}^{15}N_2$]Lys) were detected with multiple reaction monitoring (MRM) *m/z* 295.1/123.1 and 303.1/123.1, respectively. Chromatographic separation was achieved on a Luna C18(2) (100 Å, 150 × 2 mm, 3 μm) (Phenomenex Inc., Torrance CA) protected by SecurityGuard C18 guard column (4 mm L × 3 mm ID), (Phenomenex Inc., Torrance CA) using a gradient system with solvent A (10 mM ammonium formate) and solvent B (5 mM ammonium formate in acetonitrile: water (95:5) at a flow rate of 0.2 mL/min: 0 min (B 4%), 3 min (B 4%), 15 min (B 90%), 20 min (B 90%). The retention times (t_R) of 3A2MP-Lys, 5A2MP-Lys, and 3A4MP-Lys were 8.8, 9.6 and 10.2 min, respectively. The column flow was diverted away from the ESI ion source except for the time period from 5 to 12 min. The identity of the samples were confirmed using another HPLC-method. Chromatographic separation was achieved on a LUNA Phenyl-Hexyl column (150 × 2 mm, 3 μm) (Phenomenex Inc., Torrance, CA) protected by a SecurityGuard C18 guard column (4 mm L × 2 mm ID), using a gradient system with solvent A (10 mM ammonium formate) and solvent B (acetonitrile) at a flow rate of 0.2 mL/min: 0 min (B 15%), 6 min (B 15%), 9 min (B 60%), 16 min (B 90%). The retention times (t_R) of 3A2MP-Lys, 5A2MP-Lys, and 3A4MP-Lys were 3.3, 3.8 and 4.4 min, respectively.

To generate the calibration lines, human serum albumin samples (9 mg) in ammonium carbonate (50 mM, 2 mL) were spiked with different amounts of 3A2MP-Lys, 5A2MP-Lys, and 3A4MP-Lys (0.00, 0.17, 0.34, 0.85, 1.70 and 3.40 pmol) along with 3A4MP–[$^{13}C_6{}^{15}N_2$]Lys (3.40 pmol) and worked up as described in the paragraph "digestion of albumin". The calibration lines for 3A2MP-Lys, 5A2MP-Lys, and 3A4MP-Lys were generated over the range of 0.0–3.40 pmol/9 mg albumin. The concentration levels were plotted against the peak area ratio of the analytes against the peak area of the internal standard (e.g. peak area ratio 3A4MP–Lys/3A4MP–[$^{13}C_6{}^{15}N_2$]Lys). The regression coefficients $r^2 > 0.99$ were found using the regression option "linear and 1/x weighting factor". The limit of the quantification (LOQ) following this work up and analysis procedure was 17 fmol/mg albumin for 3A2MP-Lys, 5A2MP-Lys, and 3A4MP-Lys. The signal/noise ratio was >10.

Results

New lysine adducts of TDI were synthesized from N_α-Boc-protected lysine and 2-methyl-3-nitrophenyl isocyanate and 2-methyl-5-nitrophenyl isocyanate (Figure 2). The Boc group was released with trifluoroacetic acid (TFA) and the nitro group was reduced with palladium on carbon and ammonium formate to yield *N*-[({3-amino-2-methylphenyl}amino)carbonyl]-lysine (3A2MP-Lys) and *N*-[({5-amino-2-methylphenyl}amino)carbonyl]-lysine (5A2MP-Lys), respectively.

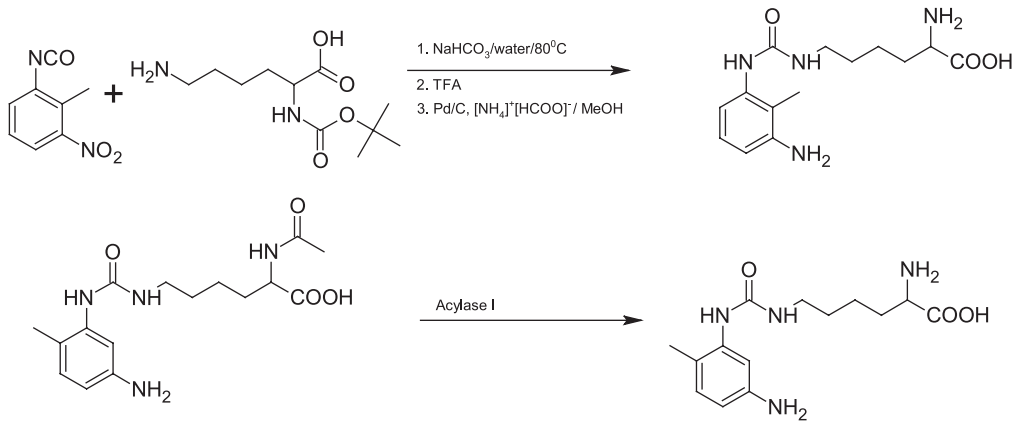

Figure 2. Procedure for the synthesis of the albumin adducts TDI-Lys.

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N-[(3-Amino-4-methylphenyl}amino)carbonyl]-lysine (3A4MP-Lys) was obtained after de-protection of the N_α-acetyl group with Acylase I from N^α-acetyl-N^ε-[({3-amino-4-methylphenyl}amino)carbonyl]-lysine (Figure 2). The products were characterized with MS and NMR. The aromatic protons could be assigned according to the coupling pattern and the chemical shift: cis coupling constant > meta coupling constant > para coupling constant. The protons of lysine were assigned according to the data from the literature (Sabbioni, Hartley, and Schneider 2001). The MS properties were similar for all three compounds. The positive electrospray ionization (ESI) MS/MS spectra of the molecular ions showed the main fragment at m/z = 173, which corresponds to N_ε-isocyanato-lysine which was generated after the cleavage of TDA from 3A4MP-Lys, 2A3MP-Lys and 5A2MP-Lys. The minor ion at m/z = 123 corresponds to protonated TDA. In negative ESI MS/MS the only fragment corresponds to the loss of lysine which yields the 2-methyl-3-aminophenyl isocyanate, 2-methyl-5-aminophenyl isocyanate, and 4-methyl-3-aminophenyl isocyanate as the major fragment at m/z = 145.2. The corresponding isotope-labeled standard for 3A4MP-Lys was synthesized with [$^{13}C_6{}^{15}N_2$]Lys. The α amino group was protected as copper complex (Caldwell, Holt, and Milligan 1971). After reaction with the 4-methyl-3-nitrophenyl isocyanate the copper complex was released using sodium sulfide as the reagent (Nowshuddin and Reddy 2006). The identity of the isotope-labeled compound, 3A4MP-[$^{13}C_6{}^{15}N_2$]Lys, was confirmed by MS and by comparison of the retention time and the UV-spectra with the ones of the unlabeled compound, 3A4MP-Lys. The enantiomeric purity of all final products was not investigated.

A LC–MS/MS method and workup procedure was developed for the determination of TDI-Lys adducts present *in vivo*. The albumin purification step and the digestion step with pronase was optimized previously for the determination of lysine adducts of MDI in rats (Kumar, Dongari, and Sabbioni 2009) and humans (Sabbioni, Dongari, and Kumar 2010). The solid phase extraction method had to be changed since the TDI-Lys adducts are more hydrophilic than the lysine adducts of MDI. For the LC–MS/MS analyses two methods with different gradient systems and HPLC-columns were developed.

The new standards were applied to the analyses of biological samples from 10 workers which were accidentally exposed to TDI. Blood samples were collected 26 days after exposure. Albumin was purified using a Cibacron™ Blue F3G-A affinity chromatography column (HiTrap blue), and/or by ammonium sulfate precipitation. An aliquot of albumin was digested with pronase in the presence of the internal standard 3A4MP-[$^{13}C_6{}^{15}N_2$]Lys. After solid phase extraction the samples were analyzed with LC–MS/MS (Figure 3). Only a few samples were above the LOQ of the method. In the samples 47, 50A, and 50B, the levels of 3A4MP-Lys were 29, 461 and 189 fmol/mg. In the samples 50A, and 50B, the levels of 5A2MP-Lys were 109 and 67.3 fmol/mg, and the levels of 3A2MP-Lys were 269 and 99.7 fmol/mg (Figure 3). The peaks were quantified with the MRM transition m/z = 295.1/123.1. The identity of the compound was additionally supported by the transition m/z = 295.1/173.1. The samples were analyzed on a reversed phase C18 column. For structural confirmation the compounds were also analyzed on a reversed phase Phenyl-Hexyl column (see experimental part).

The enzymatic hydrolysis, work up and LC–MS/MS analysis was repeated five times for the samples 50A and 50B. For the sample 50A, 3A4MP-Lys, 5A2MP-Lys and 3A2MP-Lys were obtained with 461 ± 13 fmol/mg (coefficient of variation (cv) = 2.7%), 109 ± 5.8 fmol/mg (5.3%) and 269 ± 18 fmol/mg (6.6%), respectively. For the sample 50B, 3A4MP-Lys, 5A2MP-Lys and 3A2MP-Lys were obtained with 189 ± 5.3 fmol/mg (2.8%), 67.3 ± 3.3 fmol/mg (4.3%), and 99.7 ± 2.1 fmol/mg (2.1%), respectively. Sample 50A and 50B were taken from the same person on day 28 and 56 after the accident. Plotting the adduct levels on a logarithmic scale against the time on a linear scale the following half-lives were estimated for the albumin-adduct levels: 21.7 days for 3A4MP-Lys, 19.6 days for 3A2MP-Lys and 40.3 days for 5A2MP-Lys. The half-lives of the 3A4MP-Lys and the 3A2MP-Lys adducts are in the range of the un-adducted albumin which was close to the values of the half-life of albumin, 20–25 days (Rappaport et al. 2002). The same procedure was applied for the analysis of the globin samples 50A and 50B. No adducts with lysine could be found.

Discussion

In the past Cibacron blue affinity column chromatography of albumin from rats (Kennedy et al. 1994) and guinea pigs (Jin, Day, and Karol 1993) exposed to TDI gave ambiguous results. Kennedy et al (Kennedy et al. 1995) found a 66 kDa protein in serum from rats (ca 0.2 kg body weight) which were given radiolabeled TDI: 0.19, 1.02 and 5.85 μg/L for 4 h. At the lowest dose it appears that all radiolabel was retained by the affinity chromatography as expected for albumin adducts (Kennedy et al. 1995). With increasing dose the radiolabeled material increasingly eluted with the unbound material. Therefore, at the higher concentration, another protein might bind to TDI or may be too many molecules of TDI had reacted with the albumin molecule that its binding affinity was altered (Kennedy et al. 1995). The lowest dose (0.19 μg/L) given to the rats should yield the following dose according to the formula of Alexander et al (Alexander et al. 2008): delivered dose (mg/kg) = [air concentration (mg/L) × RMV × D(×IF)]/body weight (kg). RMV = respiratory minute volume or the volume of air inhaled in one minute (L/min); D = duration of exposure (min); IF = proportion by weight of particles that are inhalable by the test species. IF was set equal to 1. The RMV for rats of 0.2 kg body weight (BW) was estimated

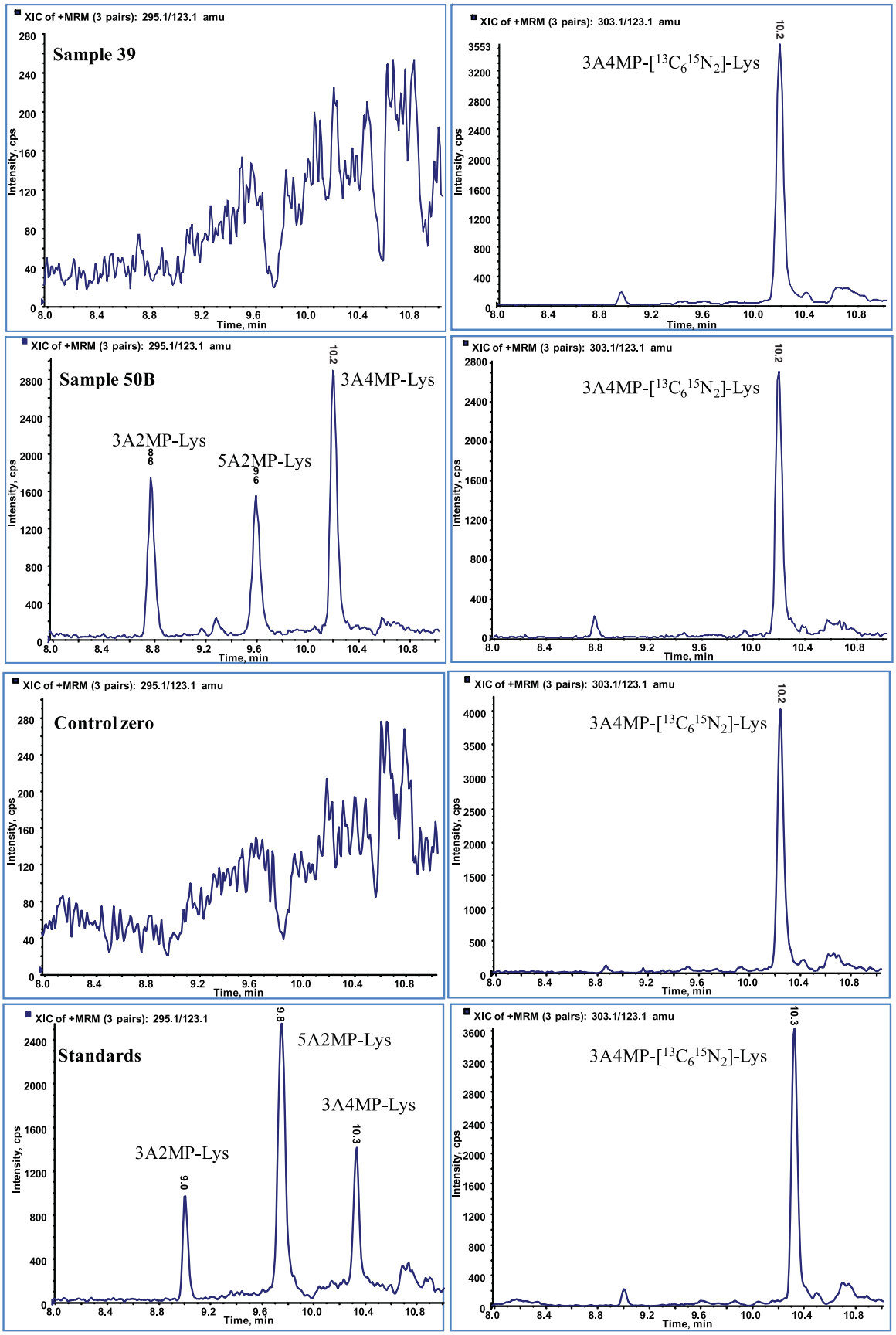

Figure 3. LC–MS/MS analysis of TDI-Lys- adducts found *in vivo*, sample 39 and sample 50B; and LC–MS/MS analysis of digested albumin spiked with the internal standard 3A4MP-[13C615N2]Lys (control sample) and digested albumin spiked with the synthetic standards 0.85 pmol (2A3MP-Lys, 5A2MP-Lys and 3A4MP-Lys).

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to be 0.154 L/min = 0.608 × BW(Kg)$^{0.852}$. The lowest exposure level (0.19 µg/L) would yield a dose of 34.7 µg/kg. Assuming that approximately 0.2% of the dose binds to albumin (see below MDI experiment) we expect approximately 400 fmol of TDI bound per mg albumin. This corresponds roughly to the highest modification level found for the human sample of the present manuscript. We estimated the percentage of isocyanate dose binding to albumin form an earlier experiment with rats exposed to 20 mg/m^3 of MDI for 6 h (Kumar, Dongari, and Sabbioni 2009). The delivered dose was estimated to be 5.55 mg/kg using the formula of Alexander (Alexander et al. 2008). In a rat approximately 980 mg albumin per kg body weight are present. Therefore a 1% modification level with MDI would yield 226.5 pmol/mg albumin. Indeed we found 44.5 pmol/mg which corresponds to 0.2% of the delivered dose. In the guinea pig experiment of Jin et al (Jin, Day, and Karol 1993)(0.6 kg body weight, 3 h, 15.3 µg/L TDI exposure) the 66 kDa protein fraction was not retained on the Cibacron blue column. This is consistent with the former experiment of Kennedy et al (Kennedy et al. 1995) since the dose for the guinea pigs was approximately 50 times higher than the lowest dose applied to the rats where all radioactivity bound with albumin was retained by the affinity chromatography. For the present work we found smaller adduct levels with albumin obtained after ammonium sulfate precipitation than with albumin obtained after HiTrap Blue purification: e.g. 3A4MP-Lys = 297 fmol/mg and 3A4MP-Lys = 467 fmol/mg, respectively. This indicates that we did not loose albumin adducts of TDI using the HiTrap column.

Just recently *in vitro* reaction of TDI with albumin showed that several lysines and the *N*-terminal amino acid reacts with 2,4-TDI or 2,6-TDI (Hettick and Siegel 2011). With the present manuscript we were able to show that lysine adducts are present also *in vivo*. The present method for the analyses of TDI-adducts is similar to the method described for albumin adducts of MDI in rats (Kumar, Dongari, and Sabbioni 2009) and humans (Sabbioni, Dongari, and Kumar 2010). For the MDI-exposed samples we analyzed also the *N*-acetylated adduct N^{ε}–[({4–[4–(acetylamino)benzyl] phenyl}amino)carbonyl]-lysine (AcMDI-Lys), which was present 25 times less than the non-acetylated adduct, N^{ε}–[({4-[4-aminobenzyl]phenyl}amino)carbonyl]-lysine (MDI-Lys). In human samples, only 29.5% workers were found to be positive for the AcMDI-Lys in contrast to the 63.9% found positive for the MDI-Lys adduct (Sabbioni, Dongari, and Kumar 2010). Therefore, we did not include the possible *N*-acetyl-TDI adducts in the present method.

Albumin is one of the potential targets involved in the etiology of sensitization reactions of isocyanates (Raulf-Heimsoth and Baur 1998; Wisnewski and Jones 2010; Wisnewski, Liu, and Redlich 2010). Therefore, albumin adducts would be a marker of exposure and a marker which is related to the mechanism of sensitization caused by isocyanates. Isocyanate exposure has been measured in several studies by determining isocyanate-specific IgE and IgG antibodies present in workers and the general population in order to have an early and reliable indicator of diisocyanate-induced asthma (Ott et al. 2007). The recent literature review of Ott et al. (Ott et al. 2007) indicates that there is considerable heterogeneity in the prevalence of specific IgE and IgG binding to diisocyanate conjugates across studies, which could be explained with differences in assay methodology and in the criteria used to define positive findings (Bernstein et al. 2002; Wisnewski et al. 2004). Bernstein et al. (Bernstein et al. 2006) demonstrated that the background prevalence of IgE or IgG binding to diisocyanate conjugates in a general population can be quite high, depending on the assay methodology and criteria used to define a positive result. Lysine adducts present in albumin modified *in vitro* with isocyanates might be crucial for the immunological response of antibodies present in exposed workers (Wisnewski et al. 2010). For the present project we developed a method to find isocyanate-specific adducts with lysine from albumin, which might be relevant to the immunogenic response observed in TDI exposed workers. This new biomarker can now be applied to populations exposed to isocyanates.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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