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Technical note

Development of sandwich ELISAs for the detection of aromatic diisocyanate adducts

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ABSTRACT

Diisocyanates (dNCOs) are highly reactive low molecular weight chemicals commonly used in the manufacturing industry. Occupational exposures to dNCOs have been shown to elicit allergic sensitization and occupational asthma. Among the most commonly used dNCOs in industry are the aromatic dNCOs, toluene diisocyanate (TDI) and methylene diphenyl diisocyanate (MDI). This study aimed to develop enzyme linked immunosorbent assays (ELISA) utilizing aromatic dNCO-specific monoclonal antibodies (mAbs) for the detection of aromatic dNCO adducts. Two sandwich ELISAs were developed. The first sandwich ELISA utilized mAb 60G2 along with an anti-human serum albumin (HSA) polyclonal antibody. This assay detected MDI-, 2,4- and 2,6-TDI-HSA adducts with limits of detection (LOD) of 2.67, <0.10, and 1.70 ng/mL, respectively. When spiked into human serum, the LOD of this ELISA increased to 34.37, 7.64 and 24.06 ng/mL, respectively. The second ELISA utilized mAbs 62G5 and 60G2 for capture and detection. This assay was capable of detecting 2,4- and 2,6-TDI-HSA adducts with LODs of <4.90 and 26.92 ng/mL, respectively, and when spiked in human serum, <4.90 and 95.93 ng/mL, respectively. This 62G5-60G2 sandwich assay was also able to detect dNCO adducted transferrin, hemoglobin, keratin and actin, but with less sensitivity than dNCO-HSA. The results of this study demonstrate potential application of these ELISAs in the identification and characterization of aromatic dNCO adducts as well as in biomonitoring occupational and environmental dNCO exposures.

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1. Introduction

Diisocyanates (dNCOs) are highly reactive, low molecular weight chemicals used in the manufacturing sector to produce polyurethane products, paints and glues. The aromatic dNCOs, methylene diphenyl diisocyanate (MDI) and toluene diisocyanate (TDI), are among the most common used in manufacturing (Allport et al., 2003). Workers handling these products without appropriate personal protective equipment may be at increased risk of developing occupational allergy and asthma. The Occupational Safety and Health Administration has recently initiated a National Emphasis Program to help protect workers from these adverse health effects associated with occupational exposures to isocyanates.

Current dNCO biological monitoring methods include the measurement of dNCO-specific antibodies in the serum and some dNCO-derived biomarkers in the blood and urine. Among these biomarkers, dNCO-derived diamines from hydrolyzed plasma and urine are commonly screened in biomonitoring studies (Gledhill et al., 2005; Budnik et al., 2011). However, detection of dNCO hydrolysis products may

Abbreviations: dNCO, diisocyanate; TDI, toluene diisocyanate; MDI, methylene diphenyl diisocyanate; HDI, hexamethylene diisocyanate; ELISA, enzyme linked immunosorbent assay; mAb, monoclonal antibody; HSA, human serum albumin.

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be limited by several confounding variables, including the lack of a standardized method for hydrolysis and the requirement for specialized instrumentation. This method also lacks specificity in that it does not distinguish between isocyanate exposure and direct exposure to diamines. Consequently, there is a need for alternative methods for the detection of dNCO exposures in the occupational environment.

dNCO haptenation to a variety human proteins following exposure has been hypothesized as a critical step in the development of dNCO sensitization and asthma. Efforts toward developing ELISA-based methods to detect dNCO-haptened proteins have remained limited due to the availability of monoclonal and polyclonal antibodies. Lemus and colleagues developed a sandwich ELISA capable of detecting as low as 3 ng of 1,6-hexamethylene diisocyanate (HDI) adducted human serum albumin (HSA) (Lemus et al., 2001). To our knowledge, no other ELISAs have been developed to assess proteins adducted by either MDI or TDI. This study aimed to develop sandwich ELISAs utilizing a set of recently produced TDI-specific monoclonal antibodies (mAbs) (Ruwona et al., 2011) for application in the biological monitoring of dNCO adducts.

2. Materials and methods

2.1. Conjugation of dNCOs to proteins

All chemicals and proteins used were obtained from Sigma Aldrich (St. Louis, MO) unless otherwise noted. dNCOs, including 4,4′-MDI (CAS 101-68-8), 2,4-TDI (CAS 584-84-9), 2,6-TDI (CAS 91-06-7), and 1,6-HDI (CAS 822-06-0) were conjugated to 0.5 mg/mL HSA (CAS 70024-90-7), human transferrin (CAS 11096-37-0), human hemoglobin (CAS 9008-02-0), keratin from human epidermis (CAS 68238-35-7) and actin from human platelet (Cytoskeleton, Inc., Denver, CO) in 0.01 M phosphate buffered saline (PBS; pH 7.4). dNCO-protein adducts were prepared by adding 10 μL of each freshly prepared dNCO/acetone solution per 1 mL of 0.5 mg/mL protein solution drop wise while vortexing to obtain final molar ratios ranging from 5:1 to 40:1 dNCO:protein. The conjugates were then incubated while vortexing for 1 h at room temperature (RT). After incubation, conjugates were dialyzed using 12–14,000 MWCO dialysis membrane (Spectrum® Laboratories, Inc., Rancho Dominguez, CA) to remove residual hydrolyzed and polymerized dNCO. Samples were stored at 4 °C until use.

2.2. dNCO-HSA-specific sandwich ELISA

A sandwich ELISA specific for aromatic dNCO-HSA was developed using the aromatic dNCO-specific mAb 60G2 (IgG_1). Briefly, Corning high protein binding 96-well plates (Corning, NY) were coated with 4 μg/mL AffiniPure goat anti-mouse IgG Fc, subclass 1 specific antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) in 0.1 M sodium carbonate buffer, pH 9.6 overnight at 4 °C. Following overnight incubation, the wells were washed three times with PBS containing 0.05% Tween 20 (PBST) and incubated on a shaker for 1 h at RT with 2 μg/mL mAb 60G2. The wells were blocked with 200 μL/well 3% non-fat dry milk powder in PBST (SMPBST) for 1 h at 37 °C. Duplicate wells containing 1 μg/mL dNCO-HSA conjugates at each molar ratio, 5:1 to 40:1, were 2 fold serially diluted in SMPBST and incubated for 1 h at 37 °C. Wells were washed and incubated for 1 h at 37 °C with biotin-conjugated affinity purified rabbit anti-HSA antibody (Rockland, Gilbertsville, PA) diluted 1/5000 (v/v) in SMPBST. Following incubation, the wells were washed and incubated for 1 h at 37 °C with alkaline phosphatase (AP)-conjugated streptavidin (Jackson ImmunoResearch Laboratories Inc.) diluted 1/5000 (v/v) in SMPBST. Binding of dNCO-HSA adducts was quantified using 0.5 mg/mL *p*-nitrophenyl phosphate in AP substrate. The optical density was determined spectrophotometrically at 405 nm using a SpectraMax M4 microplate reader (Molecular Devices, Sunnyvale, CA). The limit of detection (LOD) and limit of quantification (LOQ) for each dNCO-HSA adduct were defined as 3 and 10 times the standard deviation of 40 replicate HSA (0.5 μg/mL) control wells. This assay was additionally used to detect dNCO adducted HSA in human serum by diluting the dNCO-HSA in a 1/20 dilution of pooled human serum (Sigma Aldrich) in SMPBST.

2.3. 62G5-60G2 sandwich ELISA

A secondary sandwich assay was developed utilizing aromatic dNCO-specific mAb 60G2 (IgG_1) and TDI-specific mAb 62G5 (IgG_{2a}) (Ruwona et al., 2011). Briefly, corning high protein binding 96-well plates (Corning, NY) were coated with 4 μg/mL AffiniPure goat anti-mouse IgG Fc, subclass 2a specific antibody (Jackson ImmunoResearch Laboratories Inc.) in 0.1 M sodium carbonate buffer, pH 9.6, overnight at 4 °C. Following incubation, the wells were washed three times with PBST and incubated on a shaker for 1 h at RT with 2 μg/mL mAb 62G5. The wells were blocked with 200 μL/well SMPBST for 1 h at 37 °C. Duplicate wells containing 5 μg/mL dNCO-HSA conjugates at each molar ratio were 2 fold serially diluted in SMPBST and incubated for 1 h at 37 °C. Other dNCO-protein adducts, including transferrin, hemoglobin, keratin, and actin, were tested starting at a concentration of 25 μg/mL. Wells were washed and incubated for 1 h at 37 °C with 2 μg/mL mAb 60G2. Wells were washed and incubated for 1 h at 37 °C with biotin-SP-conjugated AffiniPure goat anti-mouse IgG Fc, subclass 1 specific antibody (Jackson ImmunoResearch Laboratories Inc.) diluted 1/5000 (v/v) in SMPBST. Following incubation, the wells were washed and incubated for 1 h at 37 °C with AP-conjugated streptavidin (Jackson ImmunoResearch Laboratories Inc.) diluted 1/5000 (v/v) in SMPBST. Binding to each dNCO-HSA adduct was quantified using 0.5 mg/mL *p*-nitrophenyl phosphate in AP substrate. The optical density was determined spectrophotometrically as previously described. The LOD and LOQ for each dNCO were defined as 3 and 10 times the standard deviation of 40 replicate HSA or 16 replicate transferrin, hemoglobin, keratin or actin (0.5 μg/mL) control wells. This assay was additionally used to detect dNCO adducted HSA in human serum by diluting the dNCO-HSA in a 1/20 dilution of pooled human serum (Sigma Aldrich) in SMPBST.

3. Results and discussion

3.1. Development of a sandwich ELISA to detect dNCO adducted proteins

Two different sandwich ELISAs were developed to detect aromatic dNCO adducts. The first ELISA utilized the IgG_1 mAb