ELSEVIER

Journal of Chromatography B, 778 (2002) 231–236

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Measurement of urinary *N*-acetyl-*S*-(*N*-methylcarbamoyl)cysteine by high-performance liquid chromatography with direct ultraviolet detection

Marcello Imbriani[a], Sara Negri[b], Sergio Ghittori[b], Luciano Maestri[b,*]

[a]*Dipartimento di Medicina Preventiva, Occupazionale e di Comunita': II Sezione di Medicina del Lavoro, Pavia, Italy*
[b]*Fondazione 'S. Maugeri', IRCCS, via Ferrata 8, 27100 Pavia, Italy*

Abstract

A new high-performance liquid chromatographic (HPLC) method is described for the determination of urinary *N*-acetyl-*S*-(*N*-methylcarbamoyl)cysteine (AMCC), the final product of the conjugation reaction between a metabolic intermediate of *N*,*N*-dimethylformamide (DMF) and glutathione. Urine samples were purified by C_{18} solid-phase extraction and then directly analysed by HPLC with an Aminex Ion Exclusion HPX-87H column maintained at 25°C and a UV detector set at 196 nm. Under isocratic conditions (2.4 m*M* sulphuric acid, flow-rate = 0.6 ml/min) AMCC eluted at 20.2 min. The reproducibility (C.V.%) was 1.3–2.7% (intra- and inter-assay, $N=5$); the accuracy was 98.0±1.7% at 10 mg/l and 101.9±1.5% at 800 mg/l (mean±SD, $N=3$). AMCC was measured in urine from 22 exposed subjects. A strong correlation was found between AMCC and environmental DMF [AMCC (mg/g creatinine) = 3.40 × DMF (mg/m^3) + 3.07; $r=0.95$], while in the urine of 20 unexposed subjects the concentration of AMCC was constantly below the detection limit of the method (0.9 mg/l in urine). The method described appears to be useful for the biological monitoring of DMF exposure.

Keywords: *N*-Acetyl-*S*-(*N*-methylcarbamoyl)cysteine

1. Introduction

N,*N*-Dimethylformamide (DMF) is a volatile liquid widely used as industrial solvent, particularly in the manufacture of synthetic leather [1]. It has been shown to cause pancreatic disorders, hepatic damage, intolerance to alcohol, and is suspected of involvement in the generation of certain occupational malignancies [2]. DMF was identified by the Environmental Protection Agency (EPA) and the Agency for Toxic Substances and Disease Register (ATSDR) as a substance which poses a significant potential threat to human health.

The absorption of DMF may occur through inhalation of vapours or by direct contact with skin. DMF is rapidly metabolized in vivo and only a small fraction of the administered dose is excreted unchanged in urine and by the gastrointestinal tract. The biological monitoring of occupational exposure to DMF is currently performed by the gas chromatography (GC) determination of *N*-methylformamide [3]; this urinary indicator is used to assess the severity of exposure to DMF during the workday [4] since it has a half-life of 3–5 h. However, this

*Corresponding author. Tel.: +39-382-556-600; fax: +39-382-556-615.
E-mail address: siclav@unipv.it (L. Maestri).

1570-0232/02/$ – see front matter
PII: S0378-4347(01)00453-4

compound is mainly a thermal degradation product of *N*-hydroxymethyl-*N*-methylformamide, the 'true' major metabolite of DMF [5–7].

An alternative pathway of the metabolism of DMF (Fig. 1) involves the conjugation of its reactive intermediate *N*-methylisocyanate with glutathione, resulting in the excretion of *N*-acetyl-*S*-(*N*-methylcarbamoyl)cysteine (AMCC), the mercapturic acid of DMF [8]. AMCC appears to be an attractive biomarker because it seems to be closely related to DMF toxicity [9]. The half life of AMCC is about 23 h [10,11]. Thus, the AMCC concentration in urine samples collected at the end of the week may correlate with the exposure to DMF during the whole working week [12].

Urinary AMCC can be measured by GC with nitrogen-selective [13] or thermionic sensitive [14] detection after the conversion of AMCC to ethyl-*N*-methylcarbamate or by GC with mass spectrometric detection (GC–MS) [14,15].

In this paper we propose a method for the direct measurement of urinary AMCC by high-performance liquid chromatography (HPLC) with UV detection. The method requires no derivatization of the compound and has the sensitivity to determine AMCC under conditions of occupational exposure to DMF.

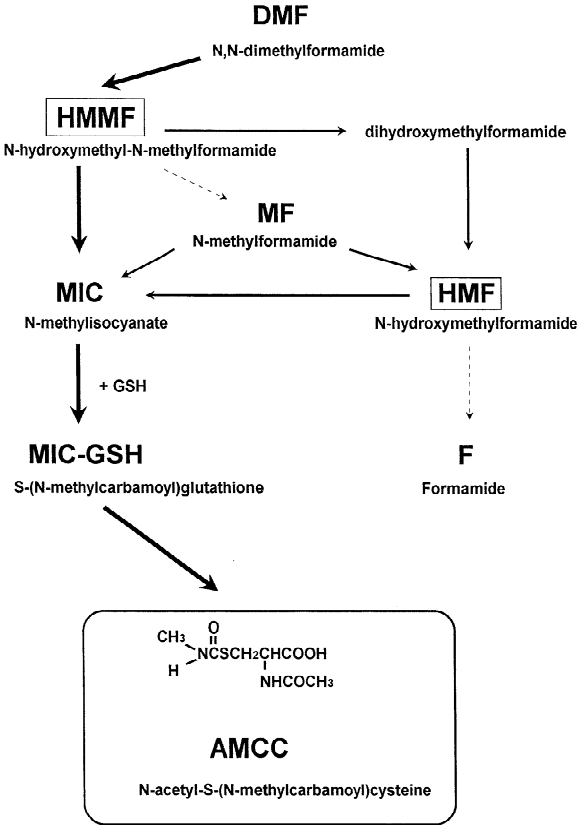

Fig. 1. Scheme of the metabolic pathway of *N*,*N*-dimethylformamide (DMF).

2. Experimental

2.1. Chemicals

Solid phase extraction cartridges (SPE, Isolute C_{18}-EC, 500 mg/3 ml) were obtained from International Sorbent Technology (IST, Hengoed, UK). The AMCC standard was obtained by custom synthesis from Alchemy S.r.l (Bologna, Italy), and the degree of purity was 97%. Water and methanol were of HPLC-grade (BDH, Poole, UK), while sulphuric acid, phosphoric acid, hydrochloric acid and all other reagents were of analytical-grade (BDH).

2.2. Apparatus

The HPLC system consisted of a Waters 600 E pump (Milford, MA, USA), equipped with an Aminex Ion Exclusion HPX-87H, 300×7.8 mm I.D., 9 μm column (BioRad, Hercules, CA, USA), a Waters 484 UV detector, a Waters Wisp 715 autosampler and a NEC APC-IV computer (Boxborough, MA, USA) for the acquisition and processing of the data. The concentration of DMF in the air of the workplace was analysed by a gas chromatograph HP 5880 A (Hewlett-Packard, Waldbrun, Germany) equipped with a nitrogen–phosphorus detector (NPD). The column (1.5 m×6 mm O.D. glass) contained 10% UCON 50-HB-5100 and 2% KOH on 100/120 mesh Chromosorb WHP. A Stat View Software was used for statistical analysis. The UV spectrum of AMCC was determined by means of a Lambda Bio 20 UV–Vis spectrometer (Perkin-Elmer, Norwalk, CT, USA).

2.3. Collection and analysis of the environmental samples

The exposure levels of the workers were determined by personal sampling. Breathing zone air samples were collected separately by means of passive samplers (TK 200, Baregio, Italy), during the 4 h of the morning and the 4 h of the afternoon.

The measurement of DMF was performed by one of the currently available methods [16]. Briefly, each sample was desorbed with methanol and analysed using the gas chromatograph. The carrier gas was helium at a flow-rate of 30 ml/min. The analysis was performed isothermally at 140°C. The temperatures of the injector and of the detector were 180 and 250°C, respectively.

2.4. Determination of AMCC in urine

The urine samples were frozen at −20°C immediately after collection and thawed just before analysis. Samples (2 ml) were acidified with 50 μl of concentrated HCl and centrifuged for 10 min at 1600 *g*. Then 1 ml was loaded onto C_{18} end-capped disposable cartridges prewashed with 3 ml of methanol and 3 ml of 1% phosphoric acid. After sample loading, the cartridges were washed with 3 ml of 1% phosphoric acid, then AMCC was eluted with an additional 3-ml aliquot of the same solution. Aliquots of 10 ml of the purified samples were directly injected onto the HPLC system.

The chromatographic analysis of the biological samples was carried out at 25°C using an isocratic elution with 2.4 m*M* sulphuric acid at a flow-rate of 0.6 ml/min. The UV detector was set at 196 nm. Under these conditions the AMCC peak had a retention time of 20.2 min.

2.5. Validation of the method

In order to obtain the calibration curves and to verify the influence of the creatinine concentration on the purification step, the AMCC standard (5–1000 mg/l) was added to the urine samples taken from two non-exposed subjects, with a creatinine concentration of 0.35 and 3.1 g/l, respectively. The samples were then purified and analysed.

The intra-assay reproducibility was calculated on three spiked urine samples with AMCC concentrations of 3.5, 55.0 and 555 mg/l, respectively. Each sample was purified and then analysed five times. The same urine samples were used also to calculate the inter-assay reproducibility: each sample was divided into five aliquots, then each aliquot was purified and analysed separately. The standard deviation of the measured amounts was divided for the mean value and the reproducibility was expressed as a percentage of the coefficient of variation (intra- and inter-assay C.V.%).

The accuracy was calculated on two concentration levels (10 and 800 mg/l). Each sample was purified and analysed three times. The amounts of AMCC found were then compared with the actual values and the accuracy was expressed as a percentage of the recovery (mean±SD). The limit of detection was calculated for a signal-to-noise ratio of 3.

3. Results and discussion

AMCC is the final product of the conjugation between glutathione and *N*-methylisocyanate — a reactive metabolite of DMF — which is considered responsible for most of the toxic effects of the solvent [9]. Therefore, determination of AMCC in urine may be useful for the biological monitoring of subjects exposed to DMF [12]. At present the methods described in the literature for the determination of AMCC require a previous reaction of the molecule before the analysis by GC with different kinds of detector [13–15].

We attempted to develop an alternative method based on HPLC–UV which needs neither derivatization nor expensive equipment. In this respect, two main problems had to be overcome: the lack of a chromophore in the AMCC molecule and its high polarity. The former characteristic obliged us to set the detector at a low wavelength (Fig. 2), but determination under these conditions is rather unspecific, since most organic compounds are detected. As a consequence, a critical step in the development of the method was the separation of AMCC from most interferences.

First, we injected urine samples diluted with the

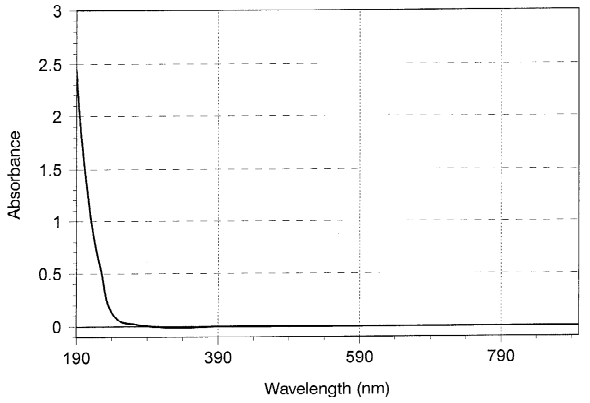

Fig. 2. Absorption spectrum of AMCC dissolved in water (AMCC concentration = 24 mg/l).

mobile phase without any purification treatment onto the HPLC system. However, complex chromatograms with very long times of analysis caused by peaks having high retention times were obtained.

The clean-up method based on solid-phase extraction (SPE) cartridges gave samples purified enough to allow a subsequent isocratic separation of AMCC from both the interfering and highly retained compounds. The SPE method developed was very easy and needed no evaporation of the sample. The recovery of the analyte was very high (mean = 95.4) and reproducible (C.V. = 1.7%). Therefore, it was not necessary to use any internal standard.

Since AMCC is a very polar compound, a suitable separation on reversed-phase analytical columns was difficult to obtain and AMCC co-eluted with several interferences (data not shown). On the contrary, the use of a resin-based column allowed us to achieve a satisfactory separation of AMCC. A HPX column gives multiple modes of interaction: ion exclusion, ion-exchange, ligand-exchange, size exclusion, reversed-phase and normal-phase partitioning [17]. The only disadvantage was the necessity of using exclusively isocratic runs since with this type of column it was difficult to perform gradient runs. Preliminary studies showed that the composition of the mobile phase (the concentration of sulphuric acid and acetonitrile) and the temperature of the column influenced the retention time of AMCC and its separation from interfering compounds. The best separation was achieved by eluting the column with a 2.4 m*M* sulphuric acid solution without any addition of acetonitrile, at the flow-rate of 0.6 ml/min and with the column temperature maintained at 25°C. Under these conditions, the peak of AMCC eluted at 20.2 min (Fig. 3). When 10 ml of sample were injected, the limit of detection was 0.9 mg/l. The calibration curve on spiked urine was linear up to 1000 mg/l of AMCC and it was not affected by the content of creatinine.

A typical calibration curve equation was:

$$Y = 2939X + 459 \quad (r = 0.999979, N = 5)$$

where Y = peak area and X = AMCC concentration (mg/l). The reproducibility was between 1.3 and 2.7% [intra- and inter-assay (C.V.%), respectively].

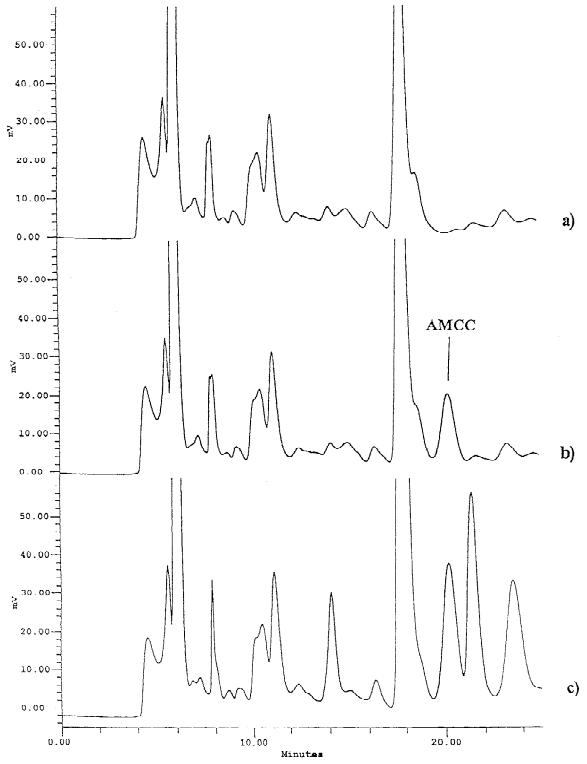

Fig. 3. Chromatograms obtained from: (a) blank urine; (b) blank urine + 25 mg/l of AMCC standard; (c) urine taken from a worker exposed to DMF (concentration of AMCC = 55 mg/l).

The accuracy was 98.0±1.7% (mean±SD) at 10 mg/l and 101.9±1.5% at 800 mg/l.

The background excretion of AMCC was evaluated on 20 random samples taken from subjects not exposed to DMF (ten smokers and ten non-smokers). To verify whether a correlation exists between the environmental concentration of the solvent and urinary concentration of AMCC, the analytical method was applied also to 22 urine from workers exposed to DMF. These samples were collected at the beginning of the following work shift, as suggested by authors [12]. Fig. 3 shows representative chromatograms of urine samples taken from exposed and unexposed subjects.

None of the urine samples taken from unexposed subjects showed measurable amounts of AMCC. This confirmed that despite the use of a low wavelength UV detection, the chromatograms were free from interfering peaks. On the contrary, AMCC was detected in all samples from workers exposed to DMF.

AMCC concentration in urine ranged from 6.0 to 506.0 mg/l (mean=97.1, SD=128.5), and after correction with creatinine, values ranged from 3.7 to 212.1 mg/g creatinine (mean=45.8, SD=51.7). Environmental DMF exposure (8h-TWA) ranged from 2.0 to 63.0 mg/m^3 (mean=12.6, SD=14.5, geometric mean=7.81, geometric standard deviation=2.52).

A strong correlation was found between DMF exposure and AMCC excretion (Fig. 4). The equation of the regression line was: AMCC (mg/g creatinine)=3.40×DMF (mg/m^3)+3.07 (r=0.95, N=22).

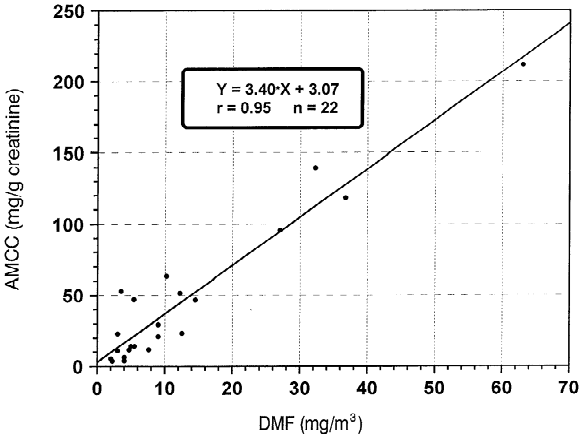

Fig. 4. Relation between DMF concentration in the air of the workplace and urinary AMCC measured at the beginning of the next workshift.

4. Conclusion

The present paper describes a new analytical method for the determination of AMCC in the urine of subjects exposed to DMF. The method is simple, fast, sensitive, selective, does not have a derivatization step and does not require expensive instrumentation. Urine samples are prepurified on SPE cartridges and analysed by HPLC on an Aminex Ion Exclusion column using isocratic runs with the UV detector set at 196 nm. The method was applied to 22 urine from workers exposed to DMF. The results showed high correlation between urinary concentration of AMCC and environmental concentration of DMF: the mean concentration of DMF was 12.6 mg/m^3, about one half of the current threshold limit value (TLV) given by the American Conference of Governmental Industrial Hygienists (ACGIH) [4].

A large number of samples can be purified simultaneously by the sample clean-up procedure, and the subsequent chromatographic separation is performed by simple isocratic runs. Therefore, the procedure appears to be suitable for biomonitoring of occupational exposure. However, it does not allow for the measurement of the background levels of AMCC in the general population. In this case, a more sensitive method should be used [14,18].

References

[1] T. Kawai, T. Yasugi, K. Mizunuma, T. Watanabe, S.X. Cai, M.Y. Huang, L.Q. Xi, J.B. Qu, B.Z. Yao, M. Ikeda, Int. Arch. Occup. Environ. Health 63 (1992) 455.
[2] R. Garnier, D. Chataigner, B. Perez-Trigalou, M.L. Efthymiou, Arch. Mal. Prof. 53 (1992) 111.
[3] R. Wrbitzky, J. Angerer, G. Lehnert, Int. Arch. Occup. Environ. Health 68 (1996) 508.

[4] Threshold Limit Values For Chemical Substances and Physical Agents and Biological Exposure Indices, ACGIH, Cincinnati, OH, 2001.
[5] C. Brindley, A. Gesher, D. Ross, Chem. Biol. Interact. 45 (1983) 387.
[6] V. Scailteur, E. de Hoffmann, J.P. Buchet, R. Lauwerys, Toxicology 29 (1984) 221.
[7] P. Kestell, M.H. Gill, M.D. Threadgill, A. Gescher, O.W. Howarth, E.H. Curzon, Life Sci. 38 (1987) 719.
[8] J. Mraz, F. Turecek, J. Chromatogr. B 414 (1987) 399.
[9] A. Gescher, Chem. Res. Toxicol. 6 (2000) 245.
[10] J. Mraz, H. Nohova, Int. Arch. Occup. Environ. Health 64 (1992) 85.
[11] A. Casal Lareo, L. Perbellini, Int. Arch. Occup. Environ. Health 67 (1995) 47.
[12] T. Sakai, H. Kageyama, T. Araki, T. Yosida, T. Kuribayashi, Y. Masuyama, Int. Arch. Occup. Environ. Health 67 (1995) 125.
[13] J. Mraz, J. Chromatogr. B 431 (1988) 361.
[14] H.U. Käfferlein, J. Angerer, J. Chromatogr. B 734 (1999) 285.
[15] A. Casal Lareo, A. Perico, P. Bavazzano, C. Soave, L. Perbellini, Int. Arch. Occup. Environ. Health 67 (1995) 41.
[16] M.E. Cassinelli, P.F. Connor (Eds.), Manual of Analytical Methods, 4th Ed., NIOSH, Cincinnati, OH, 1994.
[17] G. Santoni, P. Bavazzano, A. Perico, A. Colzi, G. Santoni, A. Medica, R. La Morgia, G. Giuliano, J. Chromatogr. B 581 (1992) 287.
[18] H.U. Käfferlein, J. Angerer, J. Environ. Monit. 1 (1999) 465.

Name: Marcello Imbriani
Nationality: Italian
Date of Birth: 26th October 1951
Marital status: Married – two dependent children
Address (work): Faculty of Medicine, Pavia University – Fondazione S. Maugeri, IRCCS – Via A. Ferrata, 4 I - 27100 Pavia, Tel. 0039-0382-592990, Fax 0039-0382-592514, e-mail: pfr@fsm.it
Education: Ph.D. Faculty of Medicine, Pavia University, 1976; Specialist in Occupational Medicine, Faculty of Medicine, Pavia University, 1979; Specialist in Respiratory Diseases, Faculty of Medicine, Pavia University, 1982.

Summary of Skills

1. Research on uptake, distribution and elimination of industrial solvents, both in experimental studies (animal, man) and in occupationally exposed workers.
2. Study of instrumental methods in laboratory and in field related to respiratory occupational risks.
3. Research on cardio-respiratory and metabolic adjustment in normal subjects, athletes and in various pathologies.

Professional Experience

1. Researcher of Pavia University, Faculty of Medicine, Department of Occupational Medicine, 1980
2. Head Physician of the Service for Respiratory Diseases of the Maugeri Foundation, 1999
3. Associate Professor of Occupational Medicine, Department of Occupational medicine, Pavia University, 2000

Publications

He co-authored nearly 130 peer-reviewed articles concerning inhalation and elimination of solvents and anesthetics and the effects of workload on the respiratory uptake of solvents and gases, and nearly 120 lectures as well as original scientific presentations at national and international meetings, chapters of books and monographs.

Other Scientific Activities

- Scientific secretariat of ''S. Maugeri Foundation'', Institute of Care and Scientific Research
- Series Editor of the Books ''Advances in Occupational medicine and Rehabilitation''
- Editor of the Journal ''Giornale Italiano di Medicina del Lavoro e Ergonomia'' (G. Ital. Med. Lav. Eng, indexed in Index Medicus)
- Managing Editor of the Journal ''Monaldi Archives for Chest Diseases'') (Monaldi Arch Chest Dis., indexed in Index Medicus)
- Managing Editor of the Journal ''Psicoterapia cognitiva e comportamentale''