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Biological monitoring of dinitrotoluene by gas chromatographic–mass spectrometric analysis of 2,4-dinitrobenzoic acid in human urine

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Abstract

The method of analysis described permits the determination of 2,4-dinitrobenzoic acid down to the lower $\mu g\,l^{-1}$ range in the urine of persons exposed to dinitrotoluene. 2,4-Dinitrobenzoic acid is the main metabolite of 2,4-dinitrotoluene and technical dinitrotoluene. After acidic hydrolysis, which served to release the conjugated part of the 2,4-dinitrobenzoic acid, the analyte was selectively separated from the urine matrix via various extraction steps and then derivatised to the methyl ester. Quantitative analysis was carried out using capillary gas chromatography and mass selective detection. 3,5-Dinitrobenzoic acid was used as an internal standard. The detection limit was 1 $\mu g\,l^{-1}$ urine. The relative standard deviations of within-series imprecision were between 5 and 6%. The relative recoveries were between 91 and 110% depending on the concentration. The analytical method developed as part of this study was used to investigate a collective consisting of 82 urine samples from persons working in the area of explosives disposal. The concentrations of 2,4-dinitrobenzoic acid determined ranged from the detection limit to 95 $\mu g\,l^{-1}$ urine. The method allowed the quantification of low-level internal exposure to dinitrotoluene.

Keywords: Dinitrotoluene; 2,4-Dinitrobenzoic acid

1. Introduction

Of the six isomers of the group of dinitrotoluenes (DNT) only 2,4-DNT is of commercial and technical importance. Technical grade DNT is a mixture of mainly 2,4-DNT and 2,6-DNT in the ratio of 80:20. 2,4-DNT is an important source material in industrial organic synthesis. Most is hydrogenated to 2,4-diaminotoluene, which is transformed into toluylene diisocyanate, a monomer in the production of polyurethanes [1]. In addition DNT occurs during the production of trinitrotoluene (TNT) as an intermediate. As a result of its gelatinising and water-repellent properties and due to its explosive potential it is added to explosive preparations [2].

Exposure to 2,4-DNT can occur over a period of time in small doses, for example with uptake of drinking water, which is contaminated with waste [3–5]. At workplaces in areas such as munitions and DNT production as well as DNT processing, short-term exposure in high doses is possible [6,7]. Another, so far neglected source of exposure is the

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disposal of old and obsolete ammunition. With the reunification of Germany 300 000 tons of munitions were taken over from the stocks of the former GDR. Since 1991 these old stocks have to a large extent been disassembled in factories [8].

The metabolism of DNT runs in two different directions as a result of the different substituents at the aromatic ring: in one direction the methyl group is oxidised and in the other the nitro group is reduced (Fig. 1). Both routes lead to the metabolic activation of the substance. Metabolites are formed which can react with macromolecules of the human body [9–11]. Reduction takes place via reactive intermediates, nitroso and hydroxylamine derivatives, to form the corresponding amine [12]. During oxidation benzyl alcohol and the reactive metabolite benzaldehyde occur intermediately [13,14]. Dinitrobenzoic acid is the end product of this reaction type. Both oxidative and reductive metabolism produce mutagenic metabolites of DNT [14–16]. The isomer mixture of DNT was classified by the DFG Commission for the Investigation of Hazardous Substances in the Work Area in the group of substances which have been found to be clearly carcinogenic in animal experiments (Category IIIA2) [17].

In the urine of occupationally exposed workers 2,4-dinitrobenzoic acid (2,4-DNBA) was identified as the main metabolite both of pure 2,4-DNT and technical grade DNT [6,7].

As DNT is absorbed readily by the skin and represents a considerable health risk to man [18,19], it is of occupational-medical and environmental-medical interest to be able to estimate the internal exposure of persons affected in a more appropriate manner. This is possible with the introduction of reliable methods of analysis which can be carried out routinely. The methods for determining the urinary metabolites of DNT published so far served to investigate highly exposed workers from the areas of production and processing of DNT. 2,4-DNBA is excreted here in the $mg\,l^{-1}$ range. The detection limits of these methods are, however, inadequate for determining the internal exposure of workers, who are exposed to explosives. The exposure situation is different in this case, as DNT is contained in explosives in much lower concentrations. Against this background, the development of a more sensitive method of analysis for the determination of 2,4-DNBA in human urine was needed, which would allow the analysis of low-level exposure in occupational-medical ranges.

In this study an analytical procedure is presented with a detection limit in the low $\mu g\,l^{-1}$ range, which is precise, reliable and suitable for routine practice. The method was used to investigate urine samples from persons working in the area of explosives disposal.

2. Materials and methods

2.1. Chemicals

2,4-Dinitrobenzoic acid (2,4-DNBA) 98% and 3,5-dinitrobenzoic acid (3,5-DNBA) 98% were obtained from Aldrich (Steinheim, Germany). Sulfuric acid 95–97%, sulfuric acid 98%, dichloromethane, methanol, sodium chloride and sodium hydrogencarbonate were obtained from Merck (Darmstadt, Germany). All chemicals and solvents used were of analytical grade.

2.2. Aqueous solution for the reextraction

0.01 *M* $NaHCO_3$ solution: 84 mg sodium hydrogencarbonate were dissolved in 100 ml bidistilled water. The pH value was 8.0.

2.3. Solution of the internal standard

A solution of 3,5-dinitrobenzoic acid in bidistilled water ($1\ mg\,l^{-1}$) was used as internal standard. For this 100 μl of an initial solution ($1\ g\,l^{-1}$) in methanol were diluted with 99.9 ml bidistilled water.

2.4. Sample preparation

At the end of a workshift or exposure phase spontaneous urine was collected in plastic bottles. The urine samples were stored deep-frozen until analysis (−18°C). Before removal of the aliquot for analysis, the samples were thawed to room temperature and shaken thoroughly.

An overview of the preparation procedure can be found in the diagram in Fig. 2. A 5 ml volume of urine was spiked with 200 μl of the internal standard

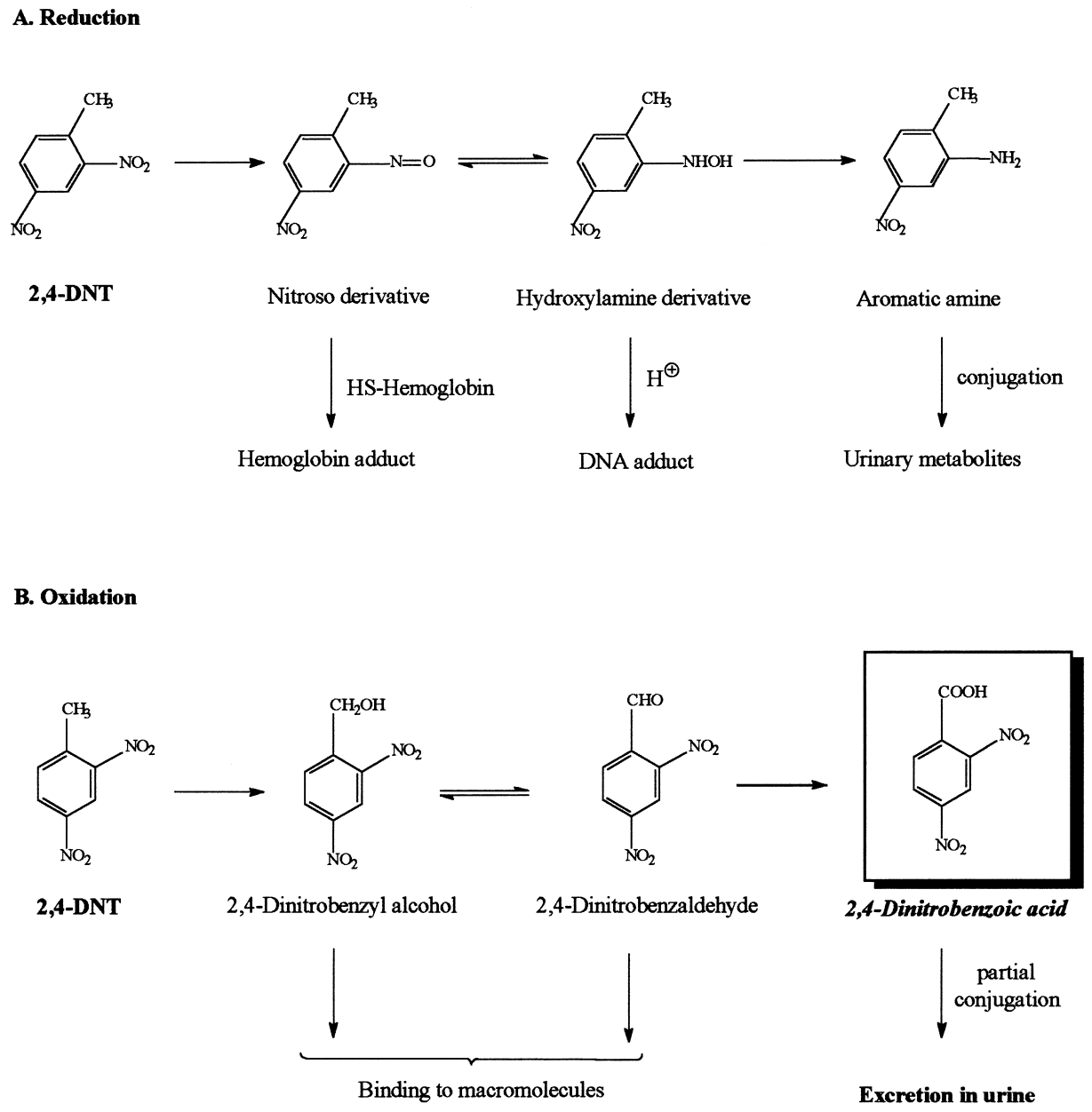

Fig. 1. Reductive (A) and oxidative (B) metabolism of 2,4-DNT with reactive intermediates. The main metabolite is 2,4-dinitrobenzoic acid.

solution (3,5-DNBA in water, 1 mg l^{-1}) and 1 ml concentrated sulfuric acid (95–97%) and hydrolysed for 1 h at 90°C in a drying cabinet. After the sample had been cooled to room temperature 500 mg of sodium chloride were added and the sample was extracted twice with 5 ml dichloromethane each time. The sample was shaken for 10 min and then centrifuged for 5 min at 1500 *g*. The organic phases were combined in a 24 ml screw neck bottle and spiked with 1 ml of a 0.01 *M* $NaHCO_3$ solution. The sample was shaken again for 10 min and centrifuged for 5 min at 1500 *g*. The organic phase was

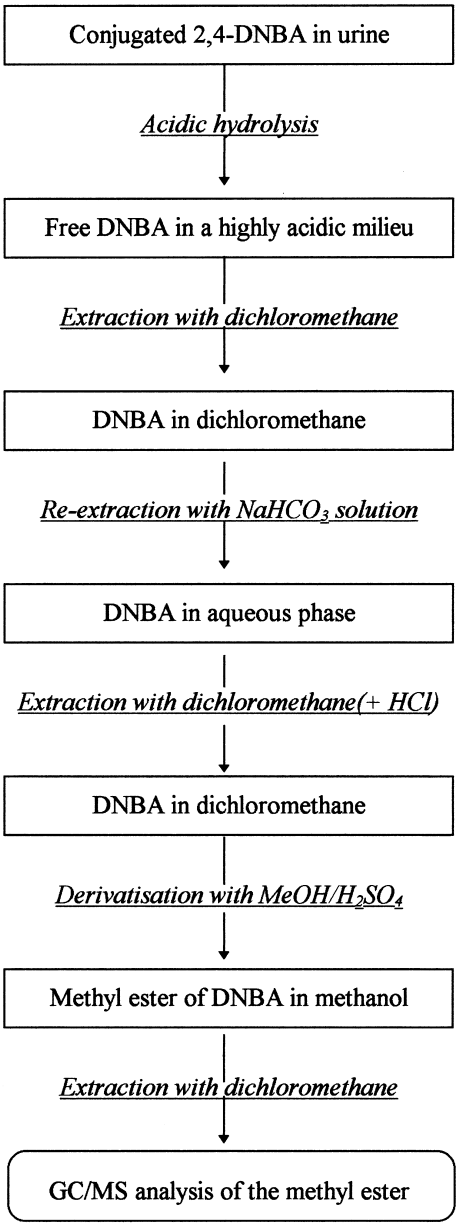

Fig. 2. Diagram of sample preparation for the analytical determination of 2,4-dinitrobenzoic acid from urine.

discarded and the aqueous solution acidified with 100 μl of a 37% hydrochloric acid. 500 mg of sodium chloride were added and the solution was twice extracted with 2 ml dichloromethane each time. The sample was shaken again for 10 min and centrifuged for 5 min at 1500 *g*. The combined organic phases were concentrated very carefully in a stream of nitrogen until they were just dry. The residue was dissolved in 1 ml of 98% sulfuric acid and 3 ml of methanol were added. For derivatisation, the samples were placed in a thermostatisable water-bath at 75°C for 1 h. After cooling, 1 ml of a saturated sodium chloride solution and 5 ml of deionised water were added to each sample. The resulting solution was then twice extracted with 2 ml dichloromethane each time including the shaking (10 min) and centrifugation step (1500 *g*). The combined organic phases were dried for 10 min over sodium sulfate. After transferral of the organic phase to 5 ml beaded rim bottles the sodium sulfate was cleaned with 500 μl dichloromethane and the organic phases were combined. The solvent volume was carefully reduced in a slight stream of nitrogen to about 200 μl and the remaining solution was transferred to a microvial. Analysis was carried out by GC–MS.

2.5. Calibration

To produce calibration solutions first of all an initial solution of 2,4-DNBA in methanol was prepared with a concentration of 1 g l^{-1}. For this 10 mg of the 2,4-DNBA were weighed into a 10 ml volumetric flask and the flask was filled to the mark with methanol. By diluting the initial solution with water, two stock solutions were produced with a concentration of 10 mg l^{-1} (S-I) and 1 mg l^{-1} (S-II). Using these two stock solutions the calibration solutions were finally produced in a concentration range from 1 to 200 μg l^{-1} by diluting with pooled urine. The calibration solutions can be kept for half a year at −18°C. They were prepared and analysed like the material investigated. Linear calibration curves were obtained by plotting the relationships of the peak areas of 2,4-DNBA to those of the internal standard against the concentrations used. For every analytical series, in addition to a quality control sample a calibration solution was analysed to check the validity of the calibration curve. The quality control samples used had a concentration of 17.6 μg l^{-1} 2,4-DNBA. The analysis of control material belonged to the internal quality control program. The results of the control samples were entered in quality control charts. These charts were continuously updated and gave information about the imprecision of

the method over a long period. The complete calibration curve had to be re-prepared if the analytical conditions changed or the results of quality control demanded it.

The calibration graphs were used to ascertain the unknown concentrations of 2,4-DNBA in urine samples of exposed persons.

2.6. Gas chromatography

Analysis was carried out on a Hewlett Packard HP 5890 Series II plus gas chromatograph fitted with a Hewlett Packard HP 7673 autosampler and a split/splitless injector operating in the splitless mode. The inlet purge-off time was 1 min. The operating temperature for the injector was 260°C. For the gas chromatographic separation a (35% phenyl)-methylpolysiloxane column was used (60 m×0.25 μm film thickness×0.22 mm I.D.), for example a BPX35 capillary column from SGE. The gas flow was 0.8 ml min^{-1} (electronic pressure control system). Helium 5.0 was used as the carrier gas.

The initial column temperature of 90°C was held for 1 min, then raised at a rate of 6°C min^{-1} to 220°C, held at this temperature for 15 min, raised at a rate of 15°C min^{-1} to 270°C and remained at this temperature for 15 min.

1 μl of the prepared samples was injected into the GC–MS system.

2.7. Mass spectrometry

The detector used was a Hewlett Packard HP 5972 MSD mass spectrometer equipped with a quadrupole mass filter in the electron impact (EI) mode. The software used was HP 5971/5972 MSD-Chemstation. EI mass spectra were obtained at 70 eV. The electron multiplier voltage was 2400 V. The MSD transfer line was heated to 300°C. For analysis of 2,4-DNBA selected ion monitoring (SIM) was used. The methyl ester derivatives were unequivocally identified both by their retention times and the masses of characteristic molecule fragments. The masses used for quantification were those with the greatest intensity (Table 1).

2.8. Human studies

Using the method described above we investigated the post-shift urine of 82 persons, 34 of whom were regularly employed in the disposal of munitions (group I) and 22 occasionally (group II). 26 persons (group III) from the same workshop who had no contact with materials containing explosives served as controls.

The stability of 2,4-DNBA in urine samples during storage was checked by Turner et al. (1985). The authors spiked blank urine with 2,4-DNBA and stored the sample at room temperature for 8 weeks. An aliquot of the same urine was stored outside in sunlight for up to 1 week. Periodic analysis indicated no significant degradation of 2,4-DNBA in both samples. Our one experiments showed that 2,4-DNBA was stable at least over a period of 2 month. Thus, it was unlikely that substantial decomposition of 2,4-DNBA occurred in deep-frozen urine samples.

3. Results and discussion

As part of this study an analytical method was developed for monitoring the exposure of workers to DNT. In the literature only few analytical methods for determining the metabolites of DNT in urine were described (Table 2). These were mainly gas chromatographic methods using mass spectrometers and electron capture detectors [6,7,20]. Smith et al. [21], however, determined spectrophotometrically

Table 1
Retention times and monitored masses for identifying the methyl esters of 2,4-DNBA and of the internal standard 3,5-DNBA

Methyl ester of the analytes	Retention time [min]	Monitored ion traces [m/z]	Quantifier [m/z]
2,4-DNBA	34.0	226[a], 196, 195, 149	195
3,5-DNBA (I.S.)	34.8	226[a], 196, 195, 149	195

In addition the masses used for quantification are given (quantifier).
[a] Molecular ions.

Table 2
Overview of studies on the biological monitoring of DNT by determination of the urinary metabolites

Author	Study	*n*	Metabolites	Range	Method
Smith et al. [21]	DNT production	278 samples	Sum of all metabolites	2–106 mg l^{-1}	Spectrophotometry
Woollen et al. [7]	Explosives industry	28 workers	2,4-DNBA	3.4–41 mg l^{-1}	GC–ECD
Turner et al. [6]	DNT production	17 workers	2,4-DNBA, 2,4-DNBAl, 2A4NB, 4A2NB	0.1–about 5 mg l^{-1}	GC–MS
Levine et al. [20]	DNT production	20 workers	2,4-DNBA, 2,4-DNBAl, 2A4NB, 4A2NB	0.1–about 60 mg l^{-1}	GC–MS
This study	Explosives disposal	56 workers, 26 controls	2,4-DNBA	1–95 μg l^{-1}	GC–MS

2,4-DNBA: 2,4-dinitrobenzoic acid, 2,4-DNBAl: 2,4-dinitrobenzyl alcohol, 2A4NB: 2-amino-4-nitrobenzoic acid, 4A2NB: 4-amino-2-nitrobenzoic acid, *n*=number of samples or of persons investigated.

the sum of all DNT metabolites. DNT and its metabolites were reduced to primary amines, then diazotized and coupled to form azo dyes. The disadvantage of this method was a relatively high background, which was caused by the excretion of primary amines such as tryptophan in human urine. Moreover antibiotics interfered with that kind of determination. The methods listed in Table 2 had detection limits of about 100 μg l^{-1}. For the analytical method developed in this study we were able to considerably lower the detection limit and guarantee a high specificity of the method. A detection limit of 1 μg l^{-1} was low enough to be able to determine low-level exposure of workers exposed to DNT.

This could be attributed among other things to the fact that we were able to achieve a high-level of esterification. 2,4-DNBA is a sterically inhibited acid, which can only be transformed slowly and with poor results. This meant that exclusively certain derivatisation agents, which do not take up much space, were suitable for esterification of the acid. Particularly suitable were methylating agents such as diazomethane, dimethylformamide/dimethylacetal, methanol/sulfuric acid and boron trifluoride/methanol. Our results showed that among the derivatisation agents listed, methanol/sulfuric acid was the most suitable. The other derivatisation agents caused either analytical interference or had poor yields of methyl esters. There were various reasons for the high level of derivatisation with the use of methanol/sulfuric acid: the esterification equilibrium was shifted to the right, as one of the initial substances, methanol, was used in extreme abundance. In addition the transformation product, water, was continually removed from the reaction mixture as a result of the added sulfuric acid. It also had to be borne in mind that the mechanism of esterification of sterically inhibited carbonic acids usually takes place via protonation of the hydroxy oxygen atom [22,23]. Water is split off and then the planar acyl-cation is formed, which can be attacked from both sides of the ring by the alcohol (e.g. methanol). By using methanol/sulfuric acid it was possible to produce the acyl-cation of 2,4-DNBA, when the residue was dissolved in sulfuric acid first. On adding methanol the acyl-cation was transformed to the ester [23].

In addition to optimising the derivatisation procedure we also tried to achieve the highest levels of extraction possible by testing several solvents. Turner et al. [6] and Levine et al. [20] used diethyl ether as the extraction agent for 2,4-DNBA and Woollen et al. [7] ethyl acetate. In our extraction experiments, however, dichloromethane was found to be the most suitable solvent both for the extraction of the free acid and also of the methyl ester in comparison to diethyl ether, ethyl acetate, chloroform, toluene, methylethylketone and cyclohexane.

Furthermore the efficiency of the hydrolysis was checked. We could show, that neither the use of a non-oxidising acid (for example hydrochloric acid) nor the longer duration of hydrolysis had any effects on the recovery of 2,4-DNBA.

3.1. Reliability of the method

The calibration curves were linear between 1 and 200 $\mu g\,l^{-1}$.

3.1.1. Precision

Within-series imprecision was determined by seven-fold analysis of pooled urine to which defined amounts of 2,4-DNBA had been added. The pooled urine was from persons not exposed to DNT. In addition the urine (actual sample) from a person was analysed seven times who was occupationally exposed to DNT.

The relative standard deviations for the spiked urine samples were 4.8% at a concentration of 20 $\mu g\,l^{-1}$ and 4.5% at a concentration of 100 $\mu g\,l^{-1}$. The seven-fold analysis of the actual sample resulted in relative standard deviations of 5.9% at a concentration of 38 $\mu g\,l^{-1}$.

Between-day imprecision was also determined using spiked pooled urine samples. The samples were prepared and analysed on 12 different days. At a concentration of 17.6 $\mu g\,l^{-1}$ a relative standard deviation of 8.2% was found. This result underlined the good reproducibility of the method. Among other things this was to be attributed to the use of 3,5-DNBA as internal standard. The isomer was particularly suitable for precision correction because of its very similar chemical properties to 2,4-DNBA. It was checked that no 3,5-DNBA was already present in the urine samples of exposed persons or non-exposed persons nor as contamination in the 2,4-DNBA standard.

3.1.2. Accuracy

Recovery experiments were carried out to check the accuracy of the method. A urine sample with a concentration of 17.6 $\mu g\,l^{-1}$ and one with 88.0 $\mu g\,l^{-1}$ 2,4-DNBA was prepared and analysed seven times. The results were determined using aqueous calibration solutions. In addition seven different individual urine samples were spiked with 2,4-DNBA (70.4 $\mu g\,l^{-1}$) and analysed.

The recovery rates determined for the aliquoted pooled urine samples were 112%±5.3% for the lower concentration and 94%±3.5% for the higher concentration. The range of the recovery rates of the seven individual urine samples was 79% to 100% (90.7%±8.3%). This showed that matrix effects only play a minor role. This was confirmed by the fact that standard solutions which were prepared in water or in urine lead to calibration curves which could be regarded as identical (Fig. 3).

In addition we attempted to estimate the analyte losses which occurred during sample preparation. Therefore, to obtain an average and representative matrix composition urine of different unexposed persons was collected over the whole day and pooled. This pooled urine was aliquoted and the samples were spiked as described above (17.6 $\mu g\,l^{-1}$ and 88.0 $\mu g\,l^{-1}$), prepared and analysed ($n=3$). The results of these measurements were compared with those of standard solutions in methanol. These solutions were derivatised without further preparation and analysed after extraction with dichloromethane. The recovery rates were between 80 and 102%. This meant that the analyte losses caused by sample preparation were slight.

3.1.3. Detection limit

Under the given conditions of sample preparation and the conditions for GC–MS, taking into consideration the three-fold signal to noise ratio, a detection limit of 1 $\mu g\,l^{-1}$ in urine was determined.

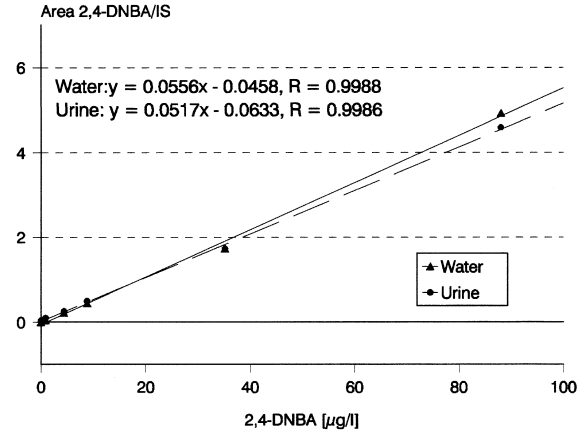

Fig. 3. A calibration curve from aqueous standard solutions and a calibration curve made on the basis of urine standard solutions in a concentration range of 1 to 88 $\mu g\,l^{-1}$.

Table 3
Results of the determination of 2,4-DNBA in the urine of workers from the area of munitions disposal

	Group		
	I	II	III
	$\mu g\,l^{-1}$	$\mu g\,l^{-1}$	$\mu g\,l^{-1}$
Mean$\pm\sigma$	12.2±20.4	2.9±4.9	d.l.
Median	5.1	d.l.	d.l.
Range	d.l.–95.0	d.l.–16.0	d.l.–5.1
90th percentile	40.0	13.9	2.2
95th percentile	73.4	15.7	4.6
Number of samples	34	22	26
Number of positive values	25	8	7

Group I: regular exposure; group II: occasional exposure; group III: control collective from the factory; d.l.=detection limit=1 $\mu g\,l^{-1}$.

3.1.4. Sources of error

Occasionally there was analytical background interference in the chromatogram. Quantitative evaluation of this unspecified substance usually yields concentrations under the detection limit of 1 $\mu g\,l^{-1}$. Further efforts are necessary to identify this substance.

To keep interference of the method at a minimum, care had to be taken that during extraction with dichloromethane no water got into the organic phase. The effectiveness of the clean-up step was reduced and the level of derivatisation was considerably lowered. In addition the solvent had to be removed very carefully and the already dry residue had not to be held in the nitrogen stream. In this case some of the internal standard 3,5-DNBA could be evaporated.

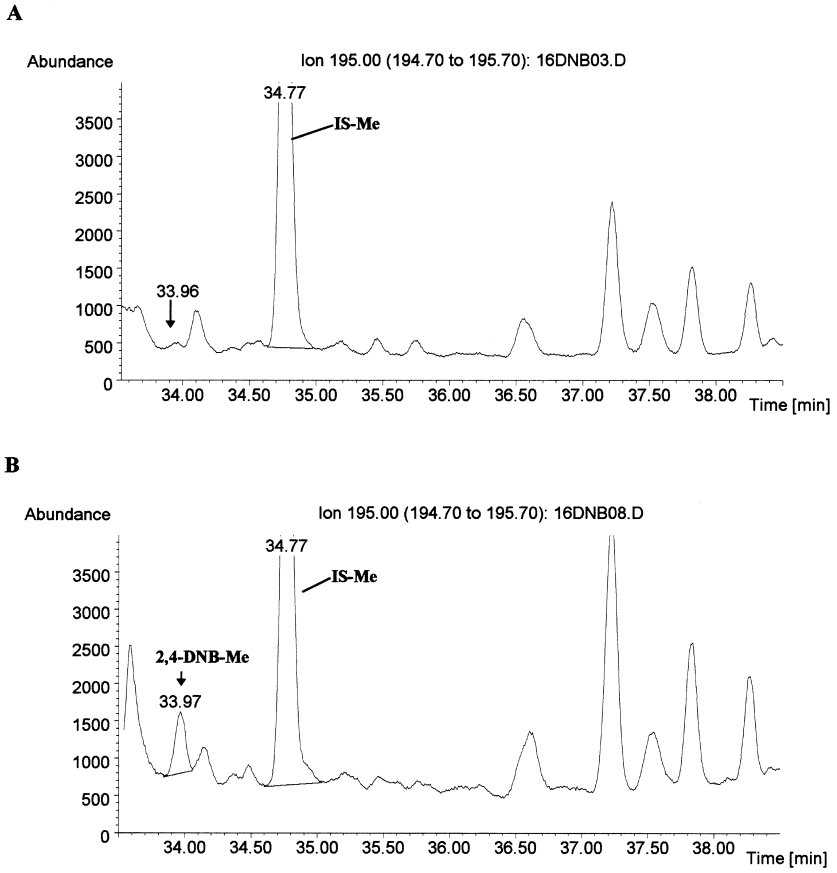

Fig. 4. Chromatograms of a GC–MS (SIM) analysis of prepared urine samples: (A) chromatogram of pooled urine from non-exposed persons, (B) chromatogram of a urine sample from a worker exposed to 2,4-DNT (group I). The concentration of 2,4-DNBA is 5.0 $\mu g\,l^{-1}$ (2,4-DNB-Me: methylated 2,4-DNBA, I.S.-Me: methylated internal standard 3,5-DNBA).

One explanation that the internal standard 3,5-DNBA was more susceptible to evaporation than 2,4-DNBA was, that 3,5-DNBA is less polar than 2,4-DNBA. The dipole moments of the two isomers are different. The pK_a values of the acids gave a hint for the different polarities. The pK_a value of 2,4-DNBA is 1.4 whereas the pK_a value of 3,5-DNBA is 2.8.

3.2. Investigation of exposed persons

With our method we investigated persons occupationally exposed to explosives. The results of biological monitoring are listed in Table 3. 2,4-DNBA could be detected in 25 of 34 urine samples (74%) from the workers regularly exposed (group I). The median value was 5.1 $\mu g\,l^{-1}$, the highest value measured 95 $\mu g\,l^{-1}$. In group II, those workers occasionally exposed, 2,4-DNBA was found in 8 of 22 urine samples (36%) and in group III, the control collective, only in 7 of 26 urine samples (27%). The median values of these two groups (II and III) were already below the detection limit. In comparison with the results published in literature (Table 2) the concentrations investigated were about 100 to 1000 times lower. This was due to the different exposure situations.

In Fig. 4 chromatograms are represented; the top one is from a prepared blank value pooled urine sample and the bottom one from a worker (group I) exposed to 2,4-DNT. The worker was found to have an internal exposure to 2,4-DNBA of 5.0 $\mu g\,l^{-1}$ urine.

4. Conclusions

The analytical method for determining 2,4-DNBA in human urine samples presented here was reproducible, sensitive and specific. The reliability data of the method such as within-series imprecision, between-day imprecision, the detection limit or recovery were good. Sample preparation was very effective and selective. Interfering matrix influences could be excluded.

With a detection limit of 1 $\mu g\,l^{-1}$, internal exposure, possibly down to the environmental range, could be quantified. According to our knowledge this has not been possible up to now.

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