Analytical Monitoring of Trinitrotoluene Metabolites in Urine by GC–MS. Part I. Semiquantitative Determination of 4-Amino-2,6-dinitrotoluene in Human Urine

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Abstract

A routine procedure for the monitoring of occupational exposure to 2,4,6-trinitrotoluene (TNT) based on the semiquantitative determination of 4-amino-2,6-dinitrotoluene (4A2,6-DNT) in urine samples by gas chromatography (GC–MS) was developed. For calibration purposes, a standard sample of 4-amino-4,6-dinitrotoluene was prepared. Urine samples were collected from munition workers in a military plant in Hradec Králové district. The samples were extracted into ethyl acetate, and analyzed by GC–MS without previous derivatization. The GC was equipped with a DB1701 column (30 m, 0.25 mm, 0.25 μm) for optimum separation. 4A2,6-DNT was detected at concentrations above the limit of quantitation (0.2 mg/L). This simple method is sufficient for the screening of high degree of TNT exposure and constitutes the first step of a broad project focused on this occupational medicine problem.

Introduction

2,4,6-Trinitrotoluene, commonly referred to as tritol or trinitrotoluene (TNT), is a yellow, odorless solid that does not occur naturally in the environment. It is widely being used as an explosive in military shells, bombs, and grenades; in industrial applications; and in underwater blasting (1). Chronic exposure to TNT leads to harmful health damage including anemia, abnormal liver function, cataract development caused by the generation of hydrogen peroxide during the reduction of nitro groups, and skin irritation. Although the participation of hydrogen peroxide in cataractogenesis by TNT has not been clarified in detail (2), the formation of this substance from TNT in incubated liver microsome fraction of male Sprague-Dawley rats was clearly demonstrated.

Harkonen and co-workers (3) found early equatorial cataracts in 12 workers (9 men and 3 women with age in the range 31 to 49 years) with a mean duration of exposure 6.8 ± 4.7 years. Short-term toxic effects of TNT were evaluated in mice, rats, and dogs. All three species showed depression of body weight and food intake and moderate to severe anemia (4). Cytotoxicity and mutagenicity are other high risk factors of handling this explosive manually. TNT penetrates the skin and is absorbed by lungs (5,6). Information on the biotransformation of TNT in experimental animals and humans dates back to the 1940s (7). In humans, dinitroaminotoluenes [2,6-dinitro-4-aminotoluene (4A2,6-DNT) and 2,4-dinitro-6-aminotoluene (6A2,4-DNT)] were the major urinary metabolites, identified by Lemberg and Callaghan (8).

Regarding the metabolic conversions, relatively detailed knowledge of aerobic and anaerobic biotransformation of this toxic substance has been accumulated. Aerobic bacterial metabolic studies can serve as useful models for the description of TNT biotransformation in the human organism as well as in dealing with environmental problems (9,10). The isomers of aminodinitrotoluene were identified as the first detectable metabolites of TNT in the pioneering work of Riefler and Smets (11,12), which constitutes the first step towards a comprehensive understanding of enzymatic biotransformation of nitroarenes (13–16).

Given the toxicity of TNT, it is extremely important to monitor all people who may come into contact with this substance on a regular basis. Apparently, the simplest way of monitoring...
the exposure to TNT is measuring the level of a stable metabolite in body fluids. In this paper, we wish to present a simple gas chromatography-mass spectrometry (GC–MS) method for screening and semiquantitation of 4A2,6-ADNT in human urine.

Materials and Methods

Chemicals and reagents

All solvents and reagents were analytical or pesticide residue analysis grade (PRA).

The standard of 4-amino-2,6-dinitrotoluene was prepared by the reduction of TNT with in situ prepared ammonium polysulfide (17); the details of the preparation will be disclosed elsewhere. The NMR data were in agreement with those published by Nielsen and co-workers (17).

Ethyl acetate was purchased from Sigma-Aldrich Ltd. (Milwaukee, WI). Hydrochloric acid (35%) was purchased from Penta (Chrudim, Czech Republic), and sodium bicarbonate was purchased from Merck KGaA (Darmstadt, Germany).

Subjects

Urine samples were collected before workshift end from randomly selected armory workers in Hradec Králové district. The group included 56 subjects (50 males and 6 females aged 24 to 58 years). The urine samples were stored at −25°C prior to GC–MS analysis.

Procedure

Calibration. Calibration solutions were prepared by the dissolution of 30 mg of 4A2,6-DNT in 25 mL of 95% ethanol. This stock solution was diluted by a mixture of pooled urine and distilled water (1:1) 20-fold and 100-fold, to give concentrations of 60.0 mg/L and 12.0 mg/L, respectively. Further calibration standards with concentrations ranging from 0.5 to 60 mg/L were prepared from these stock solutions and the urine solution. A 13-point calibration plot was made (peak area vs. concentration in mg/L, each point in triplicate). The external calibration was run during every analytical sequence of samples. Limit of detection (LOD) (0.05 mg/L) and quantitation (LOQ) (0.2 mg/L) values were determined in accord with the European Pharmacopoeia (on the basis of signal-to-noise ratio) upon analyzing solutions of lower concentrations (0.01, 0.02, 0.025, 0.05, 0.1, and 0.2) where the s/n ratio of GC–MS peaks was no higher than 10:1 (18).

Sample analysis. Concentrated hydrochloric acid (0.1 mL) was added to the urine samples (2.5 mL), and the solutions were incubated in a thermoblock at 100°C for 30 min. The samples were cooled, and then neutralized by sodium bicarbonate solution (0.15 g/sample). Following the neutralization, double extraction into ethyl acetate (2 × 6 mL), vortex mixing (2 min), and centrifugation (3500 r.p.m., 3 min) were carried out. The obtained supernatants were evaporated to dryness under nitrogen, reconstituted in of ethyl acetate (50 µL), transferred to 100-µL tubular inserts, and then placed in 0.5-mL autosampler vials. One microliter of each analytical sample was injected onto a Varian 3300 GC coupled with a Magnum ion trap MS from ThermoFinnigan (splitless mode, injector at 250°C, column DB1701, 30 m, 0.25 mm, 0.25 µm, Agilent).

Other procedures

Further corroboration of the structural identity of the 4A2,6-DNT standard was performed on a magnetic sector instrument in the electron ionization regimen and linked scan operation mode.

Results and Discussion

The purpose of this work was to develop an inexpensive method that would allow a quick screening and semiquantitative determination of 4A2,6-DNT as a marker of occupational exposure to TNT. Besides the other amine isomers (9-14), 4A2,6-DNT is one of the key diagnostic markers. The determination of 4A2,6-DNT and other metabolites in urine is possible by a variety of analytical techniques. Thin-layer chromatography, immunassay, GC, and high-performance liquid chromatography (HPLC) methods, including an MS detection, have been described in the literature (19-23). As compared to the alternative HPLC–MS technique, the GC–MS method is easy to use, generally available, and inexpensive. Internal calibration methods with a derivatization process for a more precise and sensitive determination of TNT metabolites in urine by GC–MS and GC with an electron capture detector have been described. Bader and co-workers (22) reported an original method for the analysis of nitroaromatics in urine by GC–MS, with two conceptually different internal standards (13Cd-nitrobenzene and 4-amino-3,5-dinitrotoluene). As regards these standards, the stable isotope standard is somewhat expensive, and 4-amino-3,5-dinitrotoluene is not easily available. Moreover, even though the latter substance has a different MS fragmentation pattern in comparison to

![Figure 1](image-url) The mean concentrations of 4A2,6-DNT in relation to four different job assignments.
4A2,6-DNT, it has identical separation properties under GC conditions. In principle, such non-standard way can be used for the internal calibration under the conditions of GC–MS as an alternative of the isotope dilution, but the analytical reliability data (imprecision for 4A2,6-DNT 12% within series, between days 25%) are not convincing (22). Recently, Woolen and coworkers (23) used 1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene as an internal standard. Somewhat unusually, after the extraction process, they added this substance to the final analytical sample, dissolved in ethyl acetate, due to a very low water solubility of the compound. Given these results, and because employees are commonly being rotated in various parts of the manufacture with differing levels of TNT exposure, we reasoned that a much simpler, semiquantitative screening of strongly contaminated workers bearing the highest risk of contracting a TNT exposure-related disease should be sufficient.

The measurements by Woolen et al. (23) are directly comparable with our results. They measured concentrations in the range 0.1–44 mg/L (n = 219), with the mean concentration of 9.7 mg/L (SD 7.9 mg/L). Our semiquantitative results ranged from negatives (below LOD) to 84.4 mg/L, with the mean of 11.9 mg/L (SD 9.6 mg/L). The primary results were corrected with regard to the specific gravity of urine as a method of choice for the evaluation of the influence of the renal concentrating mechanism. Hence, the urine specific gravity was determined via urinometer (24) and used in the corrective recalculations. The figures, reflecting various degrees of contamination by TNT, are in excellent agreement with the job assignments of different individuals within the manufacture. Figure 1 presents outstanding differences in the mean concentrations of 4A2,6-DNT between the manufacture staff in accord with their manufacture departments. 4A2,6-DNT was detected at concentrations above the LOD limit (0.05 mg/L, signal/noise = 3) in most of the urine samples analyzed. The LOQ limit was determined as 0.2 mg/L, with the signal/noise ratio of 10. The urine pool collected from the laboratory staff was used as a negative control sample. It is worthy to note that the normal reference range for persons potentially exposed to TNT has been set to 0–1.5 mg/L by the Czech Ministry of Health (25). Precision and accuracy data were evaluated with a view to justifying the external calibration used. The precision (within runs and run to run), expressed as the coefficient of variation (% CV) for three concentrations of the spiked standard (0.2 mg/L, 3.2 mg/L, and 10 mg/L) over a period of 10 working days was performed (% within run: 14.5, 10.3, and 8.1% run to run: 27.1, 24.8, and 21.0).

Because amine metabolites are typically excreted as N-conjugates with glucuronic acid in urine, acidic hydrolysis of the urine samples was performed before extraction. The hydrolysis was carried out at 100°C (26). Considering the aim of screening workers with a high degree of contamination, the analytical procedure involving urine hydrolysis and liquid–liquid extraction seems sufficient.

When analyzed by GC–MS in the electron impact regimen, 4A2,6-DNT from the samples produced ions at m/z 197 (molecular ion) and two characteristic ions at m/z 180 and 163. The latter are formed by a double ortho effect arising from two nitro groups attached at ortho positions to a methyl group (27,28). The key ions (m/z 197, 180, and 163), marked by an arrow from a positive analysis of a urine sample are shown in Figure 2. The peak area at the mass of m/z 180 was used for semiquantitation.

Considering the analytical reliability of our data and the clinical demand to monitor the cases with an elevated concentration of 4A2,6-DNT, we have found external calibration sufficient for the initial determination of the degree of TNT absorption.

Conclusions

A routine bioanalytical method for the monitoring of TNT metabolite levels in occupational medicine was developed and partially evaluated. Besides being inexpensive, the procedure for the determination of 4-amino-2,6-dinitrotoluene presented in this paper is sufficiently sensitive and easy to use as a convenient way of rapid assessment of occupational exposure to TNT. Our further work will include other TNT metabolites, and the results will be reported in due course.

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References


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