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Urinary metabolites of workers exposed to nitrotoluenes

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Abstract

Nitrotoluenes are important intermediates in the chemical industry. 2,6-Dinitrotoluene (26DNT), 2,4-dinitrotoluene (24DNT) and 2-nitrotoluene (2NT) are carcinogenic in animals and possibly carcinogenic in humans. Thus, it is important to develop methods to biomonitor workers exposed to such chemicals. The authors have monitored the air and urine metabolite levels for a group of workers in China exposed to 24DNT, 26DNT, 2NT and 4-nitrotoluene (4NT). The metabolites 2,4-dinitrobenzylalcohol (24DNBAlc), 2-amino-4-nitrobenzoic acid (2A4NBA), 4-amino-2-nitrobenzoic acid (4A2NBA) and 2,4-dinitrobenzoic acid (24DNBA) resulting from exposure to 24DNT were found in 89, 88, 91 and 78% of the exposed workers, respectively. The metabolites 2,6-dinitrobenzylalcohol (26DNBAlc) and 2,6-dinitrobenzoic acid resulting from 26DNT exposure were found in 99 and 86% of the exposed workers, respectively. Quantitatively, 2A4NBA, 4A2NBA and 26DNBAlc were the major metabolites. The nitrobenzoic acids were the major metabolites resulting from exposure to 2NT and 4NT and were present in 96 and 73% of the exposed workers, respectively. Air concentrations of DNT and 2NT did not correlate with the levels of metabolites in the urine. In conclusion, the dinitrobenzyl alcohols and aminonitrobenzoic acids determined in the urine provided a good marker for recently absorbed dose and were intrinsically related to the bioactivation and detoxification pathways of DNT. Air measurements were not a good measure to predict internal exposure.

Keywords: *Biomonitoring, nitrotoluene, dinitrotoluene, urine, metabolism, health effects*

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Introduction

Dinitrotoluenes (DNT) and mononitrotoluenes (MNT) are important intermediates in the chemical industry. There is clear evidence that 2-nitrotoluene (2NT) causes cancer in rats and mice (NTP 2002a). In contrast, 4-nitrotoluene (4NT) causes equivocal carcinogenic effects in rats and mice (NTP 2002b, Dunnick et al. 2003). 2,6-Dinitrotoluene (26DNT) and 2,4-dinitrotoluene (24DNT) have been classified as being carcinogenic in animals and as possibly carcinogenic in humans (IARC 1996).

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An excess of hepatobiliary cancer was found among munition workers exposed to DNT (Stayner et al. 1993). Brüning et al. (1999) found urethal tumour cases predominantly confined to miners highly exposed to DNT. It is therefore important to develop methods to monitor exposed populations. Previously, human biomonitoring studies (Levine et al. 1985, Tuner et al. 1985, Woollen et al. 1985, Smith et al. 1995, Angerer and Weismantel 1998), and metabolic studies in rat (Rickert and Long 1981, 1982, Rickert et al. 1984, Rickert 1985) identified biotransformation and excretion into urine as the most important pathway for elimination of DNT (24DNT and 26DNT). The nitro group is reduced to the corresponding amines in the gut by the microflora or in the liver. In male rats given 10 mg kg^{-1} 24DNT >85% of the dose was excreted in the urine. The major metabolites were 2,4-dinitrobenzoic acid (24DNBA) (44%), 2,4-dinitrobenzyl alcohol (24DNBAlc)-glucuronide (27%), 4-acetylamino-2-nitrobenzoic acid (4AA2NBA) (25%) and of 2-amino-4-nitrobenzoic acid (2A4NBA) (5%) (percentages were normalized to the total of all 24DNT metabolites detected). The major route of elimination of 26DNT in the rat was via the urine, with approximately 57% of the dose (Rickert and Long 1982). 2,6-Dinitrobenzyl alcohol (26DNBAlc)-glucuronide (38%), 2,6-dinitrobenzoic acid (26DNBA) (37%) and 2-amino-6-nitrobenzoic acid (2A6NBA) (25%) were detected, and accounted for approximately 95% of the urine metabolites). The metabolites of 24DNT detected in the urine of workers exposed to technical DNT were 24DNBA, 2A4NBA, 24DNBAlc, 2AA4NBA and trace levels of 4A2NBA which accounted for 32–67, 29–48, 4.4–30 and 0.3%, respectively of the average total 24DNT metabolites detected in workers (Levine et al. 1985, Turner et al. 1985, Woollen et al. 1985) (Figure 1). The metabolites of 26DNT detected in urine of the same workers were 26DNBA and 26DNBAlc, which accounted for 28–69 and 31–72% of the total 26DNT metabolites detected in workers, respectively. There have been no

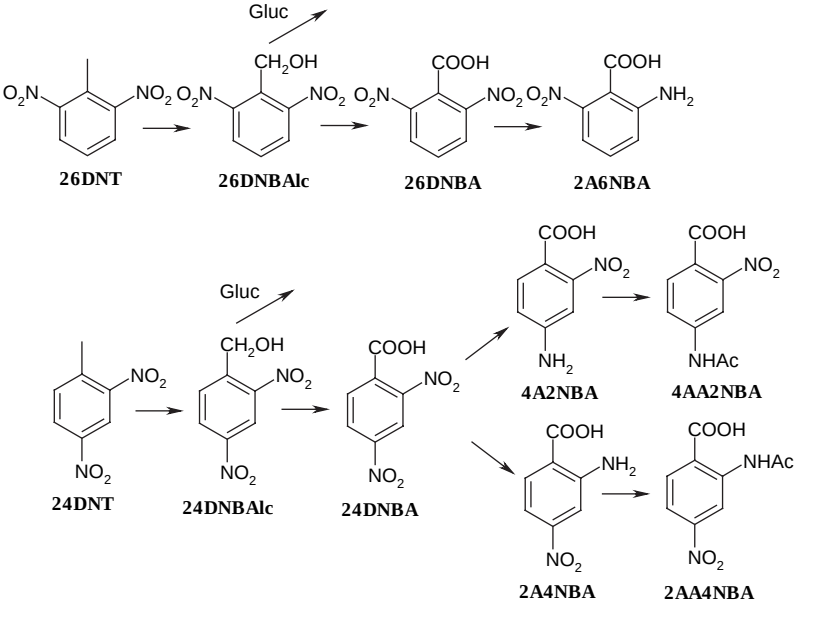

Figure 1. Major urine metabolites of 26DNT and 24DNT found in rats and/or humans. In humans, all metabolites are present, except for 4AA2NBA and 2A6NBA.

human biomonitoring studies specifically related to MNT exposure. Metabolic studies in rats have demonstrated that following administration of [^{14}C] 2NT, 3NT or 4NT, elimination of 86, 68 and 77% of the respective doses occurred through excretion into urine (Rickert 1987). The major metabolites of 2NT and 4NT identified in the rat were the nitrobenzoic acids (29% 2NBA, 28% 4NBA), nitrobenzyl glucuronides (14% 2NBAlc-glucuronide, 1.4% 4NBAlc-glucuronide), mercapturic acids (12%, S-(2-nitrobenzyl)-N-acetyl cysteine), acetylaminobenzoic acids (27%, 4-acetylaminobenzoic acid) and the nitrohippuric acid (13%, 4-nitrohippuric acid). To date, there have been five biomonitoring studies published in the literature that have reported urinary metabolite measurements in workers chronically exposed to 24DNT and 26DNT. These studies were performed on a small group of workers ($n < 28$) and the single metabolites were evaluated for few cases. The present study collected data on the inhaled exposure, the recent absorbed dose, through urinary metabolite determinations, and the chronic absorbed dose, for a much larger population (35 pre-shift workers, 80 post-shift workers and 11 controls). Previously, no inferences were made on a dose–response effect for DNT exposure in humans. Of particular interest was the investigation of dose–response relationships in these Chinese workers exposed to MNT and DNT. We were interested in defining whether biomarkers of recent exposure correlated with the risk of suffering from one or more of the health conditions, symptomatic of toxic exposure nitroarenes. Therefore, metabolite levels determined in the urine were compared with the medical status of the workers.

Materials and methods

Materials

2,4-Dinitrotoluene (99%) (24DNT), 2,6-dinitrotoluene (99%) (26DNT), 2,4-dinitrobenzoic acid (99%) (24DNBA), 3,4-dinitrobenzoic acid (99%) (34DNBA), 3,5-dinitrobenzyl alcohol (98%) (35DNBAlc), 2-nitrobenzoic acid (98%) (2NBA), 4-nitrobenzoic acid (98%) (4NBA), 2-nitrobenzyl alcohol (98%) (2NBAlc), 4-nitrobenzyl alcohol (97%) (4NBAlc), 4-nitrophenol (99%) (4NP), sodium hydrogen carbonate ($NaHCO_3$), sodium sulphate (Na_2SO_4), 4-nitrophenol glucuronide (4NP-Gluc), 1-methyl-3-nitro-1-nitrosoguanidine (MNNG) were obtained from Aldrich (Taufkirchen, Germany). Diethyl ether and methanol (MeOH) for trace analysis were obtained from Merck (Darmstadt, Germany). N,O-*bis*-trimethylsilyltrifluoroacetamide (BSTFA) was obtained from Supelco (Taufkirchen, Germany). β-Glucuronidase type B-1 from bovine liver (1 640 000 units g^{-1} protein) and β-glucuronidase type HP-2 from *Helix pomatia* (100 000 units ml^{-1}) were obtained from Sigma (Taufkirchen, Germany). Sodium acetate (NaOAc) was obtained from Fluka (Buchs, Switzerland). 2,4-Dinitrobenzyl alcohol (24DNBAlc) and 2,6-dinitrobenzyl alcohol (26DNBAlc) were synthesized according to the procedure of Gal'bershtam and Budarina (1969) and Mori et al. (1986). 2,6-Dinitrobenzoic acid (26DNBA) was synthesized according to the procedure of Mori et al. (1986). 2-Amino-4-nitrobenzoic acid (2A4NBA) was purified from crude 4-nitroanthranilic acid (70%) by column chromatography on silica using neat ethyl acetate. The product was obtained as a bright orange, crystalline solid. 4-Amino-2-nitrobenzoic acid (4A2NBA) was synthesized according to the procedure of Schmidt et al. (1999). 2-Acetylamino-4-nitrobenzoic acid (2AA4NBA) and 4-Acetylamino-2-nitrobenzoic acid (4AA2NBA) were synthesized according to the procedure of Rickert and Long (1981).

Methods

Data and urine collection. The exposed ($n=98$) and control ($n=72$) workers were employed in a factory manufacturing DNT and 2,4,6-trinitrotoluene (TNT). The factory was situated in Liaoning (Liaoning Province, China). The industrial synthesis of DNT and TNT was performed by continual batch nitration of MNT then DNT with sulphuric acid and nitric acid. The workers were grouped according to their job description and work location as follows: group leader, MNT tank, DNT tank, TNT tank, laboratory of chemical analyses, transportation of TNT to packaging, packaging, control room, disposal of waste acid, disposal of waste H_2O, and non-exposed control workers. The age of the controls was 37.4 ± 8.7 (mean $\pm$ SD) and the age of the exposed was 36.2 ± 9.5. The work years in the control group was 16.9 ± 7.6, and in the exposed group 14.9 ± 10.0. The control group consisted of 13 females and 59 males. Among the exposed workers were 29 females and 69 males. All the workers employed in the analyses section were females ($n=18$). Seven females were present in the control room.

Each participant was interviewed with a questionnaire about their general health status, exposure history, smoking and alcohol consuming habits, previous medical record and present symptoms. Sample collection, the medical examination and the questionnaire were all performed in the same week. The following examinations were performed by the medical department of the Chinese academy of preventive medicine: physical examinations: blood pressure, cardiovascular system, nervous system, and heart rate; routine blood and urine tests; liver function test (glutamic pyruvic transaminase, alkaline phosphatase, total protein, albumin, total bilirubin); electrocardiogram; ultrasonic type B examination for liver and spleen; serological assays of hepatitis B antigens and antibodies were conducted, because hepatitis B is rather common in China, and liver damage can also be caused by some of the nitroarenes.

Urine samples were collected in 50 ml plastic tubes and stored immediately at $-20^{\circ}C$. The workers were grouped according to their job description and work location as follows: group leader, MNT tank, DNT tank, TNT tank, laboratory of chemical analyses, transportation of TNT to packaging, packaging, control room, disposal of waste acid, disposal of waste H_2O, and non-exposed control workers. In total, 126 spot urine samples were collected from the factory workers. Of these, 115 were from exposed workers and 11 were from non-matched, non-exposed controls. Of the 115 exposed samples, 80 were post-shift samples, voided at the end of the 8 h work shift, and 35 were pre-shift samples, voided from a subset of workers, before commencement of their work shift.

Statistical analyses. Statistical analyses were performed with the program SPSS 10.0, Sigma Stat 2.0, and Sigma Plot 3.0. The results of the questionnaire and the medical examination were not known to the scientists performing the urine analyses and were only disclosed at the end of the analyses. All tests of statistical significance were two sided. The distributions of the metabolite levels were markedly skewed; therefore, the data were transformed logarithmically. The logarithmically transformed data showed normal distribution (Kolmogorov–Smirnov test, $p > 0.05$) only for the metabolites 26DNBAlc, 24DNBAlc, 4A2NBA, 2NBAlc, 2NBA, the sum of the mononitrotoluene metabolites (total MNT) and for the sum of the dinitrotoluene metabolites (total DNT); but excluding the control workers. By including the control workers, only 2NBAlc was normally distributed. Therefore, parametric and non-parametric tests

were performed on the data. Health effects were compared with the urine metabolite levels using the Mann–Whitney test and logistic regression analysis.

Optimization of the enzymatic hydrolysis procedure with β-glucuronidase. 4NP-glucuronide was selected as a surrogate glucuronide for potential benzyl alcohol glucuronides present in the DNT urine samples from exposed workers. The conditions for complete enzymatic hydrolysis of 4NP-glucuronide (4NP-Gluc), using different β-glucuronidases, were investigated to ascertain the optimal conditions for cleavage of glucuronides in the urine samples of DNT workers. To separate aliquots of control urine (812 μl), 0.1 vols 10 × β-glucuronidase buffer (2 M NaOAc, pH 3.5) was added with 10 μl 2NBAlc internal standard (100 μg/10 μl). The samples were then spiked with 10 μl 4NP-Gluc (1 mg μl^{-1}) and vortex mixed for 20 s. Either β-glucuronidase type B-1, from bovine liver (2000 units), or β-glucuronidase type HP-2, from *H. pomatia* (2000 units), were added to the samples and incubated in a shaking water bath at 37°C for the designated time point. The time points for hydrolysis of 4NP-Gluc were: 0, 1, 2, 4, 8, 16, 24 and 48 h. Analysis of the hydrolysis reactions was performed on a Hewlett Packard (HP) chromatographic system (HP 1100), with a diode array detector (DAD) (HP 1100). A sample aliquot (10 μl) was injected onto a LiChrospher RP-Select B column (Merck, 250 × 4 mm, 5 μm) and pre-column (4 × 4 mm). The cleaved conjugate, 4NP, and internal standard, 2NBAlc, were eluted under isocratic conditions (85:15, 10 mM $NH_4H_2PO_4$ (pH 3.6): MeOH) at 1 ml min^{-1}, ambient temperature, with a detection wavelength of 225 nm. The peak area of 4NP released from 4NP-glucuronide, with respect to internal standard, was plotted against time. The surrogate glucuronide (4NP-Gluc) had been fully cleaved by 4 h with the glucuronidase from *H. pomatia*. In contrast, the glucuronidase from bovine had failed to cleave fully the glucuronide by 48 h. Therefore, the glucuronidase from *H. pomatia* was used in all subsequent hydrolysis reactions. The time point of 4 h was selected as the optimal hydrolysis time for quick sample throughput and total glucuronide cleavage.

Generation of diazomethane for methylation of benzoic acids. An MNNG-diazomethane kit with O-ring (from Aldrich) was used to generate mmol quantities of a diazoethereal solution within a closed system. MNNG (200 mg) was placed inside the tube along with H_2O (500 μl), to dissipate any heat generated. Diethyl ether (3 ml) was placed in the outside tube and the two parts were held together with a rubber O-ring and pinch clamp. The lower part was immersed in an ice bath then 5.0 M NaOH (600 μl) was added drop-wise to the MNNG solution, through a silicon rubber septum, using a 1 ml syringe with a narrow gauge. The reaction was left for 2 h at 4°C to assure formation of an acceptable yield of diazomethane ($>60\%$).

Procedure for determination of mono- and dinitrotoluene metabolites in urine. Duplicate aliquots (1 ml) of each urine sample were pipetted into glass screw capped tubes (100 × 22 mm) with Teflon liners (Supelco, 18 mm). Then 10 μl internal standard (500 ng/10 μl 34DNBA and 35DNBAlc) were spiked into the samples. The pH of the urine was adjusted to 4.5 with 0.1 vols 10 × β-glucuronidase buffer (2 M sodium acetate, pH 3.5). The samples were vortex mixed for 30 s then 20 μl β-glucuronidase (2000 units from *H. pomatia* Type HP-2) was added and the samples were incubated in a shaking bath at 37°C for 4 h. After cooling to room temperature, the pH of the

samples was adjusted to 2 with 2 M HCl. NaCl (100 mg) was added and the urines extracted with diethyl ether (2 ml) by vortex mixing for 5 min. Following centrifugation at 3000*g* for 10 min, the samples were frozen in liquid nitrogen, then thawed at room temperature to aid phase separation. The diethyl ether layer was removed to a graduated tapered tube (98 × 15 mm). The urine phase was adjusted to pH 7.4–7.8 with $NaHCO_3$ then re-extracted with diethyl ether (2 ml) as described above. The diethyl ether layer was removed and combined with the extract from the previous step. The extracts were derivatized with 200 µl diazoethereal solution (see above) for 1 h at room temperature. The organic layer was dried through a pipette, containing anhydrous Na_2SO_4 (1 g), then reduced under a gentle stream of nitrogen, to approximately 150 µl. The samples were transferred to 200 µl micro-inserts for autosampler vials (32 × 12 mm). The samples were derivatized with neat BSTFA (50 µl), for 30 min. The samples were reduced under a gentle stream of nitrogen to the last drop. The residual was reconstituted in EtOAc (15 µl) then analysed by GC-MS.

Quantitation of mono- and dinitrotoluene metabolites in urine. The analysis was performed on an HP gas chromatographer (5890II) equipped with an autosampler (HP 7673) and interfaced to a mass spectrometer (HP 5989A). The trimethyl silyl derivatives of the nitrobenzyl alcohols and methyl ester derivatives of the nitroaminobenzoic acids, dinitrobenzoic acids and acetylaminonitrobenzoic acids were analysed by splitless injection (1 µl) onto a fused-silica capillary column (Phenomenex, ZB5, 30 m × 0.25 mm i.d., 0.5 µm film thickness) equipped with a 1 m × 0.25 mm i.d. methyl-silyl retention gap (Supelco). In all cases, the initial oven temperature, the injector temperature and the transfer line temperature were set at 90, 250 and 280°C, respectively. The oven temperature was increased at 50°C min^{-1} to 125°C, then heated at 6°C min^{-1} to 195°C, and held for 5 min. Finally, the oven temperature was heated at 50°C min^{-1} to 320°C and held for 3.5 min. Helium was used as a carrier gas with a flow rate of 1.5 ml min^{-1}. For negative chemical ionization (NCI), with methane as the reagent gas, the source pressure was typically 160 Pa, the electron energy 150 eV, 1.5 Torr, the emission current was 300 µA, the quadrupole temperature was 100°C and the source temperature was 200°C. For electron impact ionization (EI), the electron energy was 70 eV, the emission current was 300 µA, and the source temperature was 200°C.

Additional sensitivity and specificity was obtained by monitoring individual ions for each of the analytes. The dwell times for analyte masses: 225, 182, 181, 165 and 153, in group 1 (10.70–15.00 min), and 270, 226, 196, 164 and 238, in group 2 (15.00–24.00 min), were 70 ms for GC-MS with NCI-SIM (Figures 2 and 3).

Calibration of urinary metabolites of mono and dinitrotoluene. Standard solutions of known concentration (0, 25, 50, 100, 200 and 500 ng ml^{-1}) of 24DNBAlc, 26DNBA, 24DNBAlc, 24DNBA, 2A4NBA, 4A2NBA, 2NBAlc, 2NBA, 4NBAlc and 4NBA were added to control urine with internal standards 35DNBAlc and 34DNBA (500 ng ml^{-1}). The standards were taken through the assay procedure as for the exposed urines samples, then analysed according to the developed detection system described in the methods. The alcohols 24DNBAlc, 26DNBAlc, 2NBAlc and 4NBAlc were quantified against the internal standard 35DNBAlc. The acids were quantified against the internal standard 35DNBA. The correlation coefficient (r^2) determined from the regression line of the ion abundance against metabolite

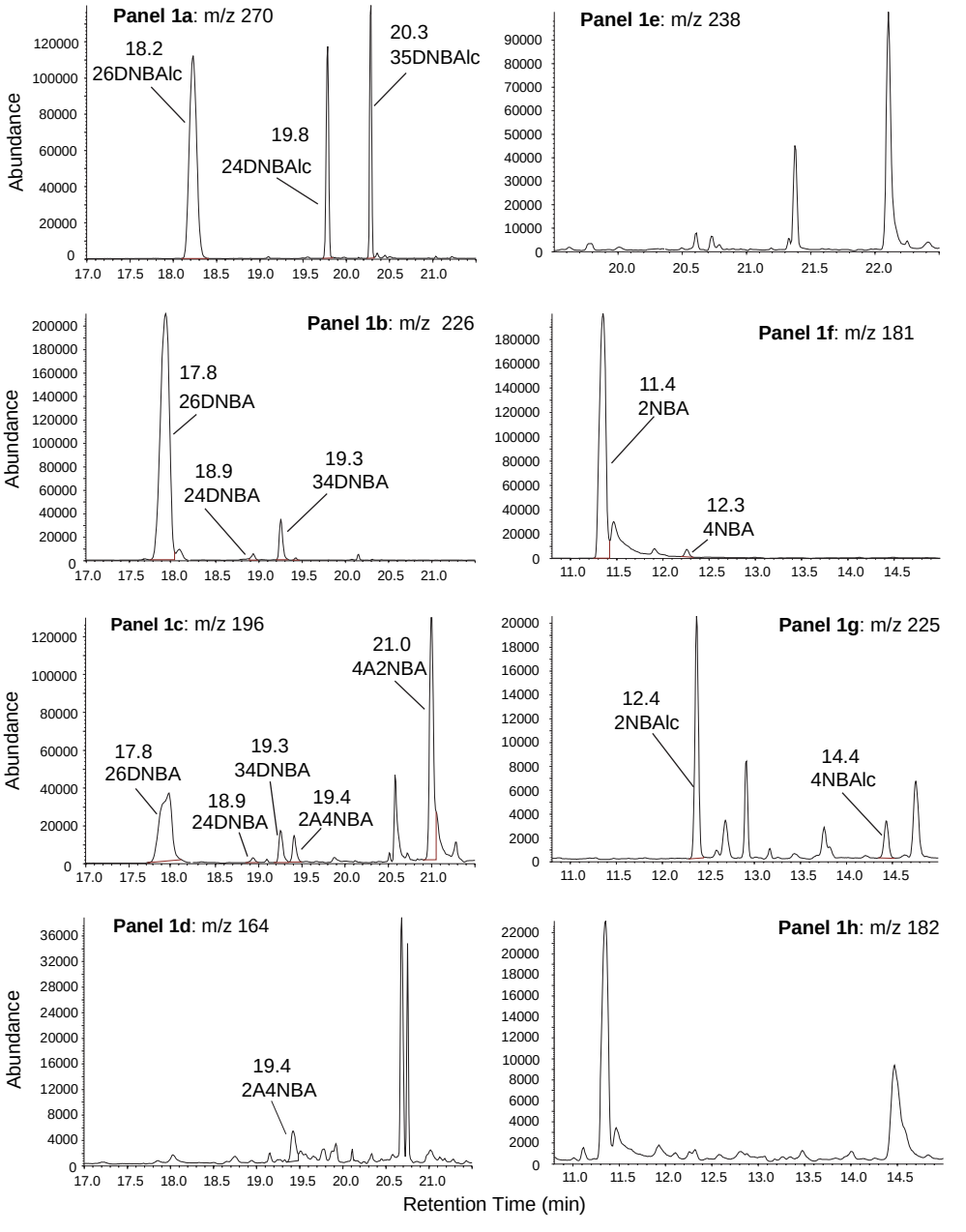

Figure 2. GC-MS chromatogram of extracted urine from a DNT-exposed worker derivatized with diazomethane and BSTFA. (1) Selected ions were monitored in NCI mode, with methane as the reagent gas. Single-ion chromatograms (SIM) of the trimethylsilyl ether derivatives of the dinitrobenzyl alcohols (*m/z* 270, 1a) 26DNBAlc and 24DNBAlc, and the mononitrobenzyl alcohols (*m/z* 225, 1g) 2NBAlc, 3NBAlc and 4NBAlc. SIM of the methyl ester derivatives of the dinitrobenozic acids (*m/z* 226, 1b) 26DNBA and 24DNBA, aminonitrobenzoic acids (*m/z* 196, 164, 1c, d) 2A4NBA and 4A2NBA, acetylaminonitrobenzoic acids (*m/z* 238, 1e) 2AA4NBA and 4AA2NBA and mononitrobenzoic acids (*m/z* 181, 1f) 2NBA and 4NBA. SIM of *m/z* 182 (1h) were acquired which corresponded to the dinitrotoluenes, 26DNT and 24DNT, respectively.

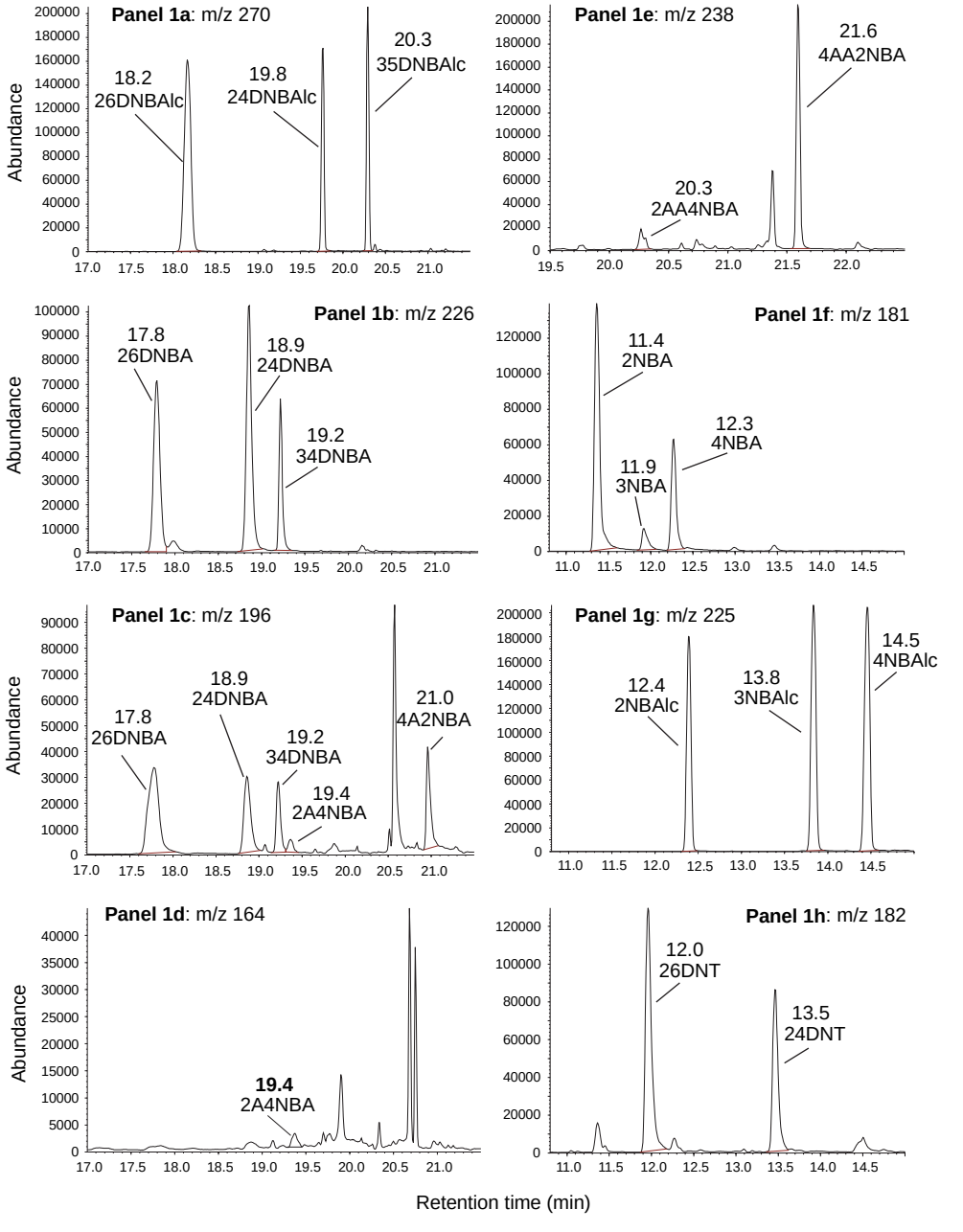

Figure 3. GC-MS chromatogram of control urine, spiked with authentic standards (500 ng ml^{-1}), then extracted and derivatized with diazomethane and BSTFA. Selected ions were monitored in NCI mode, with methane as the reagent gas. The single-ion chromatograms presented in (1a–h) have been described in Figure 2.

concentration was >0.98 for each metabolite of interest. This indicated that a linear response was obtained for each metabolite with respect to ion abundance detected in the mass analyser, over the concentration range analysed. The ion abundances acquired for quantitation of each metabolite have been described in Figures 2 and 3.

Determination of creatinine levels in workers. Urine creatinine levels were determined in workers by RP-HPLC coupled with ultraviolet light detection (254 nm). The average concentration (from two separate determinations) of creatinine was 1.47 $g\,l^{-1}$ and ranged between 0.13 and 4.62 $g\,l^{-1}$. The per cent difference between determinations fell, in the majority of cases, between 1 and 5%. For a small number of samples, the difference approached 10%. The precision associated with detection of creatinine in urine was calculated from multiple determinations of known concentrations of creatinine spiked into H_2O. From eight replicates of 0.325, 0.65, 1.3 and 2.6 $g\,l^{-1}$ creatinine spiked into H_2O the average peak areas $\pm$ the standard deviation were 0.145 ± 0.004, 0.307 ± 0.016, 0.621 ± 0.012 and 1.26 ± 0.039, respectively. The correlation coefficient, r^2, determined from the regression line between the peak area against creatinine concentration was >0.99. This indicated that a linear response was obtained with respect to ultraviolet light absorption of creatinine over the concentration range analysed. Recent publications tend to report the data without creatinine correction (Barr et al. 2005). Only urine values with creatinine values between 0.3 and 3.0$g\,l^{-1}$ are considered. In the present study, four workers (one each in the leader, MNT tank, analysis and waste group) had creatinine values <0.3 g and three workers (one in each in the leader, MNT tank, and TNT tank group) had creatinine values >3.0. Since we had no opportunity to obtain other urine samples from the workers we included all data and corrected the urine concentrations with creatinine.

Air monitoring. Air monitoring of 24DNT, 26DNT, 2NT and 4NT was performed according to the NIOSH-method 2005 (www.cdc.gov/niosh/nmam/pdfs/2005.pdf). Personal exposure monitoring (46 workers) was carried out using personal samplers from SKC. The SKC pumps were pre-calibrated with two silica gel adsorbent tubes (SKC product no. 226-10) connected in parallel. The flow rate used was 200 $ml\,min^{-1}$. The samplers were attached to the lapels of the workers to monitor the breathing zone. Each set of tubes was exchanged after 2 h to prevent overloading and breakthrough of the adsorbed material. All silica tubes were desorbed (about 1 h) in 1 ml methanol (both front and back sections). Each sample (1 μl) was analysed by GC FID (HP 9897): temperature programme: 90°C increase at 10°C min^{-1} to 200°C. Column: Ultra 1 (cross-linked methyl siloxane, 50 m $\times$ 0.32 mm, 0.52 μm film thickness). As the exposure in the factory was to a mixture of MNT and DNT, all the possible MNT and DNT isomers were investigated. Calibration lines were constructed using authentic standards; $r>0.997$ and detection limits of 5 $\mu g\,ml^{-1}$ (10 ng injected) were achieved. The extraction efficiency ranged from 90 to 92%, and adjustments were made in the calculations. The results showed that over 85% of the exposure (according to air measurements, see below for the internal dose) was from mononitrotoluenes.

Results

Development of analytical procedure

Before urinary analysis, development and validation of a number of key stages in the assay procedure was required. Initial experiments indicated that under the conditions described by Turner et al. (1985), very low recovery of the dinitrobenzoic and aminonitrobenzoic acids were observed. The recovery of each benzoic acid was

increased dramatically by the implementation of two alterations to the method procedure. By addition of NaCl to the urine enzymatic digests before extraction, and, second, the derivatization of the urine extracts, containing the benzoic acids, with the methylating agent before drying through Na_2SO_4, the recoveries of 24DNBA and 26DNBA were increased from non-detectable to 54 and 47%, respectively. By addition of NaCl before extraction of the urines, the recoveries of the alcohols, 26DNBAlc and 24DNBAlc, increased from 46 and 55% to 71 and 79%, respectively. The method of Turner et al. incorporated only one internal standard, 2-acetylaminobenzoic acid.

Since two discrete derivatizations were used for the transformation of the acids to methyl esters, and the alcohols to trimethylsilyl ethers, we selected an internal standard to control for each type of derivatization. For the benzoic acids, we selected 34DNBA as the internal standard, and for the benzyl alcohols we selected 35DNBAlc as the internal standard. The hydrolysis conditions for cleavage of glucoronides required optimization. We were unable to detect any of the dinitrobenzyl alcohol cleavage products in urine from exposed workers using bovine β-glucuronidase, according to the method of Turner et al. (1985). When the same subset of urines were re-analysed after hydrolysis with the β-glucuronidase from *Helix pomatia*, the dinitrobenzyl alcohols were detected by GC-MS. The results presented above confirmed that β-glucuronidase from *H. pomatia* was much more efficient at cleaving the glucuronide under the assay conditions described in the Methods.

Identification of metabolites in enzymatically hydrolysed urine

The urine extracts containing the derivatized metabolites were analysed in the NCI ionization mode. For increased sensitivity, the most abundant characteristic fragments, determined from the full-scan spectra, *m/z* 50–500, of authentic standards, were selected for single-ion monitoring. The retention times and mass fragments for each urinary metabolite of interest are presented in Figures 2 and 3.

Analysis of hydrolysed urine from workers exposed to MNT and DNT contained the nitrobenzoic acids 2NBA, 4NBA, 24DNBA and 26DNBA, the aminonitrobenzoic acids 2A4NBA and 4A2NBA, and the nitrobenzyl alcohols 2NBAlc, 4NBAlc, 24DNBAlc and 26DNBAlc (Figure 2). The acetylated metabolites, 2AA4NBA and 4AA2NBA, and parent compounds 24DNT, 26DNT, 2NT and 4NT, were not identified within the determination limits of the assay (10 ng ml^{-1} urine). The urinary metabolites were characterized by their mass fragments, in both NCI (Figure 2) and EI ionization modes (Figure 4), and by their retention times with respect to authentic standards (Figure 3). The methyl ester derivatized acids identified were: 2NBA, 4NBA, 26DNBA, 24DNBA, 2A4NBA and 4A2NBA. The trimethylsilyl ether derivatized alcohols identified were: 2NBAlc, 4NBAlc, 26DNBAlc and 24DNBAlc. In NCI mode the ion abundance of *m/z* fragments equal to $[M]^-$ (described in Figures 2 and 3) were acquired for quantitation of each urinary metabolite and were related to the ion abundance of internal standard. Single-ion chromatograms acquired in the EI ionization mode of a derivatized urinary extract from an exposed worker are presented in Figure 4.

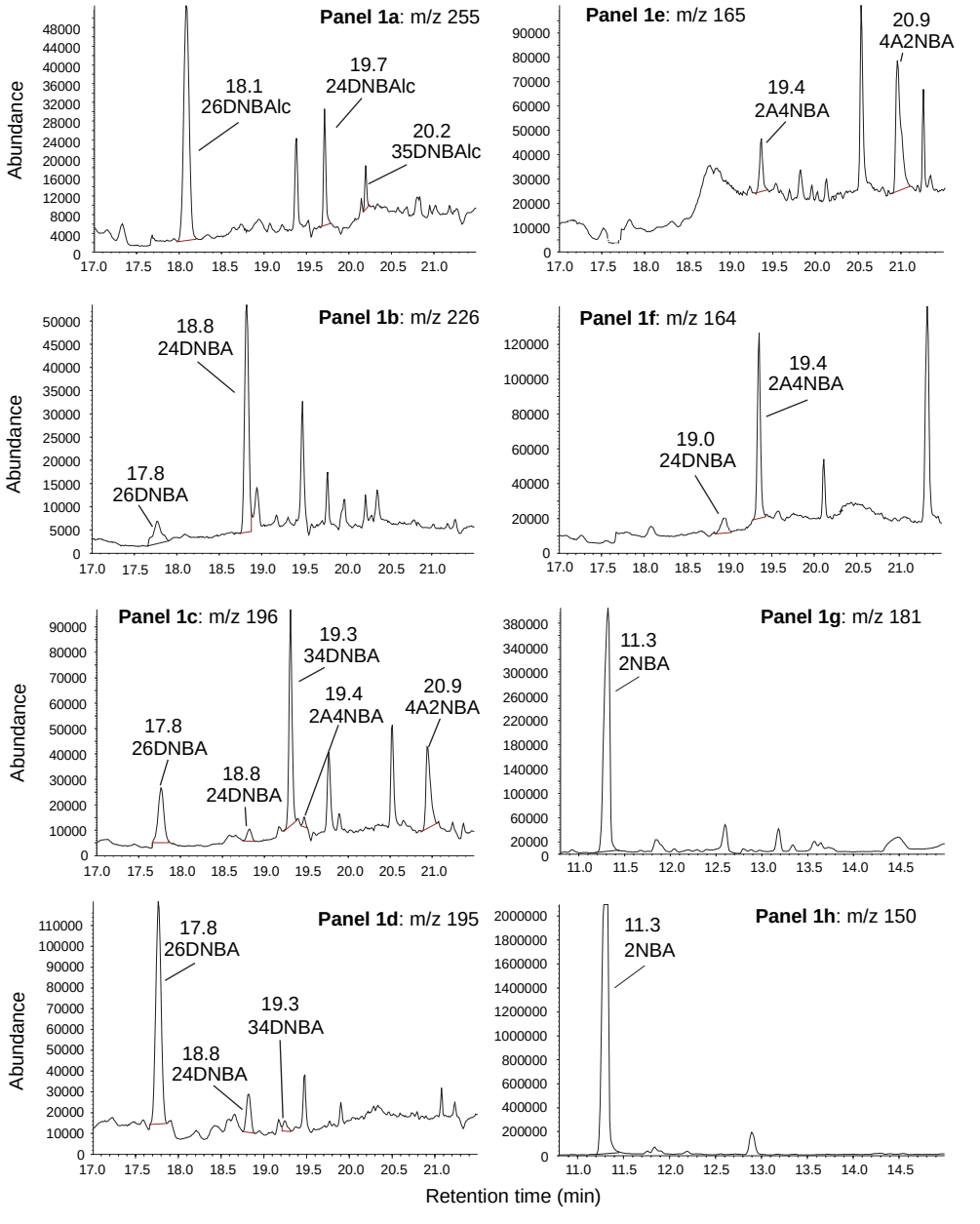

Figure 4. GC-MS chromatogram of extracted urine from a DNT-exposed worker derivatized with diazonmethane and BSTFA. Selected ions were monitored in EI mode. Single-ion chromatograms were acquired for characterization of the trimethylsilyl derivatives of each dinitrobenzyl alcohol (*m/z* 255, 1a), 26DNBAlc, 24DNBAlc and 35DNBAlc, respectively. Single-ion chromatograms were acquired for characterization of the methyl ester derivatives of each dinitrobenzoic acid (*m/z* 226, 1b), 26DNBA, 24DNBA, 34DNBA; and aminonitrobenzoic acid (*m/z* 196, 195, 1c, d) 2A4NBA, 4A2NBA, respectively. The mononitrobenzoic acids, 2NBA and 4NBA were characterized by acquisition of ions *m/z* 181 and 150 (1g, h).

Comparison of urine metabolite levels in pre- and post-shift workers

Quantitation of metabolites in urine of workers potentially exposed to mixed nitrotoluenes was performed against calibration lines of known standards. The concentration of metabolites determined in the workers was corrected against urinary creatinine levels to give a concentration in nmol g^{-1} creatinine. The metabolite levels determined in the pre- and post-shift urines, taken from 35 workers exposed to mixed nitrotoluenes, are summarized in Table I. A Wilcoxon rank-sign test showed that the metabolite levels in the sample pairs (pre- and post-shift sample of the same workers) are significantly different in the post-shift workers, except for 4NBAlc. The changes of the median and mean levels between pre- and post-shift levels were not the same for each metabolite. The mean levels in the pre-shift urines were between 4- and 22-fold lower than the levels determined in the post-shift urines. For metabolites of 26DNT, the benzyl alcohol, 26DNBAlc, was 4-fold lower in the pre- than in the post-shift urines, whereas the benzoic acid, 26DNBA, was 22-fold lower in the pre- compared with the post-shift urines. In contrast for 24DNT, the benzyl alcohol, 24DNBAlc, was 8-fold lower in the pre- compared with the post-shift urine, whereas the benzoic acid, 24DNBA, was only 4-fold lower in the pre- compared with the post-shift urines.

The difference between pre- and post-shift MNT metabolites was less pronounced than for the DNT metabolites. For 2NT, the benzyl alcohol, 2NBAlc, was 4-fold lower in the pre- compared with the post-shift urines. The benzoic acid, 2NBA, was 3-fold lower in the pre- compared with the post-shift urine. For 4NT the benzyl alcohol, 4NBAlc, was of the same order of magnitude in the pre- and post-shift urines, but the acid, 4NBA, was 9-fold lower in the pre- compared with the post-shift urines.

Urine metabolite levels in exposed post-shift workers

Urine samples from 80 exposed post-shift workers and of 11 control workers were analysed for metabolites. The median levels of the 11 controls were 0, except for 2NBAlc and 2NBA with 2.1 and 67 nmol g^{-1} creatinine, respectively. The levels were significantly different to the exposed workers according the Mann–Whitney test

Table I. Comparison of the metabolite levels (nmol g^{-1} creatinine) in paired pre- and post-shift workers ($n = 35$).

Matched pairs (nmol g^{-1})	Pre-shift (mean)	Post-shift (mean)	Pre-shift (median (25th, 75th))	Post-shift (median (25th, 75th))
26DNBAlc	214	1020	139 (0, 317)	739 (271, 1453)
26DNBA	213	4521	126 (24, 251)	3372 (989, 5035)
24DNBAlc	62	487	19 (0, 89)	349 (57, 799)
24DNBA	32	123	19 (0, 58)	52 (8.5, 153)
2A4NBA	448	3930	101 (0, 528)	1615 (655, 4033)
4A2NBA	308	3356	61 (0, 326)	2243 (439, 4297)
2NBAlc	55	213	29 (0, 60)	157 (91, 293)
2NBA	1070	2952	594 (270, 1141)	1648 (1037, 5092)
4NBAlc	6.7	6.7	0.8 (0, 7.5)	2.3 (0.3, 6.9)
4NBA	8.8	76	3.2 (1.4, 13)	42 (19, 98)

Difference between the two related groups was tested using the Wilcoxon rank-sign test. Pre- and post-shift levels were significantly different ($p < 0.0001$) with the exception of 4NBAlc ($p > 0.05$).

($p < 0.0001$). The metabolite levels of the exposed workers are presented in Table II. The median levels of the single metabolites decreased in the following order: 2NBA > 26DNBA > 4A2NBA > 2A4NBA > 26DNBAlc > 24DNBAlc > 2NBAlc > 4NBA > 24DNBA > 4NBAlc. The mean levels of the metabolites decrease in the following order: 2NBA > 26DNBA > 4A2NBA > 2A4NBA > 26DNBAlc > 24DNBAlc > 2NBAlc > 24DNBA > 4NBA > 4NBAlc. The combined level of metabolites (4A2NBA + 2A4NBA + 24DNBAlc + 24DNBA) resulting from exposure to 24DNT were 1.4-fold higher than the metabolite levels (26DNBA + 26DNBAlc) resulting from exposure to 26DNT. With respect to 26DNT-exposure, the dinitrobenzoic acid was the predominant metabolite detected in the urine and was approximately 3-fold higher than the dinitrobenzyl alcohol. With respect to 24DNT-exposure, the aminonitrobenzoic acids (2A4NBA and 4A2NBA) accounted for the majority of the 24DNT metabolites excreted. The levels of dinitrobenzoic acid and dinitrobenzyl alcohol, excreted following metabolism of 24DNT, were approximately 5- and 25-fold lower than the aminonitrobenzoic acids. With respect to the mononitrotoluenes, metabolites of 2NT were found in the highest concentrations. The nitrobenzoic acid (2NBA) was the most prevalent metabolite and was approximately 10-fold higher than the alcohol derivative 2NBAlc. Metabolites of 4NT were approximately 20-fold lower than those described for the 2NT isomer. Therefore, the most prevalent metabolites resulting from exposure to 24DNT, 26DNT, 2NT and 4NT were 4A2NBA, 26DNBA, 2NBA and 4NBA.

26DNBAlc and 26DNBA were metabolites resulting from exposure to 26DNT. 26DNBAlc and 26DNBA were observed in 99 and 86% of the exposed workers, respectively. 24DNBAlc, 24DNBA, 2A4NBA and 4A2NBA were metabolites resulting from exposure to 24DNT. 24DNBAlc, 24DNBA, 2A4NBA, and 4A2NBA were observed in 89, 78, 88 and 91% of the exposed workers, respectively. 2NBAlc, 2NBA, 4NBAlc and 4NBA were the metabolites resulting from exposure to 2NT and 4NT. 2NBAlc and 2NBA were observed in 99 and 97% of the exposed workers respectively. 2NBAlc was not present in the control workers. 2NBA, was observed in eight of the 11 control workers. 4NBAlc and 4NBA were observed in 62 and 77% of the exposed workers. Neither compounds were observed in the urine of control workers.

The relative proportion of each metabolite among workers was evaluated by calculating the ratio of the single DNT metabolite to the combined total of all DNT

Table II. Mean, median, 25th and 75th percentile levels of the urine metabolites (nmol g^{-1} creatinine) in exposed workers.

Metabolite	Mean	Median (25th, 75th)
26DNBAlc	1047	653 (285, 1285)
26DNBA	3110	1380 (283, 3757)
24DNBAlc	496	199 (39, 600)
24DNBA	101	43 (7.1, 103)
2A4NBA	2573	879 (151, 3061)
4A2NBA	2592	1348 (309, 3639)
2NBAlc	241	156 (55, 329)
2NBA	3753	2108 (917, 4548)
4NBAlc	11	1.0 (0, 5.2)
4NBA	108	30 (3.7, 85)

metabolites detected. The same calculation was performed with each of the MNT metabolites. The mean levels with standard deviation for male and female workers are listed in Table III. The metabolite ratios vary substantially among the workers. The ratios are similar between males and females except for the metabolites of 26DNT. The proportion of 26DNBA is significantly higher in males (Mann–Whitney test, $p = 0.037$).

Job related exposure to mono and dinitrotoluenes

Workers, that donated urine samples, were grouped according to their job description as follows: group leader ($n = 12$), MNT tank ($n = 14$), DNT tank ($n = 5$), TNT tank ($n = 16$), analysis laboratory ($n = 13$), transportation of TNT to packaging ($n = 2$), packaging ($n = 2$), control room ($n = 11$), disposal of waste acid ($n = 2$) and disposal of waste H_2O ($n = 3$), and control workers ($n = 11$). The results are summarized in Table IV. The median levels of each metabolite detected in post-shift urines were compared in workers grouped according to their job descriptions (Mann–Whitney test). For workers grouped into waste acid, waste H_2O or transportation of TNT, statistical analysis was not performed because the sample number was too low ($n < 5$). The highest levels of DNT metabolites were found in analysis workers and MNT, DNT and TNT tank workers. The median levels of DNT metabolites in the control room workers were very low which indicated that the risk of exposure for this job was relatively minor. The metabolite levels for group leaders and subjects packing TNT were moderately high. Each exposure group had significantly higher DNT metabolite levels compared with non-exposed factory controls and the control room workers. The differences between the median levels of all the other job group workers were not significant, except for the median levels of 26DNBAlc in analysis workers, which were significantly higher than in the other groups. The analysis workers were all females.

Table III. Comparison of the ratios of DNT and MNT metabolites in the post-shift urines of exposed workers.

	Proportions of excreted DNT[a] and MNT[b] metabolites		
Metabolite	Mean (±SD)	Male	Female
24DNBAlc	5.6±6.9%[a]	5.6±6.9%[a]	5.8±7.0%[a]
24DNBA	1.2±1.5%[a]	1.3±1.7%[a]	0.8±0.8%[a]
2A4NBA	21.6±15.7%[a]	20.4±13.8%[a]	22.8±18.1%[a]
4A2NBA	26.0±17.4%[a]	25.5±13.5%[a]	27.9±25.0%[a]
26DNBAlc	18.3±18.5%[a]	16.8±15.4%[a]	21.8±24.9%[a]
26DNBA	27.3±18.4%[a]	30.4±18.5%[a]	20.9±16.1%[a]
2NBAlc	9.2±7.8%[b]	9.1±8.3%[b]	9.6±6.7%[b]
2NBA	87.6±11.0%[b]	87.0±12.4%[b]	88.8±6.9%[b]
4NBAlc	0.4±1.1%[b]	0.4±1.3%[b]	0.3±0.6%[b]
4NBA	2.8±6.8%[b]	3.5±8.0%[b]	1.3±1.2%[b]

[a]Mean ratio, standard deviation of each metabolite against the combined total of all 24DNT and 26DNT metabolites.
[b]Mean ratio, standard deviation of each metabolite against the combined total of all 2NT and 4NT metabolites.

Table IV. Comparison of the metabolite levels (nmol g^{-1} creatinine) of 26DNBAlc and 4A2NBA among the different job groups.

	26DNBAlc	4A2NBA
Job	Median (25th, 75th)	Median (25th, 75th)
Leader	462 (264, 835)	670 (356, 1605
MNT tank	520 (242, 1248)	1420 (699, 3811)
DNT tank	1245 (510, 2281)	1348 (374, 1819)
TNT tank	866 (420, 1857)	2167 (618, 4791)
Control room	186 (60, 230)	261 (14, 2243)
Analysis	1453 (1071, 3328)	4526 (2713, 7004)
Control workers	0 (0, 4.7)	0 (0, 0)

In the present study, the alcohols were present in higher amounts in females compared with males. The differences were only statistically significant for 26DNBAlc.

Correlation between different internal dose markers

Correlation analysis was performed on the raw urine metabolite data without transformation (Pearson correlation), on the log-transformed data (log 10) (Pearson correlation) and the ranks of the data (Spearman rank analysis) (Table V). The data of the Spearman rank test were very similar to the data obtained from the Pearson correlation of the log-transformed data. Except for the correlations between isomers 26DNBAlc versus 24DNBAlc, 24DNBA versus 26DNBA, and 2A4NBA versus 4A2NBA, it appeared that outliers skewed the analyses using the raw data. The following analyses were performed using the Spearman-rank test. The best correlations were between isomers. There was a strong association between the dinitrobenzyl alcohols 26DNBAlc and 24DNBAlc, between the dinitrobenzoic acids 26DNBA and 24DNBA and between the aminonitrobenzoic acids, 2A4NBA and 4A2NBA. In general, the correlations among benzylalcohols or among benzoicacids were better than between benzyl alcohols and benzoic acids. All correlations were highly significant. Additionally, there was a good correlation between the dinitrobenzyl alcohols and the total level of DNT metabolites, which confirmed that these alcohols effectively predicted the recent absorbed dose of DNT excreted in the workers urine. The benzoic acid metabolites of 24DNT and 26DNT were strongly associated with

Table V. Correlation of urinary metabolites (nmol g^{-1} creatinine) determined in exposed workers.

Urinary metabolite	26DNBA	24DNBAlc	24DNBA	2A4NBA	4A2NBA	Total DNT
26DNBAlc	0.69	0.82	0.57	0.43	0.48	0.76
26DNBA		0.78	0.71	0.60	0.64	0.86
24DNBAlc			0.49	0.37	0.39	0.66
24DNBA				0.66	0.68	0.74
2A4NBA					0.84	0.79
4A2NBA						0.87
Total MNT						0.46

Correlation coefficients (r) were calculated with the Spearman rank test. All correlation were highly significant $p < 0.001$.

the total level of DNT metabolites. The association between the total urinary metabolites of the mononitrotoluenes and dinitrotoluenes was moderate but statistically significant. The best correlation among the MNT metabolites was found for 2NBA and 4NBA with $r = 0.60$, followed by the correlation of 2NBAlc and 2NBA with $r = 0.43$.

Correlation between external and internal markers of dose

In the exposed workers, the mean (range) 8 h TWA exposure levels (mg m^{-3}) for 24DNT, 26DNT, 2NT and 4NT were 0.043 (undetected to 0.641), 0.014 (undetected to 0.151), 0.759 (undetected to 4.29) and 0.686 (undetected to 2.40), respectively. The occupational exposure limit set by NIOSH is 0.15 mg m^{-3} for nitrotoluenes. Thus, the air levels indicated a high level of exposure to 2NT and 4NT, but an acceptable level of exposure to 24DNT and 26DNT. The results confirmed the presence of ambient levels of 24DNT and 26DNT at relative percentages of 75 and 25%, respectively. The MNT isomers, 2NT and 4NT, were found at relative percentages of 53 and 47%, respectively. The air levels of 24DNT correlated with the air levels of 26DNT, 2NT and 4NT with $r = 0.93$, 0.55, and 0.40, respectively (Spearman rank order). The air levels of 26DNT correlated with 2NT and 4NT with $r = 0.49$ and 0.32. The air levels of 2NT and 4NT correlated with an $r = 0.74$. The sum of the urine metabolites resulting from the corresponding nitrotoluene found in the air was compared with the air levels using the Spearman rank order method. The levels of 2NT, 4NT, 24DNT and 26DNT did not show any significant correlation with the corresponding metabolites ($r = 0.17$, 0.01, -0.17 and -0.12, respectively).

Relationship between internal dose of MNT and DNT and health effects in Chinese workers

Each worker was examined for non-specific adverse health effects linked with exposure to DNT, the results of which are presented in Table VI. The statistical difference of the prevalences between exposed and control workers was tested with the Chi-square test (likelihood ratio). With the exception of headache, the prevalence of illness was significantly higher in the exposed workers. The prevalences of these adverse health effects were lower than those reported by McGee et al. (1942).

The data were analysed with a non-parametric test (Mann–Whitney) and logistic regression analysis. Using the Mann–Whitney test, there was a significant relation between nausea and the median levels of the metabolites 2A4NBA ($p = 0.022$) and 4A2NBA ($p = 0.004$). Dermatitis was related to the levels of 2A4NBA ($p = 0.005$), 4A2NBA ($p = 0.016$), 24DNBA ($p = 0.019$), and 26DNBA ($p = 0.045$). Somnolence related to the levels of 4A2NBA ($p = 0.047$). Insomnia related inversely to the levels of 26DNBA ($p = 0.023$). For all other health effects, the median levels of urine metabolites were higher in the ill workers but with $p > 0.05$. Logistic regression analysis was used to calculate the odds ratio (OR) of a particular condition in the Chinese workers. The logistic regression was performed using the log-transformed urine metabolite data. The ORs for health effects in workers were greater for those workers with higher levels of metabolites. However, the ORs were only statistically significant in two cases. There was a significant dose–response relationship between the log-transformed values of the aminonitrobenzoic acids (4A2NBA, 2A4NBA) and the risk of suffering from nausea. The OR of suffering from nausea were 2.1 times

Table VI. Comparison between the prevalence (%) of adverse health effects in exposed workers and factory controls.

Health effect	Exposed ($n=98$)	Control ($n=72$)
Inertia	31.5	1.4
Nausea	23.5	0
Insomnia	21.4	8.3
Somnolence	16.3	2.8
Dizziness	9.2	1.4
Dermatitis	8.2	0
Headache	8.2	2.8

Number of exposed workers where a symptom was recorded was calculated as a percentage of the total number of exposed workers analysed. The number of factory control workers where a symptom was recorded was calculated as a percentage of the total number of factory control workers analysed.

higher (CI = 1.1–4.1) when the level of 4A2NBA levels increased by 1 log-unit ($p=0.022$). For 2A4NBA the OR of suffering from nausea was 1.7 times higher (CI = 1.05–2.9).

Discussion

For both 24DNT and 26DNT, the air levels were below the NIOSH Recommended Exposure Limit and the Occupational Safety and Health Administration (OSHA) Permissible Exposure Limits (PELs) occupational 8 h time weight averaged (TWA) exposure limits, set at 1.5 mg m^{-3}. The levels of 24DNT and 26DNT reported in this study were comparable with the 7 h TWA levels of combined 24DNT and 26DNT reported by Levine et al. (1985) (range 0.05–0.59 mg m^{-3}) and were higher than those reported by Woollen et al. (1985) (range undetected to 0.1 mg m^{-3}). For 2NT, 3NT and 4NT, the exposure levels were below the OSHA PEL and UK occupational 8-h TWA exposure limits set at 11 mg m^{-3}. For each of these compounds, the regulatory bodies identified dermal exposure as a significant route of absorption. The air levels found in the present study did not correlate with the levels of the corresponding urine metabolites. This confirms the results of former studies performed with a small number of workers, which postulated that a large proportion of the exposure is through the skin (Levine et al. 1985, Woollen et al. 1985).

The metabolite levels found in the Chinese workers in the current study were compared with the data found in the literature. Unfortunately, only values for a single metabolite (24DNBA) were available. The 24DNBA levels (0–914 nmol l^{-1} 24DNBA) found in the present Chinese workers were comparable with the levels found in German workers (5–479 nmol l^{-1}) (Angerer and Weismann 1997) involved in the cleanup of contaminated munition waste sites. The levels reported by Woollen et al. (1985) in an explosive factory were by a factor of 10 higher (2–525 μmol l^{-1}). No other metabolites were determined in these two studies. The study published by Levine et al. (1985), which investigated exposure in workers in a DNT factory, reported total 24DNT metabolite levels (0–59122 nmol g^{-1} creatinine) similar to the present study (0–65000 nmol g^{-1} creatinine). Discrete metabolite levels were not listed in this study.

For occupational health monitoring, identification of one or two metabolites would be desirable for estimation of recent exposure. The analysis time required for

identification and quantitation of all DNT metabolites would not be practical for routine monitoring. There are several criteria that need to be considered before recommending a particular metabolite as a potential disposition of the DNT, the number of exposed workers in which the metabolite can be detected, and how the metabolite reflects the total amount of metabolites excreted and exposure dose. In the previous biomonitoring studies where one metabolite was selected as a marker of recent exposure, 24DNBA was chosen (Woollen et al. 1995, Angerer and Weismantel 1998). This was most likely because these workers were exposed to technical-grade DNT that comprised predominantly of 24DNT (80% 24DNT and 20% 26DNT). Earlier work of Turner et al. (1985) and Levine et al. (1985) identified 24DNBA as the major metabolite excreted in the urine following exposure to technical-grade DNT (24DNT and 26DNT). Although we found a good correlation between 24DNBA and the total amount of DNT metabolites detected in the urine (Table V), 24DNBA was identified as a minor route in the elimination of 24DNT, and accounted for only 1.2% of the total metabolites detected in urine of DNT (24DNT and 26DNT)-exposed workers. Additionally, 24DNBA was detected in only 78% of the exposed workers compared with 26DNBA or the dinitrobenzyl alcohols (24DNBAlc and 26DNBAlc), which were detected in 86, 89 and 99% of workers, respectively. Therefore, we suggest that 24DNBA would not be an appropriate metabolite for estimating exposure at least in Chinese workers. The correlations presented in Table V suggested that determination of a number of individual metabolites could predict the combined excretion of 24DNT and 26DNT in post-shift urine, and hence the exposure and absorption of DNT in workers from their most recent work-shift. Based upon the following findings, we concluded that monitoring the aminonitrobenzoic acids in Chinese workers was an appropriate means of estimating recent exposure in workers, at least for 24DNT. The metabolites were found in $>88\%$ of the exposed workers. Excretion of the aminonitrobenzoic acids in urine was evidently an important pathway for the elimination of DNT ($>20\%$ of total DNT metabolites) and there was a strong positive association between aminonitrobenzoic acids and the total amount of DNT excreted in the urine. Since nitro group reduction was not identified in workers exposed to 26DNT, these metabolites would not be appropriate as a marker of recent exposure to 26DNT, although significant correlations were observed between the aminonitrobenzoic acids and metabolites of 26DNT. Both of the dinitrobenzyl alcohols were identified in a high percentage of exposed workers ($>89\%$) and at least for 26DNT, the benzyl alcohol was a major route of elimination from the body (17%). The association between the dinitrobenzyl alcohols and the total amount of DNT detected in the post-shift urines was strong. Therefore, we suggest that in Chinese workers, either the dinitrobenzyl alcohols or the aminonitrobenzoic acids could be employed as possible biomarkers of recent exposure from urine determinations.

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