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SHORT COMMUNICATION

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N-methylcarbamoyl adducts at the N-terminal valine of globin in workers exposed to N,N-dimethylformamide

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Abstract N,N-dimethylformamide (DMF) is a commonly used industrial solvent. The formation of some metabolites of DMF in humans occurs via N-methylcarbamoylated species (e.g. N-methylcarbamoylated glutathione). The aim of our study was to investigate whether DMF leads to N-methylcarbamoylated adducts at the N-terminal valine of haemoglobin (Hb). Therefore, Hb adduct levels of ten DMF exposed workers and ten controls were analysed by a specific and sensitive detection method using capillary gas chromatography and a mass selective detector (GC/MS). Using this method we were able to show for the first time that Hb adducts are formed during the metabolism of DMF in humans. The general population, however, shows still unidentified background levels of this adduct which are on average lower by a factor of 50. The pathway for the formation of the investigated DMF-Hb adduct in workers exposed to DMF is still unknown. As identical adducts were also found after exposure to methylisocyanate (MIC), our work indicates the formation of MIC during the metabolism of DMF. The formation of Hb adducts with DMF and its relevance for occupational health is a subject of further research.

Key words N,N-Dimethylformamide · Biochemical effect monitoring · Haemoglobin adducts · Methylisocyanate · 3-Methyl-5-isopropylhydantoin

Introduction

N,N-Dimethylformamide (DMF) is an industrial chemical with an estimated worldwide production capacity of ~250 000 tons in 1989 (GDCh, BUA-Advisory Committee 1991). It is a commonly used solvent for many vinyl-based polymers in the manufacture of films, fibres and coatings as well as a solvent for making polyurethane lacquers for clothing and accessories made of synthetic leather. Exposure to DMF may occur via inhalation and dermal absorption. The major effects which have been reported in workers exposed to DMF were gastric irritation and hepatotoxicity (Kennedy 1986). Moreover, an excess risk for testicular germ-cell tumours was identified among workers who had been exposed to a solvent mixture containing 80% DMF (Ducatman et al. 1986). Although there is only inadequate evidence for the carcinogenicity of DMF in experimental animals, DMF is possibly carcinogenic to humans (Group 2B) according to the International Agency for Research on Cancer (IARC) of the World Health Organization (WHO 1989).

The main primary metabolite of DMF is N-(hydroxymethyl)-N-methylformamide (HMMF; Mráz and Nohova 1992). The metabolic end product after oxidation of the formyl group of HMMF is N-acetyl-S-(N-methylcarbamoyl)-cysteine (AMCC; Mráz and Turecek 1987). It was postulated that the formation of AMCC may occur via a metabolic pathway, which includes the reactive intermediate methylisocyanate (MIC; Kestell et al. 1987; see Scheme 1). After the Bhopal incident with acute poisoning of MIC, it was shown that in the post-mortem blood of victims N-methylcarbamoyl adducts could be found at the N-terminal valine of haemoglobin (Hb; Sriramachari et al. 1991; Venkateswaran et al. 1992). The end product after work-up of the blood samples was 3-methyl-5-isopropylhydantoin (MIH). In our study, which was developed as a pilot study, we investigated whether such Hb adducts also could be found in workers after chronic exposure to DMF. For this purpose, we developed a capillary gas chromatography method using a mass-selective detector to determine N-methylcarbamoyl adducts at the N-terminal valine of Hb in blood samples of workers exposed to DMF in the polyacryl fibre industry.

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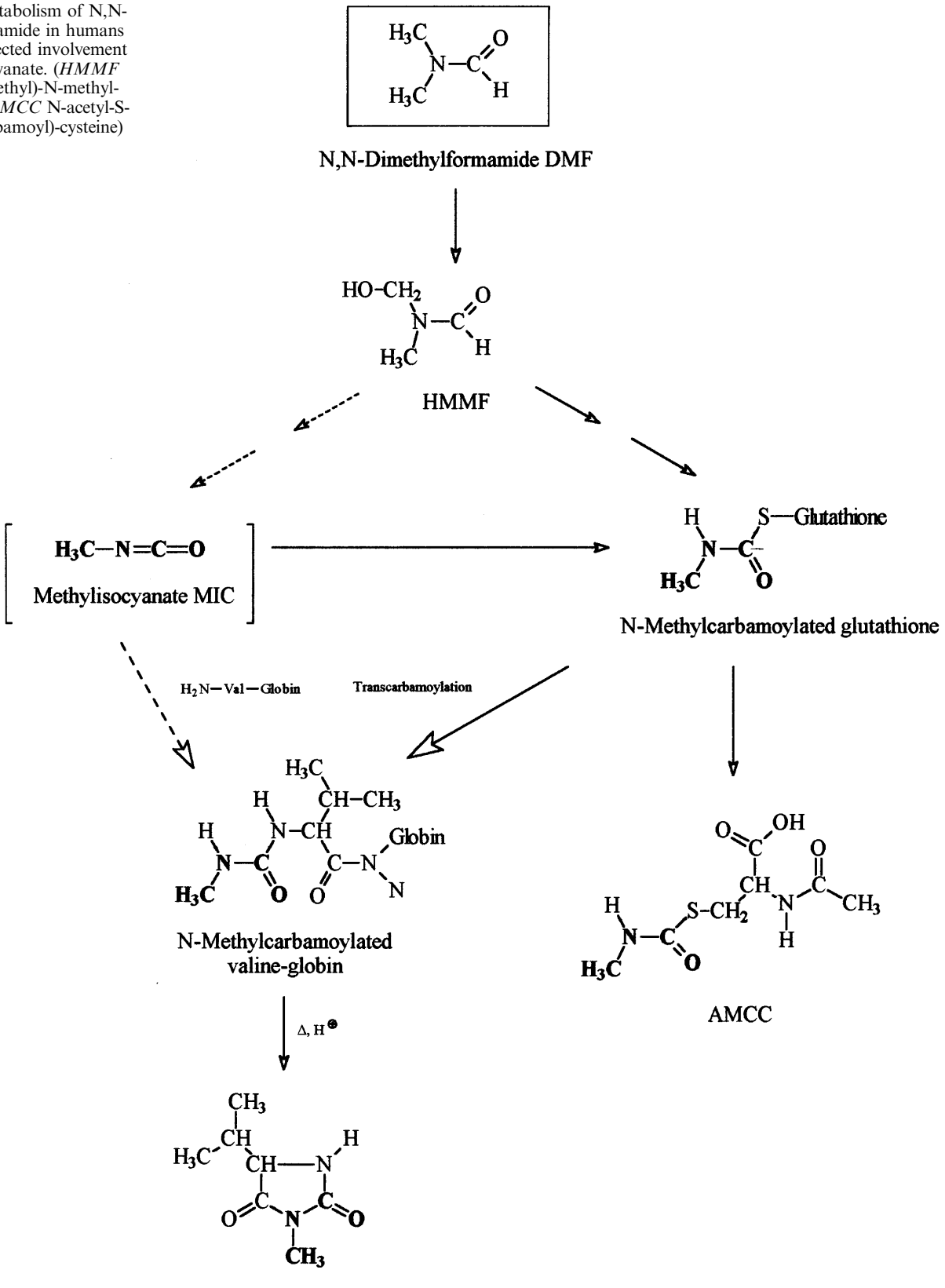
Scheme 1 Metabolism of N,N-dimethylformamide in humans with the suspected involvement of methylisocyanate. (*HMMF* N-(hydroxymethyl)-N-methylformamide, *AMCC* N-acetyl-S-(N-methylcarbamoyl)-cysteine)



Materials and methods

Synthesis of 3-methyl-5-isopropylhydantoin

MIH was synthesized by reaction of 936 mg D/L-valine (8 mmol) with 430 µl MIC (8 mmol) in 20 ml bidistilled water. The procedure and the further clean-up is described in detail in another publication (Ramachandran et al. 1988). MIH was identified by mass spectrometry (MS) and gas chromatography mass spectrometry (GC/MS) after electron impact ionization (EI). The major fragment ion of the standard is m/z 114. It is formed by a McLafferty rearrangement of the molecular radical ion m/z 156. The purity of the standard determined by nuclear magnetic resonance (NMR) spectroscopy at 400 MHz ^{1}H-NMR and ^{13}C-NMR was found to be >95%. The resulting standard was used to check the retention time and characteristic fragment ions of MIH during GC/MS/EI analysis, and was, moreover, used for calibration purposes.

Sample preparation and gas chromatography-mass selective detection

Blood samples (10 ml) were taken by venipuncture using a disposable syringe containing ethylenediaminetetraacetic acid (EDTA) as an anticoagulant. The red blood cells were separated by centrifugation; isolation and purification of globin is described in detail elsewhere (Bader et al. 1995). One hundred milligrams of globin was dissolved in 3 ml of a mixture of concentrated acetic acid and concentrated hydrochloric acid (1:1) and incubated for 1 h at 100 °C. The samples were extracted with 5 ml ethyl acetate and the organic phase was evaporated to dryness under a stream of nitrogen. The samples were dried in a vacuum desiccator over sodium hydroxide overnight and again resolved in ethyl acetate (1 ml), 1 µl of this solution was analysed by GC/MS/EI in the multiple ion detection mode (MID). Recorded ion traces for MIH were m/z 156 and 114 (ratio 1:160). GC/MS was performed on a gas chromatograph HP 5890 Series II fitted with a mass spectrometer HP 5989A. Samples analysed by GC/MS/EI were injected on a capillary column with the stationary phase DB-WAX (J&W Scientific, Folsom, USA). The column length was 60 m with an inner diameter of 0.32 mm and a film thickness of 0.25 µm. Helium was used as the carrier gas. The initial temperature of 120 °C was programmed to rise to 180 °C at 10 °C/min, held at that temperature for 25 min and then revised to 240 °C at 20 °C/min. That temperature was held for 25 min again.

Calibration process

Three millilitres of standard calibration solutions of MIH in a mixture of concentrated acetic acid and concentrated hydrochloric acid (10 mg/l–10 µg/l) were analysed similarly to the globin samples. These concentrations represent MIH levels between 1.92 µmol and 1.92 nmol MIH/g globin, respectively. The area counts of the peaks of MIH were plotted as a function of the concentration and this calibration curve was used for the calculation of the MIH content in each blood sample. The concentration of MIH is given in nmol/g globin. The detection limit calculated from a signal to noise ratio of 3:1 was 1.92 nmol MIH/g globin, which is a concentration of $\sim$10 µg/l.

Investigated subjects

Blood samples were investigated of ten male persons (six smoker, four non-smoker) who were exposed to DMF during the manufacture of fibres. The concentration of DMF in the air of the workplace was monitored during on 8-h shift by personal air samplers and ranged between 2.2 ppm and 53.7 ppm, respectively. Urine samples of these workers were also analysed to determine the total N-methyl-formamide (NMF) in urine using a method recommended by the Deutsche Forschungsgemeinschaft (DFG 1997a). The NMF levels were between 8.8 and 84.6 mg/l. Moreover, in ten control individuals (six male and four female, eight non-smokers, two smokers) without any occupational exposure to DMF the N-methylcarbamoylated Hb-adducts were determined simultaneously.

Results and discussion

In the globin samples of exposed workers as well as control individuals, the fragment ion m/z 114 could be detected. Only in the samples of the DMF exposed persons was the radical molecular ion m/z 156 also detectable. Moreover, the correct ratio between m/z 114 and m/z 156 (160:1) was observed. Although varying the GC temperature program, the retention times of the fragment ion m/z 114 for MIH remained identical in both investigated groups. GC/MS/EI-chromatograms of a control person and a worker exposed to DMF are shown in Fig. 1. In comparison to the adduct levels of the controls the N-methylcarbamoylated Hb concentrations in samples of the DMF exposed persons are up to 100-fold higher. Neither in exposed or in control persons were the N-methylcarbamoylated globin adduct levels influenced by sex and smoking habits. MIH could not be analysed in blank samples, which were also included in each analytical series. The concentrations of MIH are given in Table 1.

These results show that DMF exposure leads to identical Hb-adducts as those found after exposure to MIC (Scheme 1). The formation of N-methylcarbamoylated Hb in humans, however, is still unknown although there are two possible explanations. Firstly, MIC may be formed during the metabolism of DMF and react with the N-terminal valine in a direct manner. As there is an excess of other nucleophilic substances, e.g. sulfhydryl compounds (e.g. glutathione, etc.) in the human body, N-methylcarbamoylation of these substances is much more likely. N-methylcarbamoylated glutathione was actually determined (Slatter et al. 1991). As this molecular species is known to function as a 'carrier' substance (Pearson et al. 1991; Baillie and Slatter 1991), the second possibility for the formation of the adducts found is the transfer of the reactive N-methylcarbamoyl group from glutathione to the N-terminal valine of Hb.

The method used in our pilot study was not sensitive enough to identify N-methylcarbamoylated N-terminal valine in control persons definitively. Nevertheless, the occurrence is possible of N-methylcarbamoylated globin in individuals not exposed to DMF. However, the formation of such an adduct would afford two subsequent reactions: first of all carbamoylation of Hb by carbamoylphosphate which occurs in the human body in high amounts. Carbamoylphosphate is known to transfer the carbamoyl group to proteins in humans (Crist et al. 1973). A subsequent methylation at the nitrogen of the carbamoyl group would yield the adduct in question.

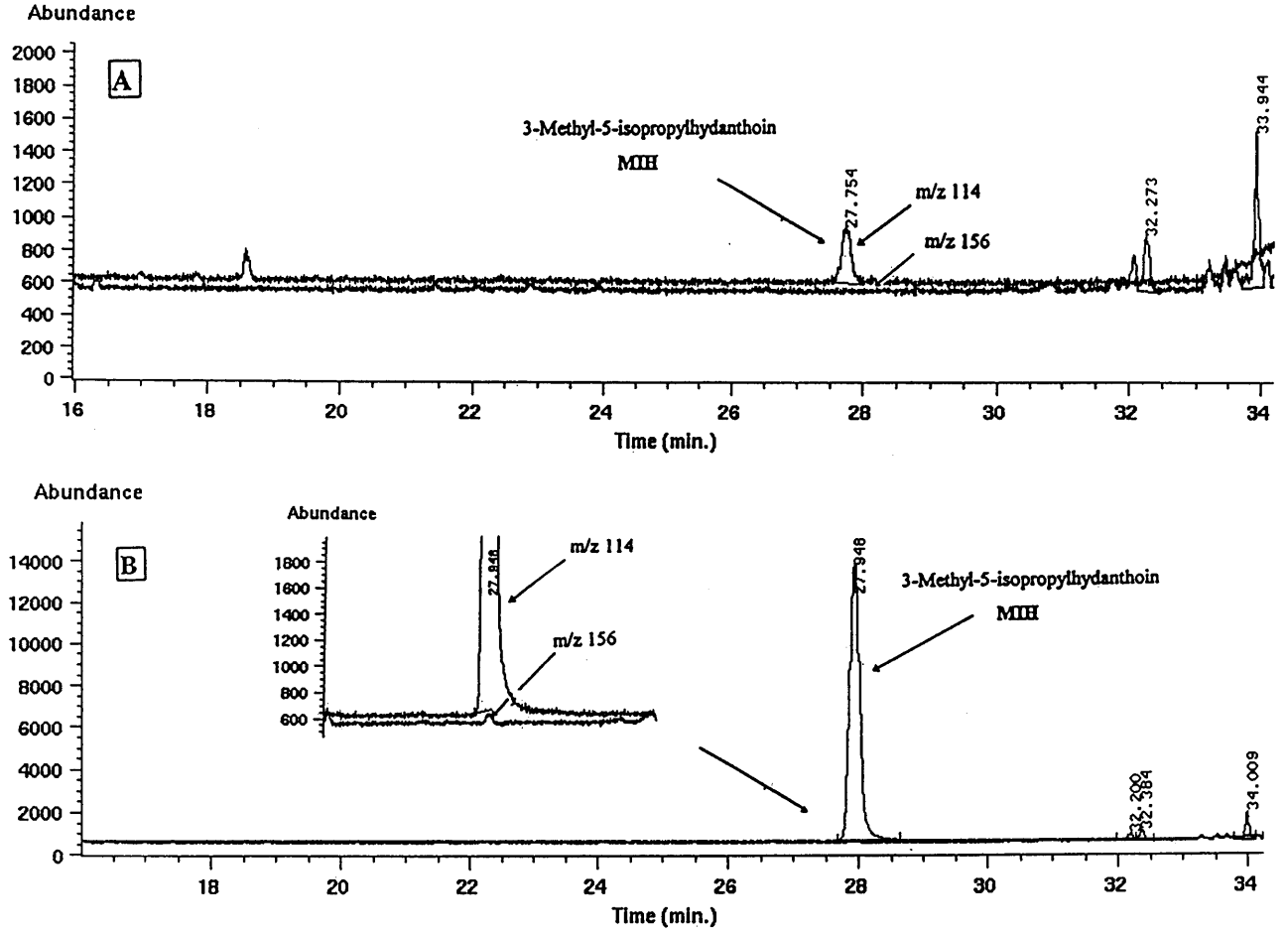


Fig. 1 Gas chromatography/mass spectrometry/electron impact ionization (GC/MS/EI) chromatogram of a globin sample of an unexposed person (**A**) and a globin sample of an N,N-dimethylformamide (DMF) exposed person (**B**) with an average DMF exposure via the ambient air of ~16 ppm. The monitored ions are m/z 156 and 114 for 3-methyl-5-isopropylhydantoin (MIH). The focused part of chromatogram B is scaled in the same manner as chromatogram A and shows in detail the signal of the radical molecular ion m/z 156 in the exposed sample

This background methylation in human Hb is a common process as well and occurs via methyltransferases or other activated methyl groups (Törnquist et al. 1988). However, the formation of N-methylcarbamoylated haemoglobin in persons not exposed to DMF needs further research.

In further studies the currently used calibration method with MIH must be replaced by a calibration method using standard substances such as N-methylcarbamoylated globin or other peptides, which could more effectively simulate the N-terminal sequence of Hb. It is common knowledge (DFG 1997b; Törnquist et al. 1992) that the latter materials are much more suitable for calibration of any kind of Hb adducts. The improved calibration technique including a complete validation of the method (using an internal standard, establishment of losses during sample preparation, within-series and between-day imprecision, recovery etc.) should be established prior to any routine application. Nevertheless, the ratio found between the N-methylcarbamoylated adduct concentrations in samples from persons exposed to DMF and those non-exposed to DMF remains the same. In summary, we were able to show for the first time that Hb adducts are formed during the metabolism of DMF in humans. The mechanism for the formation of such adducts as well as its relevance for occupational health is a subject of further research.

Table 1 Results of the analysis of N-methylcarbamoylated haemoglobin adduct concentrations in workers exposed to N,N-dimethylformamide and in non-exposed individuals. The adduct levels are given in nmol 3-methyl-5-isopropylhydantoin (MIH) per g globin

	Median (nmol/g)	Mean ± SD (nmol/g)	Range (nmol/g)
Exposed ($n = 10$)	456.21	450.76 ± 274.92	75.06–976.53
Non-exposed ($n = 10$)	6.56	7.53 ± 2.60	4.54–12.14

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