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Short Communication

High-performance liquid chromatographic determination of N-methylformamide and N-methyl-N-(hydroxymethyl)-formamide in human urine

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ABSTRACT

A reliable high-performance liquid chromatographic (HPLC) method which allows the determination in human urine of two important metabolites of N,N-dimethylformamide (DMF), namely N-methylformamide (MMF) and N-methyl-N-(hydroxymethyl)formamide (DMFOH), is reported. A single-step rapid purification of urine was performed on a C_{18} solid-phase extraction column and the eluate was injected directly on to the HPLC column. HPLC was carried out isocratically on Aminex Ion Exclusion HPX-87H column using $7.5 \cdot 10^{-4}$ *M* sulphuric acid as the mobile phase with ultraviolet detection at 196 nm. The method is specific, accurate, precise and sufficiently sensitive to be applied to the biological monitoring of MMF and DMFOH in workers exposed to DMF.

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INTRODUCTION

N,N-Dimethylformamide (DMF) is a polar solvent miscible with water and with several organic solvents that has recently encountered extensive use as a polyurethane lacquer solvent in the artificial leather industry. It has been reported that occupational exposure occurs through inhalation of the vapour and skin contact [1]. Initial metabolic studies have revealed that DMF undergoes metabolic conversion into N-methylformamide (MMF) and formamide (F) [2,3]. These two compounds were either detected in urine of exposed humans and rodents by gas chromatography (GC) and the urinary concentration of MMF was then chosen for a biological monitoring system in workers exposed to DMF. Later studies, however, revealed that in addition to MMF and F also the corresponding carbinolamine, N-methyl-N-(hydroxymethyl)formamide (DMFOH), and hydroxymethylformamide (MMFOH) were present in the urine of humans exposed to DMF [4,5]; DMFOH and MMFOH were present in urine in larger amounts but, during injection into the GC system, they were thermally decomposed to MMF and F, respectively [4,5]. As DMFOH is converted into MMF during injection, GC allows the calculation only of the sum of both metabolites (MMF plus DMFOH) and the amount of DMFOH was indirectly determined by spectrophotometric determination of formaldehyde released in the metabolic conversion of DMF into MMF [6].

The aim of this work was to develop a high-performance liquid chromatographic (HPLC) method for the determination of DMFOH in the urine of exposed workers because there are no other methods that had the required specificity and sensitivity. The method developed allows the simultaneous determination of both metabolites (MMF and DMFOH) and it is specific and has the sensitivity to determine MMF and DMFOH levels under conditions of occupational exposure to DMF.

EXPERIMENTAL

Reagents and chemicals

DMFOH was synthesized at the Military Chemical Pharmaceutic Institute (Florence, Italy) as described below. MMF was purchased from Sigma (St. Louis, MO, USA), water (HPLC grade) from Merck (Darmstadt, Germany) and methanol, chloroform, anhydrous potassium carbonate, sulphuric acid (96%), hydrochloric acid (37%) (analytical-reagent grade) and paraformaldehyde (synthesis grade) from Carlo Erba (Milan, Italy). Analytichem Bond Elut C_{18} 500-mg solid-phase extraction (SPE) columns were obtained from Varian (Victoria, Australia).

Synthesis of N-methyl-N-(hydroxymethyl)formamide [7,8]

A mixture of MMF (0.4 mol), paraformaldehyde (0.4 mol) and anhydrous potassium carbonate (0.4 g) was heated at 90°C for 1 h under magnetic stirring. After cooling to room temperature, chloroform was added and, after filtration, the solvent was removed under reduced pressure. The crude product was purified by distillation under atmospheric pressure. Yield, 92%; b.p., 137.3°C; IR (KBr, cm^{-1}), 3400, 2897, 1667, 1407; ^1H NMR ($CDCl_3$, ppm), 2.9 (s, CH_3), 4.7 (s, CH_2), 8.1 (s, CHO). The ^1H NMR data also showed the product to contain about 10% MMF. The purity of the synthesized DMFOH was also evaluated by the HPLC method, described below, and the MMF present in DMFOH, in comparison with a pure reference substance, was 8%.

Apparatus

A Waters HPCL system (Millipore, Milford, MA, USA) equiped with a Model 510 pump, a Model 715 Ultra WISP autosampler, a thermostated oven and a Model 994 variable-wavelength diode-array detector was used. Data processing was performed with Maxima 820 software on an NEC computer. A stainless-steel column (300 mm × 7.8 mm I.D.) packed with Aminex Ion Exclusion HPX-87H (9 μm) (Bio-Rad Labs., Richmond, CA, USA) was used. The mo-

bile phase was $7.5 \cdot 10^{-4}$ *M* sulphuric acid at a flow-rate of 0.9 ml/min. The oven temperature was 40°C and the injection volume was 10 μl. The detector wavelength was set at 196 nm with a band width of 3 nm; the detector sensitivity was 1 a.u.f.s. until 20 min and subsequently 0.05 a.u.f.s. Under these operating conditions the retention times obtained were DMFOH 12.6 and MMF 27.0 min.

Standard solutions

Aqueous stock standard solutions of 1.0 mg/ml DMFOH and MMF were prepared by accurately weighing the reference substances. These solutions were stable for at least one month at room temperature; their stability was assessed by determining the percentage of MMF by HPLC (at room temperature there is a very slow thermal conversion of DMFOH into MMF) and verifying the absence in the chromatogram of extraneous peaks in comparison with that of a fresh solution.

Calibration

Six solutions were prepared by dilution of the two stock solutions at the following concentrations: DMFOH, 50, 15, 5, 1.5, 0.5 and 0.2 mg/ml; and MMF, 5, 2.5, 1.0, 0.5, 0.25 and 0.1 mg/ml. To 1 ml of blank urine were added 10 μl of each of the solutions and the mixtures were briefly shaken and purified as described under *Sample preparation* below. The concentrations of the resulting six points of the calibration graph were as follows: DMFOH, 500, 150, 50, 15, 5 and 2 μg/ml; and MMF, 50, 25, 10, 5, 2.5 and 1.0 μg/ml. The dilute solutions were freshly prepared each time a calibration graph was constructed.

Sample preparation

Bond Elut C_{18} 500-mg SPE columns were conditioned with 6 ml of methanol and then with 3 ml of 1 *M* hydrochloric acid; 3.0 ml of urine sample were passed, under atmospheric pressure, through the conditioned column, the first 1 ml of eluate was discarded and the second 1 ml was collected and injected directly on to the HPLC column. Urine samples were kept at −20°C in a freezer and under these conditions DMFOH and MMF are perfectly stable. For several urines the same sample was analysed after one, two and three months at −20°C and no differences in the amounts of the two analytes were found and also the chromatographic profiles remained unaltered. Three purified urine samples were reanalysed after 48 h at room temperature and no significant differences between the two analytical results were observed.

RESULTS AND DISCUSSION

The peaks of MMF and DMFOH were identified by comparison with a chromatogram of a solution of the reference substances; the comparison was based on retention times and analysis of the UV spectra. The peaks corresponding to the two analytes were established to be pure by analysing the UV spectra collected at different times during chromatographic elution (peak purity calculated by the Waters software was >99). The specificity of the chromatographic analysis was tested by analysing blank urine before and after purification on the C_{18} SPE column performed as described above.

Before purification we obtained the chromatographic profile shown in Fig. 1, in which there are two peaks interfering with the DMFOH peak at 12.1 and 13.5 min, respectively. After purification we obtained the profile shown in Fig. 2, in which the two interferents have disappeared as they were retained on the C_{18} SPE column.

A chromatogram of blank urine sample spiked with DMFOH and MMF (50 and 10 μg/ml, respectively), prepared as described, is shown in Fig. 3. The method gave a linear response in the concentration range 2–500 μg/ml for DMFOH and 1–100 μg/ml for MMF. The values of the slope, intercept and correlation coefficient obtained by linear regression analysis of the peak-area data are reported in Table I. The chromatographic reproducibility, expressed as coefficient of variation, calculated by injecting six replicates of the central calibration solution, were 2.0% for DMFOH and 2.2% for MMF.

The accuracy was calculated by analysing six

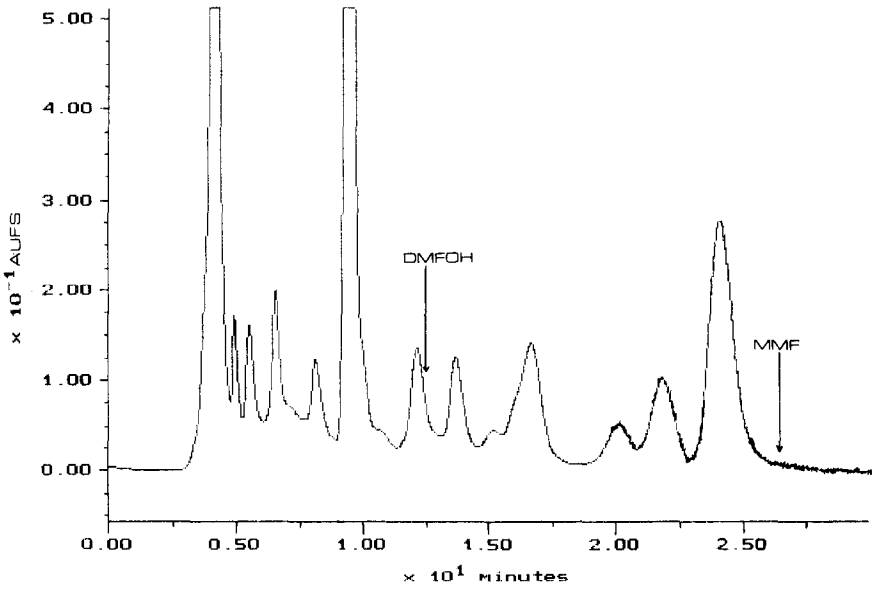

Fig. 1. Chromatographic profile of crude blank urine.

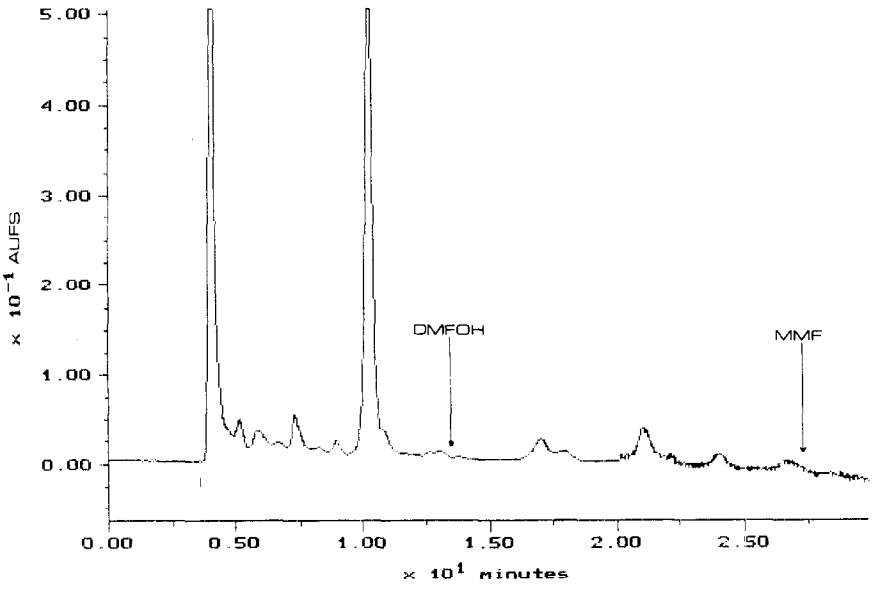

Fig. 2. Chromatographic profile of blank urine after purification on a C_{18} SPE column.

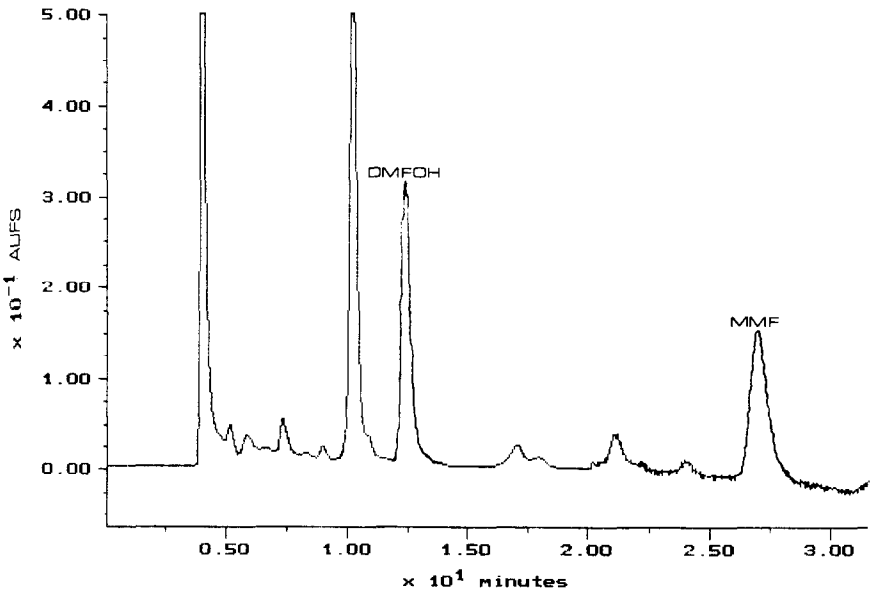

Fig. 3. Chromatogram of blank urine spiked with DMFOH (50 μg/ml) and MMF (10 μg/ml).

times on two different days a blank urine spiked with known amounts of DMFOH and MMF (50 and 10 μg/ml, respectively) by the proposed procedure. The amounts of DMFOH and MMF found were then compared with the actual values and the accuracy was expressed as a percentage of the recovery. The data showed that quantitative recoveries were obtained for both analytes and that the method was accurate (the 100% value of recovery was included between 95% confidence limits of the average value) and precise (the precision is given by the coefficient of variation of the recovery values), as shown in Table II. An analysis of variance (ANOVA) showed that differences between the results obtained on the two different days were not significant ($p > 0.05$). The limits of detection, for a signal-to-noise ratio of 3, were 1 μg/ml for DMFOH and 0.5 μg/ml for MMF.

The proposed method was preliminarily applied during a study undertaken on ten subjects exposed to about 3.7 mg/m^3 DMF (range 0.3–6.3 mg/m^3). The results ranged from 5.4 to 130.0 μg/ml for DMFOH (mean value 27.2 μg/ml, standard deviation 37.4 μg/ml) and from undetectable (two samples) to 3.6 μg/ml for MMF (mean value 1.2 μg/ml, standard deviation 1.1 μg/ml). A

TABLE I

TYPICAL PARAMETERS FOR THE REGRESSION LINE EQUATIONS FOR DMFOH AND MMF

Compound	Slope	Intercept	r^2
DMFOH	$1.9 \cdot 10^{-5}$	−0.09	0.999
MMF	$1.3 \cdot 10^{-6}$	−0.15	0.999

TABLE II

ACCURACY AND PRECISION OF INTER-DAY RECOVERY DATA

Compound	Day	Accuracy (mean ± S.D.) (%)	Precision (%)
DMFOH	1	98.7 ± 2.2	2.4
	2	97.9 ± 2.5	2.5
MMF	1	98.1 ± 2.0	2.2
	2	98.3 ± 2.3	2.4

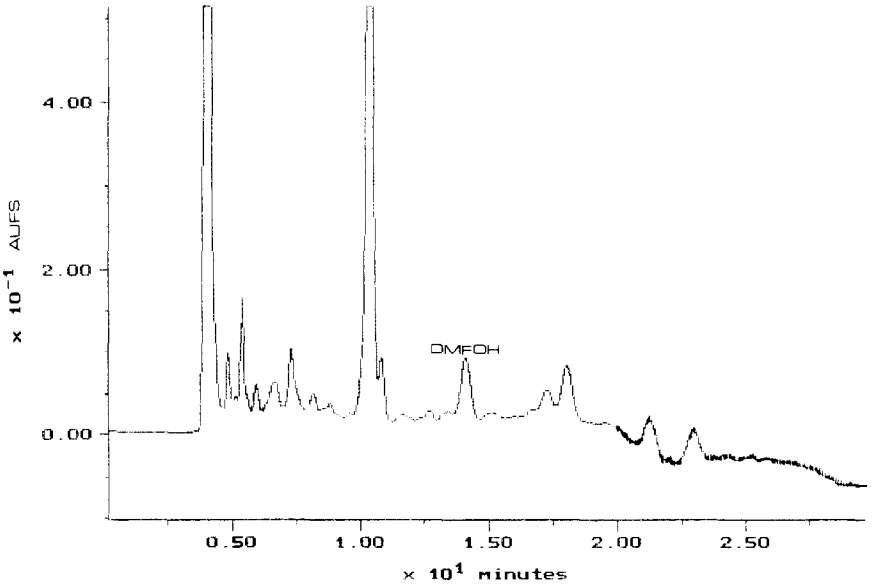

Fig. 4. Chromatogram of urine from a subject exposed to DMF. Concentration of DMFOH found = 6.9 μg/ml.

chromatogram relating to an exposed subject with low levels of DMFOH is shown in Fig. 4 (DMFOH concentration 6.9 μg/ml; MMF not detectable).

CONCLUSIONS

The proposed method allows the determination of the metabolites of DMF in human urine with a short sample preparation; the accuracy, precision, sensitivity and reproducibility are sufficient for application in studies of biological monitoring of workers exposed to DMF.

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