Journal of Exposure Science and Environmental Epidemiology (2014) 24, 113–120

www.nature.com/jes

ORIGINAL ARTICLE

Occurrence of trivalent monomethyl arsenic and other urinary arsenic species in a highly exposed juvenile population in Bangladesh

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Following reports of high cytotoxicity and mutagenicity of monomethyl arsonous acid (MMA(III)) and early reports of urinary MMA(III) in arsenic-exposed individuals, MMA(III) has often been included in population studies. Use of urinary MMA(III) as an indicator of exposure and/or health risk is challenged by inconsistent results from field studies and stability studies, which indicate potential artifacts. We measured urinary arsenic species in children chronically exposed to arsenic in drinking water, using collection, storage, and analysis methods shown to conserve MMA(III). MMA(III) was easily oxidized in sample storage and processing, but recoveries of 80% or better in spiked urine samples were achieved. Attempts to preserve the distribution of MMA between trivalent and pentavalent forms using complexing agents were unsuccessful and MMA(III) spiked into treated urine samples actually showed lower stability than in untreated samples. In 643 urine samples from a highly exposed population from the Matlab district in Bangladesh stored for 3–6 months at $\leq -70\,^{\circ}C$, MMA(III) was detected in 41 samples, with an estimated median value of 0.3 μg/l, and levels of MMA(III) above 1 μg/l in only two samples. The low urinary concentrations in highly exposed individuals and known difficulties in preserving sample oxidation state indicate that urinary MMA(III) is not suitable for use as an epidemiological biomarker.

Journal of Exposure Science and Environmental Epidemiology (2014) **24,** 113–120; doi:10.1038/jes.2013.14; published online 3 April 2013

Keywords: arsenic; exposures; speciation; urine; analysis; children

INTRODUCTION

Arsenic is a worldwide environmental toxicant[1] and a cause of lung and other cancers in populations exposed via contaminated drinking water.[2] Chronic overexposure to arsenic from drinking water is associated with hepatotoxic, neurological, gastrointestinal, cardiovascular, and respiratory effects, and possibly diabetes.[3,4] Globally, an estimated 200 million people are at risk from consuming arsenic in drinking water.[5]

In groundwater, arsenic occurs as arsenate (AsO_4^{-3}, pentavalent, INA(V)) and arsenite (AsO_3^{-3}, trivalent, INA(III)). Other dietary forms of arsenic occur as organ arsenicals in crops, fish, shellfish, and other organisms, but are not significant sources of population toxicity.[5] Arsenic toxicity is modified by human metabolism, which interconverts arsenic compounds having different toxicities. Details of the biological conversion mechanisms and processes are the subject of active investigation, but the overall pattern is thought to be alternating reduction and biomethylation steps progressing from inorganic arsenic to trimethylarsenic forms.[6] The compounds intermediate in this chain have all been reported to occur in human biological samples: inorganic arsenic (pentavalent, INA(V), and trivalent INA(III)), monomethyl arsenic (pentavalent, MMA(V) and trivalent, MMA(III)); dimethyl arsenic (pentavalent, DMA(V) and trivalent DMA(III)); and (pentavalent) trimethylarsine oxide (TMAO). In humans, DMA(V) is the predominant form in urine.

The relative toxicity among metabolites of inorganic arsenic was originally thought to decrease with increasing methylation, and this is generally the case for INA(V), MMA(V), and DMA(V). However, methylated trivalent arsenic compounds are more reactive toward tissue biomolecules than their pentavalent counterparts; MMA(III) and DMA (III) have been reported to be more cytotoxic[8] and genotoxic[9] than INA(III), and several lines of evidence have led to the hypothesis that MMA(III) may be the primary toxic species resulting from ingested arsenic.[3] Given the evidence that trivalent MMA and DMA are likely present in tissues and blood of exposed individuals, the biological levels of these compounds and their relationship to arsenic intake and genetic or other influences are important for understanding health risks from chronic arsenic exposure.[10,11]

The use of urinary arsenic concentration to ascertain exposure status has been widely practiced in studies of population health effects.[12] Measurement of specific urinary organ arsenicals as metabolites of inorganic arsenic exposure and dietary sources helps in the understanding of arsenic metabolism, excretion, and individual susceptibility to health effects.

The detection and quantification of urinary MMA(III) and DMA(III) in various populations has been reported over the past 10 years, but with highly variable findings. Some reports suggest that in exposed populations, these trivalent compounds are major or even dominant components of urinary arsenic.[13–16] Other

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Received 27 November 2012; accepted 19 February 2013; published online 3 April 2013

investigators suggest little or no DMA(III), and very low levels of MMA(III) occur in populations with a range of arsenic exposures.[10,17,18] Based on these prior reports, the usefulness of measuring urinary arsenic metabolites according to their oxidation state as a biomarker of exposure or susceptibility has been an open question of importance for planning future population studies. In addition to the particular toxicity of MMA(III),[3] the observation that DMA(III) is more reactive and harder to preserve compared with MMA (III) is also notable in assessing the value of urinary arsenic biomarkers. This study therefore focused on the detection of MMA(III) in urine.

Potential problems in the determination of MMA(III) that have been noted in the past include storage instability and measurement artifacts.[19,20] Based on the reported storage instability of MMA(III) in urine samples at –20 °C, negative findings for several of these studies[10,15,17,21–23] might be due to oxidation during storage, while findings of high levels and high proportions of MMA(III) suggest other method artifacts. Laboratory studies of MMA(III) instability have led to conclusions that it might be more prevalent than reported in urine samples from population studies, due to losses in sample handling and measurement.[24] Lindberg and co-workers[10] have suggested that MMA(III) may be too reactive toward oxidation or binding to tissue components to be suitable for use as a urinary biomarker. More recent reports have suggested that tissue levels are both more relevant and less susceptible to oxidative artifacts than are urine samples.[25]

The wide variation in reports of the frequency, proportion, and level of MMA(III) and DMA(III) in human urine may indicate variability due to a variety of factors, summarized recently by Tseng.[12] These include sample collection, preservation, and measurement; population-specific environmental or nutritional factors; or underlying genetic differences[17,26,27] related to the enzymes that control biotransformation. Resolving the questions regarding issues of sample collection/storage/analysis is therefore of prime importance in determining the biological significance of measured levels of trivalent methylated arsenic in urine.

In this report, we describe a method for determination of urinary arsenic species, characterize the ability of the method to preserve MMA with respect to its oxidation state during sample preparation and analysis, and report our findings for levels of MMA(III) and other urinary arsenic species in children chronically exposed to arsenic in drinking water.

MATERIALS AND METHODS

Chemicals

Nitric acid (trace metal grade) was from Thermo-Fischer Scientific (Waltham, MA, USA). Reagent water was prepared using a Barnstead Nanopure purification system (Thermo-Fischer) and was continually monitored to be $\leq$17 MΩ-cm. Inductively coupled mass spectrometry (ICP/MS) calibrants for inorganic arsenic and germanium were NIST traceable, obtained from Ultra Scientific (Kingston, RI, USA). Tetrabutylammonium hydroxide ion-pairing reagent (40% in water) and ammonium carbonate were obtained from Fluke (''puriss'' grade for ion chromatography; Sigma-Aldrich, St. Louis, MO, USA). Ammonium acetate buffers were prepared from glacial acetic acid and ammonium hydroxide (''Optima'' grade; Fisher).

Total arsenic was verified by ICP/MS analysis and oxidation state was verified by high-pressure liquid chromatography (HPLC) ICP/MS for all stock solutions of As species. Stock solutions (generally, 1000 mg/l) were prepared in deionized water. INA(V), arsenic pentoxide, As_2O_5, and INA(III), arsenic trioxide, As_2O_3, were ultrapure grade from Alfa Aesar (Ward Hill, MA, USA). MMA(V) was from disodium methyl arsonate hexahydrate (99.5% pure; Chem Service, West Chester, PA, USA). DMA(V) was from cacodylic acid (99.5% purity; Chem Service). Arsenobetaine (AsB) from Sigma-Aldrich (95% pure, Fluke brand) was dissolved in deionized water to make a 13 mg/l stock solution.

MMA(III) was prepared from sodium arsenite according to the method cited in Styblo et al.[28] In brief, methylarsenic (III) diiodide was prepared by reaction of aqueous methylarsonate with sulfur dioxide in the presence of excess iodide. Precipitated reaction product (estimated yield, 90%; m.p., 28 °C) was washed with cold water and dried in a dessicator. The solid diiodide was stored as a primary material at –80 °C. The yellow product dissolved in benzene was hydrolyzed by aqueous sodium carbonate, filtered, and roto-evaporated to give a residue of methylarsine oxide. An aqueous stock solution was prepared as needed as for calibrant preparation. This material in water hydrates to form MMA(III) with an initial purity of 97% by HPLC/ICP/MS and $<$1% as MMA(V).

Table 1. Instrumental conditions, ICP/MS.

Cones	Ni/Cu	
Nebulizer	MicroMist glass concentric nebulizer (Glass Expansion, Pocasset, MA, USA)	
Torch	Glass expansion, quartz standard w/platinum shield, 2.5 mm injector; 9 mm distance	
Spray chamber	Quartz, Scott type; 2 °C	
	Total arsenic assay	*Speciated (LC) arsenic assay*
Instrument	Agilent 7500CE	Agilent 7500CE with ICP/MS ChemStation B.03.03 software
Nebulizer feed (peristaltic pump)	0.1 r.p.s. ~ 400 μl/min	0.35 r.p.s. ~ 1.4 ml/min
RF power	1500 W	1550 W
Carrier gas	1.15 l/min	Ar at 0.7 l/min
Makeup ga	Not used	Ar at 0.45 l/min
Reaction gas	Helium at 4.8 ml/min	Not used
Acquisition mode	Spectral (three samples)	Time resolved (one sample)

Instrumental Analysis

Total arsenic was determined using an Agilent 7500CE ICP/MS (Santa Clara, CA, USA) with a CETAC (Omaha, NE, USA) ASX-510 autosampler. Iridium and germanium solution (1 p.p.m.; Ultra Scientific) was mixed with column effluent at zero-dead volume tee before the nebulizer; concentration after mixing was approximately 20 μg/l. Their ions ($M/Z = 72$ and 193) were monitored for system stability. The instrument was operated with the collision cell in He mode, and the $M/Z = 75$ ion was used for quantification; further operating parameters are shown in Table 1. The $M/Z = 77$ ion was also acquired, to verify that ArCl was not present. Aqueous calibrants at seven levels between 0.2 and 100 μg/l arsenic in 5% nitric acid were used (Ge internal standard) and were equivalent to a range of 1–500 μg/l in undiluted urine samples; response curves were determined using linear-weighted ($1/\sigma^2$) least squares. Over-range samples were rerun with dilution, as required. Overall method accuracy and lack of matrix effect was verified using NIST 2670a (high-range) SRM (expected: 220 ± 10 μg/l; found: 196 ± 1 μg/l) and Lyphocheck 1 (expected 35 ± 7 μg/l; found: 33 ± 2 μg/l) and Lyphocheck 2 (expected: 253 ± 50 μg/l; found: 240 ± 11 μg/l) reference materials (Bio-Rad, Hercules, CA, USA).

Arsenic species were separated using a method modified from that of Le et al.[23] by HPLC/ICP/MS. The HPLC (Agilent 1200-series) was coupled directly to the same ICP/MS system used for total arsenic. Instrument stability standards iridium and germanium were used, as described for total arsenic determination. A refrigerated sample tray was maintained at 4 °C during analysis. The column compartment was 40 °C; MMA(III) was subject to this temperature for about 2 min.

Arsenic species (10 μl injection) were separated on a reverse-phase column (Phenomenex, Torrance, CA, USA; Gemini-NX, 3μ, 150 × 4.6 mm^2 with guard column). We replaced the phosphate and malonate buffers of Le et al.[23] with volatile $(NH_4)_2CO_3$, which offered three advantages: longer column life, less degradation of ICP/MS performance due to non-volatile salts, and lower arsenic background contamination from phosphates. The elution program consisted of three-step gradients (all containing 5 mM tetrabutylammonium hydroxide and 2.5% methanol): (A) 10 mM $(NH_4)_2CO_3$, pH 9.2; 1.0 ml/min for 5 min; (B) 30 mM $(NH_4)_2CO_3$, pH 8.75;

1.2 ml/min for 8 min; and (C) 30 mM $(NH_4)_2CO_3$, pH 9.2; 1.2 ml/min for 5 min. The higher buffer strength in eluent (C) simply accelerates the return to initial pH conditions. The stabilization time was 5 min.

Calibrants for speciation analysis were prepared in a water matrix in two submixtures: reduced arsenic (AsB, MMA(III), INA(III)) and oxidized species (MMA(V), DMA(V), INA(V)). Initially, calibrants were stored at –20 °C; gradual conversion of reduced to oxidized forms was observed for these mixtures over time, particularly for the monomethyl arsenic species. Calibrant stock solutions were subsequently maintained at –80 °C and were diluted for each set of samples. Response curves and quantitation parameters were computed after adjustment for oxidation in calibrant mixtures, as follows: after analysis of the calibration series for pentavalent forms, the resulting response information was used to quantify MMA(V) and INA (V) in the trivalent calibrant solutions. The nominal calibrant levels for MMA(III) and INA(III) were recomputed based on the degree of oxidation observed. Using a high concentration stock calibration mixture for trivalent arsenic compounds that had at least 90% reduced forms, diluted calibrants were typically 80% or better trivalent species.

Quality assurance measures included water blanks (1 in 20), laboratory replicates (1 in 10), calibration check samples (1 in 10), and aqueous reference standards (1 per batch). Full calibration was performed for each batch of 60–80 samples and verified with calibrants as follows.

MMA(III) Stability Experiments

In an effort to minimize precipitates during speciation HPLC analysis, experiments were conducted using the initial HPLC mobile phase as the diluent for preparing samples. Significant oxidation of MMA(III) in standards and fortified samples stored this way led us to examine the different mobile phase components for their effect on the degree of MMA(III) oxidation observed for short-term storage of samples at –20 °C plus maintenance of the samples at 4–6 °C for the duration of the analysis batch. The conditions considered were pH, buffer strength, ion-pairing reagent, and methanol.

Stabilization of MMA(III) in urine samples using a complexing agent, sodium diethyldithiocarbamate (Na-DDC), has been reported;[29] this agent selectively complexes with trivalent metal species. Following initial laboratory experiments that showed some improvement in preservation of MMA(III) using this agent, we prepared 15 ml polypropylene screw-top tubes with 1 μM sodium DDC, 1 μM disodium EDTA, and 40 mg sodium bicarbonate. A 10 ml portion of each sample was transferred to this treated tube at collection. Unpreserved and preserved portions were handled identically in terms of temperature, storage, and shipment.

A standard addition experiment was conducted to assess redox stability of MMA(III) during in-lab storage, sample preparation, and analysis, using four urine samples with total urinary arsenic of approximately 10, 30, 200, and 300 μg/l, with corresponding percentages of MMA(V) (no MMA(III) detected) of 21%, 8%, 11%, and 10%, respectively. All four samples were used in their unpreserved version, and the two samples with highest arsenic levels were also used in their preserved version. Along with a reagent water blank matrix, each sample was fortified with 0, 1.0, 5.0, and 10.0 μg/l of MMA(III), while being maintained at 4–6 °C. The samples were prepared for analysis following the same protocol as for field samples. Instrument-ready preparations were held at 4–6 °C until analyzed, within 15 h.

Sample Collection, Transport, Preservation and Storage, and Preparation for Analysis

Urine specimens were collected following IRB-approved informed consent protocols from subjects aged 7 to17 years who were participants in a study of pulmonary and other exposure effects from arsenic in drinking water in a rural population from Matlab, Bangladesh, 2008–2011. Each child was transported to the Matlab Health Center, where they were interviewed and examined, and urine samples collected. Age, gender, height, and weight was recorded and body mass index (BMI, body weight (kg)/height (m^2)) was calculated.[30]

Urine samples were aliquoted into two to four tubes and stored at − 70 °C within 5 min. The MMA tube was always placed in the freezer immediately. As described, a portion of each sample was treated with preservatives before freezing. A small fraction (<3%) of samples had a volume of urine that was too small for the second tube; in those cases, a second void was obtained after a 1–2 h interval. The first collection was preferentially used for urinary arsenic speciation and no effect of collecting urine in two voids is expected. The samples were quarterly transported (travel time around 4 h) to Dhaka with cooling packs, which had been stored at − 70 °C overnight and shipped on dry ice as accompanied commercial airline baggage to the study center in Berkeley, CA, USA. Dry ice was added as required during airline transfers and its presence verified upon arrival in Berkeley. Samples were maintained at –80 °C from arrival until they were transferred from project storage to the analysis facility in Seattle, WA, USA by overnight carrier, maintained in a frozen state with dry ice. Typical holding times for frozen samples between field collection and lab analysis were 3–4 months.

Upon receipt at the analytical laboratory (University of Washington), samples were subdivided for total arsenic determination (1.5 ml), arsenic speciation analysis (3 × 1.5 ml), and measurement of pH, redox potential, and urinary creatinine (3 ml). Control of sample temperature was rigorous: samples were thawed in a 4 °C refrigerator and were maintained on ice during transfers and preparation for instrumental analysis, with immediate storage at –80 °C between processing. These samples typically experienced at most two thawed periods after initial freezing (once for aliquoting and once before analysis), and at no time were they allowed to reach room temperature. Samples for total arsenic and measurements other than speciated arsenic were kept at –20 °C until testing.

Samples for total arsenic determination were diluted 1:5 with 6.25% nitric. Samples for speciation analysis were centrifuged (13,550 *g*, 4 °C, 20 min), and the supernatant was transferred to polypropylene vials.

Spot creatinine concentrations were measured by the UW Medical Center clinical laboratory using the Ellman method. Specific gravity was determined by digital refractometer (Atago, Tokyo, Japan). Redox potential and pH measurements were made at 25 °C using a Delta 320 meter and electrodes from Mettler-Toledo (Columbus, OH, USA).

RESULTS

Figure 1 depicts HPLC separation for final speciation analysis conditions. Baseline resolution was achieved for the target As species; AsB has minimal retention and may be subject to interference from other unretained As compounds such as arsenocholine.

Method performance parameters are shown in Table 2. Recovery species interconversion was assessed by repeated analysis of NIST-traceable calibrants interspersed among urine samples. These data are consistent with little or no (<10%) conversion of MMA(III) to MMA(V) and INA(III) to INA(V) in the course of analysis.

Precision values for the method were determined from analysis of 40 calibrant check levels run over a period of 3 months. Instrumental replicates of samples typically had ≤5% relative percent difference when ≥2 μg/l; precision decreased at low levels. A total of 79 replicate pairs of samples were evaluated, which included many non-detectable or very low levels of arsenic, notably for INA(III) and INA(V). An overall coefficient of variation calculated for species using all results over 1 μg/l gave: AsB, 13%; INA(III), 12%; DMA, 6%; INA(V), 5%; and INA(V), 8%. MMA(III) was not detected in replicate samples.

Sample Stability Results

Storage of MMA(III) in deionized water at 4 °C showed approximately 10% conversion to MMA(V) after 24 h. Mobile phase A produced a 19% conversion. Tetrabutylammonium hydroxide alone (5 mM) gave 81% conversion, 10 mM $(NH_4)_2CO_3$ at pH 9.2 alone gave 36% conversion, and methanol alone was protective, with no apparent oxidation. Calibrant and sample storage at –80°C and at –20°C for trivalent arsenic standards and for samples fortified with MMA(III) revealed the following general observations: oxidative conversion was inconsistent under nominally constant conditions; oxidation rate was increased at low concentration of arsenic and in pure water samples compared with urine samples; extremes of pH and increased ionic strength for added buffers increased oxidation rate; and addition of methanol as an organic cosolvent decreased oxidative conversion.

The results of the standard addition study are shown in Figure 2. In general, all of the unpreserved samples showed MMA(III) response equivalent to the reference calibrant water sample; the overall recovery of MMA(III) from the aqueous standard averaged around 80%. The treated samples showed a consistently lower

recovery of spiked MMA(III), with average recoveries of approximately 55%. This overall finding indicates that the laboratory methods and conditions used allow preservation of MMA(III) levels in field samples during thawing, sample preparation, and analysis. Treatment of samples with the complexing agents used did not appear to prevent MMA(III) conversion, and in fact accelerated it.

Field Results

Mean urinary specific gravity values did not differ significantly by age, gender, height, weight, or well water arsenic level; urinary arsenic concentrations as reported have not been corrected for diuresis. Of a projected study total of 660 urine samples, total and species data were available for 643, and of these, full subject data are available for 636.

Subjects were well distributed by age and gender, as shown in Table 3. Older age categories show some gender difference in total arsenic intake as indicated by mean urinary arsenic values. Urinary arsenic levels increased with age for males; for females, small increases were seen with age for the lowest three age categories, but declined in the 15–17 years age group. In this oldest group, gender differences in total urinary arsenic were pronounced: more than a threefold elevation in male subjects *versus* female subjects.

Samples treated with preservatives showed a compressed and lower pE range (4–5) and higher pH values (8–9) than untreated samples. Total arsenic results show very good agreement between treated and untreated samples, and were also in close agreement with the summed urinary arsenic species results ($r = 0.995$), with total arsenic and sum of urinary species agreeing within about 8 μg/l on average.

Urinary arsenic and BMI, stratified by gender, and age is shown in Table 3; summary of arsenic results are shown in Table 4. DMA was the dominant form of urinary arsenic, with lesser amounts of MMA(V), and inorganic arsenic. An observed trend in species distribution as a function of total urinary arsenic was slight decreases in the % DMA (81.1% in the lowest exposure range of 0–$<$10 μg/l, decreasing to 76.3% in the highest exposure range of $>$500 μg/l), accompanied by increases in INA (7.6–12.2%) and MMA (7.4–11.1%). This would be consistent with reduced methylation efficiency at higher arsenic loads. The arsenite/arsenate ratio (0.72 increasing to 3.7) increased over these concentration ranges.

Chromatographic peaks other than our target compounds were observed in a minority of samples. These likely represent other arsenical constituents, as has been described previously. In all cases where these extra peaks were seen, they constituted a minor fraction of the total response.

PEAK	NAME	RT (minutes)	AMOUNT μg/L
A	AsB	1.60	20
B	MMA (III)	2.03	20
C	INA (III)	2.71	20
D	DMA (V)	3.92	5
E	MMA (V)	9.21	1
F	INA (V)	11.71	1

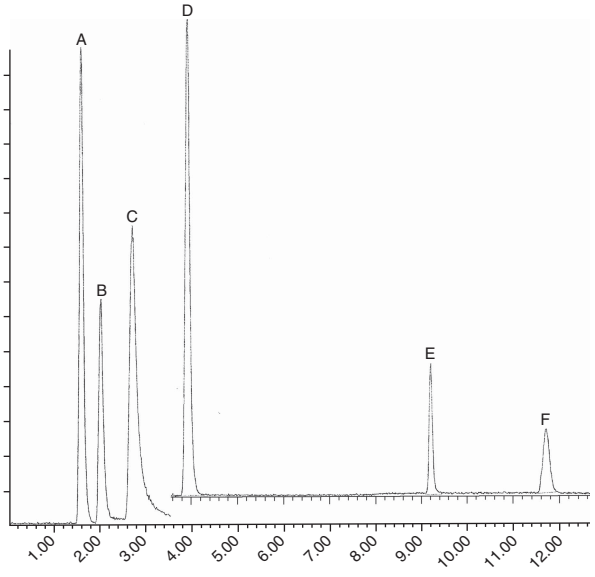

Figure 1. Aqueous standard — arsenic species. The figure is a composite of mass chromatograms ($M/Z = 75$) for a trivalent arsenic mixture (0–3.5 min) and a pentavalent arsenic mixture (3.5–12.5 min). Species: (A) arsenobetaine, 20 μg/l; (B) methyl arsonite, 20 μg/l; (C) arsenite, 20 μg/l; (D) dimethyl arsinate, 5 μg/l; (E) methyl arsonate, 1 μg/l; (F) arsenate, 1 μg/l.

DISCUSSION

Analytical Methods

In general, our approach to arsenic speciation using reverse-phase HPLC with tetrabutylammonium hydroxide ion-pairing is similar to that of several other investigators.[23] The distinctive features of this work are: (1) highly resolved separation of MMA(III) from other forms of urinary arsenic; (2) careful maintenance of low temperatures for samples and standards before and during analysis; (3) segregation of MMA(III) and INA(III) from MMA(V) and INA(V) in calibrants, permitting more sensitive and quantitative tracking of oxidation in calibrants stored at $-80\,^{\circ}$C and during analysis; (4) characterization MMA(III) in a large sample set reflecting highly exposed children. In contrast, in this field study we were not able to achieve the instantaneous flash-freezing of urine samples to liquid nitrogen temperatures or the very short holding times reported in some other studies. The overall performance of the analytical methods was comparable to a variety of other reported methods.

Table 2. Method performance.

	Analyte						
	AsB	*MMA(III)*	*INA(III)*	*DMA*	*MMA(V)*	*INA(V)*	*Total arsenic*
Retention time (min)	1.60	2.03	2.71	3.92	9.21	11.71	—
Detection limit[a] (μg/l)	0.3	0.3	0.6	0.5	0.2	0.2	1
LQL[b] (μg/l)	1	0.5	1	5	1	2.5	1
UQL[b] (μg/l)	100	170	225	500	100	250	500
Coefficient of determination (r^2)	0.998	0.995	0.996	0.999	0.998	0.998	0.999
Precision (standards, 5 μg/l, $N = 40$)	8%	8%	11%	8%	8%	6%	

Lower and upper quantitation limits are expressed in terms of original urine sample concentration (taking sample dilution into account). In several recalibrations, r^2 values are typically 0.99 or better for each compound. [a]3σ blank. [b]Based on calibrants, expressed as equivalent urine concentration.

Preservation of MMA(III)

Varying degrees of instability of MMA(III) in solution and the role of sample matrix, temperature, and pH have been noted previously. Arsenic species (excluding trivalent methylated forms) in aqueous samples are reported to be less stable than either fresh or reconstituted freeze-dried urine,[31] but several reports indicate that MMA(III) is more unstable in urine than in water.[13,19,22] Of equal significance, variable stability in different urine samples has been noted.[13] Using MMA(III)-fortified urine samples, Gong et al.[19] found 30% loss after 1 day, at both 4 °C and –20 °C. The losses of MMA(III) from urine over 1 to 3 days at 25 °C reported by Gong and his co-workers[19] are particularly significant because that is the time scale and temperature for samples during the actual analysis. Complete conversion was seen by the end of day 3; from the graphics provided, it appears that losses of 85% or so might have occurred within as little as one day. Gong et al.'s[19] study is also significant in characterizing the effect of storage at –20 °C, given the number of field studies where samples have been stored for months before analysis. The observation is based on one sample, and the variability in MMA(III) stability with different samples is yet to be explained or taken into account. None of these studies characterized sample pH or used preservatives.

The importance of pH has been noted with respect to methylation level and redox status of inorganic arsenic[31] Yehiayan et al.[32] characterized the effect of pH on freshly prepared MMA(III) in deoxygenated water and found that short-term stability was high for pH values of 4 and above. The more reactive glutathione complex ($MMA(GS)_2$) showed sharply decreasing half-life with increasing pH above 2 (the lowest pH tested). For actual field study samples, exclusion of oxygen is not a practical preservation method, but these observations do highlight the inherent tendency toward oxidation for urinary MMA(III).

By maintaining low sample temperature and optimizing sample preparation, we obtained $\geq$80% recovery of MMA(III) added to urine over the one day of analysis and saw only minor differences in stability between spiked water and four different urine samples. We noted significantly lower preservation in samples treated with complexing agents intended to bind to and stabilize MMA(III). This may simply be the effect of increased ionic strength on oxidation kinetics. In general, we conclude that chemical stabilization to "freeze" MMA(III)/MMA(V) ratios is not a feasible approach, in a situation of redox disequilibrium.

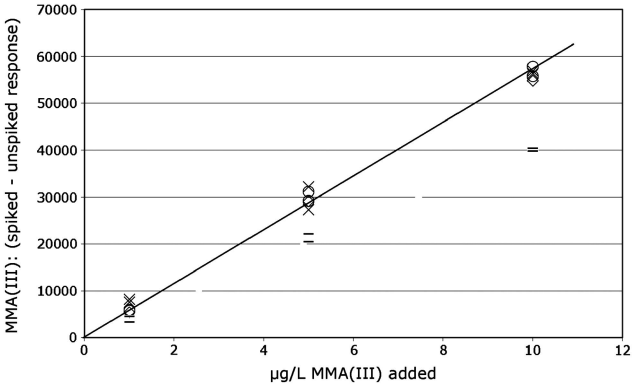

Figure 2. Monomethyl arsonous acid (MMA)(III) standard additions. × = two untreated samples, high unfortified arsenic levels; ○ = two untreated samples, low arsenic unfortified levels; – = two treated samples, high unfortified arsenic level; ◇ = ion-free water.

Urinary Arsenic and MMA(III) in Population Samples

The pattern of urinary arsenic metabolite concentrations is consistent with a study population with exposure to environmental arsenic, and indicates that historical exposure to contaminated wells has continued in many instances. Of the 643 samples reported, 40% were above 50 µg/l in combined urinary arsenic species.

Detectable MMA(III) was found in a total of 41 of 643 cases, and of these, only 8 were above the formal lower quantitation limit of 0.5 µg/l (Table 4). Two samples had MMA(III) above 1 µg/l (1.02 and 1.30 µg/l). Two slight method changes were adopted partway through the study, which may have had an effect on MMA(III) detection: initially, cold packs for the 4-h transit between Matlab and the research facility in Dhaka were – 20 °C, rather than – 70 °C. Also, the speciation analysis used 1:5 dilution with 3% methanol in deionized water and 50 µl injections; later, the samples were run undiluted using 10 µl injections. Of the 643 samples reported here, 317 were assayed using the earlier methods in which MMA(III) was detectable in three cases. The remaining 38 samples with detectable MMA(III) were among the 326 samples handled according to the revised methods. From the levels found, and these observations, we conclude that MMA(III) was initially present in relatively few samples and at barely detectable levels, and that it is also very susceptible to losses in storage and handling.

Table 3. Subject characteristics (BMI and urinary arsenic by age and gender).

		Age (years)			
	All	*7–8*	*9–11*	*12–14*	*15–17*
Overall					
N	636	183	165	215	73
Mean BMI[a] (σ, SD)	15.2 (1.9)	14.2 (1.3)	14.5 (1.4)	15.9 (1.8)	17.7 (1.9)
Mean total arsenic (µg/l) (σ)	80.9 (134)	66.3 (104)	78.9 (118)	87.6 (149)	102 (180)
Males					
N	327	98	71	119	39
Mean BMI (σ)	15.2 (1.6)	14.3 (1.0)	14.5 (1.1)	15.7 (1.6)	17.3 (1.5)
Mean total arsenic (µg/l) (σ)	94.4 (161)	66.3 (104)	94.5 (143)	102 (183)	141 (225)
Females					
N	309	85	94	96	34
Mean BMI (σ)	15.3 (2.2)	14.1 (1.6)	14.5 (1.6)	16.0 (2.1)	18.1 (2.2)
Mean total arsenic (µg/l) (σ)	66.6 (96.0)	65.2 (111)	67.1 (92.9)	70.3 (87.2)	58.2 (92.8)

[a]Body mass index: body weight (kg)/height (m^2).

Table 4. Summary of urine results.

	Total urinary arsenic (μg/l)							
	0–<10	*10–<25*	*25–<60*	*60–<100*	*100–<200*	*200–<500*	*≥500*	*Overall*
N	60	152	220	84	73	37	17	643
Average specific gravity	1.0033	1.0061	1.0087	1.0111	1.0114	1.0096	1.0148	1.0084
Urinary arsenic species								
INA(III) %	3.2	5.9	6.7	7.4	7.6	9.2	9.6	8.1
INA(V) %	4.4	3.8	3.3	3.8	3.4	2.7	2.6	3.1
Inorganic %	7.6	9.7	10.0	11.2	10.9	11.9	12.2	11.2
MMA(V) %	7.4	7.7	7.9	8.1	8.9	10.6	11.1	9.5
DMA(V) %	81.1	78.5	78.2	78.8	78.4	77.0	76.3	77.6
AsB %	3.9	4.1	4.0	1.8	1.7	0.5	0.3	1.6
Samples with detectable MMA(III)	0	1	2	7	12	12	7	41
Detection frequency (%)		0.66	0.91	8.33	16.4	32.4	41.2	6.38
Average, detectable MMA(III) as % of arsenic species		0.81	0.91	0.36	0.17	0.15	0.10	0.24

Table 5. Prior reports of urinary arsenic species and MMA(III).

Year	*Population description[ref.]*	N *(urine samples)*	*Assay method*	*Detection limit*	*Range, total urinary arsenic*	*MMA(III): frequency of detection; concentration*
2000	Adults, Romania, drinking water arsenic exposures (3–160 μg/l)[21]	58	(A)	2 μg/l	Up to 64 μg/l (c)	0 of 14 in low exposure group, 5 of 16 in highest exposed group; ≤7 μg/l
2001	Adults in Mexico with exposure to As in drinking water (30–1100 μg/l in the region)[13]	6	(B)	1.2 μg/l	42–1211 μg/l	5 of 6; ≤12 μg/l
2001	Adults in India; three exposure groups with progressively higher arsenic in drinking water (up to 248 μg/l)[14]	428	(C)	0.14–0.33 μg/l	51–1487 μg/l (a)	49%; ≤30 μg/l (a)
2004	Adults in West Bengal given controlled amounts of arsenic in water, up to 163 μg/l[15]	41	(C)	0.14–0.33 μg/l	Up to 390 μg/l (b)	100% (c); 9.4 μg/l (a)
	Adults in West Bengal with 2 years avoidance of arsenic contaminated water[15]	25			Average =218 μg/l	3 of 25; 2.4 μg/l (c)
2005	28 Adults in Mexico with low As exposure[16]	28	(B)	Not stated	9.1–106 μg/g creatinine (b)	"98% of samples had detectable methylated trivalent arsenicals"
	76 Adults in Mexico with high exposure in drinking water[16]	76			52–1398 μg/g creatinine (b)	≤102 μg/g creatinine
2007	Indigenous adult women, Argentina; up to 200 μg/l As in drinking water1[17]	147	(D)	0.1 μg/l	Genetic subgroup geometric means 228–285 μg/l	0 of 147
2007	Adults, Hungary, Romania, Slovakia; drinking water risk[10]	415	(D)	0.1 μg/l	Not given	2 of 415; ≤0.25 μg/l
2008	Children, Brazil, arsenic affected region[18]	15	(C)	Not given; <0.4 μg/l (c)	17–55 μg/l	12 of 15; ≤2.0 μg/l
2011	Adults, Zimapan and Lagunera regions, Mexico[38]	257	(B)	Not given; <0.5 μg/l	2.3–233.7 μg/l	67%, 100% in the two regions; ≤2.4 μg/l (0.4 avg.)
2011	This study: children (6–17 years), Bangladesh	643	(C)	0.2 μg/l	3.3–1127 μg/l	41 of 643; ≤2 μg/l

Assay methods: (A) HPLC/HG/AFS; (B) HG AAS with pH control; (C) HPLC/ICP/MS; and (D) HPLC/HG/ICP/MS. *Comments*: (a) Based on group averages, not individual samples; (b) based on the sum of inorganic arsenic and its metabolites; and (c) not directly provided in the paper, but estimated from data provided.

Prior findings of MMA(III) in human urine are summarized in Table 5. Studies before 2004 have been reviewed by Francesconi and Kuenelt.[20] Excluded are reports that do not directly apply to population exposure assessment. Early reports of MMA(III) in urine described subjects following administration of chelating agents (DMPS) before urine collection;[22,23] for the three untreated subjects tested, no MMA(III) was detected above the 2 μg/l detection limit. Reports of urinary arsenic species for patients undergoing arsenite treatment for blood disorders, where doses are extraordinarily high compared with environmental exposures and where the collection and management of samples is accomplished within a clinical environment, also show variable findings, from 25% detection at trace levels[10,33] to 75% detection of MMA(III),[34] for patients with urinary arsenic ranging from 1000 to 13,000 μg/l.

Population studies having environmental arsenic exposure fall into two major groups: those reporting widespread occurrence of

MMA(III) (and in many cases DMA(III)) at levels well above 2 μg/l, and those reporting few or no occurrences of these reduced methylated arsenic species in the μg/l range. Notably, the five earliest studies cited in Table 5 found frequent detection of MMA(III) with concentrations spanning a combined range of 1–30 μg/l.[13–16,21] In contrast, more recent population studies (the studies from 2007 and 2008 cited in Table 5, plus this report) found infrequent MMA(III) detection above 0.1 μg/l and low concentrations (<0.1–2.0 μg/l combined range).[10,17,18] Rabieh and co-workers,[18] using methods with high sensitivity for MMA(III), were able to detect it in 12 of 15 urine samples from children with modest arsenic exposure (as indicated by total urinary arsenic levels of 16–55 μg/l), but for only two of these cases did the MMA(III) level exceed 1 μg/l, and the MMA(III) accounted for 0–4% of detectable urinary arsenic species. In a study of Michigan residents with slightly elevated arsenic exposures (drinking water arsenic levels below 20 μg/l), MMA(III) was not detected above 0.14 μg/l in any of 387 individuals.[35–37] One recent study in regions of Mexico with elevated arsenic in drinking water found that a majority of samples had detectable MMA(III), but at levels generally below 1 μg/l (geometric mean $= 0.1$ μg/l).[38]

Our study represents the largest group reported thus far, and features children and significant arsenic exposure. The methods used, including low temperatures for sample preservation and preparation, rapid freezing upon collection, minimal sample treatment before analysis, and species separation within a few minutes of the start of analysis, all would minimize losses by oxidation of MMA(III). Yet, only two samples had urinary MMA(III) at concentrations above 1 μg/l, out of 643 cases tested. While the presence of MMA(III) at sub-μg/l levels is clearly related to total urinary arsenic, our findings add to the weight of evidence that MMA(III) is not present as a significant metabolite of inorganic arsenic exposure, even for populations with high levels of arsenic — only 1.2% (8 of 643 cases) had detectable MMA(III).

CONCLUSIONS

We have investigated the conversion of MMA(III) to MMA(V) in aqueous standards in fortified urine samples. Low temperature and addition of methanol reduces the rate of oxidation, while addition of acids, bases, or salts increases the rate of oxidation. Attempts to preserve samples by the addition of DDC complexing agent actually reduced MMA(III) recovery. Analysis of urinary arsenic species in samples having total urinary arsenic of 5 to >1100 μg/l showed few cases of detectable MMA(III), of which only 0.3% exceeded 1 μg/l arsenic as MMA(III), which is consistent with several recent reports. The finding from this study of mostly undetectable MMA(III) levels in well-preserved samples, along with similar findings from other recent studies including reports of patients undergoing arsenite treatment for leukemia, lead us to question earlier reports of prevalent MMM(III) concentrations in the p.p.b. range in urine samples. The wide variation in published observations of the prevalence and proportion of MMA(III) among urinary arsenic species is consistent with its instability to redox processes; based on the current knowledge, we conclude that measured values of urinary MMA(III) are not suitable for use in epidemiological studies as markers of biological levels of MMA(III). Urinary markers of arsenic exposure (specific as to methylation level but not differentiated as to oxidation state) continue to be useful elements of field studies; further efforts to improve the sensitivity, convenience, and comparability of results are needed.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This work was supported by the National Institutes of Health Grant Nos. R01-HL081520 and P42-ES04705. We acknowledge with gratitude the commitment of NIH to our research efforts.

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