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Determination of hemoglobin adducts in workers exposed to 2,4,6-trinitrotoluene

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Abstract

2,4,6-Trinitrotoluene (TNT) is an important occupational and environmental pollutant. TNT can be taken up through the skin and by inhalation. It is therefore essential to have fast and reliable methods to monitor human exposure. In rat experiments, it has been shown that TNT binds covalently to blood proteins and to tissue proteins. Hemoglobin (Hb) adducts of TNT are markers for the internal dose and possibly for the toxic effects of TNT, e.g. cataracts. In the present paper we introduce a new efficient method to quantify Hb adducts of TNT. Precipitated Hb was hydrolyzed with base in the presence of the surrogate internal standard 3,5-dinitroaniline (35DNA). The released 2-amino-4,6-dinitrotoluene (2ADNT) and 4-amino-2,6-dinitrotoluene (4ADNT) were quantified against 35DNA by gas chromatography–mass spectrometry with negative-ion chemical ionization. Hb of 50 workers and controls from a Chinese munition factory were investigated. The Hb adduct levels ranged from 3.7 to 522 ng for 4ADNT and from 0 to 14.7 ng for 2ADNT per gram of Hb. However, in control samples from Germany no Hb adducts of 4ADNT or 2ADNT could be found.

Keywords: Hemoglobin adducts; 2,4,6-Trinitrotoluene

1. Introduction

2,4,6-Trinitrotoluene (TNT) was the most important explosive used during the second world war. In TNT-exposed humans the notable toxic manifestations have included aplastic anemia, toxic hepatitis, and cataracts [1–3]. Similar conditions have been observed in animals. In China, chronic occupational exposure to TNT caused mainly hepatomegaly and cataract. The incidence of hepatomegaly was 41% and that of cataract was 79% in the TNT workers with prolonged exposure, whereas anemia was rarely found [4]. In mammalian systems the principal metabolites of TNT are 2-amino-4,6-dinitrotoluene (2ADNT) and 4-amino-2,6-dinitrotoluene (4ADNT); smaller amounts of 2,4-diamino-6-nitrotoluene are also formed [1,2,5]. This pattern has been repeatedly found in rat urine [6], rabbit blood [7], and urine from munitions workers [8,9]. Additionally, urine from TNT-exposed humans and rats has been shown to be directly mutagenic [9].

Earlier studies showed that the urine metabolite levels were too high in comparison to the environmental levels [8,9]. It has been deduced from these data, that absorption through the skin must be the main pathway of exposure. Therefore, biological monitoring of workers is preferable as a method of assessing exposure rather than environmental moni-

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toring. Urine metabolites usually indicate only recent exposures (up to 48 h post exposure [10]). Hemoglobin (Hb) adducts are an indicator of exposure over the last four months, assuming that the adduct is stable and that the lifetime of the erythrocytes is not affected. The mechanism of adduct formation between aromatic amines or nitroarenes with Hb involves the reaction of the metabolite nitrosoarene with cysteine residues to form a sulphinamide [11–15]. Evidence for such a reaction in vitro has been obtained by Ringe et al. [12]. Sulphinamide adducts are readily hydrolyzed under mild conditions, yielding the parent amine. Hb adducts are dosimeters for the internal dose and possibly for the target dose leading to toxic effects.

Two methods, HPLC and a competitive inhibition enzyme-linked immunoassay (CI-ELISA), have been developed for measuring the human TNT–Hb adducts [15]. Only 4ADNT was found to be bound to Hb. 2ADNT could not be detected. The results of CI-ELISA and HPLC analysis showed a good correlation ($r=0.77$). In addition it could be demonstrated that the prevalence of cataract and the degree of lenticular damage increased with the levels of TNT–Hb. The present publication introduces a gas chromatography–mass spectrometry (GC–MS) method for the detection of TNT–Hb adducts. Workers from a Chinese munitions factory studied previously [16] were investigated.

2. Experimental

2.1. Chemicals and reagents

2ADNT and 4ADNT were obtained from Promochem (Wesel, Germany). 3,5-dinitroaniline (35DNA) and 2,4-difluoroaniline was purchased from Aldrich (Steinheim, Germany). Dichloromethane (Pestanal) was obtained from Riedel-de Haën (Seelze, Germany), pentafluoropropionic anhydride (PFPA) from Pierce (Oud-Beijerland, Netherlands), diethyl ether (No. 921), sodium hydroxide (No. 6498) and water (No. 15333) from Merck (Darmstadt, Germany). d_9-4-Aminobiphenyl (d_9-4-ABP) was synthesized in our laboratory [17].

2.2. Isolation of Hb from blood and determination of Hb adducts

EDTA blood (1–2 ml) was centrifuged for 5 min at 2000 *g*. After removal of plasma, red blood cells were washed three times in equal volumes of 0.9% NaCl solution and lysed by the addition of four volumes EDTA solution (10^{-4} *M*; pH 7.5). The cell debris were removed by centrifugation. Hb was precipitated with ethanol from lysed erythrocytes. The precipitate was washed with ethanol–water (8:2), ethanol, ethanol–diethyl ether (3:1) and diethyl ether. The dried Hb (40–50 mg) was dissolved in 0.1 *M* NaOH (3 ml) in new screw-top glass tubes (16×100 mm) fitted with PTFE liners (16×100 mm). The ethyl acetate solution of 35DNA (20 ng, 20 μl) and 2,4-difluoroaniline (1 μg, 10 μl) was added to the basic Hb solution (pH>12). After 1 h in a shaking bath at room temperature dichloromethane (5 ml) was added. The mixture was vortex-mixed for 1 min, centrifuged for 5 min at 3000 *g*, and frozen in liquid nitrogen. The thawed organic layer was transferred to a graduated tapered tube (98×15 mm) and concentrated down to 200 μl in a speed evacuator. The residue was then transferred to a microinsert (200 μl, 12×32 mm), autosampler (Hewlett-Packard 7276) vials and evaporated very carefully under a stream of nitrogen at 25°C. At the disappearance of the last drop the stream of nitrogen was stopped. The residue was then taken up in 10 μl ethyl acetate containing 2 ng of the PFPA derivative of d_9-4-ABP.

2.3. Identification and quantification by GC–MS

The analyses were performed on a Hewlett-Packard chromatograph (HP 5890II) equipped with an autosampler (HP 7276) and interfaced to a mass spectrometer (HP 5989A). The amines (1 μl) were analyzed by splitless injection on to a fused-silica capillary column (Rtx-5MS, 15 m×0.25 mm I.D., 0.5 μm film thickness: Restek corporation, Bellefonte PA, USA) with a 1 m×0.25 mm retention gap (Analyt, Müllheim, Germany). In all cases the initial oven temperature, the injector temperature and the transfer line temperature were set at 50, 220 and 220°C, respectively. The oven temperature was

increased at a rate of 50 °C/min to 200°C held for 1.8 min and then increased at 30 °C/min to 300°C. Helium was used as the carrier gas with a flow-rate of 1.5 ml/min. The elution order of the amines is represented in Fig. 1. For negative-ion chemical ionization (NCI), with methane as the reagent gas, the source pressure was typically 160 Pa, the electron energy was 160 eV, the emission current was 300 μA and the source temperature was 200°C. For the identification and quantification of 2ADNT, 4ADNT and 35DNA the molecular ions 197 and 183 were monitored. The ion fragment at m/z at 180 was registered for further identification of 2ADNT and 4ADNT. The molecular ion m/z 304 of d_9-4-ABP-PFPA was monitored. The dwell time for all ions was set at 50 ms. The retention times of d_9-4-ABP-PFPA, 4ADNT, 35DNA, and 2ADNT were 5.73, 6.59, 6.70, and 6.92 min respectively.

2.4. Standard solutions

Stock 1% solutions of all amines were stored in ethyl acetate at −25°C. The final dilutions of the standard mixtures were freshly prepared at the beginning of each week and stored at −25°C.

3. Results and discussion

3.1. Recovery and precision

The precision and accuracy of the method were determined by spiking Hb (50 mg) solutions ($n=5$) with three concentrations of 2ADNT and 4ADNT (200 pg, 1 ng, and 10 ng) in the presence of the surrogate internal standard 35DNA (20 ng). The recoveries were compared with standard solutions containing the three amines. The recoveries and coefficient of variance calculated against the surrogate internal standard (35DNA) for 200 pg, 1 ng and 10 ng of 2ADNT were 98±8%, 106±7%, and 116±8%; and for 4ADNT 88±7%, 93±6%, and 99±6%. Quantification against the volumetric internal standard PFPA-d_9-4-ABP yielded higher coefficients of variation (up to 14%). Therefore, PFPA-d_9-4-ABP was not used for the quantification of the samples. However, it can be used to assess the overall recovery (107±11%) of 35DNA. The presence of Hb–adducts of 35DNA was tested in the workers with the highest Hb adduct levels of TNT. No 35DNA could be found.

3.2. Precision of Hb adduct determinations

The precision of the hydrolysis of Hb adducts was determined by hydrolyzing 40-mg aliquots of Hb ($n=5$) from an exposed worker. The concentrations of 2ADNT and 4ADNT found were 7.4 and 92.8 ng/g Hb respectively. The coefficient of variation for these analyses were ±4% and ±2%.

3.3. Covalent binding of arylamines to Hb

Hb (50 mg) was precipitated with and without the presence of 10 ng 2ADNT and 10 ng 4ADNT and washed with the sequence of organic solvents presented in Section 2.2. This experiment, performed in duplicate was a test to see if non-covalently bound arylamines were eliminated using the given procedure for the isolation of Hb. The precipitated Hb was hydrolyzed in the presence of the surrogate internal standard. No 2ADNT and 4ADNT could be detected. Therefore, the preparation procedure described for the isolation of Hb eliminates non-covalently bound amines.

3.4. Detection limit and quantification

In the NCI mode the detection limits of the standard compounds are in the femtogram range. The determination limit from the present experiments was found to be 40 pg for 2ADNT and 4ADNT per analysis of 50 mg Hb. For larger amounts of Hb the determination limit should be re-evaluated. The limits might be the same or slightly higher. The samples were quantified against calibration curves obtained from Hb (50 mg) spiked with a constant amount of the surrogate internal standard 35DNA (20 ng) and with different levels of 2ADNT and 4ADNT (0.2, 1, 5 and 10 ng). The following calibration curves were obtained: 2ADNT: $y=1.806x-0.0108$, $r^2=0.998$; 4ADNT: $y=1.984x-0.0122$, $r^2=0.999$; y corresponds to the peak-area

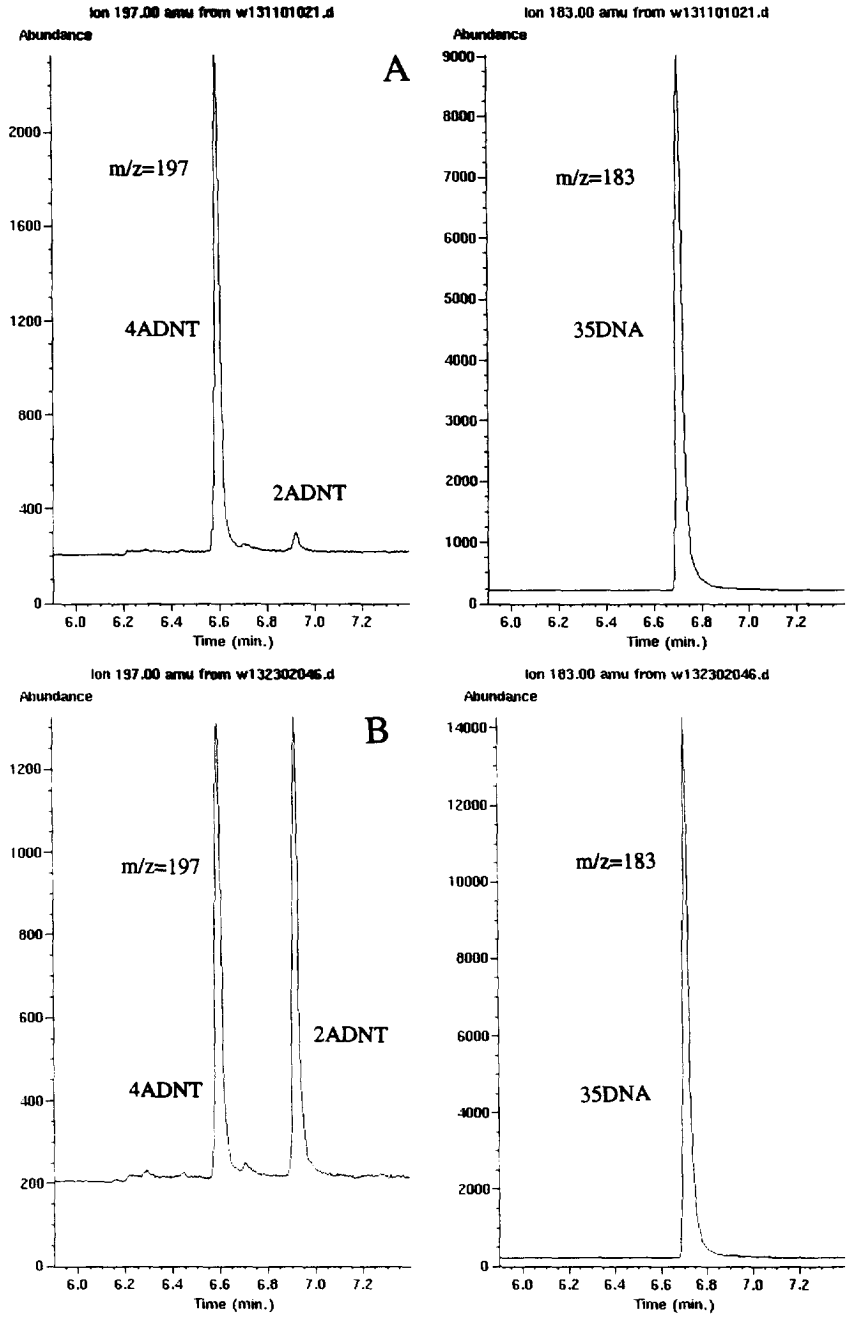

Fig. 1. Chromatograms of Hb (50 mg) extracts from (A) an exposed worker (packing) and (B) a sample spiked with 2ADNT (1 ng), 4ADNT (1 ng) and 35DNA (20 ng). Single ions were monitored in the NCI mode, with methane as the reagent gas (see Section 2). Single ions at *m/z* 183, 197 and 180 were monitored. 2ADNT (0.16 ng) and 4ADNT (2.55 ng) were detected in the Hb (50 mg) extract of the exposed worker.

ratio of 2ADNT/35DNA and 4ADNT/35DNA and x corresponds to the amount ratio of 2ADNT/35DNA and 4ADNT/35DNA.

3.5. Determination of TNT–Hb adducts in munitions workers

The workers of this study were mostly involved in the production of satchel charges for mining in a Chinese TNT factory. The processes involved were grinding, pressing, screening, filling and packing. The time of exposure was 8 h per day and five days per week. The factory controls were fire fighters, white collar workers, security guards and the director. The blood was collected by the Medical Department of the factory. Plasma and the erythrocytes were transported on dry ice to Germany. The biological samples were prepared following a standard method from our laboratory [15]. The samples were hydrolyzed with sodium hydroxide according to methods for the hydrolysis of sulphinamide adducts of arylamines ([13,15] and literature cited therein). The extracts were analyzed without derivatization, since low levels of nitroarenes can be detected by NCI. Chromatograms of Hb extracts from an exposed worker and from a sample spiked with standards are presented in Fig. 1. The 4ADNT levels of the workers were up to 522 ng/g Hb. The highest levels were found in the screening and loading group. For 2ADNT the highest level was 14.7 ng/g Hb. This is the first time that Hb adducts of 2ADNT have been detected in human samples. The ratio between the two adducts is not constant. Similar results were obtained by Woollen et al. [8] and Ahlborg et al. [9] for the urine levels of 2ADNT and 4ADNT. Hb adducts of TNT were found in all factory controls (e.g. fire department, administration). This demonstrates that there is a general contamination of the factory. Hb of our laboratory workers was free of 2ADNT and 4ADNT.

The Hb adducts determined in the present study were compared with the air levels and skin levels determined by Liu et al. [16] (Table 1). The air and skin concentrations were measured in the same workplace, but at a different time point. The values are presented in order to indicate the exposure levels at the workplaces. The adduct levels in exposed workers are more related to skin contamination than to air concentration, indicating that skin contamination is the main source of internal dose, which could be lowered through provision of simple personal protective equipment.

Covalent Hb, plasma protein and protein adducts in various tissues were found in rats dosed radiolabelled TNT [18]. Therefore, Hb adducts of TNT might be a biomarker of toxic effects. Cataracts are sometimes the first and only sign of adverse health effects in TNT-exposed workers. Health records show that 29 out of 126 exposed workers from this factory have developed cataracts [16]. Liu et al. [16]

Table 1
Hemoglobin adducts in TNT workers

	Concentration (ng/g Hb)		n[a]	Inhaled[b] (mg TNT/8h)	Skin exposure[b,c] (mg TNT)
	4ADNT	2ADNT			
Grinding	227.8±26.0	8.9±0.4	2	4.1	218.4
Loading	174.0±115.6	7.3±3.3	12	37.6	317.8
Screening	98.3±70.4	5.8±1.8	3	28.6	145.3
Pressing	96.6±53.4	5.3±0.4	4	2.7	125.2
Filling	81.8±26.0	6.4±4.3	5		
Packing	35.7±17.1	2.5±1.1	8	3.6	66.6
Mixing	35.7±5.5	n.d.[d]	2		
Controls	10.4±6.3	2.8[e]	14		

[a] Number of workers.
[b] These values were measured at the same workplace in a former study [16].
[c] Total amount of TNT per body surface.
[d] n.d. = not detected.
[e] This is the average value found for two persons. In 12 controls no 2ADNT was present.

found that the prevalence of cataracts correlates with the increase of TNT–Hb adducts. No cataracts were found when the TNT–Hb level was below 30 ng/g Hb (as determined by ELISA), even for individuals who had been employed for up to twenty years in this factory. The three subjects with the highest level of adducts (>300 ng/g Hb, as determined by ELISA), were all diagnosed with cataracts. Possibly similar protein adducts of TNT occur with lens proteins of exposed workers. This will be investigated in the near future. The presence of the two Hb adducts indicates that 4-(N-hydroxyamine)-2,6-dinitrotoluene and 2-(N-hydroxyamine)-4,6-dinitrotoluene are biologically available. These intermediates might be formed by reduction of TNT in the gut, in the liver and in the erythrocytes [18,19]. In vitro experiments with erythrocytes and TNT showed that 2% of the dose is bound to Hb [18]. Therefore, it is conceivable that a small amount of TNT adducts found in vivo might result from the reactions of TNT in the erythrocytes [15]. This is not the case for less reducible nitroarenes such as nitrobenzene [4]. Toxic damage from TNT might not be caused by protein adducts alone but also from oxidative stress [19–21]. Extensive biochemical and morphological changes in rats after a single injection of TNT were indicative of peroxidation [21] possibly caused by the cyclic oxidation and reduction of the nitro group [20]. Recently it has been postulated that cataracts result from oxidative damage of the lens [22]. Therefore, further mechanistic studies have to be undertaken to show which is the cause of cataract formation. Nevertheless, TNT–Hb adducts are a biomarker for exposure. It is thus possible to screen people exposed to such compounds.

4. Conclusions

The procedure presented here has been successfully applied to the biomonitoring of a group of over fifty workers exposed to TNT. The simplicity of this method will make it possible to routinely monitor Hb adducts from people living or working in contaminated areas. If very low exposures are expected more Hb could be used for the analyses. Lower determination limits could be achieved by derivatization of the amino group with pentafluoropropionic acid anhydride.

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