Published in final edited form as:

Chem Res Toxicol. 2019 April 15; 32(4): 557–565. doi:10.1021/acs.chemrestox.8b00262.

Di-Lysine-Methylene Diphenyl Diisocyanate (MDI), a Urine Biomarker of MDI Exposure?

Adam V. Wisnewski[*,†], **Ala F Nassar**[†], **Jian Liu**[†], and **Dhimiter Bello**[‡]

[†]Department of Internal Medicine, Yale University, New Haven, CT, USA

[‡]Department of Public Health, University of Massachusetts, Lowell, MA, USA

Abstract

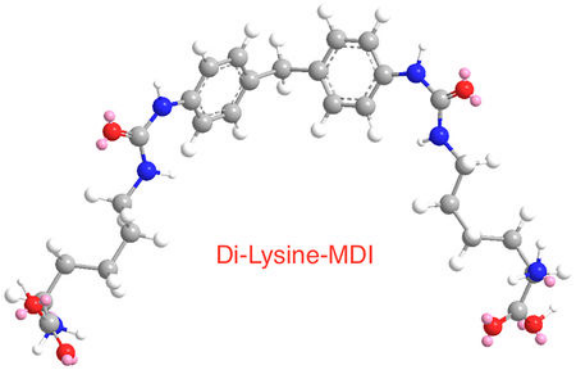
Biomonitoring methylene dipheny diisocyanate (MDI) in urine may be useful in industrial hygiene and exposure surveillance approaches toward disease (occupational asthma) prevention, and in understanding pathways by which internalized chemical is excreted. We explored possible urine biomarkers of MDI exposure in mice after respiratory tract exposure to MDI, as glutathione (GSH) reaction products (MDI-GSH), and after skin exposure to MDI dissolved in acetone. LC-MS analyses of urine identified a unique 543.29 *m/z* $[M+H]^+$ ion from MDI exposed mice, but not controls. The 543.29 *m/z* $[M+H]^+$ ion was detectable within 24 hours of a single MDI skin exposure and following multiple respiratory tract exposures to MDI-GSH reaction products. The 543.29 *m/z* $[M+H]^+$ ion possessed properties of di-lysine-MDI, including (a) an isotope distribution pattern for a molecule with the chemical formula $C_{27}H_{38}N_6O_6$, (b) expected collision induced dissociation (CID) fragmentation pattern upon MS/MS, and (c) a retention time in reverse phase LC-MS identical to synthetic di-lysine-MDI. Further MDI-specific western blot studies suggest albumin (which contains multiple di-lysine sites susceptible to MDI carbamylation) as a possible source for di-lysine-MDI, and the presence of MDI conjugated albumin in urine up to 6 days post respiratory tract exposure. Two additional $[M+H]^+$ ions (558.17 and 863.23 *m/z*) were found exclusively in urine of mice exposed to MDI-GSH via the respiratory tract and possessed characteristics of previously described cyclized MDI-GSH and oxidized glutathione (GSSG)-MDI conjugates respectively. Together the data identify urinary biomarkers of MDI exposure in mice and possible guidance for future translational investigation.

Graphical Abstract

[*]Corresponding Author: Adam V. Wisnewski, PhD, D(ABMLI), Yale University / PO Box 208057, 300 Cedar Street / TACS-415, New Haven, CT 06524 / USA, Adam.Wisnewski@Yale.edu, Phone: (203)-737-4054, Fax: (203)-785-3826.

SUPPORTING INFORMATION

Data include LC-UV-MS/MS and partial ^{1}H-NMR of MDI-GSH reaction products, Expected MS/MS CID-fragmentation for di-lysine-MDI and general schematic of it's chemical synthesis, Expected isotope distribution for (glutathione-MDI) urine molecules from mice exposed to MDI-GSH, MS/MS of urinary 558.17 and 863.23 *m/z* $[M+H]^+$ parent ions.



Keywords

methylene diphenyl-diisocyanate; MDI; urine; biomarker; exposure

INTRODUCTION

Diisocyanates are widely used chemicals in many different industries including construction, foundries, mining, automobile and polyurethane manufacturing.[1–6] Exposure can cause asthma through mechanisms that remain unclear and recognition of chemical sensitivity can be challenging for multiple reasons, including lack of diagnostic markers (e.g. chemical-specific IgE) and delayed responses that occur outside the workplace.[7–11] There is no cure for diisocyanate asthma and long-term prognosis for affected workers may be poor despite cessation of exposure.[12–16] Thus, surveillance to ensure minimal exposure represents an important approach to disease prevention in workplaces where the chemicals are used.[17–19]

One urinary biomarker of diisocyanate exposure has been described in the literature to date, based on levels of a hydrolyzable diisocyanate derivative.[20–23] Hydrolyzed/derivatized diisocyanate can be quantitated by gas chromatography (GC) coupled to mass spectrometry (MS), e.g. GC-MS.[20–22, 2425] Urine levels of hydrolyzable diisocyanate (the corresponding diamine that results when diisocyanate conjugated molecules are treated with acid or base at elevated temperatures) have been associated, to varying degrees, with workplace exposure to diisocyanate.[26–28] However, the method lacks specificity (cannot distinguish diamines, of questionable carcinogenicity),[23] is somewhat labor intensive, and provides limited information on the route by which the chemical reached the urine.[23, 25]

Two blood biomarkers for diisocyanate exposure have been described in the literature to date based on (1) a hydantoin formed by acid hydrolysis of purified diisocyanate conjugated hemoglobin[29, 30] and (2) a partially hydrolyzed diisocyanate-lysine conjugate obtained by protease treatment of purified diisocyanate conjugated albumin.[31, 32] These biomarkers have been associated with diisocyanate exposure in animal and numerous clinical research studies.[29, 30, 32–34] However, blood biomarker processing requires overnight (acid hydrolysis/enzyme digestion) steps and invasive, albeit minimally so, collection techniques that may limit frequent (e.g. daily) surveillance.

The present study was designed to better characterize urinary biomarkers of MDI, with a focus on low molecular weight (LMW) compounds that might be readily measured without extensive purification and processing. Mice were exposed via the respiratory tract to reversibly reactive MDI-GSH reaction products, a technique shown to avoid scrubbing of the upper airways and effectively target MDI to the lower airways.[35–40] Mice were also dermally exposed to MDI dissolved in the organic solvent, acetone. Twenty-four hour and "spot" urine samples from exposed vs. control animals were compared using LC-MS and MS/MS approaches to discover exposure biomarkers. Urine and airway fluid proteins were also analyzed with an α-MDI mAb to identify MDI conjugated proteins. The results are discussed in the context of our contemporary understanding of diisocyanate biochemistry, particularly it's reactivity with GSH and di-lysine in human albumin,[41–46] and relevance to human workplace exposure surveillance.

EXPERIMENTAL PROCEDURES:

Caution: Methylene diphenyl diisocyanate (MDI) is hazardous and a well-recognized immune-sensitizing chemical. Nitrile gloves, protective clothing, and goggles should be used for personal protection.

Chemicals, Reagents, and Equipment. Reduced glutathione, acetonitrile, formic acid, trifluoroacetic acid, sodium chloride, and 4,4′-methylenebis(phenyl isocyanate) (CAS # 101–68–8), N-α-(tert-butoxycarbonyl)-L-lysine, n-hexane and trifluoroacetic acid were from Sigma-Aldrich (St. Louis, MO). Mono- and dibasic sodium phosphate, ethyl acetate, diethyl ether, and dicholromethane were from JT Baker (Phillipsburg, NJ). Rat anti-mouse IgG_1 and peroxidase-coupled streptavidin were from BD Biosciences (San Jose, CA). Metabolic cages made by Techniplast were obtained through Harvard Apparatus (Hollister, MA). Syringe filters, 13 mm Acrodisc with 0.2 μm pore size, were from Pall Corporation (Ann Arbor, MI). Sodium dodecyl sulfate (SDS) polyacrylamide gels (4–20% gradient), nitrocellulose, and reducing gel electrophoresis buffer were from Bio-Rad (Hurcules, CA). Tris-HCl was from American Bioanalytical (Natick, MA). Phosphate buffered saline (PBS) was from Gibco (Grand Island, NY). Extra dry (99.8%) Acros brand acetone and SuperSignal West Femto Maximum Sensitivity enhanced chemiluminescence substrate were obtained through Thermo Fisher Scientific (Rochester, NY). Protein G Sepharose 4 Fast Flow was from GE Healthcare (Piscataway, NJ). Fujifilm Corporations X-Ray film was obtained through Dot Scientific (Burton, MI) and processed using a Kodak X-Omat 2000A processor from the Eastman Kodak Company (Rochester, NY). The LC-MS and MS/MS were run on a 1290 model Infinity LC system coupled to a 6550 model Q-TOF system using a rapid resolution HT Zorbax Eclipse Plus C18 column (2.1 × 50 mm, 1.8 μm), with MassHunter software for acquisition and analysis, all of which were from Agilent Technologies (Santa Clara, CA). Amicon Ultra 0.5-mL Centrifugal Filters Ultracel 3K (UFC500324) were obtained from Merck Millipore Ltd (Billerica, MA). HPLC/MS-grade water (Water Optima LC/MS-W6–4) was from Fisher Scientific (Fairlawn, NJ). Disposable 1cc (50mg) Sep-Pak Vac C18 cartridges (WAT054955) were from Waters (Milford, MA). An Epson (Long Beach, CA) Perfection Scanner was used to digitize developed film. Chemical structures were drawn with ChemDraw Professional v.17.

Preparation of MDI-GSH and control reaction products. MDI was reacted with GSH as previously described.[42] Briefly, MDI was dissolved in acetone to yield a 10% weight/volume (w/v) solution. The stock 10% MDI in acetone was then further diluted 100-fold into 10 mM reduced glutathione (GSH) dissolved in 200 mM sodium phosphate buffer pH 7.4, to achieve a final [MDI] of 0.1% w/v or ~4 mM, well below the starting concentrations typically used typical occupationally.[47] The reaction mixture was rotated end-over-end for 2 hours at 37°C, after which time the samples were microcentrifuged (10,000 *g*), 0.2 μm filtered and stored on ice until used within 1 hour of preparation. The total MDI-GSH reaction mixture and control solutions used in the present study were similar to those previously published[36, 42, 48] and are characterized by LC-UV, MS/MS, and ^{1}H-NMR in supporting information (Figs. S1–S3). The reaction mixture contained primarily bis(GSH)-MDI (865.25 *m/z* $[M+H]^+$ ions), and lower levels of mono(GSH)-MDI in cyclized form (558.17 *m/z* $[M+H]^+$ ions), along with unreacted GSH and limited amounts of GSSG. Control solutions were equivalent amounts of GSH treated with a 1:100 dilution of acetone without MDI, or MDI in acetone reacted in phosphate buffer without GSH at 37°C for 2 hours and identically processed.

Lower respiratory tract exposure of mice to MDI-GSH or control solutions.

Mice were lightly anesthethetized with isoflurane and 50 μL of GSH-MDI reaction products, or control solutions were delivered intranasally as previously described.[36, 49] Mice were exposed once per day for 5 days and housed in triplicate in metabolic cages to permit collection of 24-hour urine samples (on blue ice). In some studies animals were maintained in metabolic cages up to 7 days post exposure. All experiments were simultaneously run with MDI and control exposures, and repeated on three separate occasions with N=3 mice per group.

MDI skin exposure of mice. Mice were dermally exposed to 1% MDI as previously described. Animals were shaved on the back one day before (-1d) exposure, and 50 uL of 1% MDI (w/v) in acetone was applied under light isoflurane. Animals housed in metabolic cages were exposed once on day 1 and again on day 4, or in pilot studies for four consecutive days. All animal experiments were conducted in accordance with policies of the National Institutes of Health Care and Use of Laboratory Animals and were approved by Yale University's Institutional Animal Care and Use Committee.

Processing of 24-hr urine samples for LC-MS/MS analysis. Two hundred fifty microliters of urine samples were subject to solid phase extraction (SPE) with disposable C_{18} columns according to the manufacturer's instructions. Samples were first conditioned by addition of trifluoroacetic acid to 0.06%, and the eluent obtained with 60% acetonitrile was speed-vacced to dryness and resuspended in 30 μL of a 0.1% formic acid solution in preparation for LC-MS and LC-MS/MS. In later studies, the SPE step was omitted and instead urine was mixed 1:1 with 0.1% formic acid and filtered through a 3 kDa molecular weight cut-off spin column before LC-MS and LC-MS/MS analyses.

LC-MS and LC-MS/MS analysis. Samples were analyzed using the Agilent system described above in the Chemicals, Regents, and Equipment Subsection and in prior reports.

[50] Samples were mixed 1:1 with water containing 0.1% formic acid, and 3 or 10 μL (for SPE or untreated samples respectively) was loaded and eluted with water/0.1% formic acid and increasing concentrations of acetonitrile, also containing 0.1% formic acid. In some later studies 10 uL of 3kDa filtered urine was directly loaded onto the column. The acetonitrile gradient followed a linear generally increasing to 40% acetonitrile by 3 minutes, followed by increase to 98% by 4.5 minutes and return to 2% acetonitrile by 6 minutes, although gradients varied slightly as studies evolved. Positive electrospray ionization (ESI) was performed using the following parameters: gas temp- 280°C, gas flow- 11 l/min, nebulizer-40 psig, sheath gas temp- 350°C, sheath gas flow-11, Vcap-4000 V, nozzle voltage-2000 V, fragmentor voltage– 175 V, skimmer voltage 65 V, octopole RF peak voltage 750 V. For MS/MS analyses, the collision energy was automatically set using Agilent MassHunter Acquisition software according to the formula, slope × (*m/z*)/100 + offset; with the slope of 5 and offset of 2.5. The *m/z* values of all ions present in the mass spectra were corrected against two reference ions (purine, $[M+H]^+$ *m/z* 112.9856 and 1H, 1H, 3H tetra(fluoropropoxy)phosphazine, $[M+H]^+$ *m/z* 922.0097). The data acquisition range was from 110–1700 *m/z*. Quantitation of selected $[M+H]^+$ ions were calculated from extracted ion chromatograms (EICs), based on the area under the curve (AUC) of peaks with defined *m/z* ratios and retention times.

Chemical synthesis of di-lysine-MDI. Di-lysine-MDI was synthesized using the methods of Kumar et al[31] with minor modifications. Briefly, N-α-(tert-butoxycarbonyl)-L-lysine (N-Boc-Lys) (250 mg, 1.0 mmol) was dissolved in 0.25 M sodium bicarbonate (5 mL, pH 8.3). 4,4'-MDI (0.5 mmol) in 1,4-dioxane (2.5 mL) was added dropwise and the mixture was stirred overnight. The reaction mixture was concentrated in vacuo to approximately 5 mL, washed successively with n-hexane (3 × 5 mL) and ethyl acetate (6 × 5 mL), before acidification (pH 3–4) and back-extraction with ethyl acetate (3 × 10 mL). Ethyl acetate extracts were dried on anhydrous magnesium sulfate, filtered, and evaporated in vacuo. Subsequently, bis(boc-Lys)-MDI was dissolved in dichloromethane (2.5 ml) and trifluoroacetic acid (2.5 ml), stirred for 2 h at room temperature, and evaporated under reduced pressure. The residue was precipitated from, and washed with, diethyl ether. Yields were <10% of the starting material, but of >90% purity based LC-MS total ion, A210 and A254 chromatograms. The structures of the synthesis products were further corroborated using NMR spectroscopy. ^{1}H NMR (400 MHz, Methanol-d_4) δ 7.24 (d, J = 8.5 Hz, 4H), 7.06 (d, J = 8.5 Hz, 4H), 3.95 (t, J = 6.3 Hz, 2H), 3.83 (s, 1H), 3.21 (t, J = 6.5 Hz, 4H), 2.08 − 1.96 (m, 2H), 1.96 − 1.86 (m, 2H), 1.59 – 1.53 (m, 4H), 1.40 – 1.21 (m, 4H). ^{13}C NMR (101 MHz, Methanol-d_4) δ 170.41, 157.27, 137.44, 128.74, 122.63, 119.24, 52.46, 40.10, 38.79, 29.78, 29.48, 21.85.

Bronchoalveolar Lavage (BAL). Lavage of lower airways was accomplished as previously described with 3 × 0.8 mL of phosphate buffered saline (PBS).[36, 49] BAL was centrifuged at 10,000 *g*, and the supernatant was collected for Western blot.

MDI-specific Western blot. Electrophoresis and Western blots of urine and BAL fluid from MDI-GSH exposed and control mice were performed using 4–20% gradient SDS polyacrylamide gels under reducing conditions followed by transfer to nitrocellulose.[49, 51, 52] Blots were probed with DA5, an IgG1 MDI-specific monoclonal antibody (mAb) as

previously described.[53] Before analysis, the BAL and the urine were depleted of endogenous mouse IgG1 (using protein G coated sepharose beads) to eliminate background signal from secondary reagents (biotinylated anti-mouse IgG1), as previously described.[49] Following SDS-polyacrylamide electrophoresis (SDS-PAGE), the treated (protein G depleted) BAL and urine were transferred to nitrocellulose for Western blot. Five μg/mL of anti-MDI (mAb DA5) was incubated with nitrocellulose overnight in dry milk at 4°C, followed by development with biotinylated anti-mouse IgG_1, streptavidin-conjugated peroxidase, enhanced chemiluminescent substrate, exposure to film and traditional film processing. Western blot of BAL samples was performed using 50 nanoliters/lane from individual animals, while urine blots used 2 μL/lane. Scanned films were saved as .tif files and imported into PowerPoint for annotation.

Statistical Analyses. Statistical significance of differences in urinary molecules was calculated based on the area under the curve (AUC) at defined retention times for $[M+H]^+$ ions with a given *m/z* using an unpaired Student's *t*-test or with analysis of covariance when data from more than one experiment were pooled.

RESULTS:

Identification of a 543.29 *m/z* $[M+H]^+$ ion uniquely expressed in MDI exposed mouse urine.

LC-MS total ion current and base peak chromatograms of urine from MDI exposed mice vs. controls were overall very similar (data not shown). However, initial LC-MS subtraction analysis[50] identified a unique 543.29 *m/z* $[M+H]^+$ ion in spot urine samples from mice repeatedly exposed to MDI (once/day for 4 days via the respiratory tract or skin) but not control animals (Fig. 1). We subsequently quantitated the 543.29 *m/z* $[M+H]^+$ ion in 24-hour urine samples from mice housed in metabolic cages. As shown in Figure 2A, following a single dermal exposure, urinary 543.29 *m/z* $[M+H]^+$ ion levels peaked within 24 hours and declined slightly in the ensuing 48 hours. Following a second skin exposure on day 4, urinary 543.29 *m/z* $[M+H]^+$ ion levels again peaked 24 hours later, and then declined over the ensuing week. In mice exposed to MDI-GSH via the respiratory tract (Fig. 2B), the 543.29 *m/z* $[M+H]^+$ ion was undetectable within 24 hours of exposure, but became elevated following multiple daily exposures, and remained elevated up to one week post exposure cessation. Thus, a specific 543.29 *m/z* $[M+H]^+$ ion is uniquely found in the urine of MDI exposed, but not control mice.

Evidence the 543.29 m/z $[M+H]^+$ ion in MDI exposed mouse urine is di-lysine-MDI.

As shown in Fig. 3A and 3B, the isotope distribution pattern of the mouse urinary 543.29 *m/z* $[M+H]^+$ ion matched the theoretically predicted isotope distribution pattern for a molecule with the formula $C_{27}H_{38}N_6O_6$. Hulst et al[54] described a similar 543.29 *m/z* $[M+H]^+$ ion among pronase digests of human skin callus exposed to MDI ex vivo, and based on MS/MS data suggested the molecule was di-lysine-MDI with chemical conjugation to the ε-amine of the lysines, as depicted in Fig 3C. MS/MS fragmentation pattern of urinary 543.29 *m/z* $[M+H]^+$ from MDI exposed mice in the present study was also consistent with di-lysine-MDI (Fig. 4, and supporting information Fig. S4 for expected fragmentation pattern). The prominent 173.09 m/z $[M+H]^+$ daughter ion likely results from cleavage of one lysine

attached to one NCO group of MDI, leaving behind a 371.21 *m/z* fragment. The 225.10 and 199.12 *m/z* $[M+H]^+$ daughter ions likely reflect partially and completely hydrolyzed MDI. Daughter ions expected for lysine (147.11 *m/z*), and lysine + C=O from MDI, minus lysine's carboxylic acid moiety (127.09 *m/z*) are also observed.

To provide further evidence the 543.29 *m/z* $[M+H]^+$ ion in urine from MDI exposed mice was di-lysine-MDI, we chemically synthesized di-lysine-MDI, with chemical conjugation to lysines ε-NH_2s, using the approach described in the methods and depicted in supporting information Fig. S5. LC-MS/MS analysis of the synthetically purified di-lysine-MDI (Fig. 5) demonstrated its purity based on total ion, A_{210} and A_{254} chromatograms, the expected *m/z* of its $[M+H]^+$ parent ion, and its major daughter ions in MS/MS. The 543.29 *m/z* $[M+H]^+$ ion in urine from mice exposed to MDI (via the respiratory tract and the skin) displayed identical retention times as that of synthetic di-lysine-MDI (Fig. 6). Together, LC-MS/MS data on urine from MDI exposed mice and chemically synthesized di-lysine-MDI, along with previously published MS/MS studies of skin exposed to MDI in vitro, provide evidence supporting the di-lysine-MDI molecular model proposed in Fig. 3C for the 543.29 *m/z* $[M+H]^+$ ion.

MDI conjugated proteins: a potential source of di-lysine-MDI.

Specific di-lysine sites in albumin have been identified as preferred reaction targets for diisocyanates in vitro[45, 46, 55], and albumin has been described as a major target for diisocyanate in exposed human airway fluid.[52] Thus, we probed for MDI-conjugated proteins in airway fluid of mice exposed to MDI-GSH via the respiratory tract, and further evaluated the potential presence of MDI conjugated protein in 24-hour urine samples. Following 4 daily exposures to MDI-GSH, but not control solutions, the airway fluid contained high levels of MDI-conjugated to an ~68 kDa protein, the major protein present (Fig. 7). The urine of MDI-GSH exposed mice also contained MDI conjugated exclusively an ~68 kDa protein, with levels highest following multiple (4) daily exposures, then declining post exposure. Together the present data are highly consistent with in vitro studies describing direct, and GSH-mediated, MDI carbamylation of albumin (the major protein in airway fluid albumin)[52, 56], and suggest excretion of MDI-albumin in the urine up to 6 days post exposure cessation.

Urinary MDI-GSH reaction products post respiratory tract exposure.

LC-MS/MS analysis of urine from mice exposed via the respiratory tract to MDI-GSH contained specific $[M+H]^+$ ions not found in mice dermally exposed to MDI (in acetone) or control mice. One molecule was a 558.17 *m/z* $[M+H]^+$ ion with (a) an isotope distribution expected for a molecule with the chemical formula $C_{25}H_{27}N_5O_8S$ (see supporting information Fig S6), (b) a CID fragmentation pattern upon MS/MS (supporting information Fig S7) expected for mono(GSH)-MDI in a cyclized formation; daughter ions of partially hydrolyzed and fragmented MDI (225.10 and 106.06 *m/z*), MDI with the thiol group from GSH (283.05 *m/z*), cys-gly from GSH (179.05 *m/z*), or loss of glycine from the parent ion (483.14 *m/z*), and (c) an LC-MS with multiple retention times (Fig. 8) identical to that observed with cyclized MDI-GSH synthesized in vitro as previously described.[42] The MDI-GSH reaction mixture to which the mice were exposed contained low levels (see supporting

information Fig S1) of cyclized GSH-MDI we hypothesize may have been excreted unchanged, or formed in vivo.

A second molecule uniquely found in urine from mice exposed via the respiratory tract to MDI-GSH was an 863.23 *m/z* $[M+H]^+$ ion with (a) an isotope distribution pattern (supporting information Fig S6) predicted for a molecule with the chemical formula $C_{35}H_{42}N_8O_{14}S_2$, (b) a CID fragmentation pattern upon MS/MS (supporting information Fig S8) consistent with that expected for MDI-GSSG (daughter ions with 788.21, 564.11, 334.07, 259.04, and 130.05 *m/z*) and (c) an LC-MS retention pattern matching MDI-GSSG synthesized in vitro (Fig. 8), as previously described.[48]

DISCUSSION.

The present study was designed to identify potential low molecular weight (LMW) urinary biomarkers of respiratory tract and skin exposure to MDI. The study employed a recently defined model for delivering reactive MDI to the lower airways of mice, as reversibly reactive GSH conjugates[35, 36], and evaluated mice dermally exposed to MDI in organic solvent. One unique molecule in the urine of mice exposed to MDI, via either the respiratory tract or skin, was detected by reverse phase LC-MS/MS in positive mode as a 543.29 *m/z* $[M+H]^+$ ion with properties of di-lysine MDI ($C_{27}H_{38}N_6O_6$), as shown in Figure 3C. The 543.29 *m/z* $[M+H]^+$ ion was detectable within 24 hrs of a single dermal exposure, and following repetitive daily respiratory tract exposures. The data provide guidance for future clinical investigation to determine if urinary di-lysine-MDI levels reflect occupational exposure of human workers, and provide in vivo confirmation of in vitro studies linking MDI, GSH and di-lysine motifs in albumin.[36, 42, 43]

The present data are in excellent agreement with, and expand upon, findings of Hulst et al. of a molecule with a monoisotopic mass of 542.285 amu, among pronase digested samples of human skin callus exposed to MDI ex-vivo.[54] The molecule was predicted to be di-lysine-MDI, with chemical conjugation of the two lysines ε-NH_2 groups. A urinary molecule in MDI exposed mice in the present study possesses the same characteristics as the di-lysine molecule described by Hulst et al[54] and further, shares the same retention time in LC-MS/MS as synthetic di-lysine-MDI.

The finding of di-lysine-MDI as an MDI exposure biomarker fits well with the reactivity of MDI defined in vitro and through clinical studies of albumin from occupationally exposed workers.[24, 26, 33, 34] MDI (and other diisocyanates) directly carbamylate human albumin in vitro on the side chain of specific lysines, especially di-lysines.[45, 46] Diisocyanate-GSH reaction products also carbamylate specific lysine side chains of albumin in a pH and temperature dependent manner.[41, 42] In exposed workers albumin appears to be a dominant carrier protein for diisocyanates, and “mono” lysine conjugated to partially hydrolyzed MDI has been previously described as a blood diisocyanate exposure biomarker.[24, 32, 34] Together with the published literature on diisocyanate reactivity with lysine, the present findings provide compelling evidence supporting the potential for di-lysine-MDI to serve as a urinary biomarker of MDI exposure.

The identification of molecules with properties of MDI conjugated to GSH or GSSG, in cyclized formation, in urine of mice exposed via the respiratory tract to MDI-GSH reaction products suggest a possible mechanism for chemical “detoxification” and removal from the body. Such molecules may be stabilized by MDI conjugation to the amine terminus(i) of GSH, which may promote chemical excretion (vs. potentially pathogenic carbamylation of self protein) and have been described previously in microsomal studies of MDI.[48] Cyclized MDI-GSH was a minor component of the MDI-GSH reaction products to which mice were exposed in this study, and may have been eliminated unchanged. Like di-lysine-MDI, the relevance of cyclized MDI-GSH and MDI-GSSG to occupational exposure in human workers will require translational investigation.

The significance of the present findings should be interpreted with recognition of the studies strengths and weaknesses. The strengths of the study include, controlled in vivo exposures, LC-MS/MS methodology for separating/characterizing LMW urine components, chemical synthesis of a potentially novel urinary MDI exposure biomarker (di-lysine MDI), and an MDI-specific mAb to identify MDI-conjugated proteins. However, small sample volumes limited purification and orthogonal NMR verification of the urinary 543.29 m/z $[M+H]^+$ ion as di-lysine-MDI. Dose, timing, and formulation (GSH conjugate vs. organic solvent) varied for respiratory tract vs. skin exposure in this exploratory study and urine collection was standardized by time interval (24 hr) rather than creatinine levels, limiting quantitative comparisons of exposure route. Perhaps most importantly, MDI metabolism in humans remains unclear and potential species differences may exist in mice.

In summary, we used murine models of MDI respiratory tract and skin exposure to identify potential reaction products / metabolites of the chemical excreted in the urine. We identified a single urinary molecule in mice exposed dermally or through the respiratory tract, with properties of di-lysine-MDI. The data are in good agreement with known reactivity of MDI with di-lysine groups of albumin, which could be a source for di-lysine-MDI, and are strikingly similar to data on urinary styrene-globin biomarkers by Mraz et al.[57, 58] Methodology for identifying di-lysine-MDI in urine is straightforward and rapid, through direct LC-MS/MS analysis of filtered (3kDa) urine, without overnight sample processing (hydrolysis/derivatization, purification, enzymatic digestion) described for previously published diisocyanate exposure biomarkers. Together, the data highlight a newly recognized biomarker of MDI exposure in mice that may be applied toward human exposure surveillance if translational across species.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

We would like to acknowledge Drs. Terence Wu and Mousumi Ghosh of Yale University’s West Campus Analytical Core, and the staff of Yale’s LSOG Animal Care Facility for their help and support with the study.

FUNDING INFORMATION

The work was funded by a grant (OH010941) from the Center for Disease Control via the National Institute of Occupational Safety and Health.

ABBREVIATIONS LIST

AUC	Area Under the Curve
BAL	Bronchoalveolar Lavage
CID	Collision Induced Dissociation
EIC	Extracted Ion Chromatogram
HDI	Hexamethylene diisocyanate
GC	Gas Chromatography
GSSG	Oxidized glutathione
GSH	Reduced glutathione
LC	Liquid Chromatography
LMW	low molecular weight
mAb	monoclonal antibody
MDI	Methylene diphenyl diisocyanate
MS	Mass Spectrometry
m/z	mass/charge ratio
PAGE	Polyacrylamide Gel Electrophoresis
SDS	Sodium Dodecyl Sulfate
SPE	Solid Phase Extraction
TIC	Total Ion Chromatogram
TDI	Toluene diisocyanate
UV	ultraviolet

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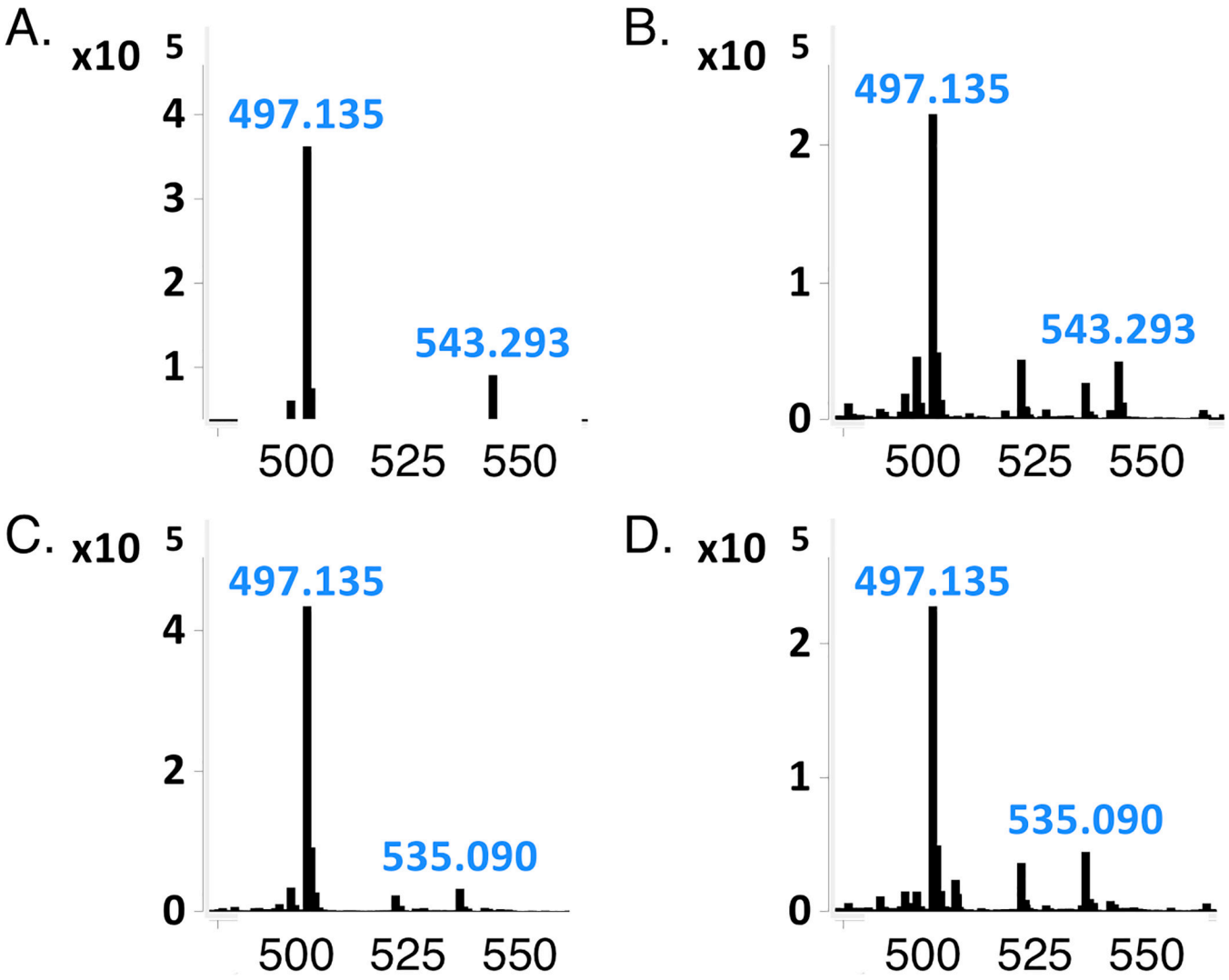

Figure 1.

Unique $[M+H]^+$ ions in urine of mice exposed to MDI. LC-MS analysis of urine from mice exposed to MDI-GSH via the respiratory tract (A), MDI in acetone via the skin (B), or control MDI reaction products via the respiratory tract (C) or skin (D) was performed and portions of spectra from fractions with retention time ~2.4 min are shown, highlighting a 543.29 $[M+H]^+$ ion unique to MDI exposed animals. Y-axis represents ion intensity and X-axis is ratio of mass/charge (*m/z*).

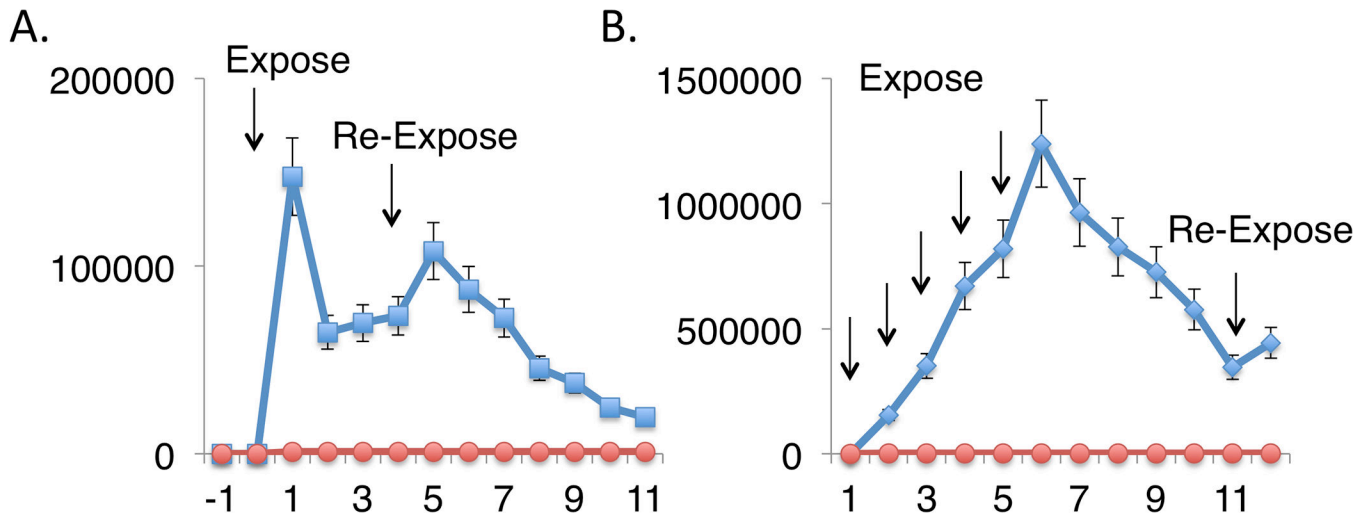

Figure 2.

Quantitation of 543.29 *m/z* $[M+H]^+$ ion in urine from MDI exposed mice. The amount of 543.29 *m/z* $[M+H]^+$ ion in 24-hr urine samples of MDI exposed mice was quantitated based on the integrated area under the curve (AUC) of extracted ion chromatograms. The mean and standard error are shown from 3 independent experiments with N=3 exposed (blue squares) or control (red circles) mice. Y-axis depicts AUC and X-axis depicts time in days.

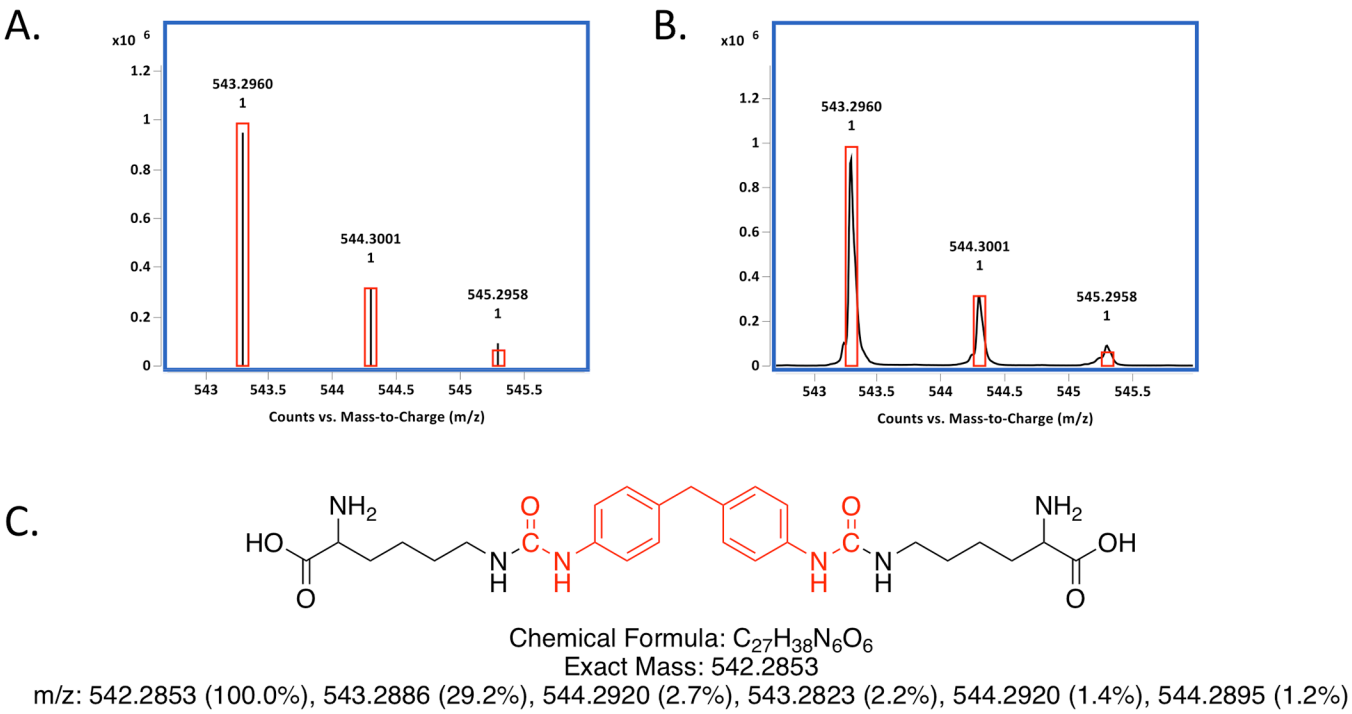

Figure 3.

Predicted isotope distribution pattern and chemical structure for the 543.29 *m/z* $[M+H]^+$ ion in urine of MDI exposed mice. Red boxes depict the signal expected for a molecule with the formula $C_{27}H_{38}N_6O_6$, in LC-MS chromatograms (A) with the background subtracted or (B) raw data. The predicted structure of di-lysine-MDI, with chemical cross-linkage of the lysines' ε-NH_2 is shown in Panel C.

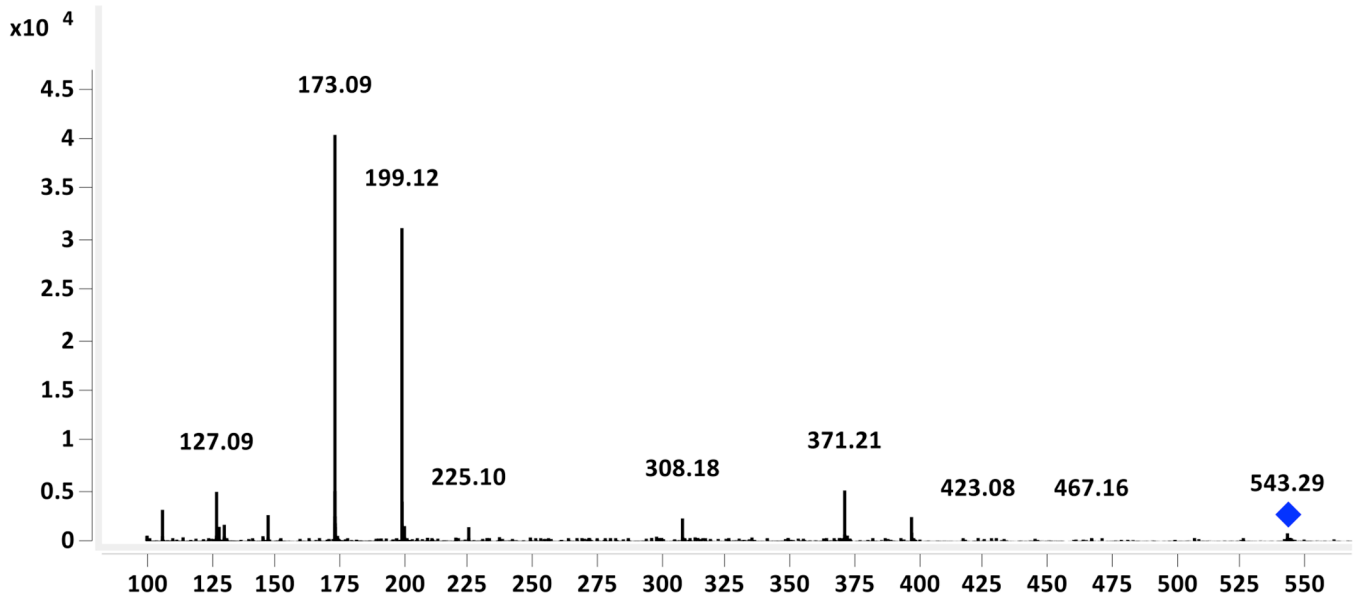

Figure 4.

MS/MS of the 543.29 *m/z* $[M+H]^+$ parent ion uniquely found in urine from MDI exposed mice. The prominent 173.09 *m/z* $[M+H]^+$ daughter ion likely results from cleavage of one lysine attached to one NCO group of MDI, leaving behind a 371.21 *m/z* fragment. The 225.10 and 199.12 *m/z* $[M+H]^+$ daughter ions likely reflect partially and completely hydrolyzed MDI. Additional daughter ions are described in the text.

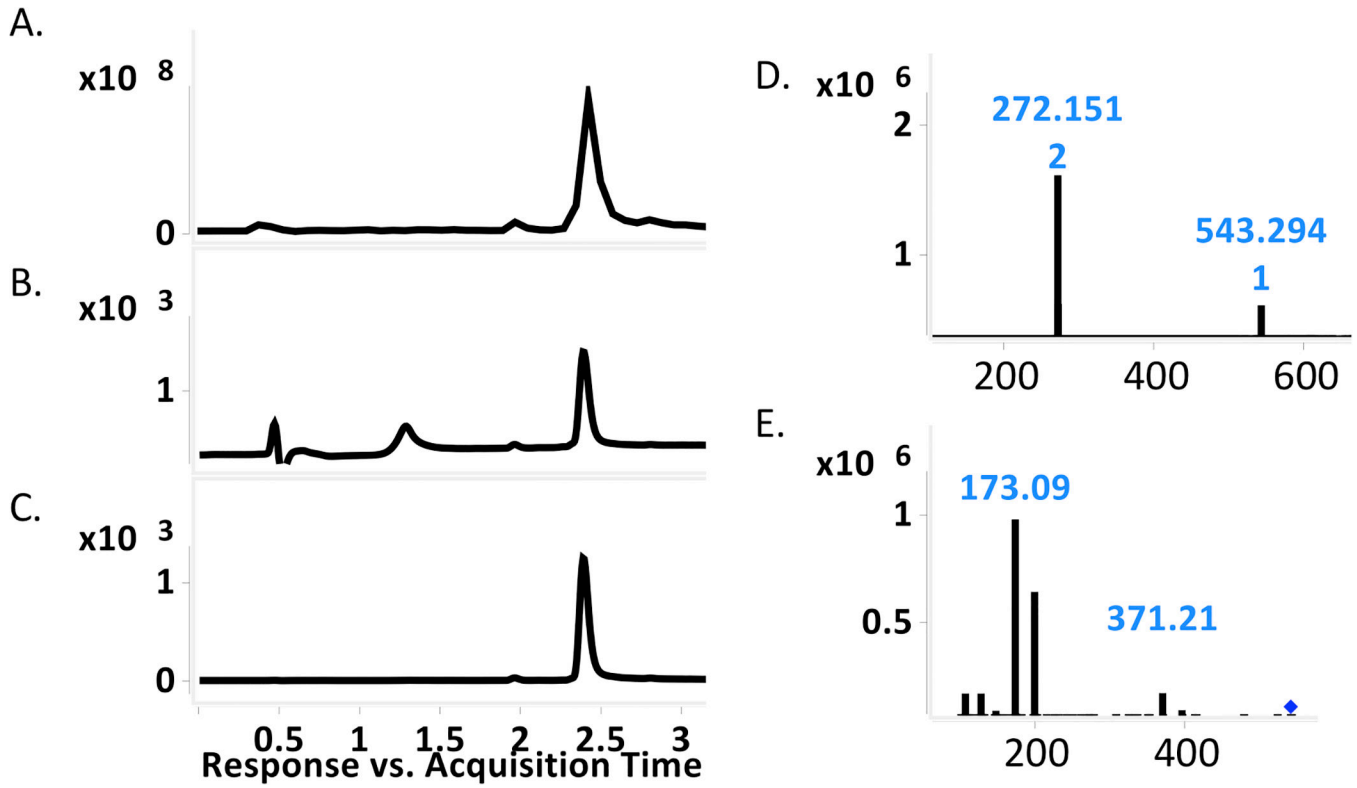

Figure 5.

Characterization of synthetic di-lysine-MDI. Chemically synthesized di-lysine-MDI was generated as described and characterized by LC-MS and MS/MS. LC-MS total ion (A), A_{210} (B) and A_{254} (C) chromatograms are shown, with acquisition time in minutes (X-axis) and ion intensity (Y-axis). MS of the major peak with ~2.4 min retention time is shown in (D), with the peak mass and charge labeled, and MS/MS of the 543.29 *m/z* $[M+H]^+$ parent ion (E) with *m/z* of dominant daughter ions labeled (Y-axis depicts ion intensity and X-axis depicts *m/z*).

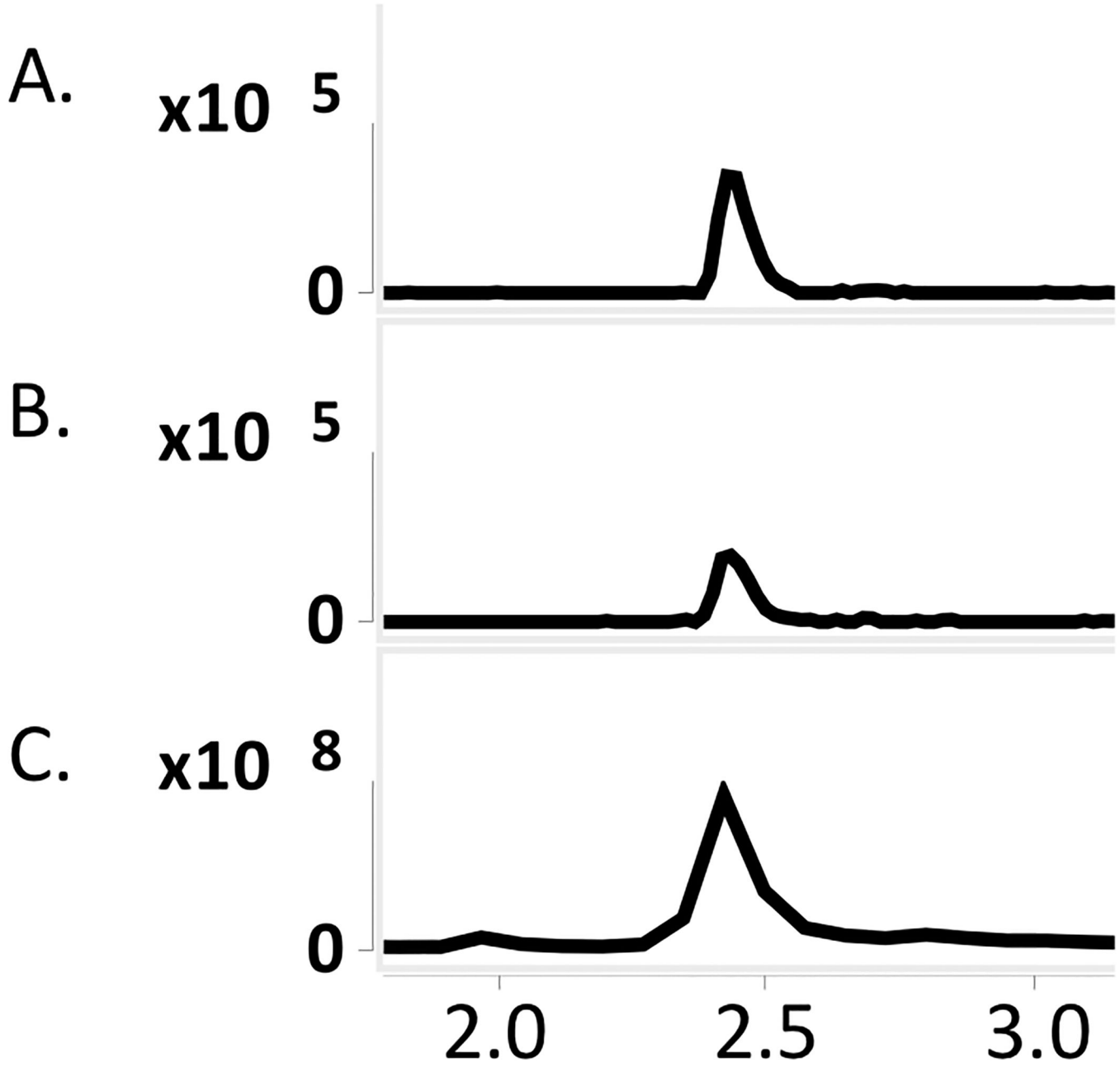

Figure 6.

The urinary 543.29 *m/z* $[M+H]^+$ ion exhibits the same retention time as synthetic di-lysine-MDI. LC-MS extracted ion chromatograms (EICs) for the 543.29 *m/z* $[M+H]^+$ ion are shown for urine from mice exposed via the respiratory tract to MDI-GSH (A) and via the skin to MDI in acetone (B). The EICs are compared with the TIC of chemically synthesized di-lysine-MDI (C). The Y axis represents ion intensity and the X-axis reflects retention time in minutes.

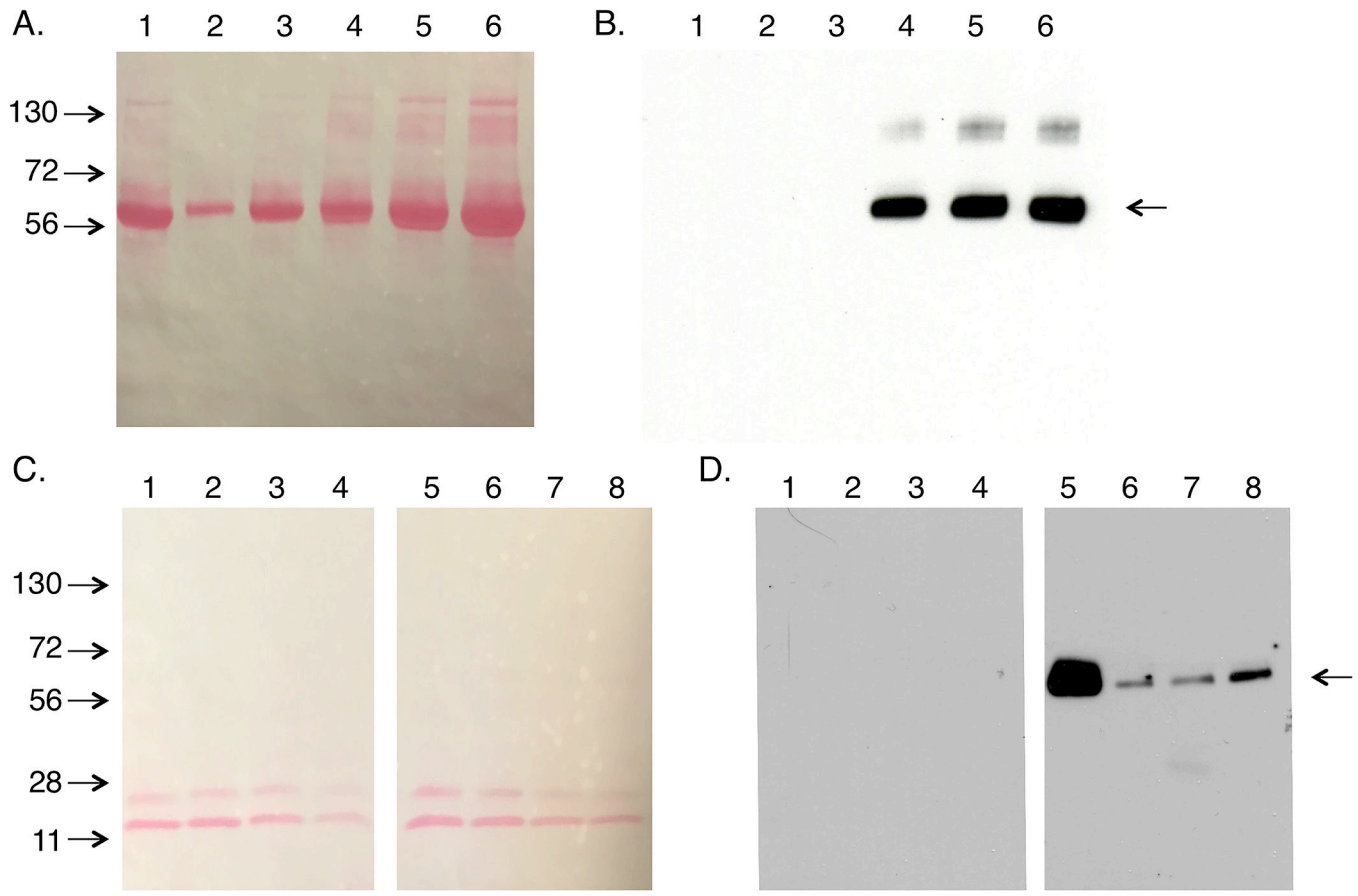

Figure 7.

MDI conjugated proteins in BAL fluid and urine. BAL fluid (A and B) and urine (C and D) were subject to Western blot. Nitrocellulose membranes were pre-stained with Ponceau-S (A/C) before probing with MDI-specific mAb DA5 (B/D). BAL fluid was from 3 control (1–3) and 3 MDI-GSH exposed (4–6) mice, obtained following 4 daily exposures. Urine samples were from mice following 4 daily exposures (lane 1/5), 1 and 6 days post exposure (lanes 2 and 3/lanes 6 and 7), and 24 hours following one additional exposure on day 10 (lane 4/8) to control MDI reacted without GSH (lanes 1–4), or MDI-GSH (lanes 5–8). Molecular weight markers (kDa) shown on left and major MDI-conjugated protein highlighted by arrow on right.

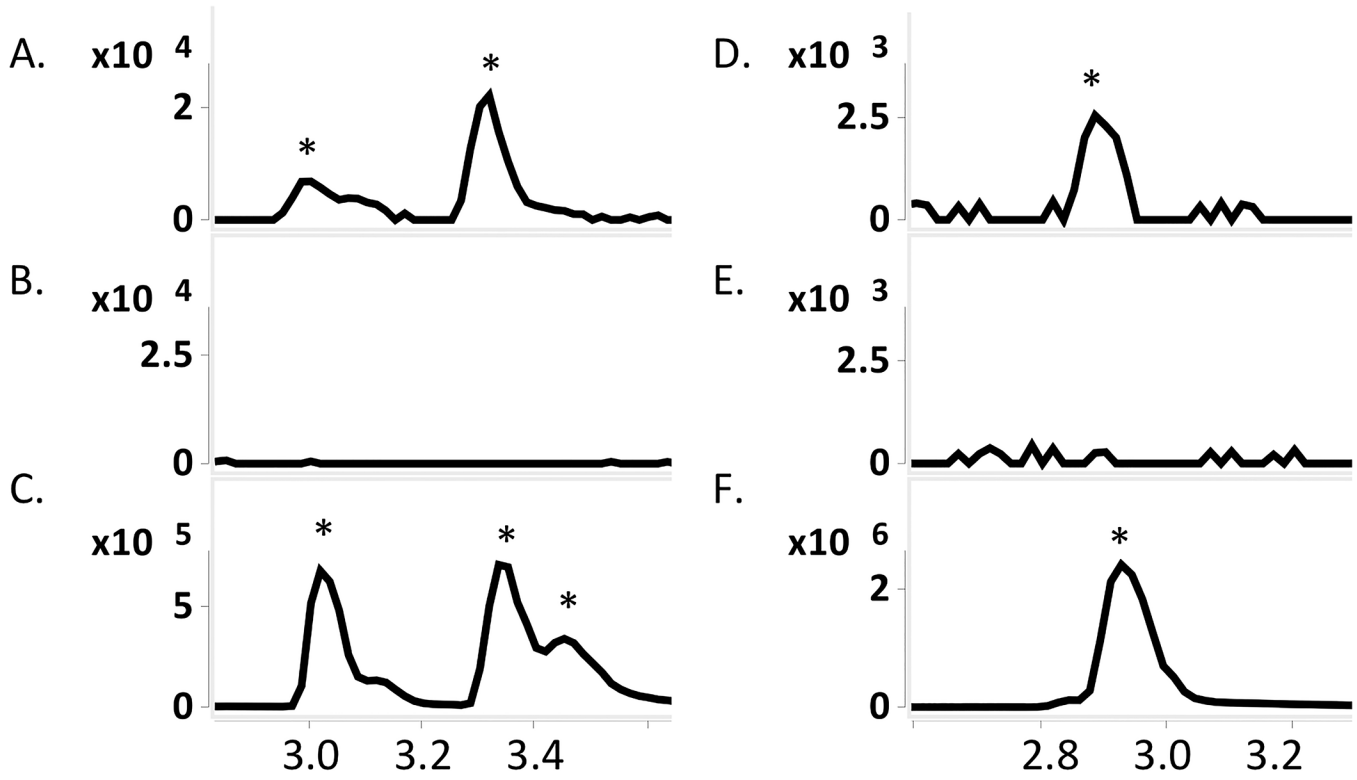

Figure 8.

Molecules with properties of cyclized GSH and GSSG-MDI in urine of MDI exposed mice. LC-MS extracted ion chromatograms for 558.17 *m/z* $[M+H]^+$ (A-C) or 863.23 *m/z* $[M+H]^+$ (D-F) ions in urine of mice exposed to MDI-GSH reaction products via the respiratory tract (A and D) or via the skin to MDI in acetone (B and E). Similar analysis of synthetic GSH and GSSG-MDI reaction products are shown in C and F for comparison.