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S-(acetamidomethyl)mercapturic acid (AMMA): A new biomarker for occupational exposure to N,N-dimethylacetamide☆

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ABSTRACT

N,N-dimethylacetamide (DMA) is used in the textile and plastics industry as a solvent alternative to more toxic N,N-dimethylformamide. Here we studied toxicokinetics of two major urinary metabolites of DMA, namely, S-(acetamidomethyl)mercapturic acid (AMMA) and N-methylacetamide (NMA). Urine samples were collected from workers exposed to DMA in a factory manufacturing acrylic fibers. AMMA and NMA were determined by HPLC/MS and GC/MS, respectively. The working scheme in the factory consisted of periods of three consecutive working shifts alternated regularly with two days off work. In the first stage of the study, NMA and AMMA were determined in urine samples collected before, in the middle, and at the end of one working shift. In the second stage, urine was collected five times during three consecutive days after a two-day rest: before and at the end of the first and second working shifts and before the third shift. It was found that the end-of-shift NMA levels were several folds higher than the pre-shift levels of the same day and dropped significantly until the next shift. On the other hand, there were no significant differences in AMMA levels before and at the end of the same shift but a continuous rise during the three-day working period was observed. Median values of NMA concentrations at the end of working shifts were between 10.1 and 17.3 mg/g creatinine, median AMMA concentrations in the second or third day of the working period varied between 12.4 and 38.1 mg/g creatinine. The approximate half-lives of NMA and AMMA (means) in the exposed workers were about 9 and 29 h, respectively. Thus, while NMA in the end-of-shift urine samples remains a preferential biomarker of DMA exposure during that shift, AMMA determined at the end of a work-week reflects cumulative exposure over the last few days. Further studies are needed to determine AMMA concentrations corresponding to the threshold limit value of DMA.

1. Introduction

N,N-dimethylacetamide (DMA) is a solvent used in the textile industry for the production of polyacrylate synthetic fibers as well as in the synthesis of resins and plastics. Though the number of industrial settings handling DMA is limited, these often employ up to several hundreds of workers. Toxicological studies on animals revealed hepatic, renal, pancreatic, pulmonary, gastric, intestinal and bone marrow damages after exposures to DMA at high doses [1]. Moderate liver damage with morphological and functional alterations [2], hand tremor, arterial hypertension and headache [3] were described in workers occupationally exposed to DMA. Cognitive alterations were reported in patients receiving DMA as a single therapeutic agent at a dose of 300 mg/kg [4]. DMA is absorbed in the body by inhalation and through the skin [5–7]. Both American Conference of Governmental Industrial Hygienists (ACGIH) [8] and Deutsche Forschungsgemeinschaft (DFG) [9] underline the remarkable dermal absorption of DMA by assigning the "skin" notation.

The reported metabolic products of DMA in humans are acetamide, N-methylacetamide (NMA) [1,10,11], and N-acetyl-S-(acetamidomethyl)-L-cysteine or S-(acetamidomethyl) mercapturic acid (AMMA), which was identified in our previous investigation [12]. Most of NMA is in fact an analytical artifact due to thermal demethylation of the true urinary metabolite N-hydroxymethyl-N-methylacetamide in the heated injection port (250 °C) of the gas chromatograph [12], however, part of N-hydroxymethyl-N-methylacetamide is still converted to NMA in the body. Conjugation of NMA with glutathione followed by mercapturic acid pathway produces AMMA as an ultimate metabolite which is excreted with urine (Fig. 1). For biological monitoring of occupational exposures to DMA, ACGIH [8] and DFG [9] recommend the measurement of NMA in urine samples

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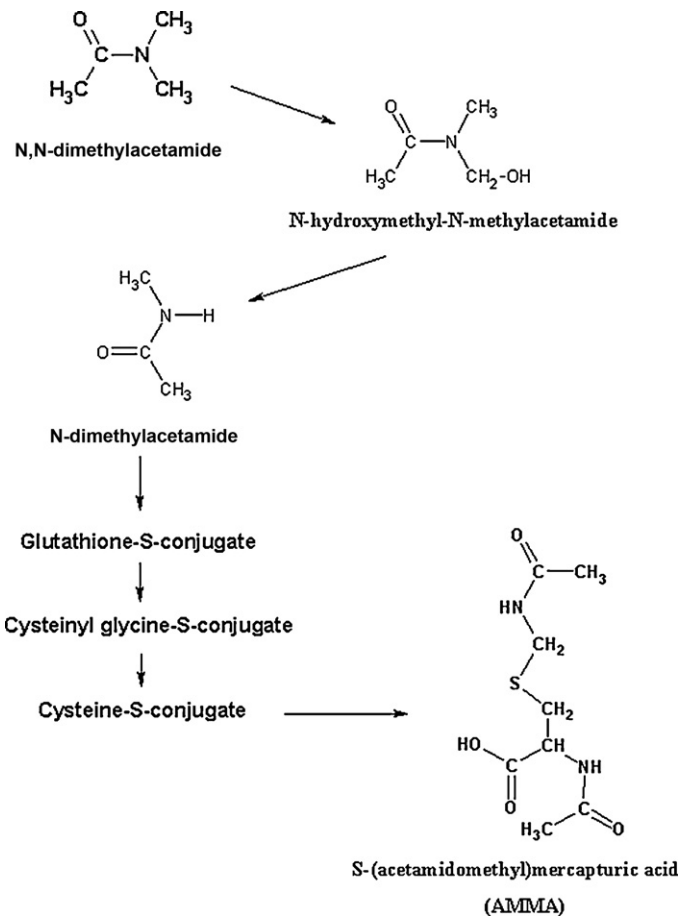

Fig. 1. The principal metabolic pathways for DMA in humans.

collected at the end-of-shift at the end of work-week with the limit of 30 mg/g creatinine.

The aim of this study was to characterize and compare the toxicokinetics of AMMA and NMA as major metabolites and biomarkers of DMA.

2. Materials and methods

2.1. Chemicals

N,N-dimethylacetamide, N-methylacetamide, and methanol (>99%) were obtained from Carlo Erba (Milan). S-(acetamidomethyl)mercapturic acid was synthesised from S-acetamidomethyl-L-cysteine (Fluka) according to Threadgill and Gledhill [13]. N-acetyl-S-(N-methylcarbamoyl)cysteine was synthesised as suggested by Mraz [14].

2.2. Study design

The study was conducted in a factory employing DMA in the production of acrylic fibers. Exposure of workers to DMA occurred during manual operations for ca. 30 min in which they were using 50% aqueous solution of DMA heated to 50 °C, and later by operating the machinery for extrusion of the acrylic fibers. In our previous article the work operations in the factory were described in detail [12]. Scheme of the working shifts was as follows: three morning shifts (from 6 AM to 2 PM), three afternoon shifts (from 2 PM to 10 PM), and three night shifts, always with two days off work between the three-day working periods.

Thirty-five workers were we enrolled in the first stage of the study. Each subject provided three urine samples within one day: before, in the middle, and at the end of a morning working shift. The sampling days were selected irrespective of whether it was the first, second, or third day of the three-day working period. (Only later we realized that the sampling day is an important factor for the toxicokinetic considerations.)

In the second stage of the study, 17 workers provided five urine samples in pre-determined time during three consecutive working days: before and at the end of the first working shift, before and at the end of the second working shift, and finally before the third working shift. All involved workers took a shower and changed their clothing after each working shift to avoid post-shift exposure to DMA due to contaminated skin or clothing.

2.3. Urine analyses

Urine samples were collected into 150 ml glass vials and stored at 4 °C until analysis which was carried out within seven days. Urinary NMA was measured by a GC/MS method described previously [15]. AMMA analysis was performed with a microHPLC/MS method as follows. After adding N-acetyl-S-(N-methylcarbamoyl)cysteine (AMCC) as an internal standard (100 mg/l) a 0.5 ml aliquot of the urine sample was passed through a SAX cartridge (500 mg) conditioned with methanol and water. AMMA was eluted with three 0.5 ml-volumes of formic acid (0.5 M). This eluate (0.2 μl) was used for the analysis. Urinary concentrations of NMA and AMMA were expressed in mg/g creatinine to correct for fluctuations in the urine excretion rate as suggested by ACGIH [8]. Urine samples with creatinine concentration outside the range of 0.3–3 g/l were excluded from the statistical analysis. In the first stage of the study 8 of 105 urine samples had creatinine level higher than 3 g/l (no urine samples with creatinine lower than 0.3 g/l). In the second stage of the study 11 of 85 urine samples were outside the reported range (4 lower and 7 higher than the range).

2.4. Equipment

AMMA was determined using a microHPLC (1100 series, Agilent Technologies) connected to a mass spectrometer (LC/MSD TRAP SL Agilent Technologies) through an electrospray source. The separation was carried out on a Zorbax SB C18 column (5 μm, 150 × 0.5 mm) using the mobile phase methanol-2 mM formic acid (5:95) at a flow rate of 20 μl/min and a column temperature of 25 °C (isocratic conditions). The injection volume was 0.2 μl. The MS operated in negative ion mode with a scan range from 100 to 500 *m*/*z*; nitrogen was used as both nebulizing and drying gas. The MS parameters were as follows: source temperature 325 °C, nebulizer gas flow (4 l/min), nebulizer pressure 15 psi, capillary voltage 3500 V, trap drive (33.5 V).

Identification and quantitation of AMMA was based on monitoring $[M-H]^-$ ion at mass-to-charge ratio (*m*/*z*) = 233 (real mass 234) with retention time of 4.4 min. The internal standard (AMCC) with retention time of 7 min, was monitored using ion *m*/*z* 219 $[M-H]^-$ for confirmation and its daughter fragment ion *m*/*z* 162 $[M-H]^-$ for quantification.

Accuracy was 99.8%; precision at 20 and 80 mg/l was respectively 4.4% and 0.6%. Calibration curves with points at 0, 20, 40, 60, 80, 100 in three non-consecutive working weeks yielded comparable results with slopes ranging between 0.037 and 0.043 with correlation coefficients between 0.9986 and 0.9994. Detection limit (LOD) of AMMA was 1.5 mg/l: it was calculated from six injections of blank urine as suggested by Miller and Miller [16].

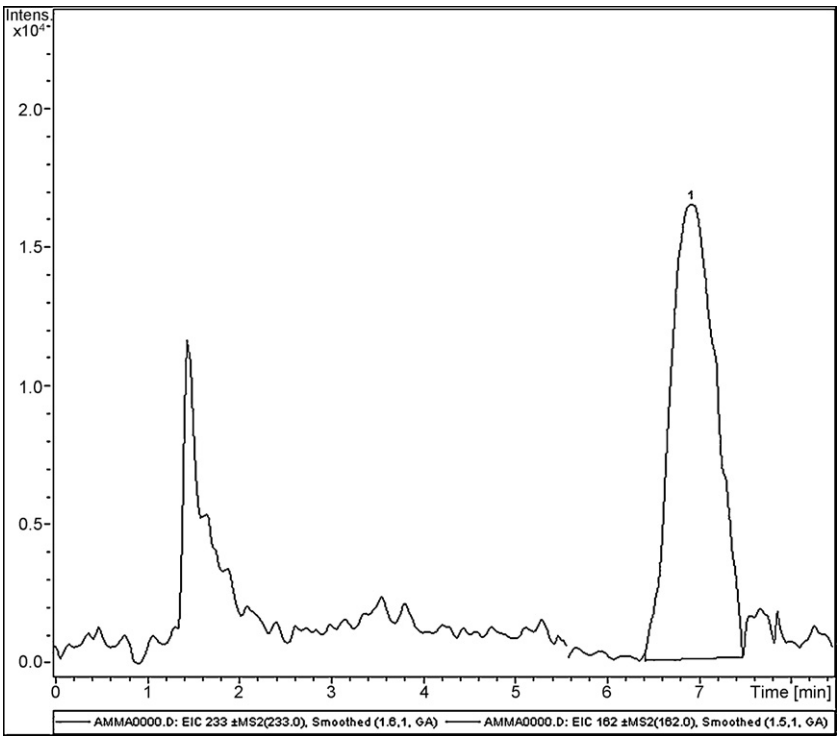

Fig. 2. HPLC/MS chromatogram of a processed urine sample from a subject not exposed to DMA; the sample was added with AMCC (100 mg/l) as internal standard. (Retention time: 7 min).

2.5. Statistical analysis

Statistical analysis was performed using Statgraphics® software (Statistical Graphics System, ST-SC Inc.). As urinary concentrations of AMMA and NMA were not normally distributed, most of the statistical analyses were carried out using non-parametric tests, i.e., the Mann Whitney Wilcoxon (W) test for comparison of two groups of data, Kruskal–Wallis and Friedman tests for comparison of three or more groups of data (matched data in case of Friedman test). A difference was considered statistically significant when $p < 0.05$.

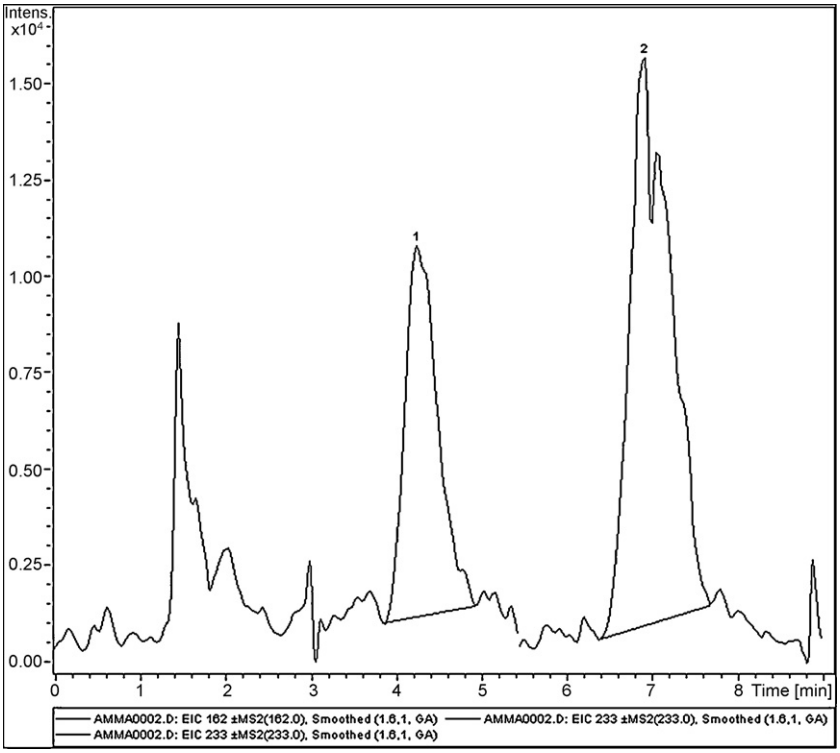

Fig. 3. Typical chromatographic pattern of a processed urine sample (containing AMMA 18 mg/l and I.S.) from a worker exposed to DMA.

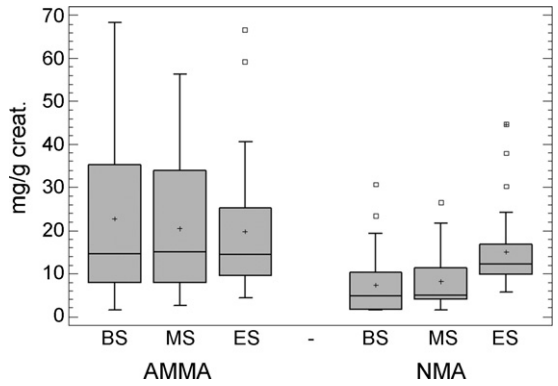

Fig. 4. AMMA and NMA concentrations (mg/g creatinine) in urine samples collected before (BS), in the middle (MS) and at the end of the same working shift (ES). Only NMA increases significantly during the working shift ($p < 0.001$).

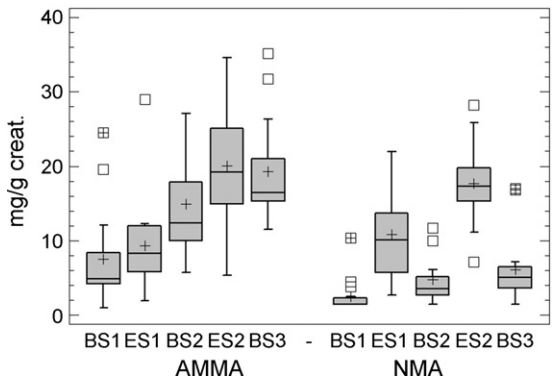

Fig. 6. AMMA and NMA concentrations (mg/g creatinine) in urine samples collected before (BS) and at the end of the shifts (ES) during three consecutive days (designated by the indices 1, 2 or 3).

3. Results

Fig. 2 shows the chromatogram of a urine sample from a subject not exposed to DMA added with 100 mg/l of I.S. (retention time: 7 min). Fig. 3 shows the chromatogram of a urine sample from a worker exposed to DMA: the AMMA concentration (retention time: 4 min) resulted 18 mg/l. Very similar chromatograms were obtained with standard samples added with AMMA.

Urinary concentrations of AMMA and NMA obtained within the first stage of the study when urine samples were collected before, in the middle, and at the end of the same morning shift, are shown in Fig. 4. Median concentrations of AMMA did not increase significantly throughout the shift. The concentrations of NMA in the same urine samples were statistically higher at the end of the shift than before and in the middle of the shift ($p < 0.001$). Median concentrations of AMMA and NMA were respectively 14.5 and 4.9 mg/g creatinine in the pre-shift urine samples, and 14.4 and 11.9 mg/g creatinine in the end-of-shift urine samples. Thus, urinary NMA concentrations increase rapidly and significantly during a working shift, while AMMA concentrations over this period remain almost constant suggesting different kinetics of the two metabolites.

Whereas each column in Fig. 4 represents the entire set of the pre-shift, middle-shift and end-of-shift urine samples, Fig. 5 shows the above pre-shift and end-of-shift data broken down according to whether the urine samples were collected on the first day or the second-third day after returning from a two-day rest. Here, pre-shift urinary concentrations of NMA on the second or third day (BS2) were much higher than on the first day (BS1). The rise of NMA during the first working day (from 1.8 to 10.3 mg/g creatinine) was striking and statistically significant while the increase during the second or third day (from 9.6 to 14.5 mg/g creatinine) was not significant.

Toxicokinetics of AMMA differs from that of NMA. Concentrations of AMMA in the pre-shift and end-of-shift urine on the first day after the rest are low and of similar value (medians: 8.2 and 10.1 mg/g creatinine), while in the following days they are also similar but significantly higher (medians: 38.1 and 27.8 mg/g creatinine).

In the second stage of the study, NMA and AMMA were measured in five urine samples collected during three consecutive working days (Fig. 6). Median concentration of AMMA in the three consecutive pre-shift urine samples rose progressively from 4.9 to 12.4 and 16.4 mg/g creatinine with a statistically significant difference between the first and the following days, whereas no significant rise was observed during the same working day. In contrast to AMMA, median concentrations of NMA were low in all pre-shift samples (1.5, 3.5 and 5.1 mg/g creatinine) and high in the end-of-shift samples (10.1 and 17.3 mg/g creatinine). However, in both the pre-shift and end-of-shift samples, the values of the second-third day were significantly higher than those of the first day.

4. Discussion

In our previous study, we identified the mercapturic acid AMMA as a novel metabolite of DMA [12]. This adds, at least formally, another similarity to the biotransformation pathways of DMA and N,N-dimethylformamide (DMF). Primary metabolites of both DMA and DMF are the corresponding N-hydroxymethyl-N-methylamides which are rapidly excreted in

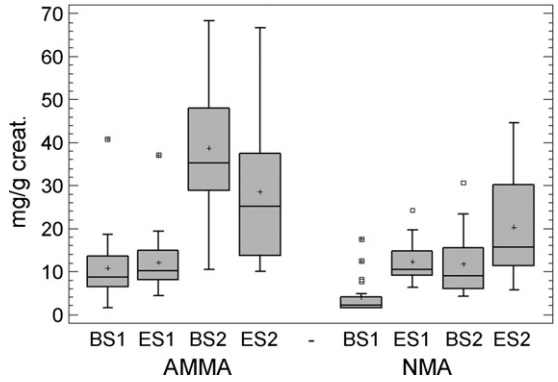

Fig. 5. AMMA and NMA concentrations (mg/g creatinine) in urine samples collected before (BS) and at the end of the same working shift (ES) when data is broken down according to the working day (designated by the indices 1 or 2).

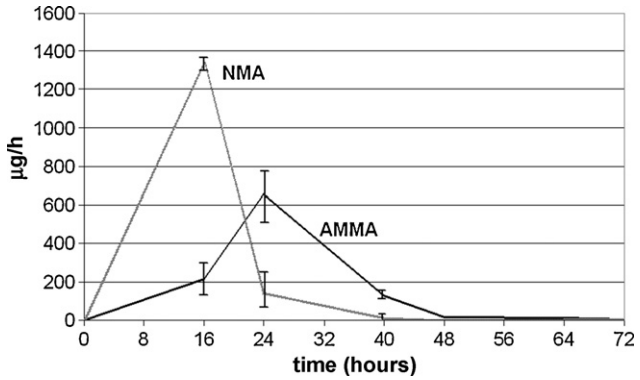

Fig. 7. Kinetics of AMMA and NMA in urine of rats after a gavage administration of DMA (200 mg/kg body weight).

urine and undergo thermal decomposition to the corresponding N-methylamides during gas chromatographic analysis. DMA and DMF also produce specific mercapturic acids, AMMA and N-acetyl-S-(N-methylcarbamoyl)cysteine (AMCC), respectively. However, the metabolic pathways leading to AMMA and AMCC are different. Formation of AMCC proceeds through the reactive intermediate methylisocyanate which is believed to cause the toxic effects due to exposure to DMF. Many cases of acute hepatotoxicity or disulfiram-like effect after ethanol intake (flushing) were reported in workers exposed to DMF. Occupational exposure to DMA causes temporary moderate effects on liver function [17,18] but neither severe hepatotoxicity nor episodes of flushing were described in the literature [5] or observed by us. DMA and DMF may be used equally in technological applications. E.g., for manufacturing of acrylic fibers DMF is employed in Germany while DMA in Japan and Italy. However, the currently recognized differences in biotransformation support the use of DMA which is metabolized to glutathione conjugate without involvement of the toxic isocyanate.

No data on the toxicokinetics of AMMA were reported before this paper. In an initial study of Perbellini (not published), four rats were administered 200 mg/kg body weight of DMA by gavage, and urine samples collected for 72 h were analyzed for NMA and AMMA as described in Section 2. Mean urinary concentrations of both compounds are shown in Fig. 7. The NMA levels in rats increased and decreased faster (half-life about 2.5 h) then those of AMMA (half-life about 6.5 h). The urinary elimination of AMMA was well described by a first-order kinetics starting from its peak moment (one compartment model).

In order to have an approximate value of the half-life of AMMA in humans we used data from the second stage of the study. Assuming that the urinary concentration of AMMA on the third working day was close to its steady state (Fig. 6), we compared the AMMA values in pre-shift urine samples of the third working day with AMMA values in the pre-shift urine samples of first working day (considering two days at rest). Considering the first-order kinetics of AMMA excretion between these two time points, AMMA half-life was assessed to 29.4 h (mean value of 13 workers, S.D. = 6.6 h). Data from 4 workers were excluded as creatinine concentration in their urine was outside the accepted range. In a similar way, the half-life of NMA was calculated by using NMA concentrations in urine samples collected at the end of the second working day and before the third working day (of the same working period). The mean NMA half-life was 8.7 h (S.D. = 1.9 h). Thus, the DMA metabolites were excreted somewhat more slowly than those of DMF (NMF: 3.8 h, AMCC: 23.1 h) [19].

There are considerable different kinetics of NMA and AMMA urinary excretion between male rats after gavage and workers after inhalation. We think that these differences can be mainly related to these two topics: the absorption after a single bolus of DMA is certainly more rapid than long lasting lung absorption in human subjects during a work shift; moreover, xenobiotic metabolism in rats is often more active and faster than in humans.

In conclusion, our results highlight AMMA as an important metabolite of DMA occurring in urine at a similar or even higher level than NMA. AMMA can be used as a biomarker of exposure to DMA many hours after the end of the working shift. Further studies are needed to determine AMMA concentrations corresponding to the threshold limit value of DMA. Lower toxicity of DMA compared to DMF yet comparable soluting properties support the preferential use of DMA in technological applications.

Conflict of interest

The authors declare no conflict of interest.

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