

Stability of Arsenic Species and Insoluble Arsenic in Human Urine¹

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Abstract

Urinary arsenic species are important short-term biomarkers that have been used in epidemiological studies. However, the stability of soluble arsenic species and the amount of arsenic lost during sample pretreatment remain unclear. The objective of this study is to evaluate the stability of soluble arsenic species in urine and aqueous standards, as well as to assess the amount of insoluble and soluble arsenic lost during pretreatment (centrifugation and filtration, respectively). High-performance liquid chromatogram inductively coupled plasma mass spectrometry was used to speciate arsenic species {Arsenite [As(III)], arsenate [As(V)], monomethylarsonic acid [MMA(V)], monomethylarsonous acid [MMA(III)], dimethylarsinic acid [DMA(V)], and arsenobetaine [AsB]} in aqueous standards and in urine samples. The arsenic levels in both freshly collected urine samples (pH = 5.5–7.0) and National Institute of Standards and Technology Standard Reference Material 2670 toxic elements in frozen-dried urine (pH 4.4) remained constant up to 6 months when stored at -20°C . In an aqueous solution mixed with 10 $\mu\text{g/liter}$ of As(III), As(V), MMA, and DMA standards, and stored at 4°C , As(III) and As(V) were stable only up to 4 weeks, and MMA and DMA remained stable up to 4.5 months. The same phenomenon was observed for 100 $\mu\text{g/liter}$ mixed aqueous standards. There was no significant loss of arsenic species in urine (<5%) when passed through a 0.45- μm filter. The amounts of insoluble arsenic in urine lost during centrifuge ranged from 1/2 to 1/17 of soluble arsenic. These findings indicated that the urinary matrix plays an important role in stabilizing arsenic species. Also, the loss of insoluble arsenic in urine during centrifuging results in underestimation of arsenic exposure, and may explain the lack of an association

between arsenic exposure and the risk of health outcomes reported in some epidemiological studies.

Introduction

Arsenic is ubiquitous in the earth's crust and biosphere. Since the nineteenth century, arsenic has been widely used in the manufacture of glass, feed additives, pigment for aniline dye, wallpaper, soap, medicine, wood preservatives, pesticides, metalloids, and semiconductor applications. Humans may be exposed to arsenic via ingestion, inhalation, or, to a lesser extent, skin absorption. Previous studies in humans have shown that exposure to arsenic may lead to cancer of liver, kidney, bladder, prostate, lymphoid, skin, lung, and colon, as well as to other adverse health effects (1–3). The reported order of arsenical toxicity reflected by carcinogenesis and vascular disorders is MMA(III)³>As(III)>As(V)>MMA(V) = DMA(V; Refs. 4, 5). MMA(III) has a very short half-life and converts to MMA(V) in a short time (6); thus, it appears in trace amounts in urine and is difficult to measure. Hence, the MMA discussed throughout this paper refers to MMA(V).

Feldmann *et al.* (7) evaluated the stability of arsenic species in freshly collected urine samples. They found that the freshly collected urine samples were stable for at least 2 months when stored at temperatures under either 4° or -20° . But scarce time points and lack of urinary pH information limited the reliability of their data. In addition, several studies have assessed the stability of arsenic species in aqueous samples but did not measure organic arsenic (MMA and DMA), and most studies lacked the urinary pH information (8–11). Some researchers (9, 10, 12) add HNO_3 to stabilize the arsenic species. However, acidification of aqueous samples leads to changes of arsenic distribution immediately.

Urinary arsenic species are important short-term biomarkers and have been used in many epidemiological studies. However, detailed stability information of organic arsenic in aqueous standards is lacking. In particular, no study has assessed the amount of insoluble arsenic in urine. The objective of this study is to assess the stability of soluble arsenic species in urine and aqueous standards, as well as the amount of insoluble and soluble arsenic lost during the sample pretreatment steps (centrifuge and 0.45- μm filter, respectively) to improve a reliable exposure biomarker data for epidemiological research.

Received 2/4/02; revised 6/19/02; accepted 7/4/02.

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¹ This work was supported by NIH Grants ES 05947 and ES 00002.

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³ The abbreviations used are: MMA(III), monomethylarsonous acid; As(III), arsenite; As(V), arsenate; MMA(V), monomethylarsonic acid; DMA(V), dimethylarsinic acid; AsB, arsenobetaine; HPLC-ICP-MS, high-performance liquid chromatogram inductively coupled plasma mass spectrometry; MMA, monosodium acid methane arsonate; NIST, National Institute of Standards and Technology; SRM, Standard Reference Material; HGAAS, hydride-generation atomic-absorption-spectrometry; CV, coefficient of variation.

Table 1 Settings of HPLC-ICP-MS for arsenic analysis

Instrument	Parameter	Settings		
HPLC	Mobile phase (16)	15 mmol/liter tartaric acid (EM Science), use 14% NH ₄ OH to adjust pH to 2.9		
	Column	Anion-exchange column: Hamilton PRP X-100 (4.1 mm identifier sequence × 25 cm, 10 μm particles; Hamilton)		
	Injection volume	100 μL (Rheodyne Model 9125; Catati)		
	Flow rate	1.5 ml/min		
ICP-MS	ICP system			
	RF power	1050 W (forward)		
	Sample and skimmer	Nickel		
	Torch	Quartz		
	Nebulizer	Cross-flow		
	Spray chamber	Double-pass		
		Parameter	Total arsenic analysis	Arsenic speciation
		Gas flow rate (liter/min)		
		Plasma		
		Ar-Ar plasma	14.85 Ar	15.00 Ar
	N ₂	0.15	0	
	Auxiliary	0.8	0.8	
	Nebulizer			
	Ar-Ar plasma	0.85–0.95	0.85–0.95	
	Data acquisition			
	Dwell time (ms)	200	100	
	Sweeps per reading	6	1	
	Readings per replicate	1	1	
	Number of replicates	5	2000	
	Points per spectral peak	1	1	
	Mass analyzed			
	Analyte	⁷⁵ As	⁷⁵ As	
	Internal standard	¹²⁸ Te (250 μg/liter)	¹²⁸ Te (20 μg/liter)	

Materials and Methods

Speciation of Arsenic Species

The stock solution (1000 μg/liter) of As(III) was prepared by dissolving appropriate amounts of sodium arsenite [As(III), NaAsO₂; J. T. Baker, Phillipsburg, NJ] in 4 grams/liter NaOH (Merck). The 1000 μg/liter stock solutions of As(V), MMA, DMA, and AsB were prepared by dissolving appropriate amounts of disodium hydrogen arsenate heptahydrate [As(V), AsHNa₂O₄·7H₂O; Fluka], MMA (AsH₄NaCO₃; Chemical Service), DMA (C₂H₇AsO₂; Fluka), and arsenobetaine (CH₃As⁺CH₂COOH, Community Bureau of Reference, Geel, Belgium) in deionized water. All five of the stock solutions were stored at 4°C in the dark and reprepared every month. Stability of this storage method over several months has been confirmed (13). The working solutions were prepared daily by serial dilutions of the 1000 μg/liter stock solutions with deionized water to give the following mixed calibration standards: 1, 10, 40, 100, and 150 μg/liter. A 20 μg/liter tellurium in 5% HNO₃ prepared from 1000 μg/ml (Baker Instra-Analyzed Reagent; J. T. Baker) was used as the internal standard.

An isocratic pump (Kontron HPLC pump 420) with an anion-exchange column (Hamilton PRP X-100 column; 4.1-mm identifier sequence × 25 cm, 10 μm particles; Hamilton) and corresponding Hamilton guard column (2.3-mm identifier sequence × 25 mm) was used for separation. The injection valve (Rheodyne Model 9125; Rohnert Park, CA) with a 100-μl injection loop was used for sample introduction. The HPLC mobile phase was a solution of 15 mmol/liter tartaric acid (pH 2.9; EM Science; Ref. 14) at a flow rate of 1.5 ml/min. This solution was filtered through a 0.45-μm filter (Waters GHP Arcordisc Minispike; Milford, MA) and degassed

before use. A 10% HNO₃ (nitric acid; Fisher, Optima) solution was used to wash the nebulizer of ICP-MS between injections to prevent clogging. All of the glassware used for laboratory work was cleaned with 10% HNO₃ (OmniTrace; Merck). The ICP-MS used in this study was SCIEX ELAN 5000 (Perkin-Elmer, Norwalk, CT) with a cross-flow nebulizer. Effluent from the column was mixed on-line with the internal standard via a zero-dead-volume mixing tee. The settings for the HPLC-ICP-MS system are listed in Table 1. The HPLC-ICP-MS was optimized by using a 10 μg/liter arsenic standard before sample analysis. Data from the HPLC-ICP-MS was imported into the ACD/Chrom Manager Package (Toronto, ON, Canada), and the deconvolution program was applied for peak area analysis.

Total Arsenic Analysis

Calibration standards for total arsenic determination were prepared from NIST Certified Standard (Gaithersburg, MD) NIST 3103a arsenic standard solution. The NIST SRM 1643d (trace element in water) and NIST SRM 2670 (toxic metals in frozen-dried urine) were used as the calibration verification standard and quality-controlled standard for total arsenic determination, respectively. A solution of 250 μg/liter tellurium in 5% HNO₃ was used as the internal standard for quantification of total arsenic. The total arsenic was determined by HGAAS with detailed information described elsewhere (15).

Urine Sample Collection and Pretreatment

The urine samples were collected in polypropylene specimen containers and stored at -20°C. Because of the salt and organic content in urine, a 10-fold dilution was necessary to reduce the

Table 2 Method parameters for arsenic speciation in urine by HPLC-ICP-MS

	As(III)	As(V)	MMA	DMA	AsB
Retention time (min)	11.6	19.7	7.0	8.3	6.6
Limit of detection ($\mu\text{g}/\text{liter}$) ^a	0.32	0.25	0.10	0.29	0.10
Absolute detection limit (ng) ^b	0.032	0.095	0.010	0.029	0.010
Relative standard deviation (%) ^c	5.8	6.0	7.9	6.0	5.3
Linear range ($\mu\text{g}/\text{liter}$)	1.0–150	1.0–150	1.0–150	1.0–150	1.0–150
Slope of the calibration curve [counts/(s \times $\mu\text{g}/\text{liter}$)]	29099	48457	53791	59895	14464
Correlation coefficient (R^2) of the calibration curve	0.9999	0.9998	0.9998	0.9997	0.9995
Loss during filtration (%) ^d					
10 $\mu\text{g}/\text{liter}$	-2.73	1.83	1.04	-1.33	1.26
Urine + 10 $\mu\text{g}/\text{liter}$	1.65	-1.49	3.55	4.46	2.73
Spiked recovery (%)					
5 $\mu\text{g}/\text{liter}$	101.3	98.5	103.7	99.2	100.2
10 $\mu\text{g}/\text{liter}$	98.7	103.2	101.9	102.8	102.6

^a The limit of detection (LOD) was calculated by three times of SD of 10 measurements of the 0.1 $\mu\text{g}/\text{liter}$ mixed arsenic aqueous standard.

^b Injection volume is 100 μl .

^c The relative standard deviation (RSD) equivalent to reproducibility ($\text{CV} = 100\% \times \text{SD}/\text{mean}$) was evaluated by 10 measurements of 2 $\mu\text{g}/\text{liter}$ mixed arsenic aqueous standard.

^d The 10 $\mu\text{g}/\text{liter}$ arsenic aqueous standard was passed through the 0.45- μm filter to see how much arsenic is retained on the filter.

matrix effect. To measure the soluble arsenic in urine, samples were thawed at room temperature and then centrifuged at $3000 \times g$ and 4° for 30 min. The precipitate was discarded, and the supernatant was passed through a 0.45- μm filter (Waters, GHP Arcordisc Minispike) to remove particulate material.

Stability of Arsenic Species

The HPLC-ICP-MS was used to study the stability of seven freshly collected urine samples from volunteers, the NIST SRM 2670 (normal level) standard solution, and 2 mixed arsenic aqueous standards [10 and 100 $\mu\text{g}/\text{liter}$ of each arsenic species: As(III), As(V), MMA, and DMA]. An AsB standard was not available when we did the stability test. The urine samples and arsenic standards were aliquoted into several microcentrifuge tubes and stored at -20 and 4°C , respectively. The pretreatment of urine samples is the same as described above. All of the urine samples were diluted 10-fold and analyzed for arsenic species weekly throughout 6 months. To study the transformation between As(III) and As(V), and control for the interference from the other arsenic species in the solution, we repeated the above experiment using solutions with single species: 100 $\mu\text{g}/\text{liter}$ of As(III) and 100 $\mu\text{g}/\text{liter}$ of As(V).

Loss During Pretreatment

Loss During Centrifugation. To measure insoluble arsenic, we collected 50 ml of urine and collected the precipitates after centrifuge. The precipitate was dissolved in 3 ml of 70% HNO_3 (Mallinckrodt, St. Louis, MO), and then microwave digestion was applied. The amount of arsenic in precipitates was measured by HGAAS.

Loss During Filtration. A 10 $\mu\text{g}/\text{liter}$ arsenic aqueous standard [As(III), As(V), MMA, DMA, and AsB], a urine sample, and a spiked urine sample were used to evaluate the amount of arsenic lost during filtration. These solutions were separated into two aliquots, one remained untouched and the other was passed through a 0.45- μm filter. The recovery of each arsenic species in the filtrate was then calculated. Because it is not plausible to inject an unfiltered urine sample directly into the HPLC column, we used filtered urine samples as the unfiltered ones and compared with refiltered urine to measure the loss during filtration.

Results

Speciation and Quantification

After reviewing the relevant literature, we found that most studies using a phosphate buffer had difficulties in separating AsB and As(III), and had problems with clogging in the nebulizer of ICP-MS (16). We modified the method of Zheng *et al.* (14) for speciation of arsenic species in water samples for urine sample analysis. We added an internal standard (20 $\mu\text{g}/\text{liter}$ tellurium) and the NIST SRM 2670 arsenic urine standard to improve quantification. Factors taken into consideration for the selection of separation method include buffer solution, pH of the buffer, limit of detection of the method, and the linear range. The limits of detection in this study (Table 2) are either better than or similar to other reported methods (14, 16–25). The linear range for five arsenic species is 0–150 $\mu\text{g}/\text{liter}$, and we expect that most of the urine samples (diluted 10-fold) with arsenic would fall within this range (usually below 100 $\mu\text{g}/\text{liter}$).

To study the effect of pH on separation, we changed the buffer pH from 2.0 to 6.0. When $\text{pH} < 2.9$, serious tailing of As(V) was observed. At the higher pH, all of the arsenic species started to overlap. The optimal pH for this analysis is 2.9. Although the buffer pH (2.9) is not close to that of human urine, the distribution of arsenic species stayed the same except at very high pH as shown by Zheng *et al.* (14). The effect of buffer concentration on the resolution of the arsenic species was also tested. We changed the buffer concentration from 5 to 30 mmol/liter. The higher the buffer concentration, the faster the arsenic species eluted and started to overlap with each other (data not shown). Therefore, 15 mmol/liter of buffer was applied throughout the experiment.

Limits of detection for As(III), As(V), MMA, DMA, and AsB by this method were 0.32, 0.25, 0.10, 0.29, and 0.10 $\mu\text{g}/\text{liter}$, respectively. The recovery of each species in the spiked (5 and 10 $\mu\text{g}/\text{liter}$ of each five arsenic species) urine and aqueous sample were between 95 and 105%. Method parameters for arsenic speciation in urine by HPLC-ICP-MS are described in Table 2.

In addition, we analyzed five arsenic species in a NIST SRM 2670 elevated level and a normal level. Because no certified values for each arsenic species were available, the levels of arsenic in these two urine standards were compared

Table 3 Determination of NIST SRM2670 of human urine by HPLC-ICP-MS

	Arsenic species					Sum of species	Certified value	pH	Literature
	As(III)	As(V)	MMA	DMA	AsB				
SRM 2670 ($\mu\text{g/liter}$) (Normal level)	2.5 ± 0.2	1.5 ± 0.2	8.3 ± 0.7	47.2 ± 1.3	13.5 ± 0.9	72.9 ± 1.9	60	4.5	This work
	15.0 ± 3.3	2.9 ± 0.7	9.5 ± 3.0	48.2 ± 2.4	21.2 ± 3.7	96.8 ± 6.3			
	<0.4	6.9 ± 0.8	9.4 ± 0.9	47.5 ± 1.6	16.0 ± 1.1	79.8 ± 2.3			
SRM 2670 ($\mu\text{g/liter}$) (Elevated level)	<2.5	ND ^a	16.0 ± 0.6	52.1 ± 2.2	15.5 ± 1.0	84.4 ± 3.3	480 \pm 100	4.4	This work
	3.2 ± 0.4	397 ± 15	8.6 ± 0.7	47.4 ± 1.5	13.5 ± 0.6	489 ± 20			
	13.1 ± 4.5	390 ± 52	10.9 ± 2.1	51.6 ± 3.4	24.7 ± 0.7	490 ± 52			
	<0.4	354 ± 17	9.2 ± 0.9	50.7 ± 1.8	16.2 ± 1.1	430 ± 17			
	<2.5	428 ± 12	15.9 ± 0.8	50.4 ± 1.9	15.6 ± 0.4	514 ± 14			Goessler <i>et al.</i> (28) Ritsemis <i>et al.</i> (29) Zheng <i>et al.</i> (14)

^a ND, below detection limit.

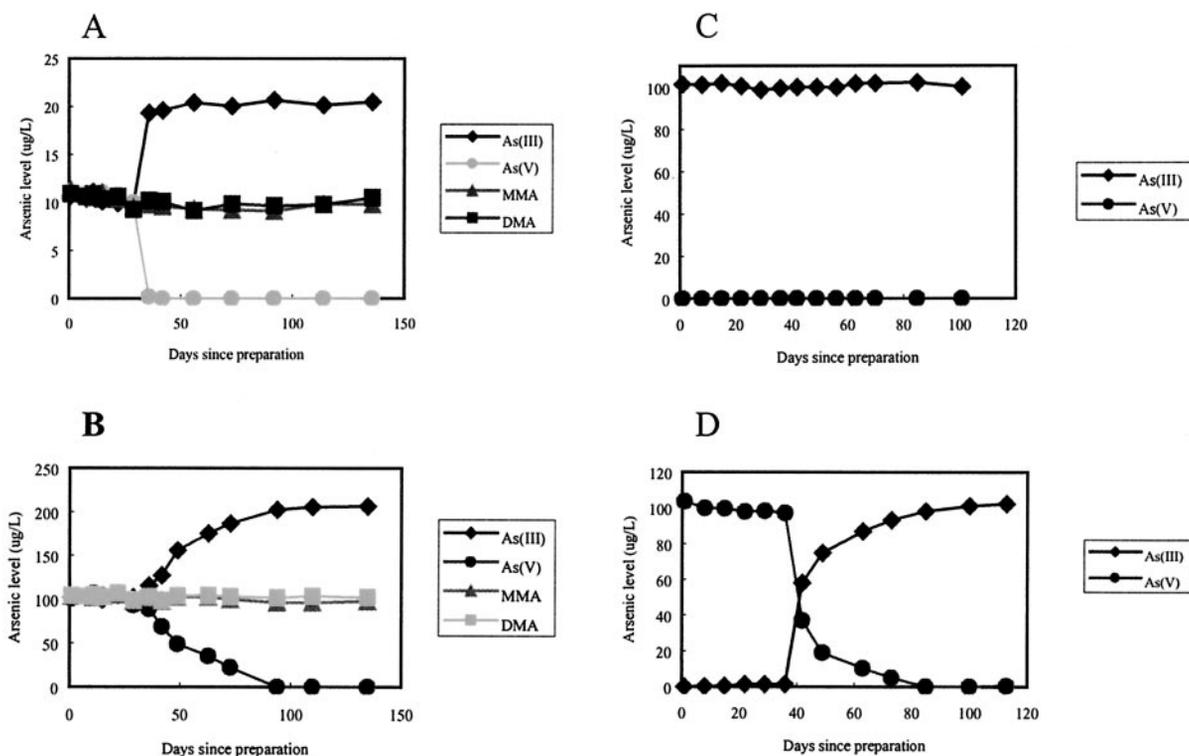


Fig. 1. Stability of 10 $\mu\text{g/liter}$ (A) and 100 $\mu\text{g/liter}$ (B) arsenic aqueous standards [As(III), As(V), MMA, and DMA; pH 6.0; 4°], and 10 $\mu\text{g/liter}$ (C) and 100 $\mu\text{g/liter}$ (D) arsenic aqueous standards [As(III) and As(V); pH 6.0; 4°]. In A, the level MMA and DMA keep stable throughout 4.5 months. The level of As(III) increased after a month from preparation, whereas the level of As(V) decreased after that. After As(V) reduced into As(III), the level of both species remain stable. In the lower level of arsenic, the shorter time is needed for the conversion. Thus, this reaction was completed earlier than 100 $\mu\text{g/liter}$ arsenic aqueous standards. In B, the level of MMA and DMA keep stable throughout 4.5 months. The level of As(III) increased after a month because preparation, whereas the level of As(V) decreased after that. After As(V) reduced into As(III), the level of both species reached stable after 3 months from preparation. In C, 100 $\mu\text{g/liter}$ As(III) aqueous standard was used to test if As(III) will oxidize into As(V) under (pH 6.0) 4°. As(III) did not oxidize into As(V) throughout 3 more months. In D, 100 $\mu\text{g/liter}$ As(V) aqueous standard was used to test whether As(V) will reduce to As(III) under (pH 6.0) 4°. As(V) reduced to As(III) after a month from preparation, and the reaction was completed after 3 months from preparation.

with the results from other laboratories (Table 3). Our results are in agreement with data from other groups.

Stability of Arsenic Species

To control for the effects of MMA and DMA on distribution, we compared the stability of As(III) and As(V) in mixed solutions [As(III), As(V), MMA, and DMA; Fig. 1, A and B] to the individual As(III) and As(V) aqueous standards (Fig. 1, C and D). We found that the presence of MMA and DMA did not affect the distribution of As(III) and As(V) in aqueous solution.

But if the arsenic methylation enzymes (or specific microorganisms) are present, inorganic arsenic may be methylated to organic arsenic (*e.g.*, MMA and DMA). The concentration of As(III) and As(V) species in the 10 $\mu\text{g/liter}$ mixed arsenic aqueous standards were stable only up to day 29. The reduction of As(V) to As(III) began thereafter and was complete by day 36 (Fig. 1A). We observed a similar effect but at a slower rate with a 100 $\mu\text{g/liter}$ standard: a significant change from As(V) to As(III) occurred after day 36 and was complete by day 94 (Fig. 1B). For both aqueous standards, MMA and DMA con-

Table 4 Six-month average level of arsenic species in freshly collected urine samples (Storage temp = -20°C)

Subject	pH value	Six-month average level of arsenic species ($\mu\text{g}/\text{liter}$)			
		As(III)	As(V)	MMA	DMA
1	5.5	2.60 \pm 0.30	0.40 \pm 0.15	4.37 \pm 0.34	15.79 \pm 0.50
2	7.0	0.31 \pm 0.06	1.41 \pm 0.14	3.27 \pm 0.43	10.32 \pm 0.72
3	6.0	1.29 \pm 0.12	0.49 \pm 0.23	4.08 \pm 0.52	19.12 \pm 0.93
4	5.5	1.06 \pm 0.07	0.64 \pm 0.28	1.54 \pm 0.18	16.57 \pm 0.82
5	6.0	1.90 \pm 0.22	2.47 \pm 0.07	1.68 \pm 0.11	16.84 \pm 0.93
6	5.5	0.45 \pm 0.07	1.20 \pm 0.20	1.56 \pm 0.08	13.67 \pm 0.90
7	6.0	1.26 \pm 0.03	1.55 \pm 0.12	2.32 \pm 0.20	9.56 \pm 1.34

Repeated measure once per week throughout 6 months.

CV for As(III): 2.4 to 19.4%, As(V): 2.8 to 37.5%, MMA: 5.1 to 13.1%, and DMA: 3.2 to 14.0%.

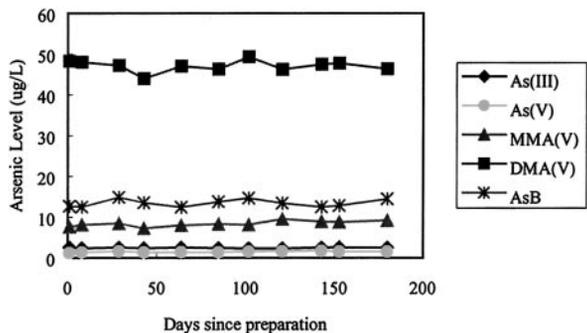


Fig. 2. Stability of NIST SRM 2670 [normal level; As(III): $2.5 \pm 0.2 \mu\text{g}/\text{liter}$, As(V): $1.5 \pm 0.2 \mu\text{g}/\text{liter}$, MMA: $8.3 \pm 0.7 \mu\text{g}/\text{liter}$, DMA: $47.2 \pm 1.3 \mu\text{g}/\text{liter}$, and AsB: $13.5 \pm 0.9 \mu\text{g}/\text{L}$; pH 4.4; -20°). The level of all five of the arsenic species remained stable during the experiment.

concentrations remained stable up to 4.5 months since preparation. The 100 $\mu\text{g}/\text{liter}$ individual As(III) aqueous standard (pH = 6.0; 4°) was stable for at least 3 months (Fig. 1C). The rate of reduction of As(V) to As(III) in 100 $\mu\text{g}/\text{liter}$ As(V) individual aqueous standard (Fig. 1D) is the same as in the mixed arsenic aqueous standard.

The average concentrations of seven urinary arsenic species in freshly collected samples (pH 5.5 to 7; -20°) were stable over 6 months [Table 4, CV for As(III): 2.4–19.4%, As(V): 2.8–37.5%, MMA: 5.1–13.1%, and DMA: 3.2–14.0%]. The NIST SRM 2670 (normal level, pH 4.4; -20°) was stable at 6 months, and the average concentrations were: As(III), $2.5 \pm 0.2 \mu\text{g}/\text{liter}$; As(V), $1.5 \pm 0.2 \mu\text{g}/\text{liter}$; MMA, $8.3 \pm 0.7 \mu\text{g}/\text{liter}$; DMA, $47.2 \pm 1.3 \mu\text{g}/\text{liter}$; and AsB, $13.5 \pm 0.9 \mu\text{g}/\text{liter}$ (Fig. 2).

Loss During Pretreatment

Loss During Centrifuge. The amount of insoluble arsenic was analyzed from four urine samples. The ratio of soluble to insoluble total arsenic in urine ranges from 2 to 17 (Table 5).

Loss During Filtration. For the 10 $\mu\text{g}/\text{liter}$ arsenic mixed aqueous standard, the percentage of loss of insoluble arsenic species after passing through the 0.45- μm filter was within $\pm 5\%$ for As(III), As(V), MMA, DMA, and AsB (Table 2).

Discussion

We found that arsenic species in freshly collected urine (pH 5.5–7) and SRM 2670 (pH 4.4) remained stable up to 6 months when stored at temperatures -20° or less. In aqueous arsenic

standards (stored at 4°), MMA and DMA remain stable for at least 4.5 months, whereas As(V) reduces to As(III) within 4 weeks after preparation. The finding that stability of arsenic species in urine was longer than in the aqueous standards may be because of the complex matrix and pH of urine, as well as the sample storage temperature. The amount of insoluble arsenic in urine lost during centrifuge is considerable for some urine samples and should be noted. This study provides new information on: (a) the loss of insoluble arsenic in human urine; (b) the stability of MMA and DMA in aqueous standards, and that DMA and MMA does not affect the distribution of inorganic arsenic; and (c) the importance of detailed monitoring of urinary arsenic species and pH information. If the amount of insoluble arsenic is also associated with health effects, then the discovery of insoluble arsenic in urinary precipitates provides possible clues to the heterogeneity seen in epidemiological studies.

Stability of Arsenic Species in Urine. Feldmann *et al.* (7) evaluated the stability of arsenic species in freshly collected urine samples. They found that the freshly collected urine samples were stable for at least 2 months (under both 4° and -20°), shorter than our observation of 6 months. The change of arsenic species among some of their subjects may be because of the few time points for sample measurement (months 1, 2, 4, and 8), system change of the analytical machine, and failure to use an internal standard to help quantification. Our study measured arsenic levels weekly, providing a better picture of the stability of arsenic species in both aqueous and urine samples. Moreover, Feldmann *et al.* (7) did not record urinary pH values, an important factor for stabilizing arsenic species in both human urine and aqueous standards. The normal pH range of human urine is 4.5–8. We found a range in pH from 5.5 to 7 among the seven urine samples collected, and the sample pH did not change over 6 months.

It has long been known that As(V) and As(III) undergo interconversion in aqueous solution depending on the pH, temperature, oxygen content, light, and the presence of other substances. However, we did not find transformation between As(III) and As(V) in human urine (both freshly collected urine samples and SRM 2670), and only low concentrations of As(III) and As(V) were present in most freshly collected urine samples. The possible explanations for these phenomena include: (a) the complex matrix of urine stabilizes the distribution of arsenic species; and (b) most As(V) is reduced to As(III) in blood, then some of As(III) is methylated into less toxic forms (MMA and DMA), and finally, the remaining As(III) is retained in the keratin of skin, hair, or gastrointestinal tract.

Stability of Arsenic Species in Aqueous Standards. Feldmann (8) reported that As(III) disappeared completely from aqueous

Table 5 Loss during centrifuge

Subject no.	Supernatant (soluble As, $\mu\text{g}/\text{liter}$)					Total As ^a	Precipitate ^b (Insoluble As, $\mu\text{g}/\text{liter}$)	Original sample ^c ($\mu\text{g}/\text{liter}$)	Ratio of soluble As: insoluble As
	As(III)	As(V)	MMA	DMA	Sum		Total As	Total As	
1	0.53	0.53	0	8.38	9.44	13.99	7.27	16.24	1.92
2	0.09	0.65	0	7.16	7.90	8.24	3.67	10.37	2.25
3	0.82	1.68	1.66	14.60	18.76	27.31	4.57	40.62	5.98
4	0.44	1.11	0.47	7.47	9.49	22.88	1.31	37.04	17.47

^a Arsenic level includes inorganic arsenic and other organic arsenic in addition to MMA and DMA analyzed by HGASS.

^b Precipitate obtained after centrifuge, after microwave digestion, HGAAS was applied for analysis.

^c No filtration or centrifuge applied and then HGAAS was used for analysis.

solution in ~ 4 days at 1 and 10 $\mu\text{g}/\text{liter}$ levels, in ~ 7 days at 100 $\mu\text{g}/\text{liter}$ level, and in ~ 18 days at the 1000 $\mu\text{g}/\text{liter}$ level under room temperature. Cheam and Agemian (9), Aggett and Kriegman (10), and Hall *et al.* (12) tried to use HNO_3 to stabilize the aqueous arsenic species. However, because the distribution of arsenic species changed right after acidification, this is not a preferable way of sample storage and, thus, was not tested in our study. Hall *et al.* (12) also reported that 0.5 and 5.0 $\mu\text{g}/\text{liter}$ As(III) and As(V) aqueous standard could remain stable for at least 11 days when stored at 5°C. Because of the lower concentrations of arsenic in that study, a shorter stability of arsenic is expected. Their results are consistent with ours. They also found that 0.5–20.0 $\mu\text{g}/\text{liter}$ As(V) was reduced to As(III) within 2 days if stored at room temperature. Usually, we preserve samples in portable freezers immediately after collection. Therefore, the stability of arsenic species under conditions of room temperature was not evaluated in our study. The studies discussed above (7–9) were limited in that they did not monitor organic arsenic (MMA and DMA) and lacked information on the urinary pH.

Loss During Centrifuge. One important finding is that the amount of insoluble arsenic in urine lost during centrifuge is 1/2 to 1/17 of soluble arsenic. Freshly collected urine samples usually formed precipitates after a couple of weeks of storage, and the time varied between samples. The components of precipitation in urine may be changed by food intake, metabolism rate, disease type, and so forth. Because of the complexity of precipitation, it is difficult to determine contents either by using the techniques of nuclear magnetic resonance, X-ray diffraction, or MS. A medical reference (26) reported that, for alkaline urine, amorphous phosphate salts and phosphates are possible constituents in the cloudy and milky precipitates, respectively, whereas in acidic urine, urates form the precipitates.

Loss During Filtration. The percentages of arsenic species in aqueous standards and urine samples lost during filtration are within a reasonable range (<5%). Therefore, the levels of soluble arsenic species of interest in the samples did not change after passing through a 0.45- μm filter.

With our modifications, this HPLC-ICP-MS method becomes a powerful tool for urinary arsenic speciation, because we use tellurium as an internal standard to help quantification, and we use a NIST SRM 2670 and 1643d to compensate for any ionization efficiency changes and changes in the nebulization efficiency. The HPLC-ICP-MS method has been adopted for a decade; the results between different instruments for the same urine samples (18, 19, 27) are consistent, and show that the HPLC-ICP-MS is a reliable method and should not affect the distribution of arsenic species. Because large amounts of chloride in urine may result in interference of argon chloride (ArCl) in HPLC-ICP-MS analysis, this possibility was evaluated by

injecting 0.15 M NaCl (the normal level in human urine). The result showed that chloride does not have significant interference on any of the five arsenic species studied.

Total urinary arsenic concentration as measured by HGAAS excludes insoluble arsenic. For subjects 1 and 2 the summation of the level of total arsenic (supernatant) and total arsenic (precipitate obtained after centrifuge) was slightly higher than the total arsenic (original samples, without any pretreatment), indicating that all of the soluble arsenic had been measured, and the small difference could be ignored. For subjects 3 and 4, the summation of the level of total arsenic (supernatant) and total arsenic (precipitate obtained after centrifuge) is lower than the total arsenic (original samples, without any pretreatment). This result indicates that some insoluble arsenic could not be measured if no microwave digestion was used before analysis by HGAAS. The portable equipment, Arsenator, used to measure the current high-level arsenic exposure of drinking water in Bangladesh measures only soluble arsenic. Also, the simple filter system using activated alumina does not remove insoluble arsenic. Although the amount of insoluble arsenic might be lower in drinking water than in urine because of fewer precipitates, it may be important to develop a convenient tool to assess the role of insoluble arsenic in both urine and water in human health. In addition, the amounts of total soluble arsenic were consistently greater than the summation of four arsenic species (sum), indicating that there were other organic arsenic species that could be measured by the HGAAS method. Thus, it is important to differentiate the meanings of “total soluble arsenic” and “the sum of all arsenic species” for studies that attempt to relate arsenic methylation ability to a health outcome.

In summary, arsenic species in human urine is stable for at least 6 months, longer than inorganic arsenic in aqueous standards. The loss of arsenic species in both urine samples and aqueous standards during filtration is negligible. However, the amount of insoluble arsenic lost during centrifuge is noteworthy. Therefore, this report provides valuable information on an important biomarker, urinary arsenic species, for epidemiological studies. Additional research should include: monitoring the long-term stability of arsenic in urine samples (*e.g.*, >1 year), monitoring the pH range that could maintain the distribution of arsenic species in urine, determining the contents in precipitates, and possibly developing a standard method to measure insoluble arsenic in human urine.

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