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Short communication

Analysis of urinary *N*-acetyl-*S*-(*N*-methylcarbamoyl)cysteine, the mercapturic acid derived from *N*,*N*-dimethylformamide

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Abstract

Human biotransformation of the industrial solvent *N*,*N*-dimethylformamide gives raise to *N*-acetyl-*S*-(*N*-methylcarbamoyl)cysteine (AMCC) which has the longest half-life (about 23 h) among urinary metabolites of *N*,*N*-dimethylformamide. It could be used for monitoring industrial exposure over several workdays, by measuring it in urine samples collected at the end of the working week. This is consistent with the suggestions of the American Conference of Governmental Industrial Hygienists, which established a limit of 40 mg/l for the year 2000. An easy, cheap and user-friendly method has been developed for determination of urinary AMCC. Unlike currently available methods, it requires neither a time-consuming preparation phase nor gas chromatographic analysis with a nitrogen-phosphorus or mass detector. The method uses high-performance liquid chromatography (HPLC), with an UV detector at 436 nm. A 10-μl volume of urine is added to a carbonate–hydrogen carbonate buffer and mixed with a dabsyl chloride solution in acetonitrile. The reaction between AMCC and the reagent is performed at 70°C for 10 min. The 'dabsylated' product is stable for at least 12 h. After brief centrifugation, the solution is ready for HPLC analysis using a C_{18} column (250×4.6 mm, 5 μm). The method is sensitive (detection limit 1.8 mg/l) and specific. It identified urinary AMCC in urine of 40 subjects not exposed to *N*,*N*-dimethylformamide with a median concentration of 3.9 mg/l. In urine samples from 20 workers exposed to *N*,*N*-dimethylformamide (5–40.8 mg/m^3), AMCC concentrations ranged from 16 to 170 mg/l. Industrial toxicology laboratories with limited instrumentation will be able to use it in the biological monitoring of workers exposed to *N*,*N*-dimethylformamide.

Keywords: *N*-Acetyl-*S*-(*N*-methylcarbamoyl)cysteine; Mercapturic acid; *N*-*N*-Dimethylformamide

1. Introduction

N,*N*-Dimethylformamide is a highly polar industrial solvent, used in the production of various polymers, coating agents, printing inks, pharmaceutical products and adhesives. Occupational exposure to *N*,*N*-dimethylformamide, via inhalation or dermal absorption, can lead to irritating and toxic effects at different sites. *N*,*N*-dimethylformamide is a suspected carcinogen [1].

N-Acetyl-*S*-(*N*-methylcarbamoyl)cysteine (AMCC), an important metabolite of *N*,*N*-dimethylformamide, is generated during experimental human

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exposure to this solvent [2]. AMCC is one of the mercapturic acids (*N*-acetyl-L-cysteine *S*-conjugates), increasingly used in biological monitoring of human exposure to environmental and industrial chemicals. Mercapturic acids are formed from glutathione (GSH) *S*-conjugates. Although this pathway can lead to different end products, the formation of mercapturic acids is the predominant route in most species, including man. Most mercapturic acids are excreted with a relatively short half-life, allowing direct evaluation of occupational exposure [3].

In the liver, biotransformation of *N*,*N*-dimethylformamide and its derivative *N*-methylformamide seems to give rise to methylisocyanate as a reactive metabolite [4].

The metabolic pathway which forms AMCC from *N*,*N*-dimethylformamide is summarised in Fig. 1.

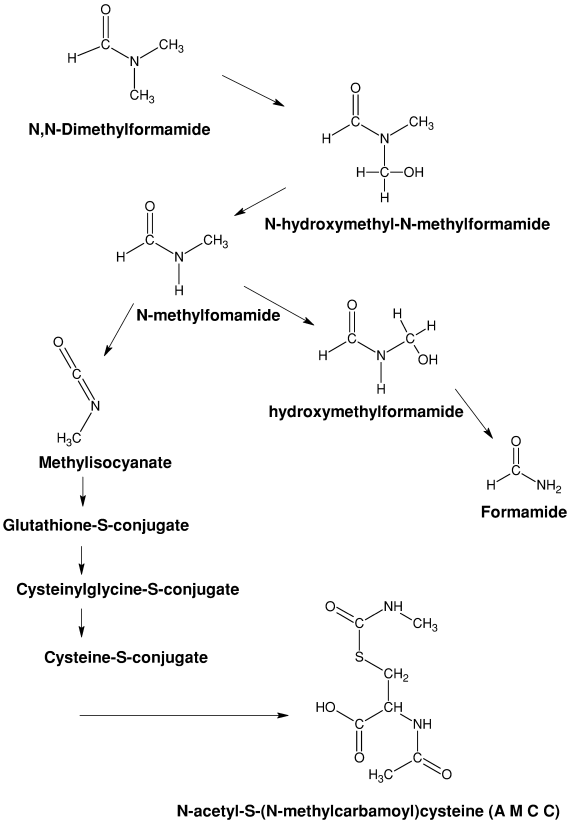

Fig. 1. Metabolic pathway of *N*,*N*-dimethylformamide in human subjects and synthesis of mercapturic acid (AMCC) after formation of methylisocyanate.

Oxidation of one methyl group of *N*,*N*-dimethylformamide forms *N*-hydroxymethyl-*N*-methylformamide. Other metabolites of *N*,*N*-dimethylformamide are *N*-methylformamide, hydroxymethylformamide, and formamide. A possible derivative of *N*-methylformamide is methylisocyanate, with which glutathione can easily react to form a glutathione *S*-conjugate. Indeed, in experimental rats, about 25% of intraperitoneally injected methylisocyanate appeared in 24-h urine collections as AMCC [5].

This metabolic pathway may favour the hepatotoxicity of *N*,*N*-dimethylformamide and of some of its metabolites [6].

N-Methylformamide is the common urinary indicator for biological monitoring of occupational exposure [7,8]. The concentration of this metabolite in urine samples collected at the end of the workshift indicates the severity of exposure during the workday; the half-life of *N*-methylformamide in the body and in urine being fairly short (3–5 h) [9]. Since the half-life of AMCC is about 23 h [9–11], it tends to accumulate in the human body. Its analysis may enable us to evaluate occupational exposure over several days, and perhaps an entire working week [12].

In 2000 the American Conference of Governmental Industrial Hygienists (ACGIH) [7] established that AMCC can be used as a biological index for occupational exposure to *N*,*N*-dimethylformamide. The limit in urine samples collected prior to the last shift of the working week was set at 40 mg/l.

Current methods for measurement of AMCC in urine are quite expensive, requiring a long preparation phase or complex instrumentation. Mraz [13] has proposed a method for AMCC in urine, involving breakdown of the AMCC molecule to ethyl *N*-methylcarbamate. The latter is then quantified using a gas chromatograph connected to a NP detector. The limit of detection (LOD) for AMCC was 0.2–0.4 mg/l, with a coefficient of variation (C.V.) between 10 and 23%. The method of Casal Lareo et al. [14] is time-consuming. Urine samples are extracted, dried and derivatized with BSTFA (*N*,*O*-bis(trimethylsilyl) trifluoroacetamide). Quantification of AMCC is then performed by gas chromatographic–mass spectrometric analysis. The LOD for AMCC was 1 mg/l, with a C.V. of 13–15%.

Ghittori et al. [15] have proposed a HPLC method with UV detection at 196 nm, after processing the AMCC with solid-phase extraction methodology; the LOD for AMCC was 0.9 mg/l. Each analysis takes about 80 min. Kafferlein and Angerer [11] have developed a method for simultaneous determination of *N*-methylformamide (NMF) and AMCC, which is broken down to ethyl-*N*-methylcarbamate. The purification of urine samples is time-consuming; separation and detection are achieved by gas chromatography with a thermoionic sensitive detector, LOD for AMCC being 0.5 mg/l. These data were confirmed by mass spectrometry.

Against this background, we propose a cheap, user-friendly method for assay of AMCC, which is derivatized with dabsyl chloride and analysed by HPLC with an UV detector at 436 nm.

2. Materials and methods

2.1. Reagents and standards

AMCC was synthesised as suggested by Mraz [13]. The dabsyl chloride was purchased from Pierce. Anhydrous sodium hydrogen carbonate and anhydrous sodium carbonate (laboratory grade purity), water and acetonitrile for HPLC were obtained from C. Erba (Milan, Italy).

2.2. Instrumentation

A Hewlett-Packard (HP) 1090 L HPLC chromatograph with HP 1040A detector was used. ASPEC XL (from Gilson) was used for the automatic injection; the volume of the injection loop was 100 μl. A ChemStation with HP software was used for acquisition and processing of data. The chromatographic separation was obtained through a C_{18} column (Gilson; 250×4.6 mm, 5 μm), with a LiChrospher 100 RP-18-5 μm guard column (Gilson; 40×4 mm, 5 μm). The same results were achieved with a LiChrospher 100 RP-18 chromatographic column (Merck; 250×4.6 mm, 5 μm). Analysis was carried out at ambient temperature, using acetonitrile and water as the mobile phase. The acetonitrile concentration, which was 30% at the beginning of the chromatographic run, was increased to 80% in 23 min. The flow-rate was set at 1 ml/min. AMCC determination was performed by recording the UV response of the eluate at 436 nm.

2.3. Preparation of the sample

A buffer solution of carbonate–hydrogen carbonate (pH 9.27) was prepared with a mixture of: (a) 2 ml anhydrous sodium carbonate 0.2 *M* solution; (b) 23 ml anhydrous sodium hydrogen carbonate 0.2 *M* solution; and (c) 100 ml of water. A 10-μl volume of urine was added to 250 μl of the carbonate–hydrogen carbonate buffer and 250 μl of dabsyl chloride, dissolved in acetonitrile at a concentration of 6 m*M*. After mixing and briefly shaking, the sample was placed in a thermostat for 10 min at 70°C. It was then centrifuged for 3 min at 1300 cfr, after which the supernatant was ready for injection into the HPLC.

The volume of urine in the buffer was based on preparatory trials, evaluating linearity of the analytical response by adding 5, 7.5, 10, 20 and 30 μl of AMCC solution in water and urine (100 mg/l). Water solutions of AMCC from 5 to 30 μl caused no modification of the analytical response, which was constantly linear. By contrast, increasing the volume of urine affected the reaction between AMCC and dabsyl chloride. When 30 μl of urine were added to the buffer, the result was close to 30% of that obtained with the water solution. When 10 μl of urine were added, results were between 90 and 95% of those obtained with the water solution. Analytical response was not affected by pH.

The method was tested on 60 urine samples: 40 from subjects not exposed to *N*,*N*-dimethylformamide (25 men, 15 women; 19 smokers, 21 nonsmokers), and 20 from male workers exposed to the solvent in a synthetic leather factory. Individual workers' exposure was measured by personal diffusive samplers.

2.4. Preparation of calibration curve

The calibration curve was obtained starting from a solution of AMCC in water (3 g/l). AMCC solutions of 5, 10, 20, 50, 100, and 200 mg/l in water and urine were obtained. The calibration curves for AMCC in urine on three different days gave linear

regressions between the peak areas and AMCC concentrations, with correlation coefficients higher than 0.999. The three regression lines were: $y=0.276x+4.4$ $r=0.9994$; $y=0.279x+1.8$ $r=0.9994$; $y=0.254x+1.03$ $r=0.9991$. These were close to the calibration curve of AMCC in water ($y=0.32x+2.5$ $r=0.9992$).

2.5. Validation of the method

Reliability and repeatability of the method were evaluated by calculating coefficients of variation for determinations of AMCC solutions in urine (5 and 50 mg/l). Five determinations a day were performed on three different days (during two different weeks). Accuracy was estimated by closeness of agreement between the result of a measurement and the true value of the measurand.

2.6. Limit of detection

Detection limits were calculated from six injections of blank urine, as suggested by Miller and Miller [16]. The LOD was 1.8 mg/l.

3. Results and discussion

3.1. Chromatographic separation

Fig. 2 shows the chromatograms of three different urine samples: (A) a sample from a subject not exposed to dimethylformamide; (B) the same sample, with 5 mg/l of AMCC added; (C) a sample from a worker exposed to dimethylformamide. The AMCC retention time was about 20 min.

In the urine of the non-exposed subject (Fig. 2A), as in all the urine samples obtained from the 40 subjects not exposed to dimethylformamide, there was a small peak at the same AMCC retention time.

In the 40 non-exposed subjects, variability of this peak was limited (Table 1). There was no statistical difference in AMCC concentration between non-smokers and smokers, or males and females. Kafferlein and Angerer [11] also report a small quantity of AMCC in urine samples obtained from 42 subjects not exposed to *N*,*N*-dimethylformamide, with a mean concentration of 0.04 mg/l. These authors confirmed the background levels of AMCC in the general population by analysing urine extracts through a gas chromatographic technique; of the two detectors they used, one was thermoionic sensitive and the other mass-spectrometric. Results are appreciably lower than ours. No information concerning the possible endogenous or exogenous sources of AMCC is provided.

3.2. Validation of the method

The accuracy, reliability and repeatability of the method are indicated in Table 2. The coefficients of variation were equal to or less than 10%.

3.3. Stability of AMCC in urine

Five urine samples with added AMCC (range, 5–200 mg/l) showed no statistical differences between analysis just after collection and after 8 days at 4°C.

Ten urine samples, obtained from workers exposed to *N*,*N*-dimethylformamide and stored for 3 months at −20°C, gave similar results to those within a few days of collection. The second measurement showed an average increase of about 2% over the first.

3.4. Urinary AMCC concentration

In urine samples from 20 workers exposed to dimethylformamide (5–40.8 mg/m^3), AMCC concentrations ranged from 16 to 170 mg/l (average, 63.5 mg/l; median, 59.1 mg/l; geometrical mean, 55.2 mg/l). ACGIH [7] has recently included AMCC among indices of occupational exposure to *N*,*N*-dimethylformamide, with a limit of 40 mg/l in urine samples collected before the last shift of the working week. The suggested value is related to an environmental threshold value (time-weighted average) of 10 ppm and potential skin absorption.

4. Conclusions

The present paper describes a method for analys-

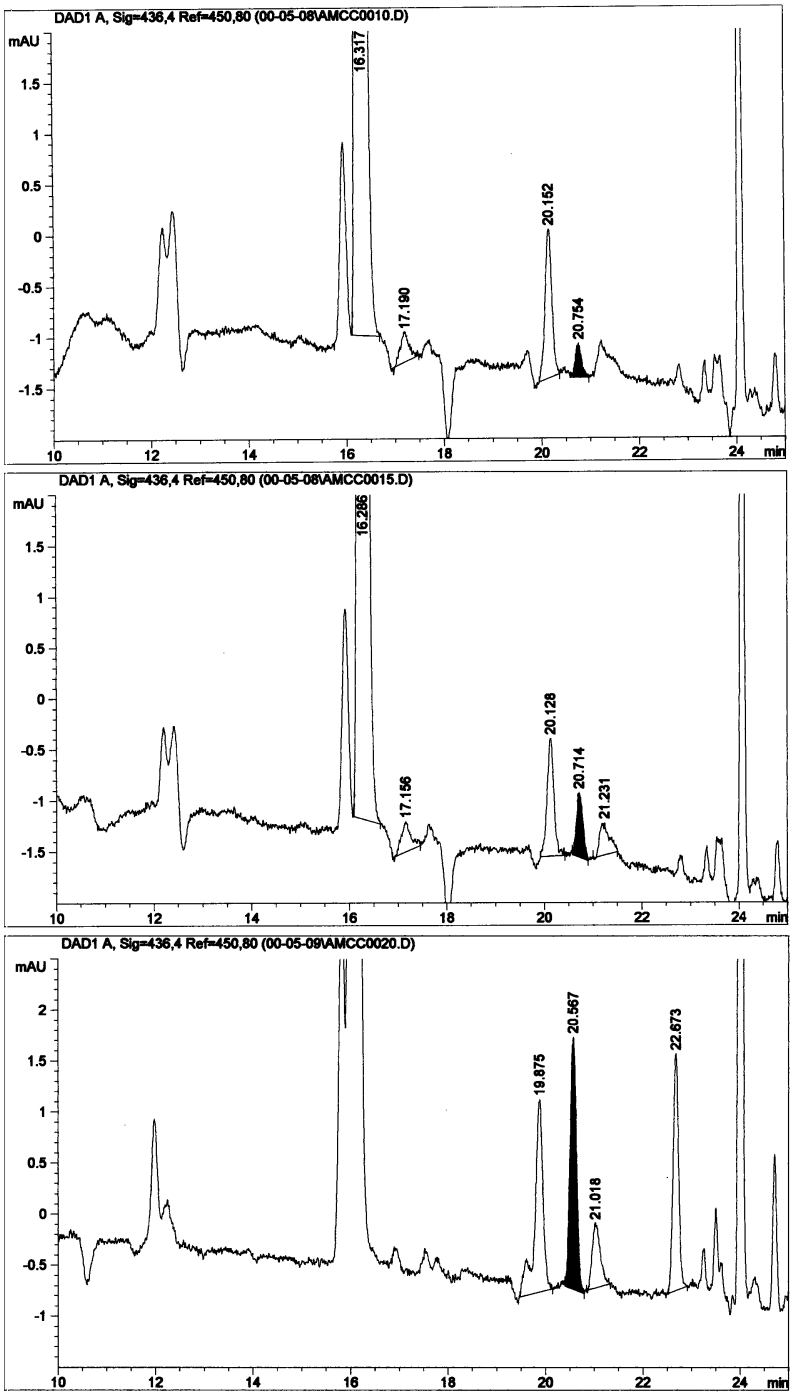

Fig. 2. Chromatograms of three different urine samples: (A) a sample from a subject not exposed to *N*,*N*-dimethylformamide; (B) the same sample, with 5 mg/l of AMCC added (black peak); (C) a sample from a worker exposed to *N*,*N*-dimethylformamide.

Table 1
Statistical parameters of AMCC in urine samples (mg/l) from subjects not exposed to *N,N*-dimethylformamide, distinguishing between smokers and non-smokers and men and women

	All data	Non-smokers	Smokers	Men	Women
Number of samples	40	21	19	25	15
Median	3.9	3.8	4.4	3.8	5
Geometrical mean	4.4	4.5	4.2	4.2	4.6
Arithmetical mean	4.7	5	4.5	4.6	5.1
Standard deviation	2.1	2.5	1.6	1.9	2.4
Minimum	1.8	1.9	1.8	1.8	1.9
Maximum	10.5	10.5	7.3	10.3	10.5

Table 2
Accuracy, reliability and repeatability of AMCC solutions at concentrations of 5 and 50 mg/l (five determinations per day, on three different days)

	5 mg/l	50 mg/l
Accuracy (%)	110	105
Reliability (C.V.[a])	6	6
Repeatability (C.V.[a])	10	5

[a] Variation coefficient (%).

ing AMCC in urine by HPLC, with an UV detector at 436 nm. This method is not only sensitive and specific for evaluation of occupational exposure to *N,N*-dimethylformamide, but is also cheap and user-friendly. Currently available methods are expensive and time-consuming, in terms of sample preparation and analysis. The availability of an easy method for industrial toxicology will provide more information on biological monitoring of urinary AMCC in workers exposed to *N,N*-dimethylformamide.

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References

[1] International Agency Research of Cancer, Monograph Evaluation of Carcinogenic Risk of Chemicals in Humans. 47 (1989) 171.
[2] J. Mráz, F. Turacek, J. Chromatogr. 43 (1987) 399.
[3] B.M. De Rooij, J.N.M. Commandeur, N.P.E. Vermeulen, Biomarkers 3 (1998) 239.
[4] A.E. Mutlib, R.E. Talaat, J.G. Slatter, F.S. Abbott, Drug Metab. Disp. 18 (1990) 1038.
[5] J.G. Slatter, M.S. Rashed, P.G. Pearson, D.H. Han, T.A. Baillie, Chem. Res. Toxicol. 4 (1991) 157.
[6] P. Kestell, M.D. Threadgill, A. Gescher, A.P. Gledhill, P.B. Shaw, A.J. Farmer, J. Pharmacol. Exp. Thep. 240 (1987) 265.
[7] American Conference of Governmental Industrial Hygienists (2000) TLVs®-Cincinnati (OH).
[8] Deutsche Forschungsgemeinschaft. MAK and BAT values 2000. VCH, Weinheim.
[9] J. Mráz, H. Nohová, Int. Arch. Occup. Environ. Health. 64 (1992) 85.
[10] A. Casal Lareo, L. Perbellini, Int. Arch. Occup. Environ. Health. 67 (1995) 47.
[11] H.U. Kafferlein, J. Angerer, J. Chromatogr. B. 734 (1999) 285.
[12] T. Sakai, H. Kageyama, T. Araki, T. Yosida, T. Kuribayashi, Y. Masuyama, Int. Arch. Occup. Environ. Health. 67 (1995) 125.
[13] J. Mráz, J. Chromatogr. 431 (1988) 361.
[14] A. Casal Lareo, A. Perico, P. Bavazzano, C. Soave, L. Perbellini, Int. Arch. Occup. Environ. Health. 67 (1995) 41.
[15] S. Ghittori, L. Maestri, D. Cottica, L. Perbellini, P. Maraccini, M. Imbriani, in: 3rd Int. Scient. Conference of Int. Occup. Hyg. Assoc, Crans Montana, September 13–18, 1997.
[16] J.C. Miller, J.N. Miller, Statistics Anal. Chem. 4 (1986) 96.