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ORIGINAL ARTICLE

Determination of a new biomarker in subjects exposed to 4,4′-methylenediphenyl diisocyanate

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Abstract

4,4′-Methylenediphenyl diisocyanate (MDI) is the most important of the isocyanates used as intermediates in the chemical industry. Among the main types of damage after exposure to low levels of MDI are lung sensitization and asthma. Albumin adducts of MDI might be involved in the etiology of sensitization reactions. This work presents a liquid chromatography (LC)–mass spectrometry (MS/MS) procedure for determination of isocyanate-specific albumin adducts in humans. MDI formed adducts with lysine of albumin: MDI–Lys and AcMDI–Lys. The MDI–Lys levels, 25th, 50th, 75th, 90th percentile, were 0, 65.2, 134, 244 fmol mg^{-1} and 0, 30.5, 57.4, 95.8 fmol mg^{-1} in the exposed construction and factory workers, respectively. This new biomonitoring procedure will allow assessment of suspected exposure sources and may contribute to the identification of individuals who are particularly vulnerable for developing bronchial asthma and other respiratory diseases after exposure to isocyanates.

Keywords: *MDI; bronchial asthma; isocyanates; albumin adduct, biomonitoring*

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Introduction

Isocyanates are highly reactive compounds that have a variety of commercial applications. Diisocyanates such as 4,4′-methylenediphenyl diisocyanate (MDI), are used for manufacturing polyurethane foam, paints, adhesives, elastomers, coatings, insecticides and consolidation of loose rock zones in coal mining or tunnelling, and many other products (Munn et al. 2005). The high chemical reactivity of diisocyanates makes them toxic. A number of adverse effects at the cellular and subcellular level have been reported, such as irritative and immunological reactions. Inhalation of diisocyanate vapours is associated with various pulmonary ailments, such as eosinophilic airway inflammation, airway hyper-reactivity, early and late-onset asthma, exogenous allergic alveolitis and direct toxic responses (Karol 1986, Baur 1990, Mapp et al. 1994, Redlich & Karol 2002). Diisocyanates are of great concern with regard to occupational health, being considered one of the main causes of occupational asthma (Karol 1986, Baur et al. 1994, Bernstein 1996). The steady rise in asthma over the past decades suggests a relationship between increasing amounts of isocyanates in consumer products and increasing prevalence of asthma in the general population, especially children (Krone & Klingner 2005). Occupational exposure to diisocyanates may take place during their production and application in the production of polyurethane foam and other products containing monomeric or polymeric diisocyanates. The predominant route of occupational exposure is through inhalation and dermal absorption (Liljelind et al. 2010).

Arylisocyanates react directly with biomolecules and/or hydrolyse to arylamines (Figure 1). Arylisocyanates (Vock & Lutz 1997, Bolognesi et al. 2001) and arylamines (Sabbioni & Jones 2002, Delclos & Kadlubar 1997) can bind with proteins and/or DNA (Figure 1) and lead to cytotoxic and genotoxic effects. Protein adducts of arylisocyanates are believed to be involved in the etiology of sensitization reactions (Raulf-Heimsoth & Baur 1998, Wisnewski et al. 2000). Arylamine-specific adducts are of the sulfinamide type (Kazanis & McClelland 1992,

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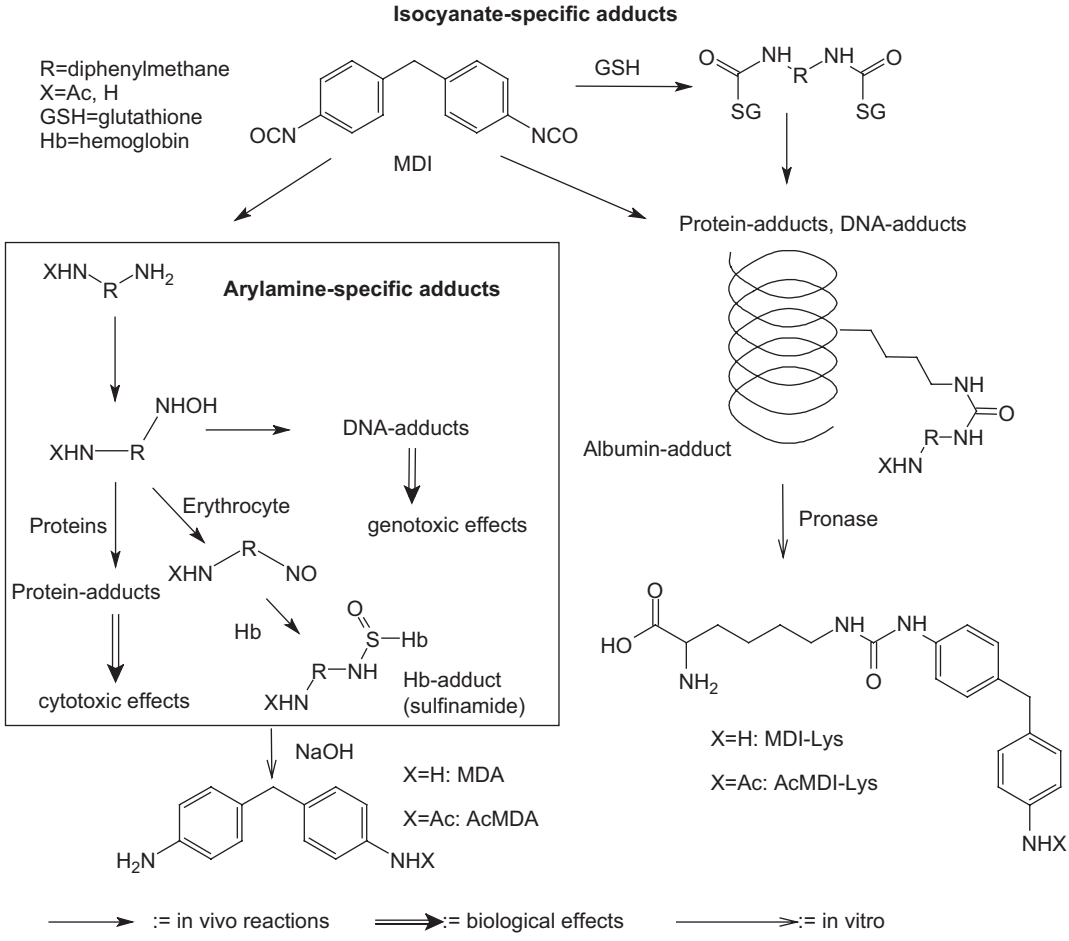

Figure 1. Adduct formation of MDI and MDA with biomolecules.

Ringe et al. 1988), which can be cleaved with mild base hydrolysis.

In contrast, isocyanates do not need any further activation to react with biomolecules (Figure 1). Important vehicles for isocyanates are their reaction products with glutathione (Pearson et al. 1991, Slatter et al. 1991). The glutathione adducts release the isocyanate moiety to react with other nucleophiles, e.g. proteins. Therefore, glutathione adducts are thought to be responsible for the transport of isocyanate to reactive sites away from the site of isocyanate uptake.

Urinary metabolites of 4,4′-methylene dianiline (MDA) and arylamine-specific adducts (Figure 1) with haemoglobin have been found in rats exposed chronically to MDI (Sepai et al. 1995). Isocyanate-specific adducts of MDI with the *N*-terminal valine of haemoglobin (Sabbioni et al. 2000) and/or with N_e-lysine of albumin have been quantified in rats (Kumar et al. 2009). The DNA adducts (Vock et al. 1996) of the nasal epithelial cell and the haemoglobin adducts (Sepai et al. 1995) correlate with the dose (Bolognesi et al. 2001) in rats chronically exposed to MDI. In rats, haemoglobin adducts have been found to correlate with the administered dose of either MDI, given by inhalation (Sepai et al. 1995), or the corresponding arylamine MDA, given by gavage (Sabbioni & Schutze 1998). MDA binds to haemoglobin (Sabbioni & Schutze 1998) and to DNA (Schutze et al. 1996) (see Figure 1).

Urinary metabolites of xenobiotics are usually detectable up to 48 h. Reaction products with blood proteins as haemoglobin and albumin can be detected over a larger time frame if the adducts are stable. Haemoglobin adducts has a lifetime of 120 days and albumin a halflife of 20–25 days (Skipper & Tannenbaum 1990). Therefore haemoglobin adducts can be detected over a longer time frame. However, albumin is one of the potential targets involved in the etiology of sensitization reactions of isocyanates (Raulf-Heimsoth & Baur 1998, Wisnewski et al. 2000). For the present project we developed a method to find isocyanate-specific adducts with albumin, which can be applied to human samples. 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

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antibodies present in workers and the general population (reviewed in Ott et al. 2007). For some chemicals known to cause occupational asthma, the presence of specific antibodies correlates well with clinical disease (van Kampen 2000). This is the case for high-molecular-weight agents in which an IgE-mediated mechanism has been identified in most instances. With diisocyanates, there appears to be less concordance between the antigenic reactivity of diisocyanate–protein conjugates and the occurrence of disease (Ott et al. 2007, Wisnewski 2007). Several research groups investigated immunological markers of diisocyanate-induced respiratory disease, in order to have an early and reliable indicator of diisocyanate-induced asthma (Ott et al. 2007). This recent literature review (Ott et al. 2007) indicates that there is considerable heterogeneity in the prevalence of specific IgE and IgG binding to diisocyanate conjugates across studies. The observed heterogeneity 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). A recent study by 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 MDI might be crucial for the immunological response of antibodies present in exposed workers (Wisnewski et al. 2010). Sites of MDI conjugation on antigenic MDI albumin products, as defined by serum IgG from MDI-exposed workers, were determined by liquid chromatography (LC)–mass spectrometry (MS/MS) analyses of tryptic digests of human albumin modified *in vitro*. The authors identified 14 MDI conjugation sites (12 lysines and two asparagines). MDI conjugated to albumin from other species exhibited minimal binding to IgG of exposed MDI workers. Therefore, Wisnecki et al. (2010) speculate that one of the lysines at positions 162, 541, 136–137 and 413–414, which differ in human albumin compared with albumin from other species, might be responsible for the immune specificity of MDI–human albumin adducts versus MDI–albumin adducts from other species. Thus, a test 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 MDI-exposed workers.

Material and methods

Workers

Blood and urine were collected from two worker groups. All biological samples were obtained at the end of the work-shift. Workers were recruited from a chemical factory (=MDI workers) (Schutze et al. 1995) and from construction sites (Sabbioni et al. 2007). The MDI workers (27 men) from the chemical industry were exposed to MDI, age median 32 (range, 20–57) (Schutze et al. 1995). The construction site workers (62 men and three women) were exposed to MDI and prepolymer products (Sabbioni et al. 2007). The median age (range) of the workers was 32.9 (20.1–59.6) years. At the beginning of the study, 40 workers (controls) had not been exposed to isocyanate products in the last 4 months and 25 workers had been exposed. The biological samples were collected from 65 workers (40 controls, 25 exposed) at the beginning of the study. After 4–7 months of exposure to isocyanate, blood samples from 19 former controls were obtained.

Chemicals

Amicon ultracentrifugal filter tubes (30K MWC; 4 ml), methanol (A454-4) for sample preparation and methanol (Optima, A456-4) for LC–MS/MS and were obtained from Fisher Scientific (Bridgewater, NJ, USA). Pronase E from *Streptomyces griseus* (#81748), ammonium formate (#17843) and sodium hydroxide were purchased from Fluka (Buchs, Switzerland). Sodium phosphate monobasic monohydrate, sodium thiocyanate, human serum albumin (#A1653), water for LC–MS/MS (Chromasolv; #39253) were purchased from Sigma-Aldrich (St Louis, MO, USA). Coomassie plus protein assay reagent for protein determination was acquired from Thermo Scientific (Rockford, IL, USA). Strata-X-33u (8B-S100-FBJ) polymeric reversed-phase columns (200 mg 3 ml^{-1}) were purchased from Phenomenex Inc. (Torrance, CA, USA). N^6-[({4-[4-aminobenzyl]phenyl}amino)carbonyl]lysine (MDI–Lys), N^6-[({4-[4-aminobenzyl]phenyl}amino)carbonyl] [$^{13}C_6{}^{15}N_2$]lysine (MDI–[$^{13}C_6{}^{15}N_2$]Lys), N^6-[({4-[4-(acetylamino)benzyl]phenyl}amino)carbonyl] lysine (AcMDI–Lys) and N^6-[4-[4-(acetylamino-3,5-dideuteriobenzyl)-2,6-dideuteriophenyl]carbonyl] lysine (Ac[$^2H_4$]MDI–Lys were synthesized as described previously (Kumar et al. 2009).

Instrumentation

A API 4000Q Trap (Applied Biosystems, Foster City, CA, USA) mass spectrometer interfaced to a HPLC (Shimadzu Prominance 20AD; Shimadzu, Kyoto, Japan) 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.

Isolation of albumin

Albumin was purified with HiTrap Blue HP column 1.0 ml (0.7 x 2.5 cm) from GE Life Sciences Inc., Westborough,

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MA, USA. The HiTrap Blue HP column was equilibrated with six volumes of binding buffer (20 mM NaH_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 NaH_2PO_4 + 2 M NaCl, 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 an Amicon ultracentrifugal filter tube (30k MWC; 4 ml) by centrifuging with 4000 rpm at 4°C for 10–15 min, and washed with water (3 x 4 ml). Samples were redissolved 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 Manufacturing (Appleton, WI, USA).

Digestion of albumin

Albumin (9 mg) in 50 mM ammonium bicarbonate, pH 8.9 (0.9 ml) was spiked with MDI–[$^{13}C_6{}^{15}N_2$]Lys (13.23 pmol) and Ac[$^2H_4$]MDI–Lys (12.02 pmol). Samples were digested and incubated with 500 µl of freshly prepared pronase E solution (6 mg ml^{-1}; 50 mM ammonium bicarbonate, pH 8.9) for 15 h at pH 8.9 and 37°C. The digest was acidified to pH 4.0 with 2 M hydrochloric acid and purified with 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 with 3 ml of 0.1% formic acid (pH 4.0). The samples were applied on the column and subsequently washed with 3 ml fractions of 0, 10, 20% methanol in 0.1% formic acid. MDI–Lys and AcMDI–Lys were eluted with 6 ml of 80% methanol in 0.1% formic acid. The eluate was concentrated to approximately 1 ml in a speed evaporator.

Quantification of albumin adducts, MDI–Lys and AcMDI–Lys, using LC–MS/MS

Shimadzu Prominance 20AD interfaced to a API 4000Q Trap LC–MS/MS (Applied Biosystems) mass spectrometer system was used for all the quantitative analysis. The MS parameter was optimized in the electrospray ionization mode (ESI). Parameter optimization was carried out with 100 pg $µl^{-1}$ solution of analyte with the flow rate of 10 µl min^{-1} in the negative ionization mode. MDI–Lys and AcMDI–Lys showed corresponding peaks at *m/z* 369.1 and 411.1 $[M–H]^-$. Quantitative optimization mode was used to maximize the signal and set the maximum suitable compound parameters for the compounds. For better resolution and sensitivity of the analyte, quadrupole mass analysers (Q1 and Q3) were set on 0.7 ± 0.1 amu resolution window.

The mass spectrometer was operated in negative ionization mode with an electrospray voltage –4500 V and a source temperature of 500°C. Nitrogen was used as ion spray (GS1), drying (GS2) and curtain gas at 40, 45 and 10 arbitrary unit, respectively. The declustering potential (DP) and collision energy (CE) for MDI–Lys and AcMDI–Lys were –75, –80 V and –28, –30V, respectively. The entrance potential (EP) for both compounds were –10 V. All data were processed using Analyst software 1.4.2 (Applied Biosystems/MDS Sciex).

The MDI–Lys and AcMDI–Lys were detected with multiple reaction monitoring (MRM) *m/z* 369.1/145.1 and 411.1/145.1, respectively. The corresponding internal standards MDI–[$^{13}C_6{}^{15}N_2$]Lys and Ac[$^2H_4$]MDI–Lys were monitored with the transitions *m/z* 377.1/153.0 and 415.1/145.1, respectively. Chromatographic separation was achieved on a Luna C18(2) (100 Å, 150 x 2.0 mm, 3 µm) (Phenomenex Inc.) protected by a C18 guard column (AJO–4287; 4 mm L x 3.0 mm ID), using a gradient system with solvent A (10 mM ammonium formate) and solvent B (methanol) at a flow rate of 0.2 ml min^{-1}: 0 min (B 20%), 3 min (B 20%), 16 min (B 90%), 20 min (B 90%). The retention times (t_R) of MDI–Lys and AcMDI–Lys were 15.2 and 15.7 min, respectively. The column flow was diverted away from the ESI ion source except for the time period from 5 to 18 min.

To generate the calibration line, human serum albumin (9 mg) in ammonium carbonate (50 mM, 2 ml) was spiked with different amounts of MDI–Lys (0.00, 0.54, 2.70 and 8.11 pmol) and AcMDI–Lys (0.00, 0.49, 2.43 and 7.28 pmol) along with MDI–[$^{13}C_6{}^{15}N_2$]Lys (13.23 pmol) and Ac[$^2H_4$]MDI–Lys (12.02 pmol) and worked up as described in the section 'Digestion of albumin'. The calibration lines for MDI–Lys and AcMDI–Lys were generated over the range of 0.0–8.10 and 0.0–7.28 pmol 9 mg^{-1} albumin, respectively. The concentration levels are plotted against the peak area ratio of the analyte against the peak area of the internal standard (e.g. peak area ratio MDI–Lys/MDI–[$^{13}C_6{}^{15}N_2$]Lys). The regression coefficient $r^2 = 0.998$ (MDI–Lys) and 0.999 (AcMDI–Lys) were found using the method of regression option 'linear and 1/x weighting factor'. The limit of the quantification (LOQ) following this work-up and analysis procedure was 7.5 and 6.7 fmol mg^{-1} albumin for MDI–Lys and AcMDI–Lys, respectively. The signal/noise ratio was >10. The limit of detection for standard compounds analysed by LC–MS/MS was 0.675 and 0.606 fmol for MDI–Lys and AcMDI–Lys, respectively.

Statistical analyses

The statistical analyses were performed with SPSS 16.0. The data were not normally distributed (one-sample Kolmogorov–Smirnov test, $p < 0.05$). Therefore, non-parametric tests were used for correlations (Spearman

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rank correlations), for the comparison of groups (Mann–Whitney test) and for the comparison of paired samples (Wilcoxon sign test).

Results

Plasma was collected from workers in the chemical industry (Schutze et al. 1995) and from workers on construction sites (Sabbioni et al. 2007). In previous studies other biomarkers had been determined in this group of workers: analysis of urinary metabolites after acid treatment (MDA–U-acid), urinary metabolites after base extraction (MDA–U-base, AcMDA–U-base) and arylamine-specific (Figure 1) haemoglobin adducts (MDA–Hb, AcMDA–Hb) released after mild basic extraction (Table 1). For the present work isocyanate-specific adducts with albumin were analysed (MDI–Lys, AcMDI–Lys) Albumin was isolated from plasma with Cibachron blue affinity chromatography. Albumin was digested with pronase in the presence of the internal standards, MDI–[$^{13}C_6{}^{15}N_2$]Lys and Ac[$^2H_4$]MDA–Lys. The digests were purified using solid-phase extraction. The concentrated extracts were analysed with LC–MS/MS (Figure 2). The best LC–MS/MS sensitivity for the quantitation of the albumin adducts found *in vivo* – MDI–Lys and AcMDI–Lys – was obtained using ESI in the negative ion mode. For the quantitation of the adducts *in vivo* isotope dilution MS was used.

The isocyanate-specific adduct with albumin, MDI–Lys, could be found in 62.5%, 63.6% and 15% of the MDI workers, exposed construction workers and control construction workers, respectively. The results were summarized in Table 1. The acetylated metabolite AcMDI–Lys was found in 4.2%, 29.5% and 0% of the MDI workers, exposed construction workers and control construction workers, respectively. The MDI–Lys levels were significantly higher in the MDI workers and the exposed construction workers compared with the control workers (Mann–Whitney test, $p < 0.01$). The MDI–Lys levels were similar in both exposed worker groups. The MDI–Lys levels, 25th, 50th, 75th, 90th percentile, in the exposed construction and MDI workers were 0, 65.2, 134, 244 fmol mg^{-1} and 0, 30.5, 57.4, 95.8 fmol mg^{-1}, respectively. The changes in the biomarker levels were compared in a group of construction workers ($n = 19$, controls) which were analysed prior to isocyanate exposure and after 4–7 months of isocyanate exposure (Figure 3). The MDI–Lys levels increased significantly (Wilcoxon sign test, $p < 0.01$).

The correlations of the biomarkers in the different populations were investigated (Table 2). All biomarkers correlate with $r > 0.9$ (Spearman rank correlation) in the rat experiment. In the construction workers the best correlations are found between the urinary metabolites and MDI–Lys. The correlation with the haemoglobin adduct is much smaller. For the MDI workers the number of subjects is probably too small in order to obtain significant correlations between the biomarkers.

Discussion

This is the first report about the presence of isocyanate-specific adducts with albumin in humans. Reactions of

Table 1. Comparison of biomarkers found in workers. MDI–Lys and AcMDI–Lys are the isocyanate-specific adducts with albumin.

	Exposed[a]	Mean ± STD Median, range	Controls[b]	Mean ± STD Median, range	MDI workers[c]	Mean ± STD Median, range	Rats[d]
MDI–Lys (fmol mg^{-1})	63.6% ($n=44$)	102.4 ± 156.8 68.8 (0–899.4)	15.0% ($n=40$)	4.33 ± 11.1 0 (0–43.8)	62.5% ($n=24$)	36.1 ± 38.6 30.5 (0–138)	10.6 ± 1.71 pmol mg^{-1}
AcMDI–Lys (fmol mg^{-1})	29.5% ($n=44$)	6.32 ± 117 0 (0–51.2)	0% ($n=40$)	0	4.2% ($n=24$)	25.6[e]	0.486 ± 0.118 pmol mg^{-1}
MDA–U-acid (pmol ml^{-1})	100% ($n=45$)	2.15 ± 3.24 1.34 (0.017–16.4)	100% ($n=37$)	0.373 ± 0.307 0.297 (0.048–1.46)	95.7% ($n=23$)	2.07 ± 1.99 1.70 (0–10.2)	65.5 ± 18.5 pmol ml^{-1}
MDA–U-base (pmol ml^{-1})	91.1% ($n=45$)	0.093 ± 0.163 0.033 (0–1.004)	25% ($n=40$)	0.0096 ± 0.57 0 (0–0.176)	95.7% ($n=23$)	0.0537 ± 0.0565 0.0565 (0–0.139)	0.214 ± 0.0263 pmol ml^{-1}
AcMDA–U-base (pmol ml^{-1})	91.1% ($n=45$)	0.794 ± 1.426 0.378 (0–7.44)	22.5% ($n=40$)	0.0229 ± 0.057 0 (0–0.304)	78.3% ($n=23$)	0.605 ± 0.658 0.54 (0–3.0)	8.28 ± 3.20 pmol ml^{-1}
MDA–Hb (fmol g^{-1})	27.3% ($n=44$)	41.1 ± 86.1 0 (0–373.3)	0% ($n=40$)	0	38.5% ($n=26$)	40.5 ± 60.4 0 (0–219)	15.3 ± 5.39 pmol g^{-1}
AcMDA–Hb (fmol g^{-1})	4.5% ($n=44$)	136.7 ± 653.3 0 (0–3743)	0% ($n=40$)	0	0% ($n=26$)	0	24.3 ± 8.88 pmol g^{-1}

All the other biomarkers have been published previously (Schutze et al. 1995, Sabbioni et al. 2007). MDA–U-acid corresponds to MDA found after acid treatment of urine. MDA–U-base and AcMDA–U-base correspond to MDA and AcMDA found after base extraction of urine. MDA–Hb and AcMDA–Hb correspond to MDA and AcMDA found after mild base hydrolysis of Hb. [a]Exposed construction workers. [b]Control construction workers. [c]Workers exposed to MDI in a chemical factory (=MDI-workers). [d]Biomarker levels found in rats exposed to 0.26 mg m^{-3} MDI (Kumar et al. 2009, Sepai et al. 1995). [e]Value of the only positive sample.

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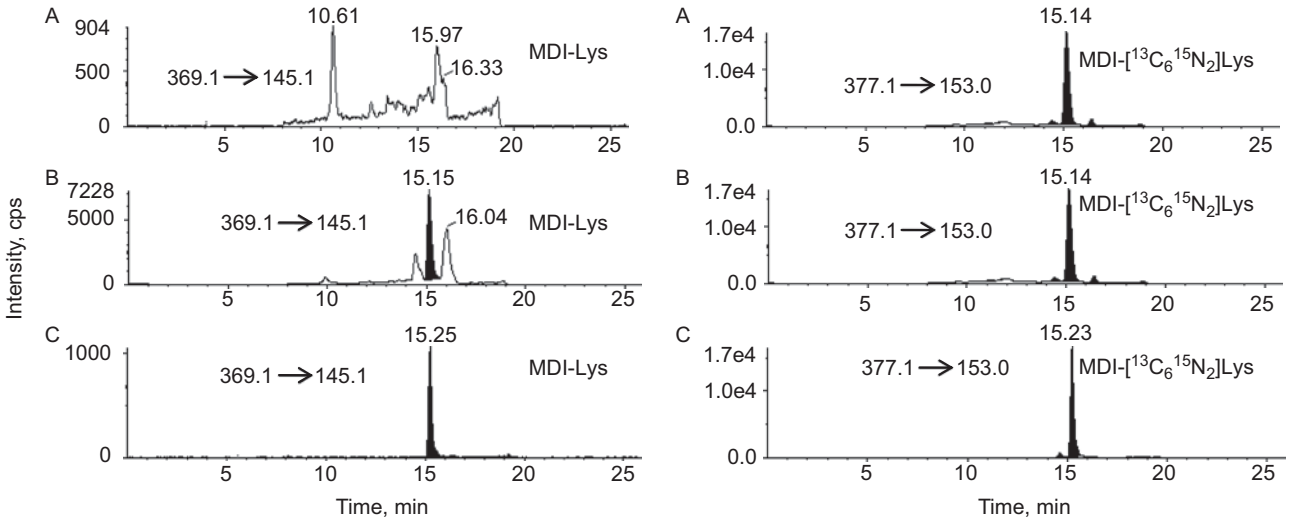

Figure 2. LC–MS/MS analysis of MDI–Lys found *in vivo* in the presence of the internal standard MDI–[$^{13}C_6{}^{15}N_2$]Lys (5 ng). (A) Control construction workers; (B) exposed construction workers; (C) MDI–Lys standard (0.2 ng).

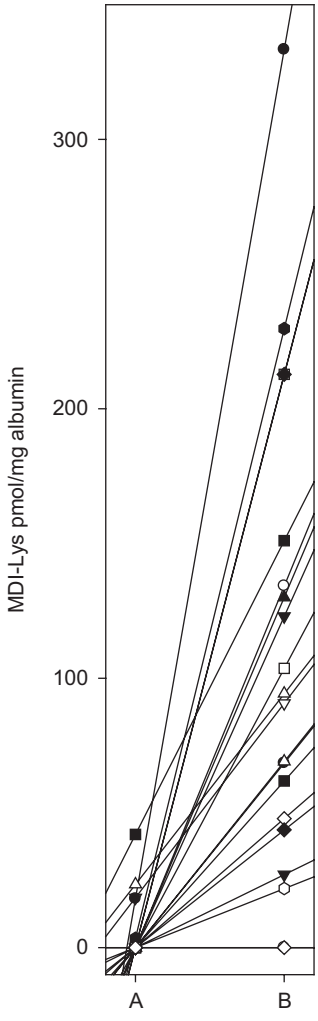

Figure 3. MDI–Lys levels in 19 workers. (A) Before working with isocyanates, and (B) after working with isocyanates in the past 4–7 months.

Table 2. Spearmen rank correlations of the biomarkers measured in different biospecimen.

	MDA–Hb	MDA–U-acid	MDA–U-base	AcMDA–U-base
MDI–Lys	0.295*[a]	0.535**[a]	0.537**[a]	0.476**[a]
	0.382*[b]	–[b]	–[b]	–[b]
	0.945**[c]	0.951**[c]	0.944**[c]	0.908**[c]

[a]Exposed construction site workers; [b]workers employed in a MDI factory (=MDI–workers); [c]rats exposed chronically to 0.26 mg m^{-3} MDI. *Correlation is significant at the 0.05 level (two-tailed); **correlation is significant at the 0.01 level (two-tailed).

isocyanates with amino groups as of the epsilon amino group of lysine yields ureas, which are very stable. Lysine adducts with methylphenylisocyanate could be cleaved only after treating the compound for several hours at 100°C (Sabbioni et al. 1997) with 0.3 M NaOH and/or 6M HCl. Therefore such adducts present in proteins are very stable. Thus, although all the isocyanate analyses listed in Table 1 were performed many years after the other measurements it is unlikely that the isocyanate biomarkers are an underestimation. How does the isocyanate-specific marker relate to the other markers measured previously? The data were compared with biomarkers presented in previous publications (Schutze et al. 1995, Sabbioni et al. 2007) for the same group of workers: analysis of urinary metabolites after acid treatment (MDA–U-acid), urinary metabolites after base extraction (MDA–U-base, AcMDA–U-base) and the arylamine-specific (Figure 1) haemoglobin adducts (MDA–Hb, AcMDA–Hb) released after mild basic extraction. The results were summarized in Tables 1 and 2. In addition the biomarker levels were compared with the levels found in rats which were exposed to 0.26 mg m^{-3} MDI for 17 h per day and 5 days per week (Sepai et al. 1995). In these rats first pathological effects were seen in the lung. Interstitial fibrosis was

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found in 79% of this exposure group and 10% bronchiolar type hyperplasia was found in this exposure group in comparison to 0% in the control group (Hoymann et al. 1995).

In the exposed construction workers, albumin adducts of MDI were present to a larger extent then MDA adducts with haemoglobin (64% vs 27% positive samples). Similar results were obtained in the MDI workers. Measuring only haemoglobin adducts would miss many workers which were exposed. Acid treatment of urine yields positive results in all exposed and control workers (Sabbioni et al. 2007). The levels were significantly higher in the exposed group. The ratio of albumin adducts (rats/humans) was greater than 100 (Table 2). For the urinary metabolites MDA–U-acid, MDA–U-base and AcMDA–U-base the ratio rats/humans was approximately 33, 3 and 12, respectively. Therefore, it appears that the rats are exposed to a larger extent to the monomers which can form the albumin adducts than the workers. The workers are probably exposed to small amounts of monomer but to a larger amount of prepolymer. These prepolymers cannot form the monomer adducts (e.g. MDI–Lys), but they could still be metabolized to the corresponding MDA monomers which would be detected in urine. In rats and humans the albumin adduct levels, MDI–Lys, are 1000 times larger than the haemoglobin adduct levels, MDA–Hb.

The ratio of the acetylated MDA varies greatly among the different biomarkers. In rats exposed to 0.26 MDI m^{-3} (Table 1), the ratio for the urinary metabolites AcMDA/MDA, the haemoglobin adducts (AcMDA–Hb/MDA–Hb) (Sepai et al. 1995), and the albumin adducts AcMDI–Lys/MDI–Lys was 40, 1.5 and 0.046, respectively. In humans the mean ratio of the urinary metabolites and the albumin adducts AcMDI–Lys/MDI–Lys was 10 and 0.06, respectively. For AcMDA–Hb the LOQ (120 fmol per sample) was six times higher than for MDA–Hb (<20 fmol per sample) (Schutze et al. 1995); therefore in all MDI-exposed workers only one sample was positive for the AcMDA–Hb adduct.

The rat data indicated that the haemoglobin adducts and the urinary metabolites are the consequence of biologically available MDA (Figure 1) which is then acetylated to a large degree (Kumar et al. 2009). From these data, the following pathways were postulated. The isocyanate-specific adducts result via the direct reaction of the isocyanate groups with albumin and haemoglobin or indirectly via the reaction of MDI with glutathione to the resulting thiocarbamate (Figure 1), which releases the isocyanate in the plasma and erythrocytes to yield the adducts with lysine of albumin. The arylamine-specific adducts result after MDI hydrolyses to MDA, which is further oxidized in the liver to its N-hydroxy arylamine and subsequently to the nitroso compound in the erythrocytes (Figure 1). The nitroso derivative of MDA and/or AcMDA yields the sulfinamide adducts, which are acid and base labile.

In conclusion, with the present method we showed that isocyanate-specific adducts of MDI with albumin can be determined in workers. MDI–Lys adducts in albumin are probably relevant for the antigenic properties of albumin present *in vivo*. With the present method the antigens used for the immunological tests can be characterized by the determination of the amount of MDI–Lys present after digestion. Thus, in future studies, the relationship between albumin adducts, immunological tests and the disease status should be investigated in MDI-exposed populations.

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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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