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# Simultaneous determination of *N*-hydroxymethyl-*N*-methylformamide, *N*-methylformamide and *N*-acetyl-*S*-(*N*-methylcarbamoyl)cystein in urine samples from workers exposed to *N,N*-dimethylformamide by liquid chromatography–tandem mass spectrometry

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**Abstract**

*N*-Hydroxymethyl-*N*-methylformamide (HMMF) and *N*-methylformamide (NMF) in urine samples from workers exposed to *N,N*-dimethylformamide (DMF) cannot be distinguished by a gas chromatographic method because HMMF is converted to NMF at the injection port of gas chromatography (GC). Total NMF (HMMF + NMF) has been measured instead. Also, the determination of *N*-acetyl-*S*-(*N*-methylcarbamoyl)cystein (AMCC), which is supposed to be related to the toxicity of DMF, needs multiple treatments to convert to a volatile compound before GC analysis. There is no previous report of a simultaneous determination of three major metabolites of DMF in urine. The aim of this study is to develop a simple and selective method for the determination of DMF metabolite in urine. By using a liquid chromatography–tandem mass spectrometry, we can directly distinguish these three major metabolites of DMF in a single run. The diluted urine samples were analyzed on Capcell Pak MF SG80 column with the mobile phase of methanol in 2 mM formic acid (10:90, v/v). The analytes were detected by an electrospray ionization tandem mass spectrometry in the multiple-reaction-monitoring mode. The standard curves were linear ($r > 0.999$) over the concentration ranges of 0.004–8 μg/mL. The precision and accuracy of quality control samples for inter-batch ($n = 6$) analyses were in the range of 1.3–9.8% and 94.7–116.8, respectively. The sum of each HMMF and NMF concentration determined by LC-MS/MS method shows high correlation ($r = 0.9927$ with the slope of 1.0415, $p < 0.0001$) with NMF included HMMF concentration determined by GC method for 13 urine samples taken from workers exposed to DMF. The excretion ratio of HMMF:NMF:AMCC is approximately 4:1:1 in molar concentration.
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*Keywords:* *N,N*-Dimethylformamide; *N*-Hydroxymethyl-*N*-methylformamide; *N*-methylformamide; *N*-acetyl-*S*-(*N*-methylcarbamoyl)cystein; LC-MS/MS

## 1. Introduction

*N,N*-Dimethylformamide (DMF) has been widely used in synthetic leather and polyacrylonitrile fiber industries as a washing solvent. Because of its high miscibility with both water and organic solvents, DMF has been important in these industries in washing off impurities, despite its toxicity. DMF has been reported to increase the chance of getting pancreatic disorders, liver dysfunction, testicular carcinoma, etc. [1–3]. The occupational exposure limit of DMF was set as 10 ppm in most of countries [4,5]. DMF is absorbed through both inhalation of the vapor and dermal contacts such as submerging hands under the DMF fluid. Therefore, ambient air monitoring by itself cannot effectively reflect the amount of real intake.

The major metabolites include *N*-hydroxymethyl-*N*-methylformamide (HMMF) and *N*-methylformamide (NMF)

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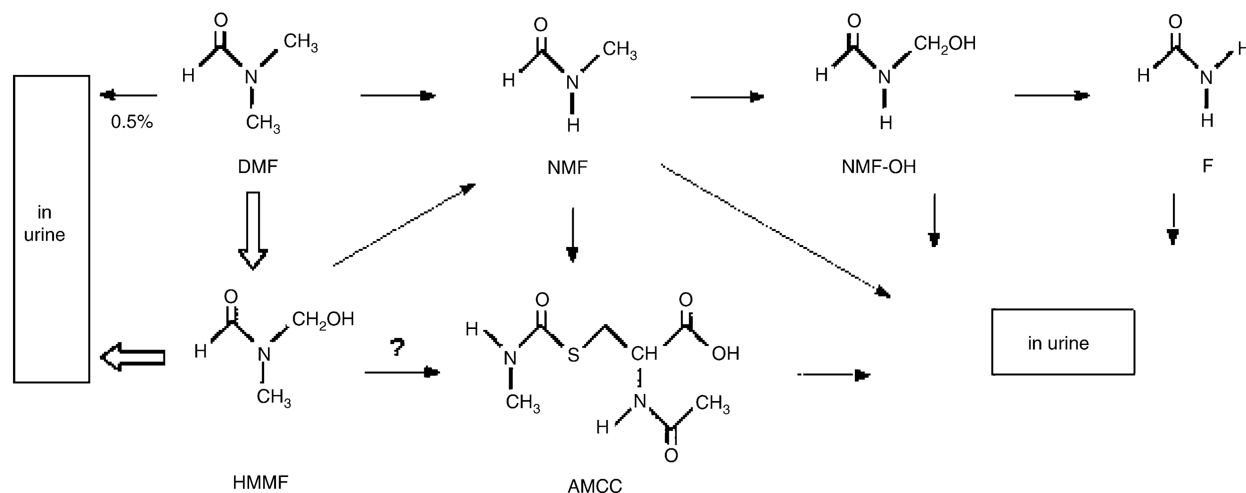


Fig. 1. Hypothetical breakdown of DMF in human, copied with a written permission from Ref. [6].

and *N*-acetyl-*S*-(*N*-methylcarbamoyl)cystein (AMCC). The American Conference of Governmental Industrial Hygienists (ACGIH) set the Biological Exposure Indices (BEI) for DMF as 15 mg of NMF and 40 mg of AMCC in one liter urine [5]. Fig. 1 shows the hypothetical breakdown of DMF in human [6]. The determination of each metabolite of DMF is important in understanding the metabolic pathway of DMF and assessing the exact amount of intake in body after a DMF exposure.

A number of methods for the analysis of the metabolites of DMF in urine were reported using gas chromatography (GC). HMMF, which is the major metabolite of DMF, was detected as the total NMF in urine because HMMF was converted to NMF during the analytical procedure by GC [6–10]. AMCC, which is supposed to be related to the toxicity of DMF and has prolonged half-life, has been focused for the field study of biological monitoring for workers [11–14]. Quantitation of AMCC in urine by GC needed multiple treatments to convert AMCC to a volatile compound. Kafferlein et al. [15] compared the suitability and accuracy of four GC methods developed for determination of urinary metabolites of DMF. Two methods were able to measure only the total NMF, and the other two methods measured both the total NMF and AMCC. The two tested methods for the determination of AMCC showed high correlation but differed in the values significantly. So, there was still a need to develop a reliable, simple and selective method for the determination of AMCC for the further application of AMCC to the field study. High performance liquid chromatographic methods were tried but under the UV detector, it was hard to surpass numerous background peaks in urine [16,17]. No method was available for simultaneous determination of three major metabolites of DMF, i.e., HMMF, NMF and AMCC in urine in a single run.

The purpose of this study is to develop a simple, reliable and selective method for simultaneous determination of the major metabolites of DMF, that is, HMMF, NMF and AMCC in urine by using a liquid chromatography–tandem mass spectrometry (LC-MS/MS). The presented method has been successfully applied to the evaluation of HMMF, NMF and AMCC in urine samples from workers exposed to DMF.

## 2. Experimental

### 2.1. Chemicals

NMF, DMF (99.9%, HPLC grade) and formic acid (96%, A.C.S. reagent) were purchased from Sigma-Aldrich Korea Chemical Co. (Seoul, Korea). Deionized water was produced by the Milipore Q system, and methanol (HPLC grade) was purchased from J.T. Baker (Phillipsburg, NJ, USA). HMMF was purchased from Research Institute for Pharmaceutical Institute of Ewha University (>95%, Seoul, Korea). AMCC (>95%) was kindly donated from Dr. Mraz from Czech [11].

### 2.2. Preparation of standard solutions and quality control samples

Ten milligrams of AMCC, HMMF and NMF were accurately weighed into a 10 ml volumetric flask, and dissolved in methanol to make standard stock solution (1 mg/ml). Aliquot amount of 1 mg/ml stock solution was diluted with water to make each working standard solutions. All solutions were stored at 4 °C in the dark when not in use. Blank urine was collected from non-exposed volunteers and stored at −20 °C in a deep freezer. Human urine calibration standards of HMMF, NMF and AMCC (0.004, 0.02, 0.04, 0.20, 1.0, 2.0, 4.0 and 8.0 μg/ml) were prepared by spiking appropriate amount of the working standard solutions into human blank urine. Quality control (QC) samples at 0.004, 0.2, 1.0 and 8.0 μg/ml were prepared in bulk. The QC samples were aliquoted (1 ml) into polypropylene tubes and stored −20 °C until analysis.

### 2.3. Sample preparation

One milliliter of blank urine, calibration standard and QC samples was diluted to 10 ml with methanol and centrifuged at 2000 × *g* for 20 min. One hundred microliters of urine supernatant was diluted with 10% methanol in 2 mM formic acid to make 500 μl solution. Diluted urine samples were filtered with Mini-UniPrep Syringeless filter (Whatman, PVDF media, 0.45 μm pore size), and 1 μl were injected onto LC-MS/MS.

### 2.4. LC-MS/MS analysis

For LC-MS/MS analysis, the chromatographic system consisted of a Hewlett Packard 1100 pump, a diode array detector and a G1329 autosampler. The separation was performed on a Capcell Pak MF SG80 (5 μm, 4.6 mm i.d. × 150 mm, Shiseido, Tokyo, Japan) using a mobile phase of 10% methanol in 2 mM formic acid at a flow rate of 200 μl/min. The analytical run time was 20 min. The eluent was introduced directly onto the tandem mass spectrometer (API 3000, Applied Biosystems/MDS Sciex, Foster city, CA, USA) through the turbo ion-spray source with typical settings as follows: curtain gas (CUR), 8; nebulizer gas (NEB), 8; turbo gas, 50 psi; ion-spray voltage 5000 V; temperature, 400 °C. Multiple-reaction-monitoring (MRM) mode using specific precursor/product ion transitions was employed for the quantitation. The molecular ions of HMMF, NMF and AMCC were fragmented at collision energy of 30 V by collision-activated dissociation with nitrogen as the collision gas. Detection of ions was performed by monitoring the transitions of *m/z* 90–60 for HMMF, *m/z* 60–42 for NMF and *m/z* 221–122 for AMCC.

### 2.5. Method validation

The percentage accuracy and precision (the relative standard deviation, R.S.D.) were evaluated by determining the analytes in QC samples at four different levels on two different days. The calibration curve was obtained by analyzing calibration standards in triplicate at eight concentrations. The lower limit of quantitation (LLOQ) was set at a level where the following criteria were met: signal-to-noise ratio ≥ 5.0 with accuracy deviation ≤ ±20% and R.S.D. ≤ 20%.

### 2.6. Application

The developed LC-MS/MS method was used in the analysis of urine samples collected from the thirteen workers exposed to DMF.

Using previously developed GC method [6], a blind experiment for 13 urine samples taken from the workers exposed to DMF was performed to compare the data with the one earned from LC-MS/MS. Varian CP-3800 gas chromatography with Thermionic Specific Detector (nitrogen-phosphorous specific detector, NPD) was used with HP Innowax (cross-linked polyethyleneglycol, 0.32 mm × 60 m, 0.15 μm) column at starting temperature of 100 °C for 2 min with temperature programming of 20 °C/min to 200 °C for 10 min. Injector temperature was set at 250 °C. One milliliter of urine was diluted with 0.2 mM acrylamide (internal standard) in methanol solution to 10 ml and centrifuged at 2000 × *g* for 20 min. Three microliters of urine supernatant was injected to GC.

These two different sets of experiment were done by different analysts as a blind test. For GC analysis, known concentrations of quality control samples earned from Korea Analytical Quality Assurance Program were analyzed to confirm the data accuracy [18].

## 3. Results and discussion

### 3.1. LC-MS/MS

The full scan mass spectra and the MS/MS spectra of all analytes were obtained from infusion of 8 μg/ml 10% methanol in 2 mM formic acid solutions of each compound at a flow rate of 10 μl/min under positive ionization conditions. To obtain the abundant protonated molecular ions ($MH^+$) without any evidence of fragmentation, declustering potentials (DP) was optimized to 20 V. $MH^+$ ions from HMMF, NMF and AMCC at *m/z* 90, 60 and 221, respectively, were selected as the precursor ions and subsequently fragmented in MS/MS mode to obtain the product ion spectra yielding useful structural information (Fig. 2). The fragment ions at

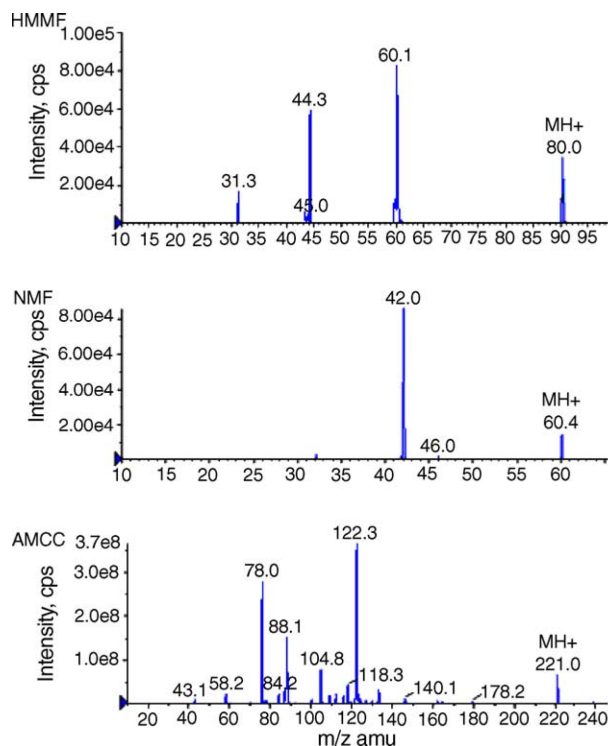


Fig. 2. Product ion mass spectra of HMMF, NMF and AMCC.

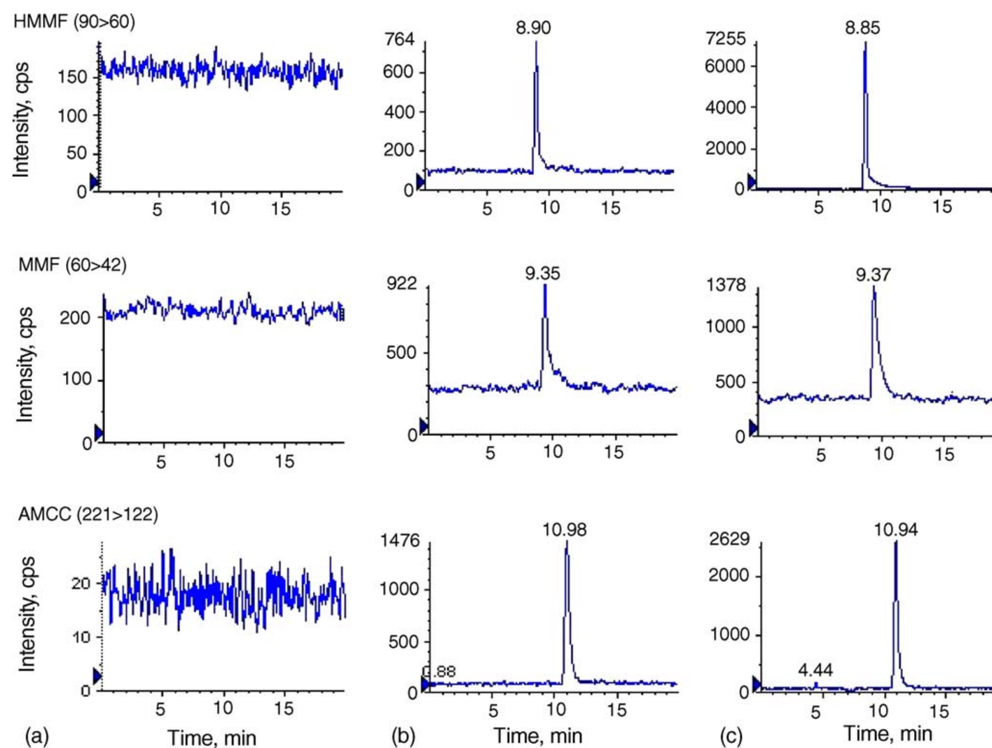


Fig. 3. MRM LC-MS/MS chromatograms of (a) blank urine sample, (b) blank urine sample spiked with HMMF, NMF and AMCC at 0.004 μg/ml and (c) an end-shift urine sample collected from a worker exposed to DMF.

*m/z* 60 (by the loss of aldehyde group from $MH^+$) for HMMF, *m/z* 42 (by the loss of water from $MH^+$) for NMF and *m/z* 122 (by the loss of methyl amino and carbonyl moiety from $MH^+$) for AMCC were produced as the prominent product ions (Fig. 2). The quantitation of the analytes was performed using the MRM mode because of its sensitivity and selectivity for the detection of analytes. The MRM transitions were selected using precursor → product ion combination at *m/z* 90 → 60 for HMMF, *m/z* 60→ 42 for NMF and *m/z* 221 → 122 for AMCC.

The protein precipitation with methanol and 50-fold dilution of urine samples resulted in minimizing the contamination and the potential suppression effects in Turbo ionspray source. Fig. 3 shows the representative MRM chromatograms obtained from the analysis of blank urine sample, blank urine sample spiked with HMMF, NMF and AMCC to make 0.004 μg/ml of target concentration, and an end-shift urine sample collected from a worker exposed to DMF. There were no signs of interference peaks that could affect the detection of analytes.

Table 1
Precision and accuracy of HMMF, NMF and AMCC in quality control samples spiked in urine

| Analytes | Theoretical concentration (μg/ml) | Intra-batch ($n = 3$) | | | Inter-batch ($n = 6$) | | |
|---|---|---|---|---|---|---|---|
| | | Mean (μg/ml) | R.S.D. (%) | Accuracy (%) | Mean (μg/ml) | R.S.D. (%) | Accuracy (%) |
| HMMF | 8 | 7.51 | 1.2 | 93.9 | 7.58 | 2.1 | 94.7 |
| | 1 | 1.14 | 1.9 | 114 | 1.12 | 6.1 | 112.1 |
| | 0.2 | 0.22 | 3.3 | 110 | 0.22 | 7.0 | 109 |
| | 0.004 | 0.0042 | 4.9 | 105 | 0.0043 | 5.4 | 107.9 |
| NMF | 8 | 7.69 | 2.6 | 96.1 | 7.73 | 1.3 | 96.6 |
| | 1 | 1.01 | 9.4 | 100.9 | 1.05 | 7.9 | 104.8 |
| | 0.2 | 0.22 | 6.7 | 112.3 | 0.23 | 6.9 | 113.8 |
| | 0.004 | 0.0046 | 9.9 | 113.8 | 0.0047 | 9.8 | 116.8 |
| AMCC | 8 | 7.75 | 1.8 | 96.9 | 7.78 | 1.5 | 97.3 |
| | 1 | 1.07 | 2.6 | 107.4 | 1.07 | 1.7 | 107.2 |
| | 0.2 | 0.22 | 3.5 | 107.7 | 0.21 | 3.8 | 105.7 |
| | 0.004 | 0.0042 | 3.8 | 105.7 | 0.0042 | 2.7 | 105 |

Table 2
Mean concentration of HMMF, NMF and AMCC in urine samples collected from 13 workers exposed to DMF determined by LC-MS/MS and GC method

| | Amount of DMF metabolites by LC-MS/MS (μg/ml) | | | Amount of NMF included HMMF by GC (μg/ml) |
|---|---|---|---|---|
| | HMMF | NMF | AMCC | |
| Mean | 15.7 | 2.6 | 9.1 | 19.1 |
| Range | 1.0–108.5 | 0.2–16.6 | 1.4–48.8 | 1.3–113.5 |
| S.D. | 1.6 | 1.8 | 1.4 | 1.6 |

### 3.2. Method validation

The calibration curves for HMMF, NMF and AMCC were linear in the range of 0.004–8 μg/ml with correlation coefficients of >0.999. Table 1 shows the accuracy and precision of three DMF metabolites for QC samples. R.S.D. of inter-batch was between 1.5 and 9.8% and percentage accuracy was between 94.7 and 116.8% in the four QC levels. The LLOQ for HMMF, NMF and AMCC was set at 0.004 μg/ml. The representative chromatograms of LLOQ are shown in Fig. 3b. The signal-to-noise ratios are about 40 for HMMF and NMF and about 10 for AMCC.

### 3.3. Application of field study

For the application of field study for biological monitoring of DMF-exposed workers, we analyzed 13 urine samples taken from the workers exposed to DMF using LC-MS/MS and GC method. By GC, HMMF was converted to NMF at high temperature of inject port and detected as NMF, and therefore, NMF concentration determined using GC reflects the sum of HMMF and NMF concentration in urine. The sum of each HMMF and NMF concentration determined by LC-MS/MS method shows high correlation ($r = 0.9927$ with the slope of 1.0415, $p < 0.0001$) with NMF included HMMF concentration determined by GC method (Fig. 4). Table 2 shows the mean concentration of three major metabolites of DMF in thirteen workers exposed to DMF. The excretion ratio in molar concentration of HMMF:NMF:AMCC is approximately 4:1:1 in urine samples taken from workers exposed to DMF. These results suggest that HMMF is a major metabolite of DMF in human and this method can be used in order to evaluate the ratio between each metabolite.

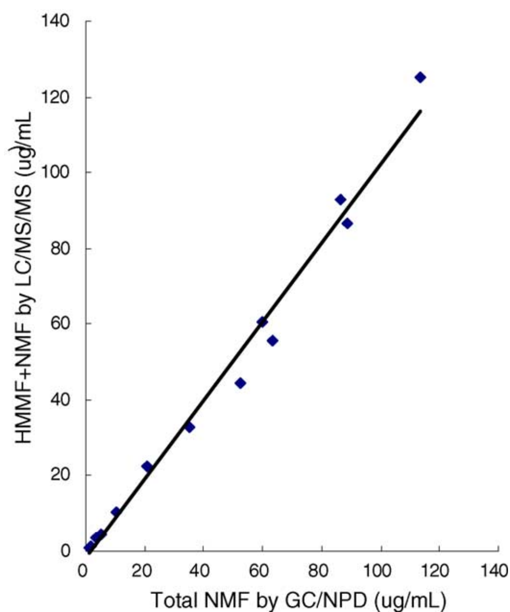


Fig. 4. Correlation between the concentrations of total NMF analyzed by GC method and the sum of HMMF and NMF determined by LC-MS/MS method in end-shift urine samples taken from 13 workers exposed to DMF.

## 4. Conclusion

A rapid and reliable LC-MS/MS method for the simultaneous determination of three major metabolites of DMF, i.e., HMMF, NMF and AMCC in human urine has been successfully developed. Sample preparation is simple procedure including protein precipitation with methanol and dilution with mobile phase. The presented method shows good precision and accuracy in the concentration range of 0.004–8 μg/ml. This method can be suitable for biological monitoring of the exposure to DMF.

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