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Concentration determination of urinary metabolites of *N,N*-dimethylacetamide by high-performance liquid chromatography-tandem mass spectrometry

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Abstract: Objectives: *N,N*-Dimethylacetamide (DMAC) is widely used in industry as a solvent. It can be absorbed through human skin. Therefore, it is necessary to determine exposure to DMAC via biological monitoring. However, the precision of traditional gas chromatography (GC) is low due to the thermal decomposition of metabolites in the high-temperature GC injection port. To overcome this problem, we have developed a new method for the simultaneous separation and quantification of urinary DMAC metabolites using liquid chromatography-tandem mass spectrometry (LC-MS/MS). **Methods:** Urine samples were diluted 10-fold in formic acid, and 1-μl aliquots were injected into the LC-MS/MS equipment. A C18 reverse-phase Octa Decyl Silyl (ODS) column was used as the analytical column, and the mobile phase consisted of a mixture of methanol and aqueous formic acid solution. **Results:** Urinary concentrations of DMAC and its known metabolites (*N*-hydroxymethyl-*N*-methylacetamide (DMAC-OH), *N*-methylacetamide (NMAC), and *S*-(acetamidomethyl) mercapturic acid (AMMA)) were determined in a single run. The dynamic ranges of the calibration curves were 0.05-5 mg/l ($r \geq 0.999$) for all four compounds. The limits of detection for DMAC, DMAC-OH, NMAC, and AMMA in urine were 0.04, 0.02, 0.05, and 0.02 mg/l, respectively. Within-run accuracies were 96.5%-109.6% with relative standard deviations of precision being 3.43%-10.31%. **Conclusions:** The results demonstrated that the proposed method could successfully quantify low concentrations of DMAC and its metabolites with high precision. Hence, this method is useful for evaluating DMAC exposure. In addition, this method can be used to examine metabolite behaviors in human bodies after exposure and to select appropriate biomarkers.
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Introduction

N,N-Dimethylacetamide (DMAC) is a highly polar solvent, characterized by its ability to mix with hydrophilic and hydrophobic solvents. DMAC is used as a reaction solvent for the production of synthetic fibers and resins and of medical chemicals, because of its high boiling and flash points and its high thermal and chemical stabilities[1)]. 10,000-100,000 tons of DMAC were manufactured and imported in Japan in 2008, the last year for which data are available[2)].

DMAC is also used as a substitute for *N, N*-dimethylformamide (DMF) because of the similarity of the molecular structures of these two compounds. DMAC is less toxic than DMF, because methyl isocyanate, a highly toxic metabolite, is produced by the metabolism of DMF but not of DMAC[3,4)]. However, a two-year DMAC inhalation study with rats and mice showed increased incidences of hepatocellular adenoma and hepatocellular carcinoma[5,6)]. In a DMAC inhalation study with pregnant

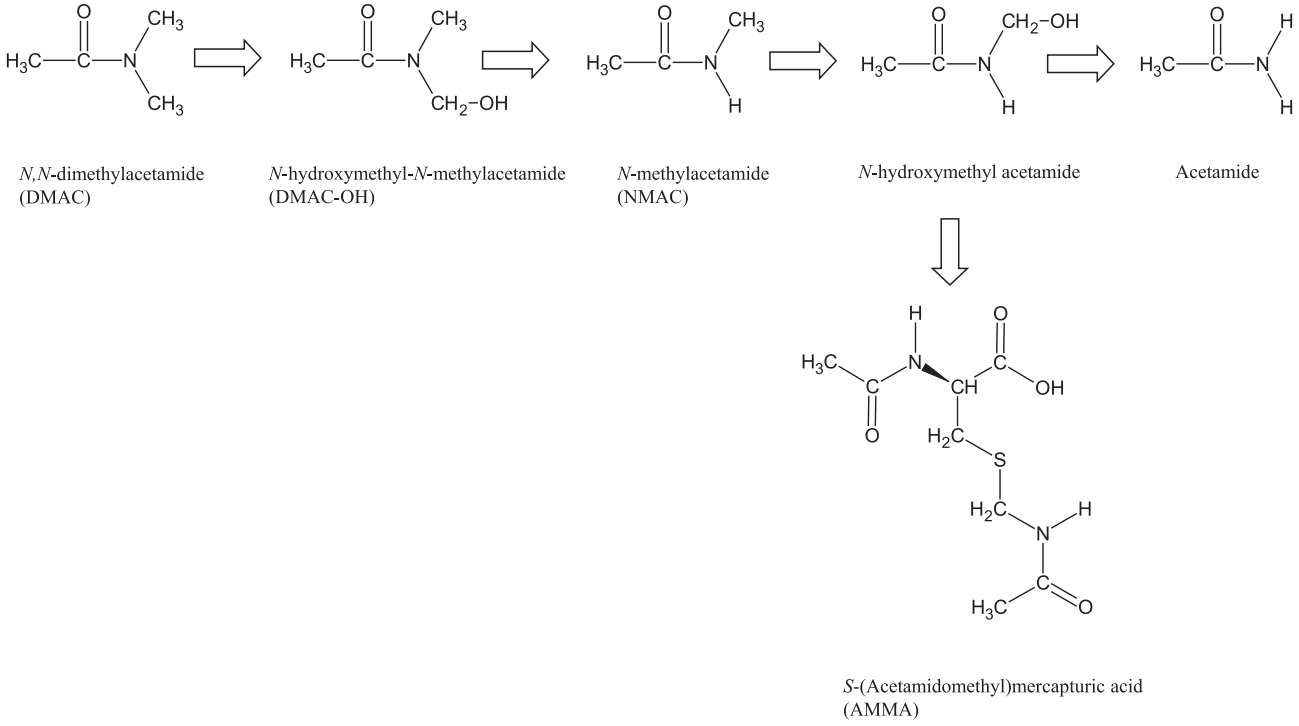

Fig. 1. Principal metabolic pathway for *N,N*-Dimethylacetamide (DMAC) in humans; from the American Conference of Governmental Industrial Hygienists Biological Exposure Indices 2011[11] with slight modifications.

rats, the most sensitive signs of developmental toxicity, such as increased liver weights and hepatocellular swelling, appeared[7].

Human studies found toxic hepatitis among DMAC-exposed workers in acrylic and urethane fiber factories in Japan and other countries[8-10].

In industrial workplaces, DMAC is absorbed primarily via the lungs and skin[11]. A human volunteer study indicated that dermal absorption was 40% of the total DMAC vapor uptake[12]. Accordingly, biological monitoring reflecting total DMAC uptake is necessary for the exposure assessment of workers.

In humans, DMAC is metabolized to *N*-hydroxymethyl-*N*-methylacetamide (DMAC-OH), *N*-methylacetamide (NMAC), *N*-hydroxymethyl acetamide (NMAC-OH), acetamide (AC), and *S*-(acetamidomethyl) mercapturic acid (AMMA) (Fig. 1)[11], among which DMAC-OH is the most common metabolite[4,11]. Occupational exposure limits based on biological monitoring of DMAC are not presently defined in Japan. Elsewhere, the recommended limits for urinary NMAC concentration, defined as a Biological Exposure Indices (BEIs) by the American Conference of Governmental Industrial Hygienists (ACGIH), is 30 mg/g creatinine[11]. Although DMAC exposure monitoring is not regulated in Japan, over 2,000 urinary samples were measured for biological DMAC monitoring every year, and 5.7% of these samples exceeded the ACGIH-recommended value of 30 mg/g creatinine for urinary NMAC[13].

The GC-based method, most commonly used for measuring NMAC in urine, was first reported by Barnes et al. in 1974[14]. The urinary NMAC level recommended as a BEI by the ACGIH was determined based on data collected by the Barnes method using GC with an injection port temperature of 200°C. However, Kawai et al. reported that DMAC-OH thermally decomposes into NMAC at this temperature[15]. The NMAC concentrations at 150°C and 200°C were 70% and 90%, respectively, of the concentration at 225°C, at which the concentrations plateaued[15]. Perbellini et al. reported that urinary NMAC concentrations at 150°C and 200°C were 38% and 83%, respectively, of the concentration measured at 250°C[4]. Previously, we conducted a similar experiment, using an aqueous standard DMAC-OH solution, and found that the concentration plateaued at 175°C[16]. Hence, the detected NMAC concentration is strongly dependent on the injection port temperature. Measurements in recent years were typically taken with an injection port temperature of 200-250°C[16], but the measured concentrations may be inaccurate because of variation in the NMAC concentrations due to the differences in temperature.

In the preliminary examination, we tried to avoid decomposition of analytes during GC analysis by modifying the molecular structures of the target compounds to increase their thermal stabilities. Derivatization was attempted using several types of alkyl chloroformates suitable for performing derivatization in urine[17-20]; however, these derivatizations were unsuccessful.

Table 1. Retention times and MRM parameters for selected precursor and product analytes.

Analyte	Retention time (min)	Precursor ion (*m/z*+; Q1)	Product ion (*m/z*+; Q3)	Collision energy (eV)
DMAC	11.49	88.1	46.1	–18
DMAC-OH	4.95	104.0	44.0	–14
NMAC	3.70	74.3	43.0	–21
AMMA	9.29	235.1	164.0	–11

A method for determining the final metabolic product, AMMA, by high-performance liquid chromatography (HPLC) was developed[3], but HPLC-based methods for determining DMAC-OH and NMAC have not been reported previously. In general, HPLC is considered unsuitable for measuring low-molecular-weight chemicals. However, its shortcomings were addressed by using a tandem mass spectrometer (MS/MS) as the detector and by selecting a column with strong retention of the target analytes. Electrospray ionization (ESI), the gentlest ionization method available, was used to prevent thermal degradation of DMAC-OH.

This study aimed to develop an analytical method that can simultaneously determine concentrations of urinary DMAC, DMAC-OH, NMAC, and AMMA.

Materials and Methods

Materials

DMAC and NMAC of guaranteed reagent grade were purchased from Tokyo Chemical Industry (Tokyo, Japan). HPLC-grade methanol and formic acid were purchased from Wako Pure Chemical Industries (Osaka, Japan) and Tokyo Chemical Industry, respectively. Water was purified using a Milli-Q water system (Millipore, Bedford, MA, USA). DMAC-OH and AMMA were synthesized by Tokyo Chemical Industry.

LC instrumentation and conditions

Separations were conducted using a Shimadzu Nexera UHPLC/HPLC system consisting of a DGU-20AR degasser, two LC-30AD pumps, an SIL-30AC autosampler, and a CTO-30A column oven (Shimadzu Scientific Instruments, Kyoto, Japan). A GL Sciences InertSustain C18 column (2.1 mm × 150 mm; 2 μm) was used, and the column temperature was 40°C. The mixture of 10 mmol/*l* aqueous formic acid solution and methanol was employed as the mobile phase, using the following gradient: 1% methanol for 0.1-10 min, 1%-50% methanol over 10-15 min, 50% methanol over 15-17 min, and 1% methanol over 17-24 min. The flow rate was 0.2 m*l*/min, and the injection volume was 1 μ*l*.

MS instrumentation and conditions

Qualitative and quantitative determination of the target compounds was conducted using an LCMS-8030 triple quadrupole MS with an ESI source (Shimadzu, Kyoto, Japan) in positive ion mode. Analyses parameters were as follows: interface voltage = 4.5 kV; interface temperature = 350°C; desolution line temperature = 250°C; nebulizer gas flow = 3.00 *l*/min; block heater temperature = 400°C; and drying gas flow = 15.00 *l*/min. Collision-induced dissociation was performed with argon gas. A multiple reaction monitoring technique, optimized using standard metabolite solutions, was applied to the fragment combination of each compound, as detailed in Table 1.

Preparation of standard solutions

DMAC, DMAC-OH, NMAC, and AMMA stock solutions were prepared by diluting the corresponding reagents to a concentration of ~1,000 mg/*l* in methanol. Standard solutions were prepared by diluting the stock solutions with water to approximate concentrations of 0.5-100 mg/*l*. Next, a blank urine sample collected from a non-exposed worker was centrifuged at 3000 rpm for 10 min using a Himac CT4D centrifuge (Hitachi, Tokyo, Japan). Subsequently, 0.01 m*l* of the above standard solution, 0.1 m*l* of the supernatant of the blank urine, and 0.9 m*l* of aqueous formic acid (10 mmol/*l*) were mixed in a tube. Consequently, these standard solutions corresponded to 0.05-10 mg/*l* of urine. Finally, a 1-μ*l* aliquot was injected into the LC-MS/MS equipment, and a standard curve was made.

Sample preparation

To confirm the applicability of the developed method, the above blank urine sample and pooled urine samples from workers handling DMAC were used. The urine samples were centrifuged at 3000 rpm for 10 min; 0.1 m*l* of the supernatant, 0.9 m*l* of aqueous formic acid (10 mmol/*l*), and 0.01 m*l* of water were mixed in a tube; and a 1-μ*l* aliquot was injected into the LC-MS/MS.

Method validation

Standard solutions of each target compound corresponding to the minimum concentrations of their respective calibration curves (DMAC: 0.047 mg/*l*, DMAC-OH: 0.053 mg/*l*, NMAC: 0.047 mg/*l*, AMMA: 0.050 mg/*l*) were measured repeatedly (n = 5). From the calibration curve, the method's limit of detection (LOD) and limit of

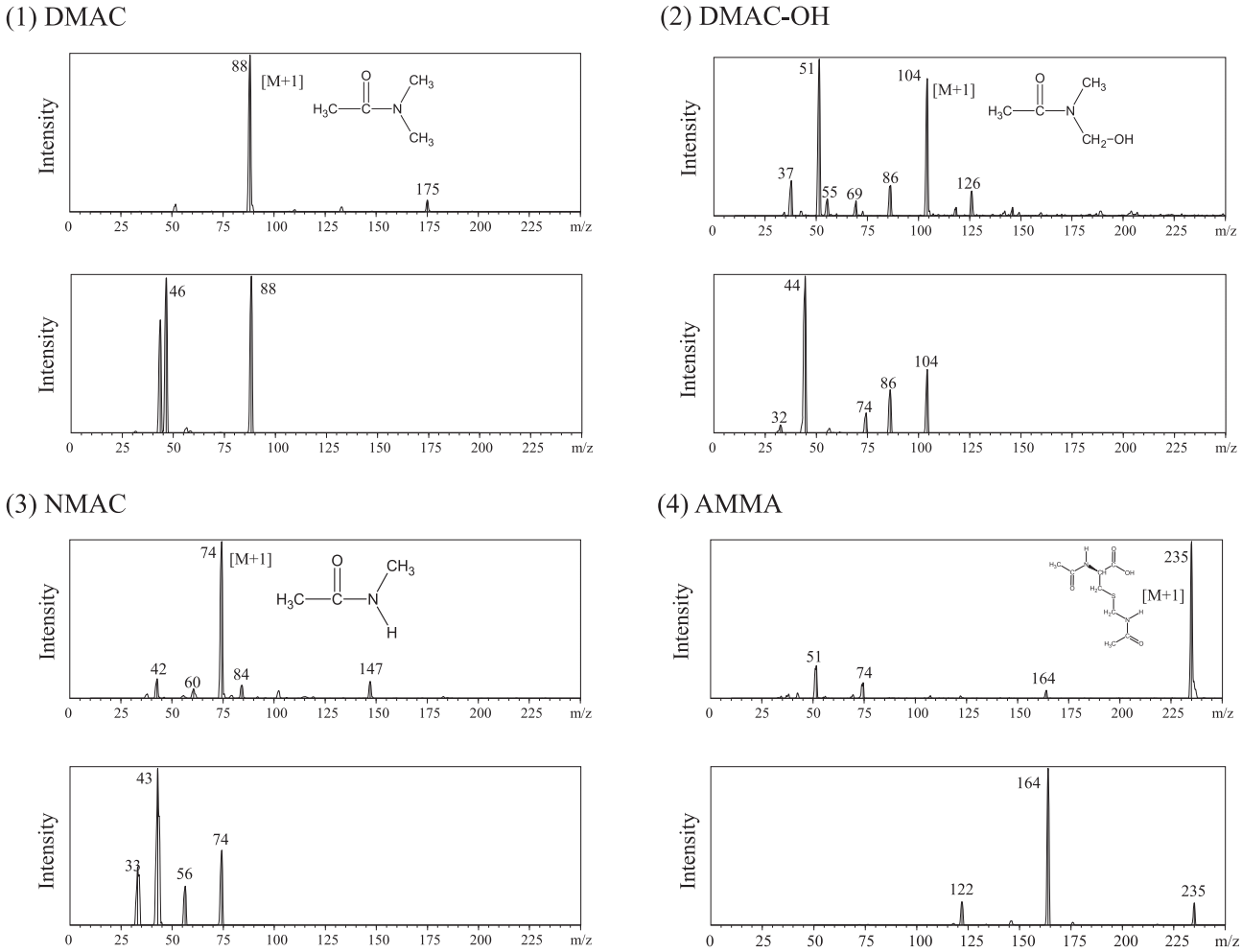

Fig. 2. Mass spectrum and molecular ion structure (top), and MS/MS spectra (below) of *N,N*-Dimethylacetamide (DMAC) metabolites in aqueous solution. (1) DMAC , (2) *N*-hydroxymethyl-*N*-methylacetamide (DMAC-OH), (3) *N*-methylacetamide (NMAC), and (4) *S*-(acetamidomethyl) mercapturic acid (AMMA).

quantification (LOQ) for all DMAC metabolites were defined as three and ten times the standard deviation ($n = 5$), respectively, of the peak area of the lowest standard.

Reliability was evaluated by determining the within-run and between-run accuracies and precisions using a spiked urine sample. Within-run accuracy was measured for three concentrations with $n = 5$ per analysis batch. Between-run accuracy was determined for three concentrations with $n = 5$ per analysis batch; each analysis was repeated three times. Within-run precision was measured for three concentrations with $n = 5$ per analysis batch. Between-run precision was determined for three concentrations with $n = 5$ per analysis batch; each analysis was repeated three times.

To evaluate between-person variability of matrix factor, urine samples of five non-exposed persons were prepared by the same procedure as described in "preparation of standard solutions," and the four metabolites were measured by LC-MSMS.

Results

Separation and identification of DMAC metabolites

Fig. 2 shows the mass spectrum and MS/MS spectra of DMAC metabolites in aqueous solution. The mass spectrum of DMAC metabolites exhibited a major ion such as a molecular cation. The MS/MS spectra for this precursor ion exhibited fragment ions.

Fig. 3 shows the chromatograms of the standard-spiked aqueous solution, blank urine, standard-spiked urine sample, and pooled urine samples of exposed workers. The InertSustain C18 column exhibited the most favorable retention and peak shapes across the four compounds in the standard-spiked aqueous solution and the urine sample. The optimal mobile phase was found to be a combination of 10 mmol/*l* aqueous formic acid solution and methanol, and the target compounds and other constituents in urine were separated using gradient elution, where the mixing ratio of 10 mmol/*l* formic acid:methanol was 99:1 (pH

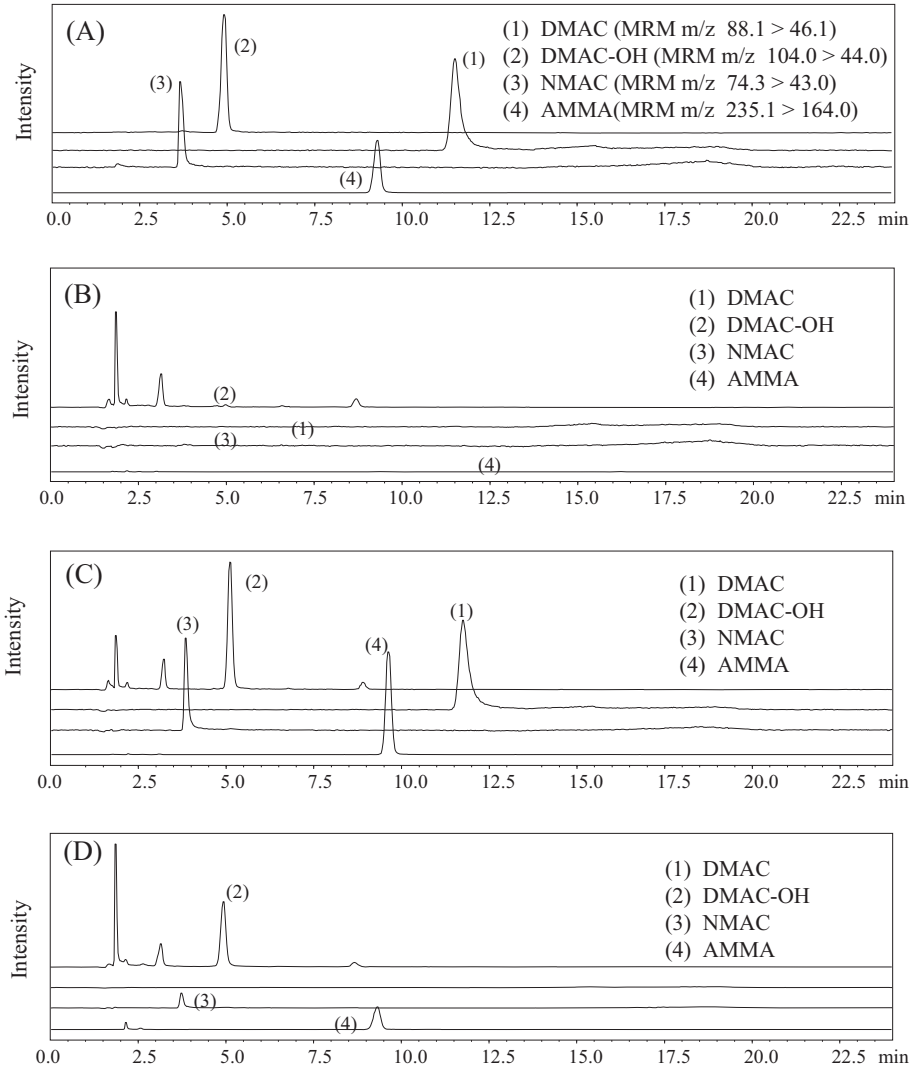

Fig. 3. MRM chromatogram of DMAC metabolites. (A) standard-spiked aqueous solution. ((1) DMAC (4.7 mg/*l*), (2) DMAC-OH (5.3 mg/*l*), (3) NMAC (4.7 mg/*l*), and (4) AMMA (5.0 mg/*l*)). (B) blank urine. (C) standard-spiked urine sample. ((1) DMAC (4.7 mg/*l*), (2) DMAC-OH (5.3 mg/*l*), (3) NMAC (4.7 mg/*l*), and (4) AMMA (5.0 mg/*l*)). (D) pooled urine samples of exposed workers.

2.3) for the first 10 min of elution.

Peaks corresponding to the four target compounds and interfering peaks were not found in the blank urine sample. Three peaks corresponding to DMAC-OH, NMAC, and AMMA were observed in the pooled urine samples of exposed workers. No interfering peaks were observed in the pooled urine samples of exposed workers.

Method validation

Table 2 shows the LOD values and the calibration curves for each metabolite. Each curve exhibited acceptable linearity within the concentration range of 0.05-5 mg/*l*, where the correlation coefficient was ≥ 0.999 for all four compounds. Table 3 shows the reliabilities of the measurements. Within-run accuracies were 96.5%-109.6%, with relative standard deviations of precision being 3.43%-10.31%. Between-run accuracies were 99.6%-111.8%, with relative standard deviations of precision being 2.91%-8.79%. Relative standard deviations of matrix factor within the concentration range of 0.05-5 mg/*l* were 10.6% (mean ± standard deviation: 75.6 ± 7.9), 13.7% (69.6 ± 9.5), 12.6% (65.1 ± 8.2), and 16.1% (126.9 ± 20.1), on average, respectively, for DMAC, DMAC-OH, NMAC, and AMMA.

Discussion

Method development

We tried various methods for separation and quantification of the target compounds by HPLC. Since the inten-

Table 2. Ranges of linearity and correlation coefficients for the proposed method.

	Detection limit (mg/*l*)	Range of linearity (mg/*l*)	Calibration curves		
			Slope ($\times 10^5$, *l*/mg)	Intercept ($\times 10^3$)	Correalation coefficient
DMAC	0.04	0.13–4.7	1.9	2.0	0.9999
DMAC-OH	0.02	0.07–5.3	1.2	8.0	0.9997
NMAC	0.05	0.16–4.7	0.82	5.7	0.9999
AMMA	0.02	0.07–5.0	0.78	6.1	0.9995

Table 3. Intra- and interday coefficients of variation for the proposed method.

Spiked urine concentration (mg/*l*)	Intraday (n = 5)[a]			Interday (n = 15)[b]		
	Mean±SD (mg/*l*)	Precision (%)	Accuracy (%)	Mean±SD (mg/*l*)	Precision (%)	Accuracy (%)
DMAC						
0.47	0.45±0.05	10.31	97.2	0.48±0.04	7.77	103.1
0.93	0.90±0.05	5.66	96.5	0.94±0.06	6.68	101.4
4.65	4.66±0.32	6.81	100.2	4.65±0.24	5.12	99.9
DMAC-OH						
0.53	0.56±0.03	5.56	106.2	0.56±0.03	5.09	107.0
1.05	1.12±0.04	3.43	106.6	1.14±0.03	2.91	108.6
5.25	5.24±0.29	5.59	99.7	5.23±0.19	3.57	99.6
NMAC						
0.47	0.49±0.02	4.80	104.8	0.49±0.02	5.04	105.2
0.94	0.98±0.05	5.44	103.8	0.99±0.06	6.23	105.6
4.70	4.69±0.22	4.75	99.8	4.69±0.21	4.39	99.7
AMMA						
0.50	0.52±0.05	10.13	105.8	0.53±0.05	8.79	106.3
0.99	1.08±0.09	8.19	109.6	1.11±0.08	6.93	111.8
4.95	4.93±0.40	8.08	99.6	4.94±0.34	6.79	99.8

[a]Intraday reproducibility analysis was performed on a single day.
[b]Interday reproducibility analysis was performed over three consecutive days five replicates.

sities of the ions generated by adding proton to the target compounds in positive mode were high, the collision energies and product ions of the target compounds were determined by automated optimization using these ions as the precursor ions. The flow rate of the nebulizer gas was evaluated to be between 1.5 *l*/min and 3.0 *l*/min; the latter was chosen because of its superior peak strength. Because ionization of the target molecules was suppressed by other constituents in urine, removal of non-target compounds was attempted using solid-phase extraction (using Mono Spin C18-CX, GL Science, Tokyo, Japan) as a preprocessing step, but it was unsuccessful. Therefore, the HPLC column and mobile phase conditions were optimized for separation of the target compounds from other constituents.

DMAC metabolites have high polarities and low molecular weights. Six HPLC columns were evaluated for the retention of these metabolites: three reverse-phase Octa Decyl Silyl (ODS) columns (Shim-pack XR-ODS (Shimadzu, Kyoto, Japan), Inertsil ODS-3 and InertSustain C18 (GL Science, Tokyo, Japan)), an HILIC column (Inertsil Amide (GL Science)), a normal-phase column (Inertsil Diol (GL Science)), and a polymer column (Capcell Pak MF C18 (SHISEIDO, Tokyo, Japan)), typically used for biological samples containing proteins.

Retention by the Shim-pack XR-ODS column was insufficient. Inertsil Amide (GL Science) and Inertsil Diol (GL Science) columns both demonstrated poor reproducibility. The peak shapes of DMAC and DMAC-OH were poor using the Capcell Pak MF C18 column and overlapped with an unknown peak. The Inertsil ODS-3 column exhibited the strongest retention among the columns

evaluated; the separation of the four metabolites was also favorable. However, the sensitivity to AMMA was extremely poor compared to that of the other metabolites. Favorable retentions and peak shapes were obtained with all four target compounds using the InertSustain C18 column.

In addition to the evaluation of the HPLC columns, the sensitivity, peak shape, and separation of the four analytes were compared by changing the combination and conditions (acid and salt concentration) of the columns and mobile phases. For the mobile phase, acetonitrile and methanol were used as organic solvents, formic acid was used as a volatile acid, and ammonium formate and ammonium acetate were used as volatile salts. Favorable peak shape and strength were obtained using the InertSustain C18 column and a mobile phase consisting of methanol and a 10 mmol/*l* aqueous formic acid solution.

The target compounds and other constituents in the urine samples were separated using gradient elution, where the mixing ratio of 10 mmol/*l* formic acid:methanol was 99:1 (pH 2.3) for the first 10 min. Using SPARC[21] (ARChem, Lionel A. Carreira, University of Georgia), the p*K*a values of DMAC, DMAC-OH, NMAC, and AMMA were estimated to be −0.30, −0.04, −0.11, and 3.37, respectively. Therefore, the pH of the mobile phase should be in the range of 1.37-2.37 to prevent deprotonation of the metabolites and to retain them on the column. Consequently, the concentration of formic acid used was relatively high.

Method validation

DMAC and two metabolites, NMAC and AMMA, have been previously measured in urine. The LOQ of DMAC in urine was calculated to be 0.05 mg/*l* using GC[4], while that using the method described herein was 0.13 mg/*l*. The LOQ of NMAC in urine using GC was reported to be 1.5 mg/*l*[4], while that in the present study was 0.16 mg/*l*. The LOQ of AMMA in urine using LC was reported to be 1.5 mg/*l*[3], while that using the method developed in this study was 0.07 mg/*l*.

The guidelines concerning bioanalytical method validation by the European Medicines Agency (EMA)[22] define acceptable within-run and between-run accuracies as less than 15%; the accuracies in the present study were below 12%. The guidelines also define acceptable within-run and between-run precisions to be 15% or less; the results obtained in this study were below 11%.

The guidelines of the EMA[22] define acceptable relative standard deviations of matrix factor to be less than 15%; the relative standard deviations of matrix factor in the present study were below 14% for DMAC, DMAC-OH, and NMAC, so our developed method can be used to quantitatively determine these three metabolites. Because variation of matrix factor for AMMA slightly exceeded the guidelines, it may be necessary to use a standard addition method for each subject.

Thus, separation and quantification of DMAC metabolites in urine is possible using the method developed in this study. However, the exposure limit recommended by the ACGIH (30 mg/g creatinine of NMAC in urine) cannot be applied directly to the NMAC concentrations measured in the present study because this limit was determined based on data collected by the Barnes method, in which DMAC-OH was thermally decomposed to NMAC. Therefore, it is necessary to determine the relationships between the exposure to airborne DMAC and the metabolite concentrations determined using the method proposed herein.

Conclusion

A high-precision method capable of measuring low concentrations of DMAC and its metabolites in urine was developed. This method can be utilized to assess DMAC exposure of industrial workers. In addition, this method can be used to determine the in-vivo behavior of metabolites after exposure and to select appropriate biomarkers.

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Conflicts of interest: None declared.

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