Identification and analysis of 2,4- and 2,6-toluenediisocyanate (TDI) adducts at the N-terminal valine of globin

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Abstract

Diisocyanates, reactive compounds used in the production of polyurethanes and potent occupational allergens, readily bind to nucleophilic groups of proteins. The stable adducts formed by the reaction of xenobiotics with blood proteins are increasingly used as exposure indicators. To extend the biomonitoring scope of 2,4- and 2,6-toluenediisocyanate (2,4- and 2,6-TDI), we investigated their adducts at the N-terminal valine of globin. Full human blood was incubated in vitro with TDI, 0.1-10 μmol/ml. Globin was isolated and subjected to Edman degradation during which the N-terminal valine adducts were detached to produce the corresponding imidazoline-2,4-diones (hydantoins). The following products were identified by GC/MS analysis: 3-(5-amino-2-methylphenyl)-5-isopropylimidazoline-2,4-dione (2-TVH), 3-(3-amino-4-methylphenyl)-5-isopropylimidazoline-2,4-dione (4-TVH), and 3,3´-(4-methyl-1,3-phenylene)-bis(5-isopropylimidazoline-2,4-dione) (2,4-TDVH), all derived from 2,4-TDI, and 3-(3-amino-2-methylphenyl)-5-isopropylimidazoline-2,4-dione (6-TVH) and 3,3´-(2-methyl-1,3-phenylene)-bis(5-isopropylimidazoline-2,4-dione (2,6-TDVH), derived from 2,6-TDI. Preparation of the reference compounds consisted of reacting TDI with valine, conversion of the products to hydantoins, and preparative chromatography. The TDI-valine hydantoins showed remarkable chromatographic properties. While in the GC analysis each of the compounds eluted as a single narrow peak, on the HPLC column 4-TVH afforded a narrow peak, 2-TVH a broad peak, and 6-TVH two well resolved narrow peaks. This was due to steric intramolecular interactions leading to hindered rotation around the C-N bond connecting the hydantoin and aromatic rings, and hence to the formation of diastereomeric rotamers in all products except 4-TVH. The individual TDI-valine adducts were determined using the corresponding hydantoins and the 5-*sec*-butyl analogue of 4-TVH as calibration and internal standards, respectively, assuming that the conversion of the ureid adducts to hydantoins was quantitative. Dosing of human blood with 2,4-TDI, 10 μmol/ml, resulted in the formation of 2-TVH, 4-TVH, and 2,4-TDVH at a level of 1.1, 1.1, and 0.4 μmol/g globin,

respectively, which accounted for 1.4%, 1.4%, and 0.5% of the total 2,4-TDI dose added to blood.

Key words: Toluenediisocyanate; Globin adducts; Hydantoins; Biological monitoring

1. Introduction

Toluene diisocyanates (TDI) are widely used in the chemical industry, namely, in the production of polyurethane foams, paints, elastomers, and coatings. Commonly, a mixture of 2,4- and 2,6-TDI isomers in the ratio of 80:20 or 65:35 is used. The electrophilic character of the isocyanate group underlies high reactivity of TDI as well as their toxic effects. TDI induce adverse health effects in the respiratory tract, thereby being the major cause of occupational asthma in the Western world [1,2]. It is believed that the molecular event resulting in sensitization is covalent binding to proteins [3-5]. Inhalation studies with guinea pigs exposed to ^{14}C-labelled 2,4-TDI have shown extensive binding to the respiratory tract, but the label was also distributed to blood and other tissues. In blood, most of ^{14}C was bound to a 60-kDa plasmatic protein, most likely albumin [6]. In guinea pig hemoglobin treated with TDI, the adducts at α and β chains, including cross-links between both chains, were detected [7]. However, systematic investigation on the binding site(s) for TDI in globin have not been carried out yet.

Isocyanates bind readily to the N-terminal amino acids of proteins to produce ureid adducts. Treatment of such adducts with hot acid results in the detachment of the isocyanate-specific cyclic products, imidazoline-2,4-diones (hydantoins) [8]. This reaction (Edman degradation procedure) has been introduced in protein chemistry to identify the N-terminal amino acids via their hydantoins with an isothiocyanate reagent. On the other hand, an isocyanate adduct with known N-terminal amino acid can be converted to the corresponding

hydantoin for the purpose of the adduct determination. For example, the adduct of methylisocyanate at the N-terminal valine of hemoglobin was determined following its conversion to 3-methyl-5-isopropylhydantoin [9]. The hemoglobin adducts persist in the organism over the whole life-time of the erythrocytes (ca. 4 months in humans). They are increasingly employed as biomarkers of cumulative exposure to the parent compounds [10].

Under physiological conditions, the reactions of the isocyanate group with nucleophiles compete with hydrolysis, the latter reaction converting isocyanate to amine. The following products can be formed between 2,4-TDI and N-terminal valine of globin: one „1:2 adduct“ in which the diisocyanate molecule cross-links two valine molecules, and two isomeric „1:1 adducts“ in which one isocyanate group is linked to valine while the other isocyanate group had been hydrolyzed to amine. 2,6-TDI produces with valine one 1:2 adduct and one 1:1 adduct. Following our preliminary reports [11,12], here we describe in detail preparation of specific hydantoins (TDI-Val hydantoins) corresponding to the above mentioned adducts. The standards were then used to establish the relationship between the TDI dose and the adduct level in human blood treated with TDI. Some of the TDI-Val hydantoins exhibit remarkable chromatographic and spectroscopic properties, reflecting the steric barriers between the conformers. Our results are consistent with and complementary to those published by Sabbioni et al. [13]. The usefulness of the TDI-Val adducts in biomonitoring of the exposure to TDI will be discussed.

2. Material and methods

2.1. Chemicals

2,4-TDI (purum, >98%), 2,6-TDI (purum, ≥97%), L-valine and L-isoleucine (purum) were obtained from Fluka. All other chemicals and solvents were of analytical grade, from various commercial sources. The analytical TLC was conducted on silica gel 60 F_{254} plates

(Merck, Darmstadt, Germany). Column chromatography was performed on silica gel L40/100 (Kavalier, Votice, Czech Republic).

2.2. Synthesis and isolation of the TDI-Val hydantoins

General procedure for the preparation of the TDI-Val hydantoins consisted of direct reaction of TDI with Val, heating of the reaction products with HCl/acetic acid, and isolation of pure compounds from the complex mixture using chromatographic methods.

For preparation of the 2,4-TDI-Val hydantoins, i.e., 3-(5-amino-2-methylphenyl)-5-isopropylimidazoline-2,4-dione (2-TVH), 3-(3-amino-4-methylphenyl)-5-isopropyl-imidazoline-2,4-dione (4-TVH) and 3,3´-(4-methyl-1,3-phenylene)-bis(5-isopropyl-imidazoline-2,4-dione) (2,4-TDVH), L-valine (20 mmol, 2.34 g) was dissolved in 100 ml water and pH was adjusted to 6 by 1 M NaOH. Dioxane (50 ml) was added, and the solution of 2,4-TDI in dioxane (0.2 M) was added dropwise while pH was kept at 6 by 1 M NaOH. The endpoint of the reaction was indicated when the TLC assay for valine (mobile phase: n-BuOH/acetic acid/H_2O 4:1:5, detection: ninhydrin) became negative, i.e., after addition of ca. 150 ml of the 2,4-TDI solution. The solvents were evaporated in a vacuum rotary evaporator, the solid was dissolved in concentrated HCl/acetic acid 2:1 (50 ml) and heated at 100 ^{o}C for 8 h. Then, 10 M NaOH (60 ml) was added with cooling, followed by 15 g of $(NH_4)_2SO_4$. Additional 10 M NaOH was added to attain pH 8. The solution was then extracted with ethyl acetate (3×100 ml). Solvent was evaporated, and the complex mixture of products was repeatedly chromatographed on a silica gel column with $CHCl_3$/MeOH/acetic acid (8:2:1 v/v) mobile phase to obtain Fraction 1, containing predominantly 4-TVH and 2,4-TDVH, and Fraction 2, containing mainly 2-TVH. The column fractionation was monitored by TLC with $CHCl_3$/MeOH/acetic acid (8:2:1 v/v) mobile phase, using UV lamp and dimethylamino-benzaldehyde spraying reagent for detection (R_F: 2,4-TDVH, 0.72; 4-TVH, 0.69; 2-TVH, 0.50). The Fractions 1 and 2 were further chromatographed by preparative HPLC on a 250 × 8

mm stainless steel column Separon SGX C18 5 μm (Tessek, Prague, Czech Republic), using PU 4100 liquid chromatograph with PU 4120 diode array detector and P 3202 data station (Philips, Cambridge, UK). Mobile phase was MeOH/H_2O 20:80 (v/v) with linear gradient to 90% MeOH (0-20 min), at a flow rate of 2.5 ml/min. Up to 10 mg of material was separated in one HPLC run. The eluate was collected and evaporated to dryness, and the products were characterized by mass spectra and NMR.

The 2,6-TDI-Val-hydantoins, i.e., 3-(3-amino-2-methylphenyl)-5-isopropylimidazoline-2,4-dione (6-TVH) and 3,3´-(2-methyl-1,3-phenylene)-bis(5-isopropylimidazoline-2,4-dione (2,6-TDVH), were prepared similarly as described for their 2,4-TDI-related analogues. The crude mixture of products was chromatographed on a silica gel column to collect a single fraction containing predominantly 6-TVH (R_F: 0.64 and 0.70) and 2,6-TDVH (R_F: 0.68), which were further separated by preparative HPLC. 6-TVH was eluted in two well-resolved peaks at a ratio of approximately 1:1.

3-(3-Amino-4-methylphenyl)-5-isopropylimidazoline-2,4-dione or 3-[4-(2-amino)tolyl]-5-isopropylhydantoin **(4-TVH)**. EI-MS: *m/z* 247 (86), 205 (7), 148 (100), 119 (4), 106 (12). ^{1}H-NMR (DMSO-d_6): δ 8.40 (bs, 1H, N*H*); 6.96 (d, J = 7.9, 1H, C(5)-*H*); 6.49 (d, J = 1.9, 1H, C(2)-*H*); 6.33 (dd, J = 7.9 and 1.9, 1H, C(6)-*H*); 5.03 (bs, 2H, NH_2); 4.09 (d, J = 3.2, 1H, hydantoin ring C*H*); 2.11 (m, 1H, C*H*(CH_3)$_2$); 2.05 (s, 3H, CH_3-aryl); 1.00 and 0.85 (d, J = 6.9, 6H, CH(CH_3)$_2$). ^{13}C-NMR (DMSO-d_6): δ 156.3 and 173.4 (*C*O); 120.7, 130.3, and 146.8 (aromatic-*C*); 114.2, 120.7, and 129.7 (aromatic *C*H); 17.0 (*C*H_3-Ar); 61.1 (hydantoin *C*5-H); 29.9 (*C*H (CH_3)$_2$); 15.7 and 18.4 (CH (*C*H_3)$_2$).

3-(5-Amino-2-methylphenyl)-5-isopropylimidazoline-2,4-dione or 3-[2-(4-amino)tolyl]-5-isopropylhydantoin **(2-TVH)**. EI-MS: *m/z* 247 (100), 205 (4), 148 (91), 120 (9), 106 (25). ^{1}H-NMR (DMSO-d_6): δ 8.38^a and 8.42^b (s, 1H, N*H*); 6.92^a and 6.93^b (d, J = 8.3, 1H, C(5)-*H*); 6.52 (dd, J = 8.3 and 2.2, 1H, C(6)-*H*); 6.32^a and 6.22^b (d, J = 2.2, 1H, C(2)-*H*); 5.0 (bs, 2H, NH_2); 4.12^a and 4.18^b (d, J = 3.0^a and 2.6^b, 1H, hydantoin ring C*H*); 2.11 (m, 1H, C*H*(CH_3)$_2$);

1.88[a] and 1.89[b] (s, 3H, C*H*$_3$-aryl); 1.00 and 0.89 (d, J = 7.1 and 6.6, 6H, CH(C*H*$_3$)$_2$); diastereomer ratio a:b = 55:45. ^{13}C-NMR (DMSO-d$_6$): δ 156.1, 156.2, 173.7, and 173.8 (*C*O); 121.9, 122.1, 131.3, 131.4, 147.1, and 147.2 (aromatic-*C*); 113.7, 114.1, 114.4, 114.5, 130.5, and 130.6 (aromatic *C*H); 16.2 and 16.4 (*C*H$_3$-Ar); 61.3 and 61.7 (hydantoin *C*5-H); 29.5 and 29.8 (*C*H(CH$_3$)$_2$) ; 15.6, 16.0, 18.2, 18.6 (CH(*C*H$_3$)$_2$).

3,3´-(4-Methyl-1,3-phenylene)-bis(5-isopropylimidazoline-2,4-dione) or 2,4-toluylene-3,3´-bis(5-isopropylhydantoin) **(2,4-TDVH).** White solid. EI-MS: *m/z* 372 (100), [M]$^+$; 330 (68), [M-C(CH$_3$)$_2$]$^+$; 273 (38), [M-NH(CO)CHCH(CH$_3$)$_2$]$^+$; 231 (42), [M-NCONH(CO) CHCH(CH$_3$)$_2$]$^+$; 174 (27), [C$_6$H$_3$CH$_3$(NCO)$_2$]$^+$; 72 (45). ^{1}H-NMR (DMSO-d$_6$): δ 8.55[a], 8.56[b], 8.48[a] and 8.52[b] (s, 2H, N*H*); 7.43[a] and 7,44[b] (d, J = 8.2[a] and 8.5[b], 1H, C(5)-*H*); 7.41 (m, 1H, C(6)-*H*); 7.31[a] and 7.06[b] (m, 1H, C(2)-*H*); 4.24[b], 4.16[a], 4.12[a] and 4.09[b] (d, J = 3.5[a] and 3.8[b], 2H, hydantoin ring C*H*); 2.12 (m, 2H, C*H*(CH$_3$)$_2$); 2.10[a] and 2.13[b] (s, 3H, C*H*$_3$-aryl); 0.87 and 1.00 (m, 12H, CH(C*H*$_3$)$_2$); diastereomer ratio a:b = 3:2. ^{13}C-NMR (DMSO-d$_6$): δ 172.66, 172.69 and 172.72 (*C*O); 155.67, 155.83 and 155.88 (NH*C*ONH); 135.7 and 136.0 (C, C(4)); 131.2, 131.5, 130.4 and 130.5 (C, C(1) and C(3)); 130.7 and 130.8 (CH, C(5)); 126.5, 126.88, 126.91 and 126.96 (CH, C(2) and C(6)); 61.35, 61.42, 61.6 and 62.0 (hydantoin ring *C*H); 29.6, 29.9 and 30.0 (*C*H(CH$_3$)$_2$); 17.1 and 17.2 (*C*H$_3$-Ar); 15.9, 16.0, 16.2, 18.47, 18.50 and 18.7 (CH (*C*H$_3$)$_2$).

3-(3-Amino-2-methylphenyl)-5-isopropylimidazoline-2,4-dione or 3-[2-(6-amino)tolyl]-5-isopropylhydantoin **(6-TVH)**. EI-MS: *m/z* 247 (100), 205 (7), 148 (73), 120 (13), 106 (25). ^{1}H-NMR (DMSO-d$_6$): δ 8.34[a] and 8.38[b] (s, 1H, N*H*); 6.92 (t, J = 7.8, 1H, C(5)-*H*); 6.67 (d, J = 8.0, 1H, C(4)-*H*); 6.35[a] and 6.20[b] (d, J = 7.7, 1H, C(6)-*H*); 5.02 (bd, J = 3.3, 2H, N*H*$_2$); 4.11[a] and 4.18[b] (d, J = 3.3[a], 1H, hydantoin ring C*H*); 2.13 (m, 1H, C*H*(CH$_3$)$_2$); 1.76[a] and 1.78[b] (s, 3H, C*H*$_3$-aryl); 1.18[a] 1.00[b], 0.91[a] and 0.87[b] (d, J = 6.9, 6H, CH(C*H*$_3$)$_2$); diastereomer ratio a:b = 54:46. ^{13}C-NMR (acetone-d$_6$): δ 157.3, 157.4, 173.4, and 173.5 (*C*O); 121.0, 121.1, 132.9, 133.0, 146.2, and 146.3 (aromatic-*C*); 115.5, 115.6, 117.9, 118.3, 126.9, and 127.0

(aromatic *C*H); 16.3 and 16.8 (*C*H_3-Ar); 62.7 and 63.1 (hydantoin *C*5-H); 31.0 and 31.4 (*C*H(CH_3)$_2$) ; 12.2, 12.3, 18.8, and 19.1 (CH(*C*H_3)$_2$) .

3,3´-(2-Methyl-1,3-phenylene)-bis(5-isopropylimidazoline-2,4-dione or 2,6-toluylene-3,3´-bis(5-isopropylhydantoin) **(2,6-TDVH)**. White solid. EI-MS: *m/z* 372 (86), 330 (54), 311 (37), 274 (72), 231 (58), 174 (30), 72 (100), ions assigned as in 2,4-TDVH. ^{1}H-NMR (DMSO-d_6): δ 8.59[a] and 8.54[b+c] (s, 2H, N*H*); 7.40, 7.31 and 7.18 (m, 3H, aromatic C-*H*); 4.25[a] (m), 4.17[b+c] (d, J = 3.6), 2H, hydantoin ring C-*H*; 2.15 (m, 2H, C*H*(CH_3)$_2$); 1.91[b], 1.87[a], 1.84[c], (s, 3H, C*H*$_3$-aryl); 1.02 (m, 6H, CH(C*H*$_3$)$_2$); 0.90 (d, J = 6.6, 6H, CH(C*H*$_3$)$_2$). Ratio of diastereomers a:b:c = 40:24:36. ^{13}C-NMR (DMSO-d_6): δ 172.76, 172.80 and 172.82 (*C*O); 155.8 and 155.9 (NH*C*ONH); 134.7, 135.1 and 135.5 (C, C(2)); 132.0, 132.15, 132.17 and 132.4 (C, C(1) and C(3)); 129.3, 129.5, 129.8 and 129.9 (CH, C(4) and C(6)); 126.6, 126.7 and 126.75 (CH, C(5)); 61.6 and 62.0 (hydantoin ring *C*H); 29.7 and 30.0 (*C*H(CH_3)$_2$); 15.9, 16.2, 16.3, 18.4 and 18.6 (CH(*C*H_3)$_2$); 12.83, 12.86 and 12.93 (*C*H_3-Ar).

3-(3-Amino-4-methylphenyl)-5-*sec*-butylimidazoline-2,4-dione or 3-[4-(2-amino)tolyl]-5-*sec*-butylhydantoin **(4-TIH)**. 4-TIH was prepared from 2,4-TDI and L-isoleucine by a procedure described for 4-TVH. White solid. EI-MS: *m/z* 261 (94): $[M]^+$, 205 (21), 148 (100), 134 (34), 106 (13).

2.3. Incubation of blood with 2,4-TDI and isolation of globin

Full human blood treated with citrate, obtained from the blood donor unit, was used for the experiments. To a blood sample (5 ml) heated at 37 ^{o}C, 2,4-TDI solution in dioxane (0.005-0.5 M, 100 μl) was added dropwise while the tube with blood was gently shaken by hand. Then, the sample was left in a shaking water bath (37 ^{o}C) for 1 h and spun down at 2500 rpm for 15 min. Plasma was removed, the erythrocytes were washed twice with saline (25 ml) and hemolyzed with distilled water (1 ml). Globin was precipitated by cold acetone containing 2% of concentrated hydrochloric acid (15 ml), washed three times with cold

acetone and once with cold diethylether, and dried in SpeedVac at room temperature for 30 min, then at 65 ^{o}C for 1 h.

2.4. Determination of the TVH adducts in globin

Samples of globin (100 mg) were placed in 10-ml ampules, and concentrated HCl/acetic acid 2:1 (5 ml) and an aqueous solution of the internal standard 4-TIH (1 mM, 0.1 ml) were added. The unsealed ampules were heated in a heating block at 100 ^{o}C for 8 h (the elongated top of the ampoule served as a reflux condenser). Care was taken at the beginning of heating, since the samples may develop foam until the globin is dissolved. After cooling, the contents of the ampules were transferred to 25-ml centrifugation tubes, 10 M NaOH (2 ml), ammonium sulphate (2 g), and one drop of phenol red were added, and more 10 M NaOH was added stepwise to attain pH 8.0-8.5 (indicated by the change of color from pink to yellow and by a pH indicator paper strip). It is important that the pH value doesn´t exceed 9, otherwise the hydantoins may decay. The neutralized hydrolysate was extracted with ethyl acetate (8 ml), the extract was evaporated to dryness and dissolved in n-propanol (100 µl). Routine analyses for 2-TVH and 4-TVH were carried out on a Chrom 5 gas chromatograph (Laboratory Instruments, Prague, Czech Republic) equipped with a fused silica capillary column HP-50+ 15 m × 0.53 mm I.D. × 1 µm film thickness (Agilent) and a nitrogen-selective detector. Carrier gas was nitrogen (30 kPa). Injector, column and detector temperatures were 250 ^{o}C, 250 ^{o}C and 290 ^{o}C, respectively. Injector was set in a split mode, 1:20. Quantitation was based on the ratio of peak heights of the analyte and the internal standard. For construction of the calibration curve, control globin (100 mg) was spiked with 2-TVH and 4-TVH (0-200 nmol in 100 µl of aqueous solution), and the samples were processed as described for authentic samples.

The calibration curves for 2-TVH and 4-TVH were found to be linear (r=0.99) within the range of 0 to 200 nmol TVH per sample, and were described by the equation *TVH*

(nmol/sample) = a + b×R (*R*, ratio of the peak heights of TVH vs. 4-TIH). With 4-TIH, 100 nmol/sample, the value of *b* was typically about 80 for both 2-TVH and 4-TVH. The coefficient *a* was usually not significantly different from 0.

2.5. GC/MS

Mass spectra were measured on a gas chromatograph type 6890 coupled to a mass spectrometric detector MSD 5973 (Agilent) with EI source at 70 eV. The samples were introduced to the detector via a ZB-35 fused silica capillary column, 18 m × 0.25 mm I.D. × 0.25 μm film thickness (Phenomenex), at a helium flow rate of 0.67 ml/min (linear velocity of 40 cm/s). Injector, fitted with a straight liner containing a plug of glass wool and heated at 270 ^{o}C, was set in a split mode (1:20). Column temperature started at 150 ^{o}C, then was increased at 20 ^{o}C/min to 270 ^{o}C, then at 40 ^{o}C/min to 340 ^{o}C, and finally was held at 340 ^{o}C for 5 min. Temperatures of transfer line and ion source were 300 ^{o}C and 180 ^{o}C, respectively. Mass detector scan range was *m/z* 50-500.

2.6. HPLC

Chromatographic behavior of the hydantoins was studied on an HPLC system type 1100 Series equipped with a G1315B diode-array detector (Agilent). The column used was Zorbax Eclipse XDB-C8, 150 × 4.6 mm (Agilent). The mobile phase was MeOH/water 50:50 pumped at a flow rate of 0.5 ml/min. The detector was set at 220 nm or 240 nm.

2.7. NMR

^{1}H-NMR spectra were measured on a Varian Mercury Plus spectrometer (300 MHz resonance frequency for ^{1}H). ^{13}C-NMR spectra were measured on a Bruker Avance DRX500 spectrometer (500 MHz resonance frequency for ^{1}H).

3. Results and discussion

3.1. Preparation of the reference TDI-Val hydantoins

The initial step in the preparation of the TDI-Val hydantoins was a direct reaction of TDI with valine in the water/dioxane solvent to obtain a mixture of the intermediate N-carbamoyl (ureid) adducts. Whereas valine is water soluble, the solubility of TDI in water is very low. Moreover, TDI rapidly decays in aqueous media due to hydrolysis and consequent cross-linking. The solubility of TDI was greatly enhanced by the addition of dioxane as a non-reactive and fully water-miscible solvent. The rates of the ureid formation and the isocyanate group hydrolysis depend strongly on pH. Thus, the profile of the TDI-Val adducts can be modified by pH of the reaction medium. At the neutral or slightly alkaline pH (7-9), formation of the ureids predominates over hydrolysis. Consequently, the major product consists of TDI and valine in the rate of 1:2. On the other hand, at low pH, TDI is preferentially hydrolyzed to the corresponding toluenediamine (TDA). In this study we especially aimed at preparing the adducts in the rate of 1:1, in which one isocyanate group of TDI was linked with valine while the second isocyanate group had been hydrolyzed. These adducts were obtained with high yield when TDI was added dropwise to the excess of valine while pH was kept at around 6. In addition to the demanded 1:1 adducts, the resulting mixture contained inevitably also the 1:2 adduct, TDA, and a bulk of other products, presumably TDI oligomers and polymers. Such material could not be fractionated by any chromatographic technique because of insolubility in common organic solvents except DMF or DMSO. Thus, the individual TDI-Val ureids have not been isolated. Instead, the mixture of the ureids was converted by Edman reaction to the corresponding hydantoins, which were extracted with ethyl acetate and feasibly fractionated by column chromatography and preparative HPLC. Pure compounds were characterized by GC/MS-EI, ^{1}H- and ^{13}C-NMR spectrometry. All three 2,4-TDI-Val hydantoins (2-TVH, 4-TVH, 2,4-TDVH) and both 2,6-TDI-Val hydantoins (6-

TVH and 2,6-TDVH) were prepared (Fig. 1). Mass spectra of the hydantoins 2,4- and 2,6-TDVH showed characteristic ions at *m/z* 372 (M^+) and fragments at *m/z* 330, 273, 231 and 174, corresponding to the combined losses of one or two propene and/or valinyl moieties. The isomeric hydantoins 2-TVH, 4-TVH, and 6-TVH gave molecular ions at *m/z* 247 (M^+) and fragments at *m/z* 205 and 148, corresponding to the losses of propene and valinyl, respectively. The isomerism of 2-TVH and 4-TVH was assigned using NMR spectroscopy.

An elegant synthetic procedure for preparation of the TVH isomers was described by Sabbioni et al. [13]. Isomeric methylnitrophenyl (mono)isocyanates were reacted with valine to afford intermediate methylnitrophenylcarbamoyl valines, the nitro group was reduced to obtain methylaminophenylcarbamoyl valines, and these were converted to TVH. Problems arising from the use of bifunctional isocyanates were eliminated, and single major products were obtained in high yields. Mass- and NMR spectra of TVH as reported by Sabbioni et al. and ourselves are consistent. This synthetic approach cannot be used to prepare dihydantoins 2,4-TDVH and 2,6-TDVH.

3.2. Relationship between the structure and properties of the TDI-Val hydantoins

The series of the positional isomers 4-TVH, 2-TVH, and 6-TVH showed remarkable chromatographic characteristics. In GC/MS, all three isomers afforded, as expected, narrow peaks, one each, which differed slightly in their retention times (Fig. 2a) and relative abundances of characteristic ions in the mass spectra. In reversed-phase HPLC, 4-TVH and 2-TVH almost coeluted, but while the peak of 4-TVH was relatively narrow (corresponding to the column efficiency), the peak of 2-TVH was enormously broad. 6-TVH eluted earlier, affording two well-resolved narrow peaks (area ratio of ca. 1:1) (Fig. 3) with identical UV spectra. TLC analysis of 6-TVH also afforded two resolved spots. The two 6-TVH fractions were separately collected, and each was immediately reanalyzed under the same HPLC conditions. Both analyses afforded two peaks with retention times, UV spectra, and area ratio

identical with those obtained in the original analysis. Reanalysis of both fractions by GC/MS afforded an identical peak. The above described properties of the TVH isomers occurred invariably on several different reverse-phase HPLC columns using neutral (MeOH/water) or acidic (0.1% trifluoroacetic acid in acetonitrile/water) mobile phases.

The above phenomenon is associated with the rotation around the C-N bond connecting the aromatic and hydantoin rings. The rotation may be hindered due to steric interactions between the hydantoin ring and the adjacent methyl group on the aromatic ring, leading to the formation of diastereomeric rotamers. Depending on the barrier of rotation, these rotamers can or cannot be separated by HPLC. In 4-TVH, free rotation around the C-N bond occurs, and thus only one isomer exists. In both 2-TVH and 6-TVH, a hindered rotation around the C-N bond gives rise to two diastereomeric rotamers. In the case of 6-TVH, the rotation is hindered enough to enable separation of these rotamers in HPLC but not sufficiently hindered to make them stable on a longer time scale. Thus, on reanalysis, the 6-TVH rotamers attained the equilibrium again. In 2-TVH, the equilibration rate of the rotamers is comparable to the analysis time in HPLC, so that only one broadened peak was observed.

GC analysis of the dihydantoins 2,4-TDVH and 2,6-TDVH was possible at a very high temperature, above 300 ^{o}C. Each compound afforded a single peak (Fig. 2a). In reversed-phase HPLC with MeOH/water mobile phase, 2,4-TDVH eluted as a rather broad and symmetrical peak, while 2,6-TDVH eluted as a peak which appeared to be composed of two components with very low resolution superimposed upon each other (Fig. 3). Further, the seeming impurities eluting around the dominant peak of 2,6-TDVH have shown identical characteristics (UV, APCI-MS, and GC/MS spectra) as the main peak. This is accounted for by existence of 2,6-TDVH in several spatial forms, with individual properties similar to those of the individual isomers of TVH.

The rotation barriers of the TDI-Val hydantoins have been studied in detail using NMR measurements at a variable temperature (details will be published elsewhere). The energy of

the rotation barrier was found to be in the order 4-TVH < 2-TVH < 6-TVH, and 2,4-TDVH < 2,6-TDVH, which is in agreement with the previous observations [13]. Thus, the variable temperature NMR spectra and the barriers of rotation calculated therefrom well explain the relative stability of the TDI-Val hydantoin rotamers, and are consistent with their chromatographic behavior.

3.3. Determination of the 2,4- and 2,6-TDI-Val adducts

Formation of the TDI adducts with biological macromolecules is a direct reaction not mediated by the metabolizing enzymes. Thus, the adducts produced in the current in vitro experiment should be representative of the pattern of adducts generated on the exposure to TDI in vivo. This study has focused on the TDI adducts attached at the N-terminal valine of globin, for which the priority analytical procedure is Edman degradation converting the adducts to the corresponding hydantoins. The procedure has been adapted to specific properties of the TDI-Val hydantoins. Globin isolated from human blood incubated in vitro with TDI was heated with concentrated HCl /acetic acid at 100 ^{o}C for 8 h. The hydrolysate was neutralized to pH 8, the hydantoins were extracted with ethyl acetate and analyzed by GC/MS-EI and HPLC. In the 2,4-TDI-treated globin, all expected compounds, namely, 2-TVH, 4-TVH, and 2,4-TDVH, were identified using the synthetic reference standards (Fig. 2b). Analogically, analysis of globin from blood incubated *in vitro* with 2,6-TDI revealed the presence of 6-TVH and 2,6-TDVH (Fig. 2c). These products were also detected when blood was incubated with „technical“ 2,4-TDI containing ca. 20% of 2,6-TDI.

Detection of the above hydantoins in globin from full blood treated with TDI confirmed that TDI can penetrate the erythrocyte membrane and form stable adducts at the N-terminal valine of globin under physiological conditions. Whereas the 1:1 adducts detected as TVH have been found also by Sabbioni et al. [13], the occurrence of 1:2 adducts (converted to 2,4- and 2,6-TDVH) is reported here for the first time. Intermolecular cross-links were previously

detected in the guinea pig hemoglobin treated with 2,4-TDI [7], however, their location at the globin chain was not identified. The finding of the 1:2 adducts is an evidence of intermolecular cross-linking of two globin molecules via their N-termini, and indicates that these termini in native erythrocytes are in close proximity to each other and/or are sufficiently flexible to allow their linking.

A simple procedure for routine determination of TVH isomers at the levels of ≥nmol/g globin has been developed. The method was sufficiently sensitive to measure TVH in globin from human blood incubated *in vitro* with 2,4-TDI over the range of 0.10-10 mM. A critical step of the procedure is neutralization of the globin hydrolysate before extraction of TVH. Since TVH are weak amines, their extraction recovery is pH-dependent, with the maximum value above pH 7. On the other hand, TVH decay through ring opening in the alkaline conditions. For example, 4-TVH and 2-TVH (1 mM) decayed substantially at pH 11 within minutes. Therefore, the neutralization of the hydrolysate with 10 M NaOH has to be carried out carefully. Ammonium sulphate was used to slow down the dependence of pH on the added volume of NaOH by buffering the system.

The globin extracts were analyzed by GC on a microbore column using the nitrogen-selective detector. Determination of TVH at ≥ng amounts (on column per injection) exhibited satisfactory chromatographic performance. It required no special attention to the cleanliness of the chromatographic system, and the calibration curves were linear. On the other hand, determination of sub-ng amounts of TVH was problematic and unsuitable for routine analysis because of peak distortion and compound losses, as reported also previously [13]. GC analysis for 2,4- and 2,6-TDVH was only semiquantitative and limited to concentrated samples. The calibration curves were strongly non-linear, and the amounts up to several ng/injection were fully retained on the column. The relationships between 2,4-TDI concentration and TVH levels on a logarithmic scale are shown in Fig. 4. The hydantoin levels produced with 2,4-TDI, 10 mM, are given in Table 1. Formation of almost equal

amounts of 2-TVH and 4-TVH indicates that reactivity of isocyanate groups in the positions of *ortho*- and *para*- is very similar.

On the analysis using nitrogen-sensitive detector or by GC/MS, the peak of 2-TVH partially coeluted with an unknown impurity whereas the peak of 4-TVH was not interfered. Thus, 4-TVH was chosen for further investigations as an index of exposure to 2,4-TDI. It has to be considered that 4-TVH is just an analytical derivative of N-(3-amino-4-methylphenyl)carbamoylvaline, which is the the true biological adduct of toxicological concern. Rigorously, the step of the conversion of the adduct to 4-TVH should also be included in the calibration procedure. However, a globin calibration standard with known level of the adduct is not available. Then, the most suitable alternative is to calibrate the method using a peptide simulating the globin adduct´s N-terminal sequence. Sabbioni et al. [13] employed the tripeptide adduct N-[(3-amino-4-methylphenyl)carbamoyl]valyl-glycyl-glycine and its deuterated analogue as calibration and internal standards, respectively. The method of Sabbioni et al. can be considered as a reference one. The current calibration procedure employs 4-TVH and its methyl homologue 4-TIH as calibration and internal standards, respectively. These are carried through the entire analytical procedure. Our method and the reference metod would afford identical results assuming that the recovery of 4-TVH from the adduct is quantitative. This assumption appears to be realistic. In a related ureid adduct, N-methylcarbamoylvalyl-leucyl-aniline, the recovery of the corresponding 3-methyl-5-isopropylhydantoin was determined to be 90% [14] or 100% [15]. This suggests that the recovery of 4-TVH from the globin adduct is also close to 100%, and that 4-TVH can be used as a practical calibration standard. The internal standard 4-TIH has very similar analytical properties as 4-TVH, therefore, it is a practical alternative to the deuterated 4-TVH internal standard.

The degree of saturation of N-terminal valine in the investigated globin samples can be assessed. Concentration of N-terminal valine in human globin (α- and β-chains, 1:1) is 65

μmol/g. In globin treated with 10 mM 2,4-TDI, with the 4-TVH level of 1.11 μmol/g (Table 1), the latter accounts for at least 1.11/65 = 1.7 % of total binding capacity of N-terminal valine, and all three adducts together account for at least (1.11 + 1.15 + 2×0.40)/65 = 4.7 % thereof. Probably an additional part of this capacity is covered by unidentified adducts in which 2,4-TDI crosslinks the N-terminal valine with other nucleophilic amino acid residues. Table 1 also shows relative yields of the particular hydantoins, expressed as % of the total 2,4-TDI dose added to blood. These were calculated on the assumption that one ml of blood contains 0.125 g of globin, according to the formula: relative yield of TVH (%) = [TVH level (nmol/g glob) × 0.125 × 100]/2,4-TDI dose (nmol/ml blood).

The relative yields of the hydantoins from TDI in the *in vitro* incubations do not appear to be high, however, the large abundance of competing nucleophilic groups in plasmatic and red blood cell proteins including globin has to be considered. The N-terminal valine is clearly a significant binding site for isocyanates in globin. This is in agreement with our earlier *in vitro* model study on the reactivity of 2,4-TDI and hexamethylenediisocyanate towards various amino acid residues [16], where the fastest reactions were observed with N-terminal amino acids and lysine residues. The N-terminal valine was also a major binding site for methylisocyanate [9] and methylphenylisocyanate [17]. In conclusion, TVH should to be seriously considered as suitable biomarkers of the cumulative internal exposure to TDI.

3.4. Applications

Besides the *in vitro* experiments with human globin described in this paper, determination of 4-TVH was carried out in rats dosed ip. with 2,4-TDI in olive oil, 0.15 mmol (26.1 mg)/kg body weight. The maximum level of 4-TVH, 14±3 nmol/g globin (n=10), was declining slowly to a pre-exposure value attained in ca. 2 months [12]. The 4-TVH levels in occupationally exposed workers and in animals exposed to 2,4-TDI by inhalation are expected to be far below the detection limit of the presented method. However, the sensitivity

of 4-TVH determination can be substantially increased by using LC/MS, or after derivatization with a perfluoroacyl anhydride followed by GC/ECD or GC/MS-NCI detection. Using these methods, 4-TVH was determined in a TDI-exposed worker and in two women with polyurethane covered breast implants [13]. Similarly, 6-TVH levels up to 0.30 nmol/g globin (mean, 0.09 nmol/g globin) were found in 14 Korean workers exposed to TDI in the production of music instruments (Sakai T., unpublished results). A GC/MS-NCI method for 4-TVH determination developed in our laboratory will be published separately.

Acknowledgements

The study was supported by grant 310/00/0934 from the Grant Agency of the Czech Republic, which is gratefully acknowledged.

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Table 1. Formation of the TDI-Val hydantoins upon incubation of full human blood with 2,4-TDI, 10 μmol/ml

Hydantoin	4-TVH	2-TVH	2,4-TDVH
Level in globin (μmol/g) [a]	1.11±0.34	1.15±0.38	0.40±0.10
Relative yield from 2,4-TDI (%) [b,c]	1.39	1.44	0.50

[a] mean ± S.D for n = 4-20

[b] 2,4-TDI dose added to full blood: 100%

[c] on the assumpion that 1 ml of blood contains 0.125 g globin

Figure captions

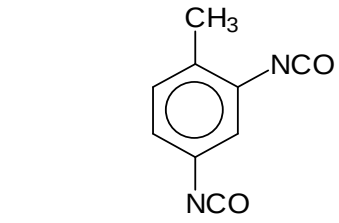
Figure 1. Structure of compounds used in the study

Figure 2. GC/MS analysis of the TDI-Val hydantoins: a) separation of the synthetic standards, b) globin (HG) from human blood dosed with 2,4-TDI, 10 μmol/ml, c) globin from human blood dosed with 2,6-TDI, 10 μmol/ml. 4-TIH was added as an internal standard. None of the compounds 2-TVH, 4-TVH, 6-TVH, 4-TIH, 2,4-TDVH, and 2,6-TDVH was detected in control globin samples. The chromatographic conditions are described in 2.5.

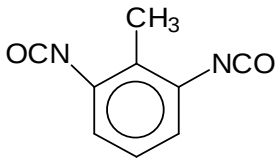
Figure 3. HPLC analysis of the TDI-Val hydantoins. Column: Zorbax Eclipse XDB-C8 4.6×150 mm, mobile phase: MeOH/water 50:50 at a flow rate of 0.5 ml/min

Figure 4. Hydantoin levels in globin from human blood incubated *in vitro* with 2,4-TDI, 0.1-10 μmol/ml

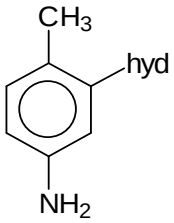
Figure 1



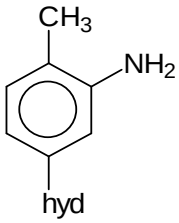
2,4-TDI



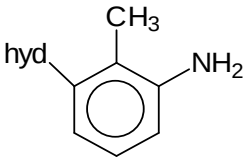
2,6-TDI



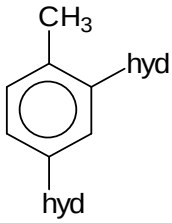
2-TVH



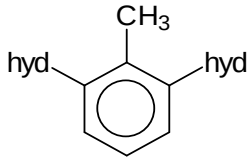
4-TVH



6-TVH



2,4-TDVH



2,6-TDVH

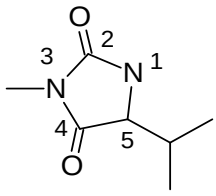


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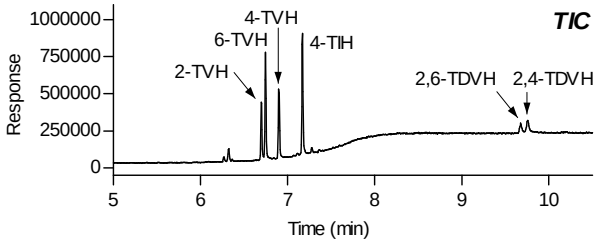
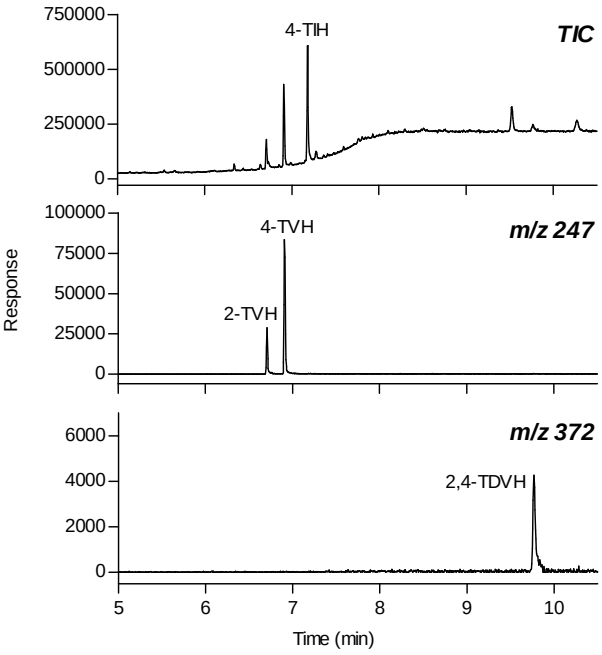
Figure 2

a) Hydantoin standards



b) HG-2,4-TDI



c) HG-2,6-TDI

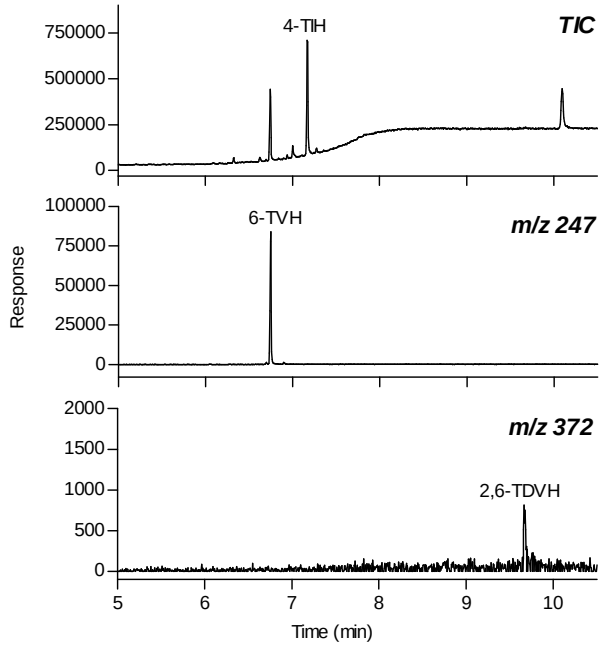


Figure 3

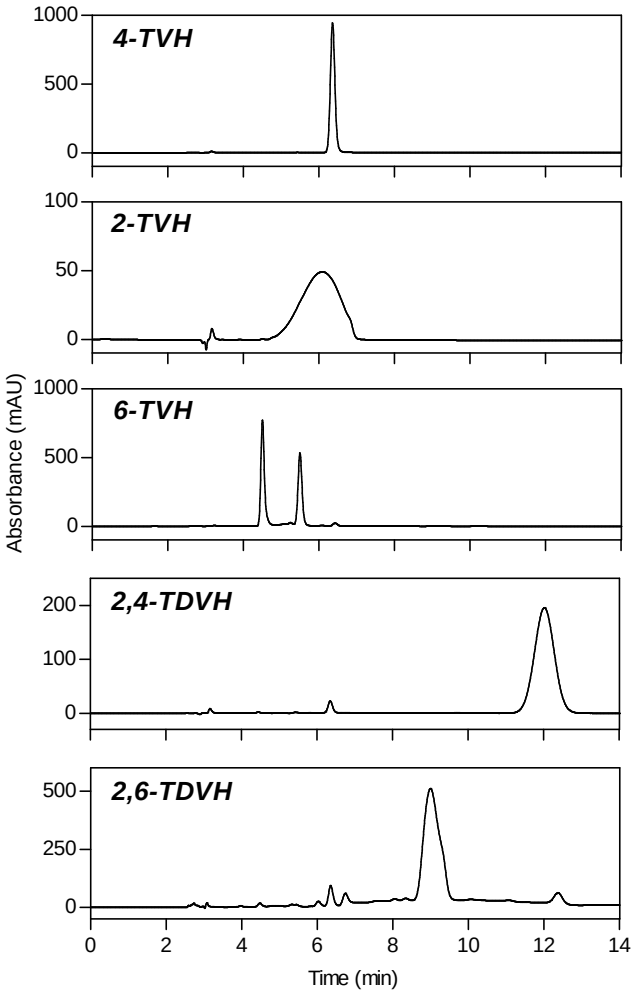
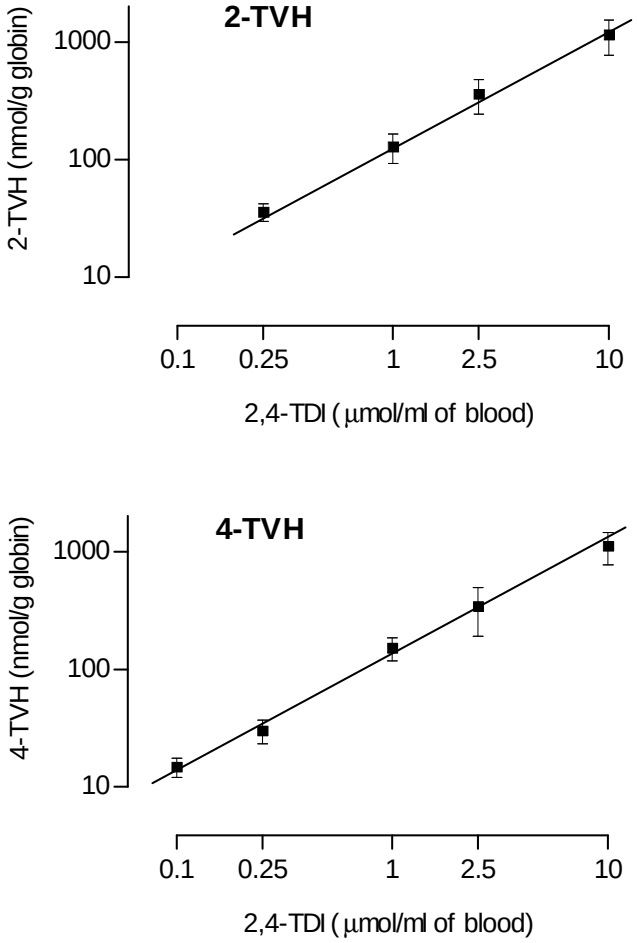

4-TVH
2-TVH
6-TVH
2,4-TDVH
2,6-TDVH
Absorbance (mAU)
Time (min)
1000
500
0
100
50
200
250
2
4
6
8
10
12
14

Figure 4


2-TVH
2-TVH (nmol/g globin)
1000
100
10
0.1
0.25
1
2.5
10
2,4-TDI (µmol/ml of blood)
4-TVH
4-TVH (nmol/g globin)
1000
100
10
0.1
0.25
1
2.5
10
2,4-TDI (µmol/ml of blood)