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Arsenic Speciation in Urine and Blood Reference Materials

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Abstract. Acute and chronic exposure to arsenic is a growing problem in the industrialized world. Arsenic is a potent carcinogen and toxin in humans. In the body, arsenic is metabolized to produce several species, including inorganic forms, such as trivalent (As^{III}) and pentavalent (As^{V}), and the methylated metabolites such as monomethylarsonic acid, (MMA^{V}), and dimethylarsinic acid (DMA^{V}), in addition to arsenobetaine (AsB) which is ingested and excreted from the body in the same form. Each of these species has been reported to possess a specific but different degree of toxicity. Thus, not only is the measurement of total As required, but also quantification of the individual metabolites is necessary to evaluate the toxicity and risk assessment of this element. There are a large number of reference materials that are used to validate methodology for the analysis of As in blood and urine, but they are limited to total As concentrations. In this study, the speciation of five arsenic metabolites is reported in blood and urine from commercial available control materials certified for total arsenic levels. The separation was performed with an anion exchange column using inductively coupled plasma mass spectrometry as a detector. Baseline separation was achieved for As^{III}, As^{V}, MMA^{V}, DMA^{V}, and AsB, allowing us to quantify all five species. Excellent agreement between the total arsenic levels and the sum of the speciated As levels was obtained.

Key words: Arsenic; speciation; ICP-MS; HPLC; urine; blood; reference materials.

Arsenic is a human toxin and carcinogen affecting the lives of millions of people worldwide [1, 2]. Once ingested most of the As is excreted from the body, but a certain fraction of it accumulates in the body giving rise to several health effects including skin and lung cancer, cardiovascular effects and hypertension. Since many arsenic compounds are present in environmental and biological materials, humans are exposed to a variety of arsenic forms possessing different degrees of toxicity. Inorganic forms of As are highly toxic (LD50 for mice are $4.5\,mg\,kg^{-1}$ and $14–18\,mg\,kg^{-1}$ for the arsenite and arsenate, respectively) [3]. *In vivo*, inorganic As is biotransformed via several steps to monomethylarsonic acid (MMA^{V}) and dimethylarsinic acid (DMA^{V}), before it is excreted in the urine [1]. Approximately, 60–80% of As species in urine is DMA^{V}, 10–20% is MMA^{V} and 10–20% is inorganic arsenic [1]. Methylated As has much lower toxicity compared to the inorganic As (LD50 for mice are $1800\,mg\,kg^{-1}$ and $1200\,mg\,kg^{-1}$ for the MMA^{V} and DMA^{V}, respectively). Arsenic usually enters the body through the ingestion of contaminated drinking water or through the food chain.

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The most common exposure to high levels of arsenic in food is via marine products in the form of arsenobetaine (AsB) or plant products in the form of various arsenosugars. Arsenobetaine has the least toxicity of the five forms investigated in the current report (LD50 for mice is 10000 mg kg^{-1}). Thus, due to the highly different toxicities of As species, analysis of total As is insufficient for complete toxicological and risk assessment evaluation [4].

In the past decade there has been numerous reports of the speciation of As compounds in both biological and environmental samples, most recently reviewed by Gong et al., Mc Sheehy et al., Karthikeyan et al., Smedley et al., and Watt et al. [5–9]. Several techniques have been proposed falling in two general categories: (1) separation hyphenated with spectrometric detection and (2) voltammetric and polarographic methods [5, 10]. In terms of the separation, liquid chromatography (reverse phase, ion exchange or ion pair chromatography) has been most widely used, while capillary zone electrophoresis, supercritical fluid chromatography and gas chromatography have been used to a lesser extent. Modes of detection involve coupling to hydride generation atomic absorption, inductively coupled plasma mass spectrometry (ICP-MS) and to a lesser extent mass spectrometry (MS). Gas chromatography mass spectrometry has been used for direct identification of As metabolites, however, this particular technique suffers from poor sensitivity. Detection by ICP-MS has been the most popular in recent years, because of its high sensitivity, large dynamic range, simplicity of the chromatographic and detection systems, and the possibility of simultaneous determination of arsenic and other trace elements. Numerous reports have shown coupling of HPLC to ICP-MS to study various environmental, geological, clinical and biological samples. From the biological perspective, speciation of As in urine has been most widely studied, but there are also reports of the speciation of As in blood, stomach contents, marine animal tissue samples and food products [5–8, 10–16].

A large number of commercially available control materials are available for the analysis of total As in human-type specimens including blood, serum, urine and tissues, but to the best of our knowledge there are none for speciated As in human blood; and the control materials for speciated As in human urine are very limited with only one report present in the literature [16]. In the current study we report the speciation of five As metabolites in several urine and blood materials, with verified levels for total arsenic.

Materials and Methods

Deionized water from a Millipore ultrapure water system was used for the preparation and dilution of all reagents, samples and standards. The arsenite (As^{III}), arsenate (As^{V}), and dimethylarsinic acid (DMA^{V}) were purchased from Sigma (St. Louis, MO) while the disodium methyl arsenate (MMA^{V}) was obtained from Chem. Services (West Chester, PA). The arsenobetaine form was obtained from Fluka (Milwaukee, WI, USA). Stock solutions of the arsenic compounds (~1000 mg As/L) were prepared by dissolving the appropriate amount of arsenic compound in deionized water. Stock solutions were standardized against an atomic absorption arsenic standard solution (SPEX Industries, Edison, NJ) containing 1000 mg as As/L using ICP-MS. The mobile phases used for the chromatographic separation consisted of 4 mM ammonium hydrogen carbonate (Fisher Scientific Pittsburgh, PA), 4 mM ammonium sulfate (Aldrich Milwaukee, WI) and 4 mM ammonium phosphate (Fisher Scientific Pittsburgh, PA) with the pH adjusted to 8.9 with ammonium hydroxide (Aldrich Milwaukee, WI). Urine and blood lyophilized control materials were purchased from Utak (Valencia, CA). Additionally, urine lyophilized control materials were also obtained from Biorad (Munchen, Germany) and Sigma (St. Louis, MO). The control materials were reconstituted in distilled deionized water following the manufacturers' protocols. All materials were kept reconstituted for a maximum of 30 days at 4 °C.

The analysis of arsenic metabolites in blood and urine was performed using HLPC coupled to inductively coupled plasma mass spectrometer (ICP-MS). The HPLC system consisted of a 200 series binary pump, a 200 series auto sampler with a 250 μL sample loop (Perkin Elmer, Norwalk, CT), a PRP-X100 guard column and a PRP-X100 250 mm × 4.1 mm i.d. (Hamilton, Reno, NV) anion exchange column. The outlet of the HPLC column was connected to a concentric glass nebulizer (Meinhard, Santa Ana, CA) and quartz cyclonic spray chamber of Elan 6100 DRC ICP-MS (Perkin Elmer, Norwalk, CT).

Urine samples were diluted 10 fold with 1% nitric acid and injected on the HPLC column. Blood samples for arsenic speciation were prepared by mixing 400 μL of 0.25 M sucrose, 50 μL of blood, and 50 μL of 10% nitric acid. The mixture was vortexed briefly (~10 seconds), sonicated for 20 minutes, vortexed briefly again and centrifuged for 5 minutes. The supernatant was then transferred into a plastic HPLC vial for analysis. The selection for sucrose was made by trial and error from several organic molecules in order to maximize the extraction efficiency of the arsenic metabolites from whole blood.

The injection volume onto the anion exchange column was 50 μL of diluted urine or blood extract. For the chromatographic separation, the mobile phase was delivered at a flow rate of 1.0 mL min^{-1}. Peak integration was measured using TotalChrom (Perkin Elmer, Norwalk, CT). An external calibration curve based on peak area was used for quantification. The external controls were 0.5, 2, 5.0 and 10.0 μg L^{-1} of each arsenic species in 1% nitric acid. All standards and samples were prepared in 1% nitric acid and the chromatographic separation was optimized using this matrix. Detection limits were calculated based on three standard deviations from 20 blank measurements (1% nitric acid). The noise levels of urine and blood matrix in 1% nitric acid were the same as the blank. For this reason the blank measurements were used for the calculation of the detection limit. Detection of the As species was achieved using ICP-MS with and without dynamic reaction cell

Table 1. Operating conditions of the HPLC-ICP-MS for arsenic speciation

HPLC parameters	
Column	Hamilton PRP-X100 (25×4.1 mm i.d.)
Mobile phase	4 mM ammonium carbonate, 4 mM ammonium phosphate and 4 mM ammonium sulfate buffer (pH 8.9)
Column temperature	ambient
Flow rate	1.0 mL min^{-1}
ICP Operating parameters	*setting*
Plasma power	1350 W
Auxiliary gas flow	1.2 L min^{-1}
Plasma gas flow	15 L min^{-1}
Nebulizer gas flow	0.98 L min^{-1}
Sampler and skimmer cones	Pt
DRC gas flow	0.0 L min^{-1} (for urine)
RPq	0.25 (for urine)
Mass spectrometer acquisition	*setting*
Monitored signal	m/z: 74.9216
Dwell time	500 ms
Scan mode	Peak Hopping
Sweeps/reading	1
Readings/replicate	1200
Replicates	1

(DRC) for blood and urine samples. The ArCl interference did not co-elute with any of the As metabolites studied and thus the DRC was turned off during the analysis. The operating conditions for the chromatographic separation and the ICP-MS detection are listed in Table 1.

To investigate the stability of each of the five metabolites during and after the sample preparation process, we designed a study using a 2 µg L^{-1} spike of each metabolite to the Utak normal level urine and the Utak level 1 blood controls. The stability study was performed from 0 to the 72 hour point and the prepared diluted urine and blood extracts were kept at 25 °C and 4 °C, respectively.

Results and Discussion

We have undertaken a study to investigate the As metabolite composition of several reference materials certified for total arsenic levels. Figure 1 shows chromatograms of 0.5, 2, 5 and 20 µg L^{-1} As^{III}, As^{V}, MMA^{V}, DMA^{V}, and AsB under standard mode of the ICP-MS (i.e. without the purging of hydrogen gas in the dynamic reaction cell). Baseline separation was achieved for all species.

Using the sample preparation reported here, the detection limits for each of the arsenic species as determined by ICP-MS are listed on Table 2. The limits of detection ranged between 0.2 and 0.4 µg L^{-1} of As with the lowest for arsenobetaine and highest for arsenate. The limits of detection obtained using our current methodology are below the levels of As in urine and blood reported in the literature (based on total As levels) [17]. The precision of the study based on the relative standard deviations of all control materials used for the quantification ranged from 2–30% depending on the As species. The precision for the method was calculated for control materials with metabolite concentrations above 1 µg L^{-1}, since the uncertainties for low level metabolites ranged up to 100%.

Figure 2 shows example chromatograms of Utak normal urine and Utak blood level 1 control materials. The normal urine level contains DMA^{V}, and AsB and small amount of the MMA^{V} and pentavalent inorganic arsenic forms. A peak at 6.4 min corresponds to the retention time of Cl^{-}, observed at m/z = 75 as ArCl. The Cl^{-} is well separated from the other components and does not affect the quantification of the As

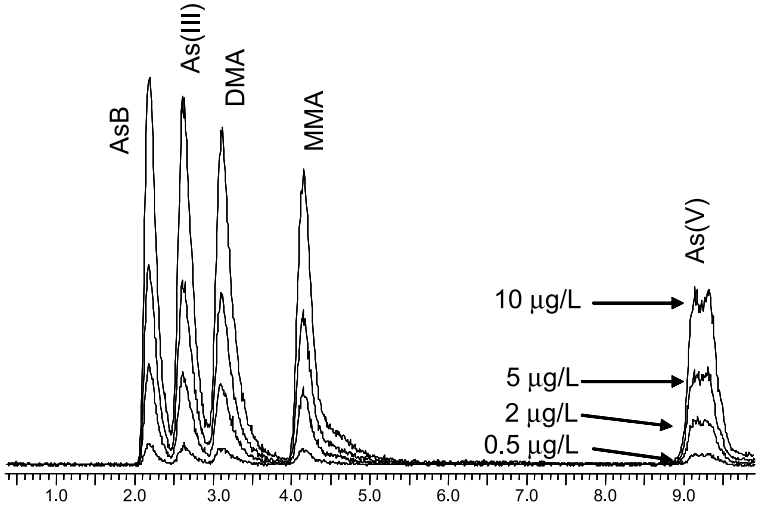

Fig. 1. Chromatographic separation of As^{III}, As^{V}, MMA^{V}, DMA^{V}, and AsB standards (0.5 µg L^{-1}, 2 µg L^{-1}, 5µg L^{-1} and 10 µg L^{-1} of each As form) using anion exchange column, 4 mM ammonium carbonate, 4 mM ammonium phosphate and 4 mM ammonium sulfate buffer (pH 8.9), flow rate at 1.0 mL min^{-1}. Detection was by ICP-MS: at m/z = 74.9216, 0.5 s dwell time and 1350 W RF power

Table 2. Detection limits and precision for As species by ICP-MS

Arsenic species	Precision (controls above 1 μg L^{-1} As)		
	Detection limits (mg L^{-1})	Urine speciation RSD	Blood speciation RSD
Arsenobetaine (AsB)	0.2	4–30%	4–16%
Arsenite (As^{III})	0.3	2–21%	1–18%
Arsenate (As^{V})	0.4	2–20%	2–30%
Dimethylarsinic acid (DMA)	0.3	6–20%	5–13%
Monomethylarsonic acid (MMA)	0.3	7–21%	8–14%

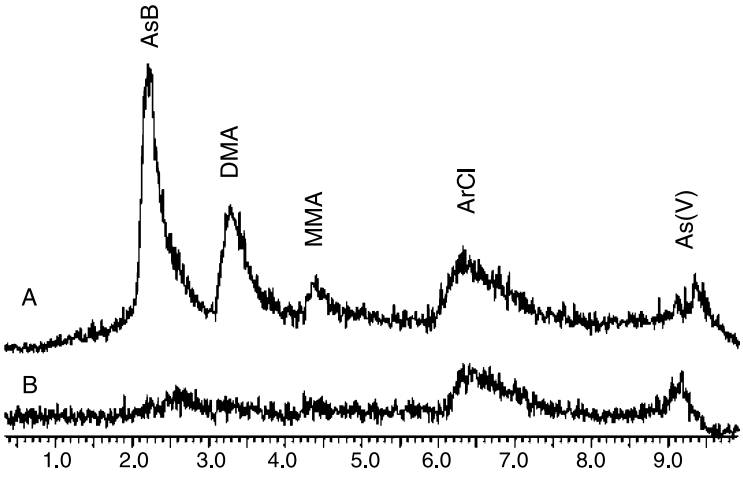

Fig. 2. Speciation of As metabolites in (A) Utak normal urine and (B) Utak blood level control verified for total As levels. Separation and detection conditions are the same as in Fig. 1

species. In the chromatogram of the blood level 1 control, small amounts of the pentavalent and trivalent inorganic As metabolites are observed.

In the current paper we investigated both external and standard addition calibration quantification for AsB, MMA^{V}, DMA^{V}, As^{V}, and As^{III}. Figure 3 shows an example comparison of the two calibration approaches for arsenobetaine in both blood and urine control materials. The correlation coefficient between the two types of quantification was 0.998, indicating that the blood and urine matrix did not affect determination of AsB using external calibration curve based on standards prepared in 1% nitric acid. For the other four metabolites similar correlation coefficients were observed (data not shown).

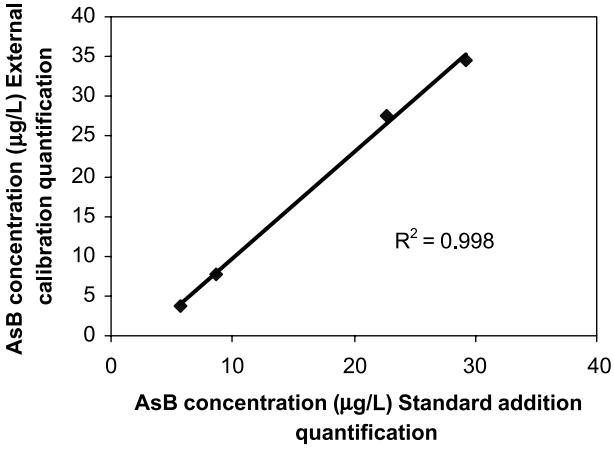

Fig. 3. Comparison between quantification for arsenobetaine in urine and blood control reference materials based on the method of standard addition calibration using 2 and 10 μg L^{-1} additions and external calibration method as shown in Fig. 1

In Table 3 we present a stability study of the five arsenic metabolites after the dilution with 1% nitric acid for urine and the extraction for blood samples. The table shows the adjusted amounts of the spike after the subtraction of the amounts of arsenic metabolites present in the control material. At time 0, i.e. injection right after the sample preparation the recoveries of 2 μg L^{-1} spike were in the range of 105–125% for blood and 70–125% for urine. After 24 hour, an increase in the arsenobetaine peak was observed and the quantities of As^{V}, MMA^{V} and DMA^{V} were slightly decreased. The formation of AsB in the blood or urine extract is unlikely. Thus, we can hypothesize that the increase might be caused by a formation of trivalent MMA or DMA which are likely to co-elute with AsB or As^{III} metabolites at the buffer pH used for our separations [18]. The long term

Table 3. Stability of As metabolites in pre-treated blood and urine control materials over 72 hour period

Time, temperature	Arsenic concentrations, $\mu g\,L^{-1}$				
	As^{III}	As^{V}	AsB	MMA	DMA
Utak blood, level 1 (lot 6909) with 2 mg L^{-1} each As^{III}, As^{V}, AsB, MMA, DMA					
0 hours	2.3 ± 0.1	2.5 ± 0.1	2.5 ± 0.0	2.1 ± 0.1	2.5 ± 0.2
24 hours, 4 °C	2.3 ± 0.1	1.2 ± 0.1	2.9 ± 0.1	1.6 ± 0.1	2.3 ± 0.2
72 hours, 4 °C	2.3 ± 0.2	2.4 ± 0.1	2.7 ± 0.2	2.3 ± 0.3	2.7 ± 0.3
24 hours, 25 °C	1.9 ± 0.3	1.8 ± 0.2	2.8 ± 0.3	1.9 ± 0.1	2.2 ± 0.3
72 hours, 25 °C	2.2 ± 0.3	1.7 ± 0.3	2.8 ± 0.2	2.2 ± 0.2	2.4 ± 0.3
Utak urine normal (lot 6361), with 2 mg L^{-1} each As^{III}, As^{V}, AsB, MMA, DMA					
0 hours	2.3 ± 0.1	2.1 ± 0.0	2.5 ± 0.2	1.8 ± 0.1	1.4 ± 0.2
24 hours, 4 °C	2.6 ± 0.2	1.6 ± 0.1	2.9 ± 0.3	1.9 ± 0.2	2.3 ± 0.4
72 hours, 4 °C	2.4 ± 0.1	1.4 ± 0.2	2.2 ± 0.3	1.5 ± 0.3	2.0 ± 0.4
24 hours, 25 °C	2.5 ± 0.2	1.0 ± 0.2	2.7 ± 0.3	1.6 ± 0.1	2.3 ± 0.4
72 hours, 25 °C	2.7 ± 0.1	1.5 ± 0.2	2.0 ± 0.4	1.9 ± 0.2	2.5 ± 0.3

stability of the As metabolites is currently under investigation in our laboratory.

Table 4 summarizes all determined concentrations from six urine and three blood reference materials. The reported concentrations are based on the average of 4 to 6 determinations. AsB and DMA^{V} were present in all control materials, which is expected from the As metabolism (in the body ~70–80% of the inorganic arsenic is converted to DMA^{V}; AsB is present in variety of marine organisms, and thus it will be high in population on a fish diet). Inorganic arsenite and arsenate were measured in both Utak blood and urine and Biorad urine. MMA^{V} was not found in any of the reference materials investigated in the current study.

Table 4. Concentrations of arsenic species in control materials ($x \pm SD$) as obtained by ICP-MS

Arsenic concentrations in $\mu g\,L^{-1}$ urine

	As^{III}	As^{V}	AB	MMA	DMA
Utak normal	0.6 ± 0.5	0.2 ± 0.2	12.1 ± 0.3	–	6.4 ± 0.4
Utak high	95.1 ± 11.8	6.7 ± 1.4	9.6 ± 1.6	–	7.4 ± 1.6
BioRad 1	0.4 ± 0.7	56.8 ± 4.0	8.1 ± 0.5	–	4.7 ± 0.3
BioRad 2	2.6 ± 0.8	141 ± 10	6.0 ± 0.4	–	4.2 ± 0.3
Sigma 1	–	–	6.5 ± 0.7	–	2.8 ± 0.2
Sigma 2	–	–	13.3 ± 0.4	–	6.5 ± 1.1

	Sum of species	Certified value for total As	Method	Lot#
Utak normal	19.3 ± 0.6	19 (range 16–22)	ICP/MS	3593
Utak high	119 ± 15	117 (range 99–135)	ICP/MS	3592
BioRad 1	70.0 ± 4.2	66 (range 43–90)	ICP/MS	69041
BioRad 2	154 ± 12	177 (range 133–222)	ICP/MS	69042
Sigma 1	9.3 ± 0.8	14 (range 8–20)	ICP/MS	070K6401
Sigma 2	19.8 ± 1.4	27 (range 19–35)	ICP/MS	070K6402

Arsenic concentrations in $\mu g\,L^{-1}$ blood

	As^{III}	As^{V}	AsB	MMA	DMA	As-X
Utak level 1	–	–	0.8 ± 0.2	–	–	0.9 ± 0.4
Utak level 2	15.8 ± 1.4	7.9 ± 0.3	1.4 ± 0.3	–	–	–
Utak level 3	50.4 ± 2.0	18.8 ± 0.1	1.4 ± 0.4	–	–	–

	Sum of species	Certified value for total As	Method	Lot#
Utak level 1	1.6 ± 0.6	<10 (range 0–10)	ICP/MS	4668
Utak level 2	25.7 ± 0.8	29 (range 23–35)	ICP/MS	4468
Utak level 3	70.5 ± 1.5	88 (range 70–96)	ICP/MS	4466

An additional As form was found in the separation of Utak blood level 1 (lot 4668). This particular form did not appear in the speciation profile of any of the other control materials. We were not able to identify this form according to its retention time.

The sum of the concentrations of the arsenic species found in the control materials was within the verified range for total As provided by Utak, Biorad and Sigma. This confirms excellent recoveries and shows that the described speciation method is sufficient for the determination of the As species present in the above commercially available control materials.

In the certification documents of the controls used in this study, we found no information regarding the species used for elevating the levels of the control materials. Thus, we provide a short discussion of that matter. From the speciation profile obtained here, is evident that for each of the control material, the elevated levels were achieved by spiking each material with the inorganic arsenic species, except for the Sigma controls which showed an increased level of DMA and AsB. The Utak material used a mix of As^{III} and As^{V} for spiking of all of their samples as shown in Table 3. For elevated Utak urine control, the ratio of As^{III} to As^{V} was 14:1, whereas Utak blood level 2 and 3 showed ratios of 2:1 and 2.7:1, respectively. Biorad on the other hand used As^{V} to increase the level of As in their material.

Conclusion

In this report we have used a simple robust method in order to demonstrate the speciation of As forms from several urine and blood commercially available control materials. Excellent agreement between the total arsenic levels and the sum of the speciated As levels was obtained.

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