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Development, validation and characterization of an analytical method for the quantification of hydrolysable urinary metabolites and plasma protein adducts of 2,4- and 2,6-toluene diisocyanate, 1,5-naphthalene diisocyanate and 4,4′-methylenediphenyl diisocyanate

CARL JOHAN SENNBRO, CHRISTIAN H. LINDH, HÅKAN TINNERBERG, CECILIA GUSTAVSSON, MARGARETA LITTORIN, HANS WELINDER and BO A. G. JÖNSSON

Department of Occupational and Environmental Medicine, Institute of Laboratory Medicine, University Hospital, Lund, Sweden

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Occupational exposure to diisocyanates within the plastic industry causes irritation and disorders in the airway. The aim of this study was to develop, validate and characterize a method for the determination of 2,4-toluenediamine (2,4-TDA), 2,6-toluenediamine (2,6-TDA), 1,5-diaminonaphthalene (1,5-NDA) and 4,4′-methylenedianiline (4,4′-MDA) in hydrolysed urine and plasma, and to study the correlation between the plasma and urinary levels of these potential biomarkers of 2,4-toluene diisocyanate (2,4-TDI), 2,6-toluene diisocyanate (2,6-TDI), 1,5-naphthalene diisocyanate (1,5-NDI) and 4,4′-methylenediphenyl diisocyanate (4,4′-MDI), respectively. Samples were hydrolysed with 0.3 M NaOH at 100°C for 24 h. The diamines were extracted, derivatized with pentafluoropropionic acid anhydride, and quantified by selected ion monitoring on gas chromatography-mass spectrometry. The repeatability and reproducibility of the method were 7–18% and 7–19%, respectively. Dialysis experiments showed that the metabolites of 2,4-TDI, 2,6-TDI, 1,5-NDI and 4,4′-MDI in plasma were exclusively protein adducts. No free diamines were found in urine, indicating that all diisocyanate-related metabolites were in a conjugated form. For each diisocyanate-related biomarker, there were strongly significant correlations ($p < 0.001$) between individual levels of metabolites in plasma and urine, with Spearman's rank correlation coefficient (r_s) values of 0.74–0.90. The methods presented here will be valuable for the development of biological monitoring methods for diisocyanates.

Keywords: biological markers, gas chromatography-mass spectrometry, diisocyanates, protein adducts.

Introduction

Diisocyanates have been used in the production of polymers since the middle of the 20th century. These compounds are bifunctional and hence can act as cross-binders in polymerization reactions. The inherent bifunctional reactivity of the diisocyanates is due to the presence of two isocyanate (NCO) functional groups. Diisocyanates react easily with compounds that can act as nucleophiles towards the carbon in the NCO group in an addition reaction. Such reactions occur with nucleophiles such as primary and secondary amines (to form ureas), alcohols (to

* Carl Johan Sennbro, Department of Occupational and Environmental Medicine, University Hospital, SE-221 85 Lund, Sweden. Tel: (+46) 46 173198; fax: (+46) 46 143702; e-mail: carl-johan.sennbro@ymed.lu.se

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form urethanes), thiols (to form thiocarbamoyls) and water (to form carbamic acids, which spontaneously decompose into carbon dioxide and amines).

Aromatic diisocyanates such as toluene diisocyanate (TDI), naphthalene diisocyanate (NDI) and methylenediphenyl diisocyanate (MDI) are today mainly used in the manufacture of rigid and flexible polyurethane foams, but are also used in the production of surface coatings, elastomers, adhesives and sealants (Klees and Ott 1999). Industrially, TDI is mainly used as a 80:20 mixture of the two isomers 2,4-TDI and 2,6-TDI, NDI is mainly used as the 1,5-NDI isomer, while the most common commercial technical grade MDI consists of a mixture of about 40–50% 4,4′-MDI, 2.5–4.0% 2,4′-MDI, 0.1–0.2% 2,2′-MDI, with the remainder being higher oligomer homologues (Klees and Ott 1999). Noxious air levels of these diisocyanates may be produced at plants manufacturing polyurethane, either by volatilization at ambient or elevated temperatures, mechanical aerosolization (e.g. spraying) or thermal degradation (e.g. welding). Occupational exposure to these and other diisocyanates can cause various acute and chronic respiratory symptoms, such as irritation of the mucous membranes, occupational asthma and rhinitis, and hypersensitivity pneumonitis (Vandenplas *et al.* 1993, Baur *et al.* 1994). Since exposure to diisocyanates mainly occurs through inhalation, the assessment of occupational exposure has mostly focused on methods for determining air levels (Purnell and Walker 1985, Streicher *et al.* 1994, 2000). However, methods for biological monitoring have several advantages compared with air monitoring. Quantification of biomarkers can give an estimation of the internal dose, which gives an alternative and complementary assessment of total exposure, including possible routes of exposure other than inhalation and taking into account individual lung ventilation. Additionally, biomarkers with long half-lives, such as protein adducts, can be used to monitor long-term exposure.

In order to identify and evaluate biomarkers, the metabolism and toxicokinetics of diisocyanates must be studied. This has been done in some detail in humans, especially for TDI (Brorson *et al.* 1991, Lind *et al.* 1996a, Day *et al.* 1997). Inhaled diisocyanates are deposited in the airways and are then systemically absorbed, probably in conjugated form, and have been determined as protein adducts with albumin and haemoglobin (Sepai *et al.* 1995, Day *et al.* 1996, Lind *et al.* 1997a,b) in the bloodstream. The inhaled diisocyanates are eliminated as low molecular weight urinary metabolites (Lind *et al.* 1996b). Urinary metabolites, plasma metabolites and haemoglobin adducts of diisocyanates have been used as quantitative biomarkers (Maitre *et al.* 1993, Sepai *et al.* 1995, Schütze *et al.* 1995, Lind *et al.* 1996a, Lind *et al.* 1997a, Kaaria *et al.* 2001, Sabbioni *et al.* 2001), but further validation of the analytical methods used is desirable. Firstly, the precision, linearity and limit of quantification (LOQ) of the analytical method need to be determined. Secondly, since a large majority of the metabolites formed *in vivo* after TDI, NDI or MDI exposure are not free diamines, the nature and characteristics of these metabolites need to be further elucidated. Furthermore, in order to be able to interpret the levels of biomarkers, comparisons to levels in unexposed reference persons, to air exposure levels, and to health effects must be investigated.

The aim of this study was to develop and validate a method for the quantification of biomarkers for TDI, NDI and MDI in urine and blood plasma from occupationally exposed workers and also to study some characteristics of these biomarkers. The method presented here is better characterized, easier, more convenient and more robust than earlier reported methods. In addition, a method for the quantification of NDI has not previously been reported.

Materials and methods

Chemicals

Sodium chloride (NaCl, pro-analysis quality [p.a.]), potassium chloride (KCl, p.a.), sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$, p.a.), potassium dihydrogen phosphate (KH_2PO_4, p.a.), disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$, p.a.), tetrasodium ethylene diamine tetra-acetic acid tetrahydrate ($Na_4EDTA \cdot 4H_2O$, 99%), disodium dihydrogen ethylene diamine tetra-acetic acid dihydrate ($Na_2H_2EDTA \cdot 2H_2O$, p.a.), sulphuric acid (H_2SO_4, p.a.) and sodium hydroxide (NaOH, p.a.) were purchased from Merck (Darmstadt, Germany). Hydrochloric acid (HCl, 37%, p.a.) was obtained from Arcos Organics (Geel, Belgium) and toluene (high performance liquid chromatography grade) was obtained from LabScan (Dublin, Ireland). Pentafluoropropionic acid anhydride (PFPA, 99%, derivatization grade), 2,4-diamine toluene (2,4-TDA, 98%), 2,6-diamine toluene (2,6-TDA, 97%), 1,5-diaminonaphthalene (1,5-NDA, 97%) and 4,4′-methylenedianiline (4,4′-MDA, 97%) were bought from Aldrich Chemicals (Milwaukee, Wisconsin, USA). The internal standard, tri-deuterium-labelled 2,4-TDA (D_3-2,4-TDA), was synthesized by Synthelec (Lund, Sweden). Water was purified to an ultra-pure grade by an ELGA Maxima HPLC Mark II (USF ELGA, Bucks, UK) purification system.

Reagents and buffers

The 0.01 M HCl solution was prepared by adding 0.4 ml of concentrated HCl in water to a final volume of 0.5 l. The 3 M H_2SO_4 solution was prepared by adding 83 ml of concentrated H_2SO_4 in water to a final volume of 0.5 l. The 0.3 M NaOH solution was prepared by adding 6 g of NaOH in water to a final volume of 0.5 l. The 0.5 M phosphate buffer, pH 7.5, was prepared by dissolving 150 g of $NaH_2PO_4 \cdot H_2O$ and 11 g of $Na_2HPO_4 \cdot 12H_2O$ in 1.0 l of water and adjusting the pH by adding either HCl or NaOH. Phosphate buffered saline with EDTA (PBS) was prepared by dissolving 8 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4, 1.4 g $Na_2HPO_4 \cdot 12H_2O$, 0.45 g $Na_4EDTA \cdot 2H_2O$ and 0.45 g $Na_2H_2EDTA \cdot 2H_2O$ in 1.0 l of water.

Materials

Test tubes with Teflon-lined screw caps and a volume of 13 ml from Labora (Sollentuna, Sweden) were used in the sample preparation. Dialysis tubing with a volume of 2 ml cm^{-1} and a cut-off of 12–14 kDa (SpectraPore, Spectrum, Houston, Texas, USA) was used for the dialysis of plasma samples. Blood samples were collected in Venoject® blood sampling tubes (Terumo Europe, Leuven, Belgium) containing sodium heparin. Urine samples were collected in polyethylene bottles. Urine for the calibration standard curves was obtained from healthy male volunteers from our laboratory. Plasma for the calibration standard curves was obtained from the local blood bank.

Apparatus

The gas chromatography-mass spectrometry (GC-MS) system consisted of a Fison 8065 gas chromatograph coupled to a VG Trio 1000 single quadropole mass spectrometer (Fisons, Manchester, UK). The GC-MS system was connected to an A200S auto-sampler (Carlo Erba Instruments, Milan, Italy) and was used for the quantification of 2,4-TDA, 2,6-TDA, 1,5-NDA and 4,4′-MDA. The injected samples were separated on a fused silica capillary GC column (30 m × 0.25 mm) with a DB-5MS stationary phase with a film thickness of 0.25 μm (J & W Scientific, Folsom, California, USA) and helium gas as the mobile phase. A Sigma 3E-1 centrifuge (Osterode am Harz, Germany) was used for liquid phase separation. An IKA Vibrax VXR shaker table (Janke & Kunkel, Staufen, Germany) was used for liquid–liquid extractions. A drying oven (Termaks 85-TS8136, Bergen, Norway) was used for hydrolysis of the biological samples. Mettler AE163 and PE 600 (Stockholm, Sweden) balances were used to accurately weigh the chemicals. A PHM84 pH meter (Radiometer Copenhagen, Lyon, France) was used to adjust the pH of the buffers.

Subjects

The biological samples were collected from a total of 169 occupationally exposed workers from 13 different plants. Workers were exposed to 2,4-TDI, 2,6-TDI, 1,5-NDI or 4,4′-MDI, or to different mixtures of these diisocyanates, as ascertained by air measurements (C. J. Sennbro *et al.*, unpublished data). Plasma samples were collected from 145 subjects. Urine samples were collected once from 82 and twice from 86 exposed subjects. The study was approved by the Ethical Committee at Lund University, Sweden, and written informed consent was obtained from the subjects.

Collection and storage of biological samples

Blood samples were collected by arm vein puncture, in Venoject® blood sampling tubes. On arrival at the laboratory, the plasma was separated from the blood cells by centrifugation. The plasma samples were then stored in polyethylene tubes at −20°C until analysis. Urine samples were collected in polyethylene bottles as one pooled sample for each worker during the last 4 h of the work shift. The samples were kept at ambient temperature until arrival at the laboratory and stored in polyethylene tubes at −20°C until analysis.

Preparation and storage of standards

Stock solutions were prepared by dissolving accurately weighed amounts of D_3-2,4-TDA, 2,4-TDA and 2,6-TDA in 0.01 M HCl, while 1,5-NDA and 4,4′-MDA were dissolved in acetonitrile. Standard solutions containing 2,4-TDA, 2,6-TDA, 1,5-NDA and 4,4′-MDA were further prepared by diluting the stock solutions in 0.01 M HCl. A standard solution of internal standard was prepared by diluting the stock solution in 0.01 M HCl to 1 μg ml^{-1}. No degradation of standard solutions of 4,4′-MDA, 2,4-TDA or 2,6-TDA were observed during 4 months' storage at room temperature or 12 months' storage in a refrigerator. For 1,5-NDA stored in a refrigerator, degradation of 25% was observed after 12 months. Calibration standards were prepared by spiking non-hydrolysed urine or plasma with the standard solutions. The final volumes of the urine and plasma calibration standards were 1.0 ml and 0.50 ml, respectively. The levels of diamines in the spiked calibration standards were in the range 0.2–100 ng ml^{-1} ($n = 6$) for each compound when plasma or urine samples were analysed.

Sample preparation and analysis of urine and plasma samples

Urine samples were worked up and analysed as double aliquots of 1 ml each. Plasma samples were worked up and analysed as double aliquots of 0.5 ml each, due to the more limited supply. The samples were hydrolysed with 2 ml of 0.3 M NaOH for 24 h in 100°C in 13 ml test tubes with Teflon screw caps. Prior to hydrolysis, brand new test tubes were preheated in 300°C for at least 24 h. The standard samples were not hydrolysed. After hydrolysis, 50 μl of the internal standard was added and the samples were extracted with 2 ml of toluene by vigorous shaking for 10 min. After extraction, the organic phase was transferred to a new tube and derivatization was performed by the addition of 20 μl of PFPA. The samples were vortexed and the excess of reagent was then removed by extraction with 2 ml of 0.5 M phosphate buffer, pH 7.5. Finally, the organic phase was transferred to a GC vial.

Each sample was injected onto the GC-MS system in splitless mode, using a sample volume of 2 μl and a split time of 1 min. The temperature of the injector was 300°C. The analytes were separated using the following temperature programme: 0–1 min: 100°C; 1–6.5 min: 100–210°C; 6.5–9 min: 210–310°C; 9–11 min: 310°C. The retention times were typically 6.3 min for 2,6-TDA, 6.6 min for 2,4-TDA and D_3-2,4-TDA, 8.3 min for 1,5-NDA and 10.9 min for 4,4′-MDA. The temperature was 200°C in the ion source and 300°C in the interface region. The compounds were analysed in the negative ion chemical ionization mode using ammonia as the moderating gas. Selected ion monitoring (SIM) was performed on the PFPA derivatives at a mass to charge ratio (m/z) of 394 and 374 for 2,4-TDA, 394 for 2,6-TDA, 430 for 1,5-NDA, 470 for 4,4′-MDA and 397 for D_3-2,4-TDA. The chromatographic peaks were quantified against a 1/concentration weighted standard calibration curve, using D_3-2,4-TDA as the internal standard for all four analytes. Different GC-MS methods have previously been evaluated (Skarping *et al.* 1994).

Analysis approval criteria

The calibration standard curve was approved if the correlation coefficient (R) was ≥ 0.990. Back-calculated values of the calibration standard curve samples, that is the deviation from the spiked concentration, were allowed to deviate up to 20%, and up to 30% at concentrations ≤ 0.5 ng ml^{-1}. The reported values of the sample concentrations (C) of 2,4-TDA, 2,6-TDA 1,5-NDA and 4,4′-MDA were calculated as a mean value of the two analysed aliquots (C_1 and C_2). If $C \geq$ LOQ, the duplicate samples were allowed to deviate 30%, calculated as $100^{\star}(C_1/C_2 - 1)$, where $C_1 > C_2$. If $C <$ LOQ, an absolute deviation of 0.2 ng ml^{-1} was allowed. If these criteria were not fulfilled, the sample was run once more and the mean value of the two closest values was reported. When detecting 2,4-TDA in urine, an

interfering compound has been observed. Therefore, this analyte was quantified with two different SIM traces. Since 2,4-TDA and the interfering compound have different fragmentation patterns, the samples containing interference could be identified. The quotients of the SIM traces for 2,4-TDA in biological samples were compared with the quotients of the standard samples and should not deviate more than 15% to be approved. If a larger deviation was observed, the lowest value was reported.

Linearity, accuracy and precision for spiked samples

Linearity was evaluated in the range 0.2–500 ng ml^{-1} for all analytes in both urine and plasma. For each batch of analysis, R and the back-calculated values of the calibration standards were checked according to the analysis approval criteria. The within-run accuracy and repeatability for non-hydrolysed spiked urine and plasma samples were determined at 10 ng ml^{-1}, analysed in 10 replicates in the same batch. Urine or plasma were spiked by adding adequate amounts of 2,4-TDA, 2,6-TDA 1,5-NDA and 4,4′-MDA. Thereafter, the samples were processed through the analytical procedure as described above, but without hydrolysis, together with a standard calibration curve.

Precision for hydrolysed samples

The repeatability and reproducibility of the method were determined by comparing all the analysed duplicate samples with a determined concentration $\geq$ LOQ of a total of 134 plasma samples and 245 urinary samples. Duplicate samples that were analysed on the same day were used to calculate the repeatability, while duplicate samples that were analysed on different days were used to calculate the reproducibility. Some samples were used for both repeatability and reproducibility. The formula

$$s = \sqrt{\frac{1}{2n} \cdot \sum_{i=1}^{i=n} (x_1 - x_2)_n^2}$$

was used to calculate the standard deviation s, where x_1 and x_2 are the results of the different aliquots analysed and n was the number of samples.

Limit of detection and LOQ

The limit of detection (LOD) for each analyte was determined by analysis of 10 chemical blanks. The LOD was defined as the concentration corresponding to the mean value of the 10 chemical blanks plus three times the standard deviation. The blanks were processed, together with a standard calibration curve, via the analytical procedure described above. The LOQ was defined as the lowest level of diamine that could be determined for hydrolysed samples from exposed workers, with a precision of 25%.

Hydrolysis conditions

Plasma and urine samples from 30 exposed workers were analysed after both acidic and alkaline hydrolysis. Duplicate samples were analysed in both conditions. The biological samples were, in the case of hydrolysis by acid, hydrolysed with 2 ml of 3 M H_2SO_4 for 16 h, corresponding to the procedure performed at other laboratories (Lind *et al.* 1996b, Kaaria *et al.* 2001). After hydrolysis, 5 ml of 10 M NaOH and 50 μl of internal standard were added, and the samples were then processed in the same way as after alkaline hydrolysis, as described in the section on sample preparation. The samples containing quantifiable levels of biomarkers were compared directly as quotients and by linear regression.

The course of hydrolysis recoveries of diamines, using alkaline conditions was studied by pooling 12 different urine and 12 different plasma samples, respectively, from exposed workers. The pooled samples were made alkaline with 0.3 M NaOH and then portioned and heated at 100°C. Samples were taken at 0, 0.5, 1, 2, 4, 6, 8, 14, 16, 18, 20, 22 and 24 h. Immediately after sampling, the internal standard was added, and the samples were then extracted with toluene. Finally, all samples were processed via the analytical procedure described in the section on sample preparation.

Stability in spiked samples

The stability of the diamines under the conditions of hydrolysis were studied by spiking pooled urine and plasma, respectively, with 2,4-TDA, 2,6-TDA, 1,5-NDA and 4,4′-MDA to a concentration of 50 ng ml^{-1}. The samples were made alkaline by the addition of 0.3 M NaOH and then portioned and incubated at 100°C. Samples were taken at 0, 0.5, 1, 2, 4, 6, 8, 14, 16, 18, 20, 22 and 24 h. Immediately after sampling, the internal standard was added, and the samples were then extracted with toluene. The samples were processed via the analytical procedure described in the section on sample preparation.

Dialysis of plasma samples

In order to characterize the molecular weight of the plasma metabolites, plasma samples from 20 exposed workers were dialysed against PBS at 4°C for 7 days. The volume of each exposed plasma sample was 0.5 ml and the volume of the PBS was 900 ml, which was exchanged five times. Samples from the same workers were stored in the same conditions in parallel for quantitative comparison of adducts after dialysis. After completion of the dialysis, the volume of the dialysate was measured and the corresponding non-dialysed samples were adjusted to equal volumes by the dialysis buffer. The samples and the dialysis buffer were then hydrolysed for 24 h after the addition of 2 ml of 0.3 M NaOH. Finally, the samples and the dialysis buffer were processed via the analytical procedure described in the section on sample preparation. The samples containing quantifiable levels of biomarkers were compared before and after dialysis.

Correlations between plasma and urinary levels of biomarkers

Correlations between the individual levels of each diisocyanate-related biomarker in plasma and urine were statistically evaluated by calculation of the Spearman's rank correlation coefficient (r_s). The samples analysed were collected on the same day for each subject.

Results and discussion

Linearity, accuracy and precision for spiked samples

The standard calibration curves for the analytes were found to be linear within the range 0.2–100 ng ml^{-1} in both plasma and urine, but not up to 500 ng ml^{-1}. When samples with a concentration exceeding 100 ng ml^{-1} are found, re-analysis should be performed by diluting the samples with 0.3 M NaOH. The correlation coefficients for the standard calibration curves were always higher than 0.990. In order to fulfil the approval criteria that back-calculated values should not exceed 20%, a $1/C$ weighted calibration curve had to be used, in order to increase the accuracy and precision for lower concentrations by adjusting the curve to fit the lower concentrations better. The accuracy and precision for urine and plasma samples spiked with 10 ng ml^{-1} of 2,4-TDA, 2,6-TDA, 1,5-NDA and 4,4′-MDA are presented in Table 1. In summary, the samples could be determined with very high accuracy and precision. The accuracy was 95–98% in urine and 93–101% in plasma, while the repeatability was 2–4% in both urine and plasma. This is consistent with other methods used at other laboratories (Maitre *et al.* 1993, Sepai *et al.* 1995, Lind *et al.* 1996b, Kaaria *et al.* 2001). These results indicate that this method is very robust and reliable when analysing spiked samples.

Table 1. The accuracy and precision for the determination of diamines in spiked non-hydrolysed urine and plasma samples at a concentration of 10 ng ml^{-1}

Analyte	Within-run accuracy (%)	Repeatability (%)
Urine		
2,4-TDA	96	3
2,6-TDA	95	2
1,5-NDA	98	4
4,4′-MDA	95	2
Plasma		
2,4-TDA	100	2
2,6-TDA	101	2
1,5-NDA	93	3
4,4′-MDA	93	2

Precision for hydrolysed samples

The performance of the method is better reflected in its repeatability and reproducibility rather than its precision and accuracy with spiked samples. Repeatability and reproducibility were calculated from all samples that contained levels $\geq$LOQ (Tables 2 and 3). These parameters were less precise for all analytes than the corresponding precision for spiked samples. This is probably due to the hydrolysis step, which may be a limiting factor for the precision of the method. A possible explanation for this is that other reactions, which may not be completely reproducible, compete with the hydrolysis. Since the precision for 1,5-NDA and 4,4′-MDA do not deviate significantly from those for 2,4-TDA and 2,6-TDA, the use of deuterium-labelled NDA or MDA as internal standards would probably not increase considerably the precision for these analytes. Repeatability and reproducibility have not been reported previously for these biomarkers. The accuracy of the method could not be determined, since this requires well-defined conjugates as standards as well as the use of deuterium-labelled conjugates as internal standards.

Table 2. Repeatability of the determination of diamines in hydrolysed urine and plasma samples from exposed workers

Analyte	Number of samples	Range (ng ml^{-1})	Median concentration (ng ml^{-1})	Repeatability (%)
Urine				
2,4-TDA	115	0.5–76	3	9
2,6-TDA	126	0.5–115	4	12
1,5-NDA	30	0.6–81	6	13
4,4′-MDA	46	0.5–78	1	18
Plasma				
2,4-TDA	59	0.6–28	6	7
2,6-TDA	72	0.6–62	5	9
1,5-NDA	16	0.5–59	9	12
4,4′-MDA	18	0.5–74	2	13

Table 3. Reproducibility of the determination of diamines in hydrolysed urine and plasma samples from exposed workers

Analyte	Number of samples	Range (ng ml^{-1})	Median concentration (ng ml^{-1})	Reproducibility (%)
Urine				
2,4-TDA	95	0.5–25	5	14
2,6-TDA	97	0.6–66	4	18
1,5-NDA	8	2–81	9	16
4,4′-MDA	27	0.5–25	1	14
Plasma				
2,4-TDA	42	0.6–31	8	10
2,6-TDA	44	0.5–35	6	10
1,5-NDA	6	0.5–59	2	7
4,4′-MDA	10	0.5–50	1	19

LOD and LOQ

The LOD values were 0.1 ng ml^{-1} for 2,4-TDA, 2,6-TDA and 1,5-NDA in both urine and plasma. The LOD for 4,4′-MDA was 0.05 ng ml^{-1} in both urine and plasma. These results are in agreement with those obtained with other methods used at other laboratories (Maitre *et al.* 1993, Sepai *et al.* 1995, Lind *et al.* 1996b, Kaaria *et al.* 2001). In both urine and plasma, the LOQs were 0.5 ng ml^{-1} for 2,4-TDA, 2,6-TDA, and 4,4′-MDA, and 0.6 ng ml^{-1} for 1,5-NDA.

Comparison of different hydrolysis conditions

The new approach of alkaline hydrolysis conditions has been recommended (Sabbioni *et al*. 1997) because it optimizes the hydrolytic release of all kinds of possible bonds between isocyanate moieties and amino acids. After alkaline hydrolysis, compared to acid hydrolysis, an average of 168%, 152%, 165% and 169% of 2,4-TDA, 2,6-TDA, 1,5-NDA and 4,4′-MDA, respectively, were found in urine, and an average of 107%, 134%, 116% and 100% of 2,4-TDA, 2,6-TDA, 1,5-NDA and 4,4′-MDA, respectively, were found in plasma. There was a significant correlation between the two methods, as shown in Figure 1. The Pearson correlation coefficients were between 0.975 and 0.998. These findings indicate that alkaline hydrolysis released slightly higher amounts of adducts compared with acid hydrolysis, but that the two methods are relatively comparable. Different hydrolysis conditions have previously been studied, including 10 M NaOH, for urine and plasma samples from exposed workers (Lind *et al.* 1996b). In that study, 3 M H_2SO_4 was chosen as the medium for hydrolysis because of the low losses of diamines observed during hydrolysis in water.

Hydrolysis

The kinetics of the release of biomarkers in biological samples from exposed workers during hydrolysis were studied. No diamines were found in non-hydrolysed plasma, and urinary levels very close to the LOD (0.1–0.2 ng ml^{-1}) were found. Since labile conjugates could be hydrolysed under the mild alkaline conditions required for sample work-up of non-heated samples, the quantified biomarkers in urine could not be interpreted unequivocally as free diamines in urine. In urine, the kinetic curve of the release of 2,4-TDA, 2,6-TDA and 1,5-NDA during hydrolysis showed a fast increase in released diamines up to about 8 h (Figure 2a). A constant concentration, within the precision of the method, was then seen up to 24 h. For 4,4′-MDA, the release was slower and the maximal concentration (C_{max}) was not reached until 24 h. Hence, a hydrolysis time of 24 h is optimal for urine samples. In plasma, the kinetics of release seemed slower for 2,4-TDA, 2,6-TDA and 4,4′-MDA, with C_{max} at 22–24 h, and faster for 1,5-NDA, which had a C_{max} at 8 h (Figure 2b). Thus, a hydrolysis time of 16–24 h is optimal for plasma samples. A hydrolysis time of 24 h was chosen for both urine and plasma samples as it is practical, since it allows hydrolysis overnight and further sample preparation the day after. Furthermore, it is an advantage to adopt a method that allows both urine and plasma samples to be prepared in parallel.

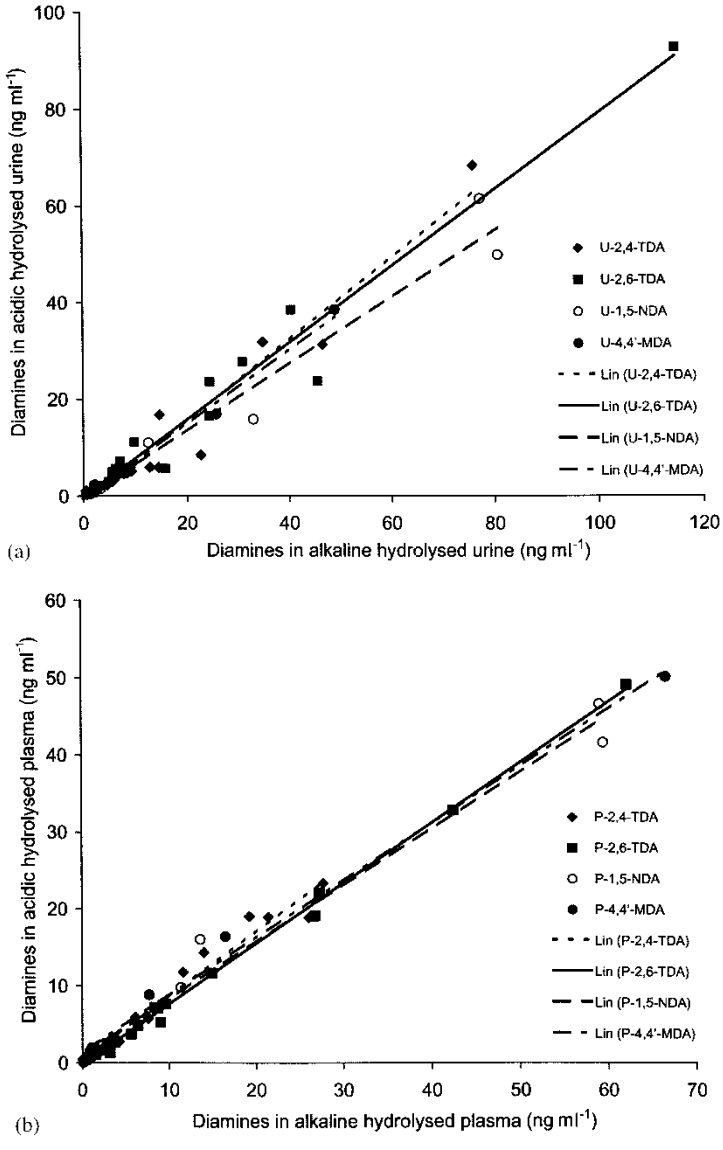

Figure 1. Comparison of hydrolysis conditions for samples from diisocyanate-exposed workers. The same samples were hydrolysed with 0.3 M NaOH (on the horizontal axis) and with 3 M H_2SO_4 (vertical axis). (a) Urine samples. Linear regression line (Lin) equations: $y = 0.9*x - 2$, $y = 0.8*x - 0.2$, $y = 0.7*x - 0.5$, and $y = 0.8*x - 0.5$ for urinary (U) 2,4-TDA, U-2,6-TDA, U-1,5-NDA and U-4,4'-MDA, respectively. All the correlations were significant ($p < 0.01$). (b) Plasma samples. Linear regression line (lin) equations: $y = 0.8*x + 0.3$, $y = 0.8*x - 0.2$, $y = 0.7*x + 0.9$, and $y = 0.7*x + 1$ for P-2,4-TDA, P-2,6-TDA, P-1,5-NDA and P-4,4'-MDA, respectively, and all the correlations were significant ($p < 0.01$).

Stability during hydrolysis in spiked samples

The stability of 2,4-TDA, 2,6-TDA, 1,5-NDA and 4,4′-MDA was studied in alkaline urine and plasma for 24 h at 100°C. In plasma, the loss of 2,4-TDI, 2,6-TDI and 1,5-NDA was about 30%, while for 4,4′-MDA no losses were observed. In urine, the loss of 2,4-TDA and 2,6-TDA was about 20% and the loss of 1,5-

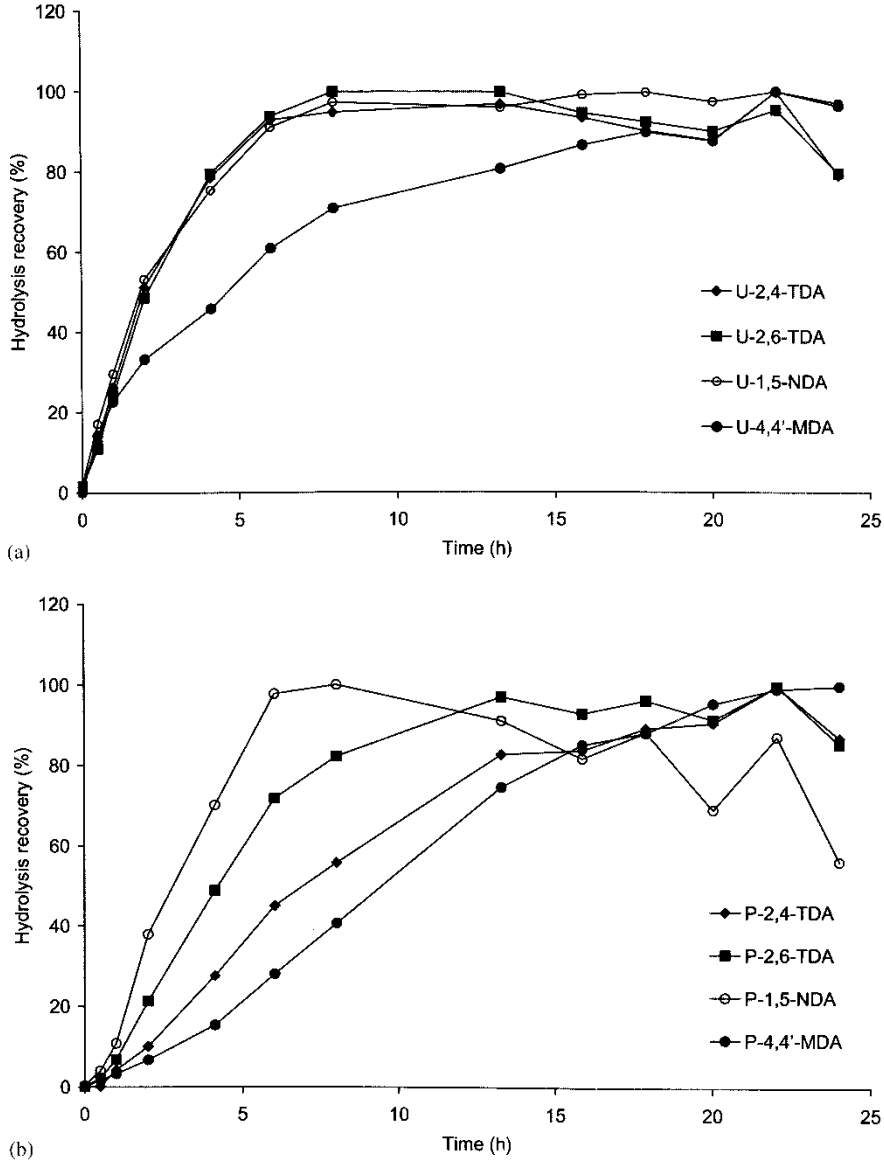

Figure 2. Course of hydrolysis recoveries of diamines 2,4-TDA, 2,6-TDA, 1,5-NDA and 4,4′-MDA in pooled samples from diisocyanate-exposed workers, using 0.3 M NaOH as the hydrolysis medium. Hydrolysis recovery was set to 100% when the maximum concentration (C_{max}) of hydrolysed diamine was reached. (a) Urine samples (U-). (b) Plasma samples (P-).

NDA was 10%, while 4,4′-MDA was stable. These results indicate that some of the hydrolysed adducts were lost by breakdown during hydrolysis, which resulted in an underestimate of the true metabolite concentrations in plasma and urine. However, as mentioned above, the true concentrations cannot be determined; the main issue is to treat all samples in the same way when comparing them.

Dialysis of plasma samples

During dialysis of the plasma samples, all low molecular weight compounds (<12–14 kDa), including possible TDI, NDI and MDI metabolites, were removed from the plasma. By comparing the amounts of released 2,4-TDA, 2,6-TDA, 1,5-NDA and 4,4′-MDA after hydrolysis of dialysed and non-dialysed samples, the relative abundance of protein adducts and low molecular weight metabolites of diisocyanates could be measured. For 1,5-NDA, apparently equal amounts were found (mean 103%, range 89–124%) in dialysed and non-dialysed plasma samples, indicating that all the metabolites found were protein bound. However, the quantified amounts of 2,4-TDA, 2,6-TDA and 4,4′-MDA after hydrolysis were larger in the dialysed samples than in the non-dialysed samples, with averages of 166% (range 113–268%) for 2,4-TDA, 155% (range 108–250%) for 2,6-TDA and 121% (range 97–171%) for 4,4′-MDA.

None of the diamines were detected in the dialysis buffer. Hypothetically, a likely explanation lies in the chemistry of hydrolysis. The diisocyanate adduct moieties might not always be released as the corresponding diamine but presumably as several other chemical species due to side reactions with molecules other than water during hydrolysis. It is likely that the levels of such molecular species that react with diisocyanate adducts during the hydrolysis may be decreased or extinguished during dialysis, leading to diminishing of the side reactions and hence to higher amounts of diamines after dialysis. This does not explain why this was not seen for NDI, but since NDI is a different chemical compound, it might differ in terms of its reactivity. Thus, it is clear that the quantified biomarkers in hydrolysed plasma samples are protein adducts, since no losses of biomarkers were observed during dialysis. This finding is in agreement with earlier results where the hydrolysable human plasma TDI metabolites from one worker were found to co-elute with serum albumin when analysed by ion-exchange chromatography and gel filtration separation (Lind *et al.* 1997b). These results do not exclude *in vivo* formation of corresponding diamines, however, since these compounds are further metabolized to reactive intermediates that – like the diisocyanates – form protein adducts, which cannot be distinguished from the corresponding diisocyanate adducts using this method. Identification of low molecular weight metabolites in plasma for any of the diisocyanates has not been reported, but any such metabolites should be rapidly eliminated by the kidneys. The nature of excreted urinary TDI, NDI or MDI metabolites has never been fully elucidated in humans. However, large quantities of low molecular weight metabolites of TDI (<5 kDa) have been found (Lind *et al.* 1996b). After mild alkaline treatment, detectable levels of free 4,4′-MDA and mono-acetylated 4,4′-MDA have been found in urine from MDI-exposed workers (Sepai *et al.* 1995, Schütze *et al.* 1995).

Correlations between plasma and urinary levels of biomarkers

Significant correlations ($p<0.001$) between plasma and urinary levels of metabolites were found for each diisocyanate-related biomarker. The r_s values were calculated to be 0.90, 0.85, 0.74 and 0.84 for 2,4-TDA, 2,6-TDA, 1,5-NDA and 4,4′-MDA, respectively. As shown in Figure 3, there was large individual variation. Apart from analytical errors (7–19%), this could be due to, for example,

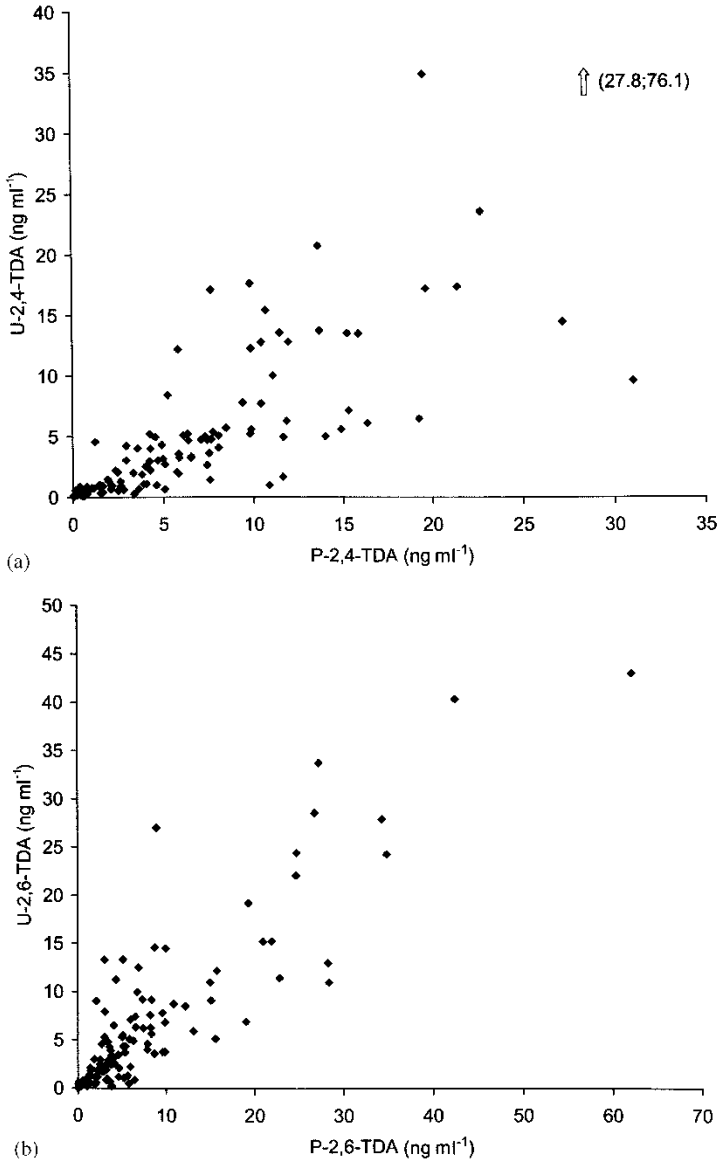

Figure 3. Correlation between levels of biomarkers in hydrolysed samples from 120 exposed workers. (a) Biomarkers of 2,4-TDI in plasma (P-2,4-TDA) and urine (U-2,4-TDA). The correlation was strongly significant ($p < 0.001$) and the Spearman's rank correlation coefficient was 0.90. (b) Biomarkers of 2,6-TDI in plasma (P-2,6-TDA) and urine (U-2,6-TDA). The correlation was strongly significant ($p < 0.001$) and the Spearman's rank correlation coefficient was 0.85.

individual differences in metabolism, but also reflects the fact that the different levels are different measurements of exposure. The plasma metabolites are protein adducts, probably mainly albumin adducts (Lind *et al.* 1997b), and thus give a relative measurement of exposure for the previous 3 weeks. The urinary levels of biomarkers, on the other hand, originate partly from the breakdown of protein adducts and partly from exposure during the day of sampling, thus giving a hybrid

measurement of exposure. This reasoning is based on a previous exposure chamber study (Brorson *et al.* 1991).

Conclusions

In this study we developed and validated a method for the analysis of several diisocyanate metabolites. Analytical methods using hydrolysis have previously been used for the biological monitoring of diisocyanates. However, by using 0.3 M NaOH as the hydrolysis medium, the less convenient handling with concentrated acid and base could be avoided. Also, one pH-adjustment step in the sample work-up could be deleted. Thus, the recovery of adducts released as diamines is increased by using the alkaline hydrolysis condition, enabling detection of lower exposure levels and making the method more robust. This method gives higher results than methods that use acid hydrolysis, but correlates well with them. Using dialysis we have shown that the hydrolysable metabolites of the diisocyanates in plasma were exclusively protein adducts. In a hydrolysis experiment we found no levels of free diamines prior to hydrolysis, and hence concluded that all diisocyanate-related hydrolysable metabolites in urine are excreted as conjugates. The correlations between the levels of hydrolysable metabolites in plasma and urine were strongly significant ($p < 0.001$), with an r_s value of 0.74–0.90. The use of these biomarkers as quantitative measures for exposure and risk will be published elsewhere (C. J. Sennbro *et al.*, unpublished data, M. Littorin *et al.*, unpublished data).

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