Determination of Monomethylarsonous Acid, a Key Arsenic Methylation Intermediate, in Human Urine

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In this study we report on the finding of monomethylarsonous acid [MMA(III)] in human urine. This newly identified arsenic species is a key intermediate in the metabolic pathway of arsenic biomethylation, which involves stepwise reduction of pentavalent to trivalent arsenic species followed by oxidative addition of a methyl group. Arsenic speciation was carried out using ion-pair chromatographic separation of arsenic compounds with hydride generation atomic fluorescence spectrometry detection. Speciation of the inorganic arsenite [As(III)], inorganic arsenate [As(V)], monomethylarsonic acid [MMA(V)], dimethylarsinic acid [DMA(V)], and MMA(III) in a urine sample was complete in 5 min. Urine samples collected from humans before and after a single oral administration of 300 mg sodium 2,3-dimercapto-1-propane sulfonate (DMPS) were analyzed for arsenic species. MMA(III) was found in 51 out of 123 urine samples collected from 41 people in inner Mongolia 0–6 hr after the administration of DMPS. MMA(III)in urine samples did not arise from the reduction of MMA(V) by DMPS. DMPS probably assisted the release of MMA(III) that was formed in the body. Along with the presence of MMA(III), there was an increase in the relative concentration of MMA(V) and a decrease in DMA(V) in the urine samples collected after the DMPS ingestion. *Key words*: arsenic speciation, biomarkers, metabolism, methylation, monomethylarsonous acid, sodium 2,3-dimercapto-1-propane sulfonate, trivalent methylarsenic species, urine metabolites. *Environ Health Perspect* 108:1015–1018 (2000). [Online 4 October 2000]

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The major human metabolic pathway for inorganic arsenic is methylation (*1–6*). Most of the inorganic arsenic, As(III) and As(V), is metabolized to dimethylarsinic acid [DMA(V)] and monomethylarsonic acid [MMA(V)] before excretion in the urine. Because the relative acute toxicity decreases from inorganic arsenite and arsenate [median lethal dose (LD_{50}) 10–20 mg/kg) to MMA(V) (LD_{50} 700–1,600 mg/kg) and DMA(V) (LD_{50} 700–2,600 mg/kg), it has been suggested that the methylation of arsenic in the body is a detoxification pathway (*2–6*). More recent research argues that the carcinogenic effects of these arsenic compounds are not well understood and may not follow the same decreasing order. Several studies suggest that methylated arsenic species, especially at the trivalent state, may be more toxic than the parent inorganic arsenic compounds (*1,7–20*). Methylation of arsenic is also implicated in the carcinogenic effects of arsenic because of the possible effects on the methylation of DNA (*21,22*).

Methylation of arsenic involves a two-electron reduction of pentavalent [e.g., As(V) and MMA(V)] to trivalent [e.g., As(III) and MMA(III)] arsenic species followed by the transfer of a methyl group from a methyl donor, such as *S*-adenosylmethionine (*23–25*). The reduction and oxidative addition sequence may be summarized in Figure 1. This methylation mechanism has been widely accepted, and the metabolites DMA(V) and MMA(V) have been consistently observed in human urine. A key intermediate for the methylation of MMA(V) to DMA(V) is the MMA(III) species. Several recent studies have indicated the presence of MMA(III) species in rat liver cytosol and hepatocytes and demonstrated the important effects of the methylated trivalent arsenic species in biological systems (*16–20,26–29*). Although DMA(V), MMA(V), As(V), and inorganic As(III) have been commonly detected in human urine (*30–43*), the key intermediate metabolite MMA(III), from the methylation of MMA(V) to DMA(V), deserves more attention; however, there is no analytical method for the speciation of MMA(III) in human systems.

This report describes the speciation of MMA(III) in addition to the usual arsenic species in human urine, As(III), As(V), MMA(V), and DMA(V). Determination of arsenic species in human urine is a measure of recent exposure to arsenic and provides useful information for a better understanding of arsenic metabolism and health effects.

Materials and Methods

Reagents and standards. Sodium arsenate [$As(O)OH(ONa)_2 \cdot 7H_2O$] and sodium cacodylate [$(CH_3)_2As(O)ONa$] were obtained from Sigma (St. Louis, MO, USA), and monomethylarsinate [$CH_3As(O)OHONa$] was obtained from Chem Service (West Chester, PA, USA). Stock solutions (1,000 mg As/L) of these arsenicals were prepared by dissolving appropriate amounts of the corresponding arsenic compounds in 0.01 M hydrocholoric acid, and standard solutions were prepared by serial dilution with deionized water. An atomic absorption arsenic standard solution (Sigma) containing 1000.0 mg As/L as arsenite in 2% KOH was used as the primary arsenic standard. Concentrations of arsenic in sodium arsenate, sodium cacodylate, and sodium monomethylarsinate solutions were standardized against the atomic absorption arsenic standard solution using both inductively coupled plasma mass spectrometry (ICPMS) and flame atomic absorption spectrometry analyses. The source of MMA(III) was the solid oxide (CH_3AsO), which was prepared following the procedure of Cullen et al. (*13*).

We used tetrabutylammonium hydroxide as an ion-pairing reagent and malonic acid as a buffer for HPLC separation. They were obtained from Aldrich (Milwaukee, WI, USA). HPLC-grade methanol was from Fisher (Pittsburgh, PA, USA). The mobile phase solutions (pH 5.8–5.9) containing 5 mM tetrabutylammonium hydroxide, 2–5 mM malonic acid, and 5% methanol were prepared in deionized water and filtered through a 0.2-µm membrane before use. Sodium borohydride (Aldrich) solution (1.3%) in 0.1 M sodium hydroxide (Fisher) was prepared fresh daily. All reagents used were of analytical grade or better.

Urine samples. One set of 164 urine samples was collected from 41 people in inner Mongolia, China. They normally drank water from wells containing 510–660 µg/L of arsenic (*44*). All the participants were asked not to consume seafood for 3 days before and during the urine sample collection period.

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They fasted overnight and were then given 300 mg of sodium 2,3-dimercapto-1-propane sulfonate (DMPS) orally. A urine sample was collected from each person before the administration of DMPS (between 11 hr before and the time of DMPS administration). We collected three urine samples from each person 0–2 hr, 2–4 hr, and 4–6 hr after the administration of DMPS. From the time of overnight fast and throughout the study, the participants did not drink well water; instead, they drank distilled water. Details on participant selection criteria and protocols for DMPS administration are described elsewhere (*44*). Informed consent was obtained from the participants before the study.

Urine samples were collected in 3-L polyethylene containers (Baxter Laboratories, Inc., Morton Grove, IL, USA). Samples were immediately frozen by placing them in a portable icebox containing dry ice. The samples were kept frozen during transportation and were stored at –20°C. The samples were stored for approximately 6 months before analysis. Samples were thawed at room temperature and an aliquot was analyzed for arsenic species using HPLC separation with hydride generation atomic fluorescence spectrometry (HGAFS) detection.

A Standard Reference Material (SRM), Toxic Metals in Freeze-Dried Urine (SRM 2670), was obtained from the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA). The freeze-dried urine was reconstituted by adding 20.0 mL deionized water as recommended by the supplier. The certified value for total arsenic concentration is 480 ± 100 µg/L in two bottles containing elevated levels of toxic metals. In the other two bottles containing normal levels of toxic metals, the concentration of arsenic is not certified, and a reference value of 60 µg/L has been provided. We used the SRM for method validation. Results for the speciation of arsenic in the SRM using the HPLC-HGAFS method were in good agreement with the certified and reference values (*45*).

Separation of arsenic using HPLC with HGAFS detection. The method of quantifying arsenic species was modified from previous methods using ion pair chromatographic separation with HGAFS (*43,46*). The HPLC system consisted of a Gilson Model 370 pump (Middletone, WI, USA) with a 5 mL/min stainless steel pump head, a Rheodyne 6-port sample injector (Model 7725i; Rheodyne, Rohnert Park, CA, USA) with a 20-µL sample loop, and a reversed-phase C18 column (ODS-3, 150 mm × 4.6 mm, 3-µm particle size; Phenomenex, Torrance, CA, USA).

The temperature of the HPLC column was maintained at 50°C. For temperature control, the separation column was mounted inside a column heater (Model CH-30; Eppendorf, Westbury, NY, USA), which was controlled by a temperature controller (Model TC-50; Eppendorf). The mobile phase was preheated to the temperature of the column by using a precolumn coil of 50-cm stainless-steel capillary tubing, which was also placed inside the column heater.

A mobile-phase solution (pH 5.8–5.9) containing 5 mM tetrabutylammonium hydroxide, 2–5 mM malonic acid, and 5% methanol was pumped through the column at a flow rate of 1.2–1.5 mL/min. Effluent from the HPLC column was mixed at two T-joints, with continuous flows of hydrochloric acid (1.8 M) and sodium borohydride (1.3%). Arsines generated were separated from liquid waste and carried by a continuous flow of argon to an atomic fluorescence detector (Excalibur 10.003; P.S. Analytical, Kent, UK) for quantitation.

The atomic fluorescence detector consisted of an excitation source, an atom cell, fluorescence collection optics, a photomultiplier tube, and a data collection unit. A quartz tube with argon/hydrogen diffusion flame was used as the atom cell for atomization. An arsenic hollow cathode lamp was used for fluorescence excitation. Atomic fluorescence (193.7 nm) was collected at a right angle with respect to the excitation light, optically filtered with a multireflectance filter to reduce scattering and background noise, and detected with a solar blind photo multiplier tube. We used a pentium computer with Varian Star Workstation software (Victoria, Australia) and an analog/digital converter board to acquire and process signals from the atomic fluorescence detector.

Results and Discussion

We have previously investigated HPLC conditions and optimized them for rapid separation of As(III), As(V), MMA(V), and DMA(V) (*46*). The speciation of these four usual arsenic compounds in urine is complete in 4 min (Figure 2A). Within this narrow separation time window, however, MMA(III) coelutes with As(III) and DMA(V). To obtain a separation of MMA(III) from As(III) and DMA(V), an improvement in the resolution between As(III)and DMA(V) is needed to allow for a wider separation window between these two species. This can be achieved by adjusting malonic acid concentration in the HPLC mobile phase. By reducing malonic acid concentration from 5 mM (Figure 2A) to 2 mM (Figure 2B), an

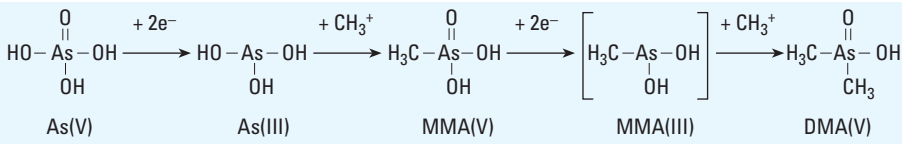

Figure 1. Summary of the reduction and oxidative addition sequence of arsenic methylation.

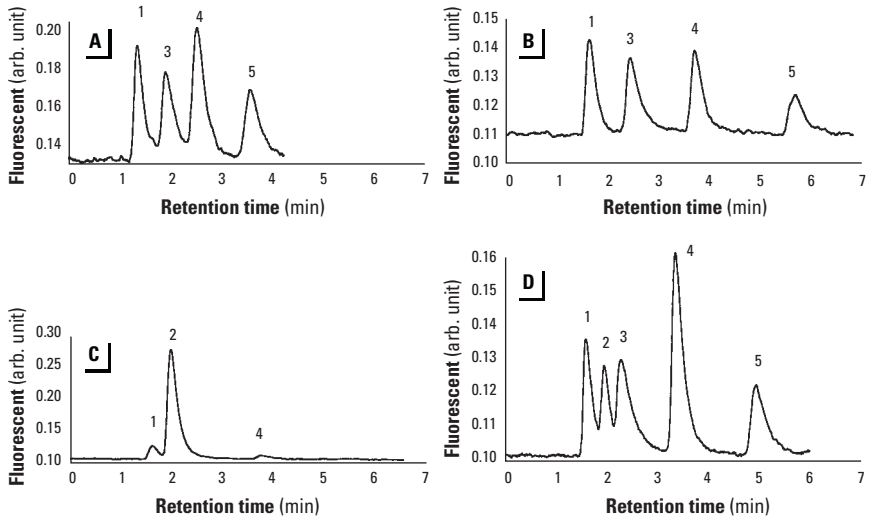

Figure 2. Chromatograms showing the separation of mixtures of As(III), As(V), MMA(V), DMA(V), and MMA(III) in deionized water. arb, arbitrary. The analyte mixtures contained As(III), As(V), MMA(V) and DMA(V) (*A*, *B*); MMA(III) standard (*C*); and As(III), As(V), MMA(V), DMA(V), and MMA(III) (*D*). For (*A*), the mobile phase contained 5 mM tetrabutylammonium hydroxide, 5 mM malonic acid, and 5% methanol (pH 5.9), and its flow rate was 1.5 mL/min. For (*B*) and (*C*), the mobile phase contained 5 mM tetrabutylammonium hydroxide, 2 mM malonic acid and 5% methanol (pH 5.9), and its flow rate was 1.2 mL/min. For (*D*), the mobile phase contained 5 mM tetrabutylammonium hydroxide, 3 mM malonic acid, and 5% methanol (pH 5.85), and its flow rate was 1.2 mL/min. Peaks labeled 1–5 correspond to As(III), MMA(III), DMA(V), MMA(V), and As(V), respectively.

extended separation time period is obtained. Under these conditions, MMA(III) (Figure 2C) can be resolved from As(III) and DMA(V). This is at the expense of a longer retention time (6 min) for the arsenic species (Figure 2B). To achieve a reasonable separation within the shortest time possible, we chose to use 3 mM malonic acid in the mobile phase. The speciation of the five arsenic compounds, As(III), As(V), MMA(V), DMA(V), and MMA(III), is complete in 5 min (Figure 2D).

Figure 3 shows chromatograms from HPLC-HGAFS analyses of a urine sample (dotted trace) and the urine sample spiked with MMA(III) and MMA(V) standards (solid trace). The retention time of the suspected MMA(III) in urine sample (dotted trace) is identical to that from the authentic MMA(III) standard. Co-injection of the MMA(III) standard with the urine sample (solid trace) demonstrates the co-elution of the suspected MMA(III) peak in the sample with that of the standard MMA(III), confirming the identity of MMA(III) in the urine sample.

The detection limit for MMA(III) in urine is 4 μg/L. We are currently improving this detection limit, and we expect to achieve a detection limit of 1 μg/L.

Figure 4 shows a series of chromatograms from the HPLC-HGAFS analyses of urine samples from a subject before and after a single oral administration of 300 mg of DMPS. No MMA(III) is observed in the urine sample collected before the DMPS treatment; inorganic As(III), DMA(V), and MMA(V) are the major arsenic species (Figure 4A). Following the administration of DMPS, MMA(III) is observed in all the three samples collected between 0–2 hr (Figure 4B), 2–4 hr (Figure 4C), and 4–6 hr (Figure 4D) after the DMPS treatment. Concentrations of arsenic species in these samples are summarized in Table 1. These are among the samples that have the highest arsenic concentrations from a total of 164 urine samples from 41 people in inner Mongolia (*44*). Before DMPS treatment, the proportions of inorganic As(III) (9%), MMA(V) (10%), and DMA(V) (81%) in the urine sample are similar to those reported in literature (*30,32,47*). After the DMPS treatment, the arsenic speciation patterns are markedly altered. The proportions of the trivalent arsenic species [As(III) and MMA(III)] is dramatically increased to 30–40%. DMA(V) is dramatically decreased to about 30%. MMA(V) is increased to about 30%. Most importantly, MMA(III), a key arsenic methylation intermediate, is observed.

To examine whether the presence of MMA(III) is due to reduction by excess DMPS present in urine, we added 100 μg/L MMA(V) and 100 mg/L DMPS to a urine sample and kept the mixture at room temperature for up to 48 hr. The reaction mixture was periodically sampled and analyzed using the HPLC-HGAFS method. No MMA(III) was detected from the mixture, although the molar concentration of DMPS is approximately 360-fold that of MMA(V). The concentration of DMPS in urine after a single oral administration of DMPS is below this level (*48*). The results confirm that the MMA(III) detected in urine samples of DMPS-treated people is not simply due to the reduction of MMA(V) to MMA(III) *in vitro*. Furthermore, a reduction of MMA(V) to MMA(III) would lead to a decreased concentration of MMA(V). But this is clearly not the case, as shown in Figure 4 and Table 1. In fact, there is an increase in the relative concentration of MMA(V) and a decrease in DMA(V). The MMA(III) species is likely formed *in vivo*. The administration of DMPS probably assists the release of MMA(III) from the body and the subsequent excretion in the urine.

We have further analyzed arsenic species in another set of urine samples from people in Romania (*29*). We also found MMA(III) in the first-morning-void urine samples from 10 people before DMPS treatment (*29*). This confirms that the presence of MMA(III) is not due to the DMPS treatment although it is possible that DMPS treatment may affect arsenic metabolism.

MMA(III) can be readily oxidized to MMA(V) during sample storage. In a preliminary study of the stability of MMA(III), we found that approximately 60% of 100 μg/L MMA(III) spiked into a urine sample was oxidized to MMA(V) after the sample was stored for 2 weeks at 4°C. The samples we

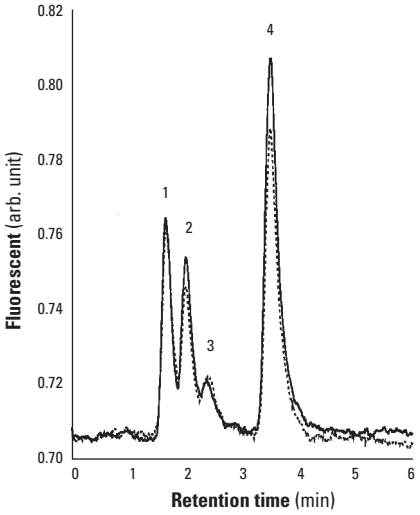

Figure 3. Chromatograms obtained from the HPLC-HGAFS analyses of a urine sample (dotted trace) and the urine sample spiked with MMA(III) and MMA(V) (solid trace). arb, arbitrary. The same conditions as shown in Figure 2D. were used. Peak identities are the same as shown in Figure 2.

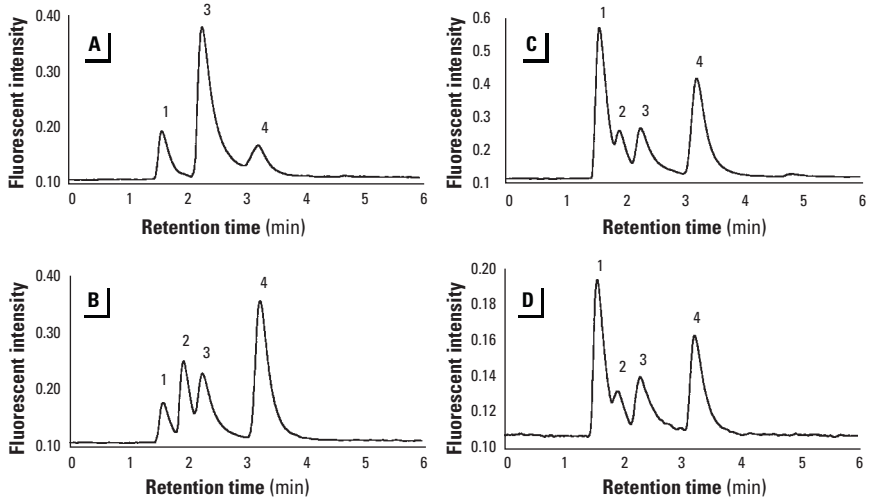

Figure 4. Chromatograms obtained from the HPLC-HGAFS analyses of a series of urine samples collected from a subject before and after a single oral dose of 300 mg DMPS (*A*) between 11 hr before and the time of the DMPS administration, and (*B*) 0–2 hr, (*C*) 2–4 hr, and (*D*) 4–6 hr after the administration of DMPS. The same conditions as shown in Figure 2D were used. Peak identities are the same as shown in Figure 2.

Table 1. Concentrations of arsenic species (μg/L) in urine samples collected from a subject before and after the administration of 300 mg DMPS.

Sample	Sampling time	As(III)	As(V)	MMA(V)	DMA(V)	MMA(III)	Sum
15A	−11–0 hr	63 ± 1	ND	66 ± 3	564 ± 9	ND	693 ± 9
15B	0–2 hr	44 ± 1	ND	239 ± 2	250 ± 2	227 ± 5	760 ± 5
15C	2–4 hr	313 ± 9	14 ± 1	311 ± 12	329 ± 5	240 ± 4	1,202 ± 12
15D	4–6 hr	59 + 1	ND	53 ± 2	72 ± 4	43 ± 3	227 ± 4

ND, below detection limit [1 μg/L for As(V) and 4 μg/L for MMA(III)]. Results are means ± 1 SD from triplicate analyses of each sample.

used in this study had been stored at –20°C for 1–6 months before arsenic speciation analyses. It is possible that the MMA(III) we detected in these urine samples is underestimated because of possible conversion of MMA(III) to MMA(V) during the sample storage. In our previous studies of the stability of As(III), As(V), MMA(V), and DMA(V), we did not observe any conversion of these species to MMA(III) (*45*).

Biomethylation is commonly assumed to be the main process of detoxifying arsenic (*2–6*) because the metabolites usually observed in urine, MMA(V) and DMA(V), are less acutely toxic and more readily excreted in urine than the inorganic arsenic species. However, there is evidence that MMA(III) could be more toxic than the inorganic arsenic species (*16–18*). The finding of highly toxic MMA(III) species in human urine reported here, together with recent studies on the toxic effects of arsenic, suggests that methylation of arsenic may not be strictly a detoxification process for humans, as previously believed. Although epidemiologic studies have demonstrated dose–response relationships between the exposure to high levels of arsenic (hundreds of microgram per liter) and the prevalence of skin, bladder, and lung cancers, health effects resulting from lower levels of arsenic ingestion have not been delineated (*25*). Studies of arsenic metabolism should contribute to a better understanding of arsenic health effects. The technique we have developed to detect trace levels of As(III), As(V), MMA(V), DMA(V), and MMA(III) in human urine is useful for metabolic and epidemiologic studies of arsenic.

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