Development and Application of a Robust Speciation Method for Determination of Six Arsenic Compounds Present in Human Urine

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Six arsenic species [arsenate, arsenite, arsenocholine, arsenobetaine, monomethyl arsonic acid, and dimethyl arsinic acid] present in human urine were determined using ion-exchange chromatography combined with inductively coupled plasma mass spectrometry (IC-ICP-MS). Baseline separation was achieved for all six species as well as for the internal standard (potassium hexahydroxy antimonate V) in a single chromatographic run of less than 30 min, using an ammonium carbonate buffer gradient (between 10 and 50 mM) at ambient temperature, in conjunction with cation- and anion-exchange columns in series. The performance of the method was evaluated with respect to linearity, precision, accuracy, and detection limits. This method was applied to determine the concentration of these six arsenic species in human urine samples (n = 251) collected from a population-based exposure assessment survey. Method precision was demonstrated by the analysis of duplicate samples that were prepared over a 2-year analysis period. Total arsenic was also determined for the urine samples using flow injection analysis coupled to ICP-MS. The summed concentration of the arsenic species was compared with the measured arsenic total to demonstrate mass balance. *Key words:* **arsenic, HPLC, ion chromatography, plasma mass spectrometry, speciation, urine.** *Environ Health Perspect* **111:293–296 (2003). doi:10.1289/ehp.5525 available via *http://dx.doi.org/* [Online 28 October 2002]**

Arsenic exists in many chemical forms with varying degrees of toxicity. The more inorganic the form, the greater the virulence. For example, arsenate (AsV) and arsenite (AsIII) are known carcinogens (*1–3*). Methylated arsenic compounds monomethyl arsonic acid (MMA) and dimethyl arsinic acid (DMA) are less toxic, and the organic arsenicals arsenobetaine (AsB) and arsenocholine (AsC), commonly found in seafood, are relatively innocuous (*4,5*). Once these compounds are ingested, they metabolize in different ways; inorganic arsenic compounds are metabolized to DMA and MMA, whereas AsB and AsC are unchanged (*4,5*) and are evidenced by their existence in urine. Speciation analysis, typically involving the coupling of a liquid chromatographic method to a highly sensitive detection method with multielement capabilities, such as inductively coupled plasma mass spectrometry (ICP-MS), is necessary to provide information about individual arsenic species.

Our goal was to employ a mobile phase that would separate the aforementioned six arsenic species in human urine in a single chromatographic run (including the internal standard) in a reasonable time period (< 30 min). An appropriate buffer that would conform to these criteria was selected. To have a high sample throughput, it was also mandatory that the mobile phases not deposit a significant amount of salt on the sampling interface of the mass spectrometer.

There have been many studies involving speciation by the coupling of ion-exchange chromatography (IC) to ICP-MS (IC-ICP-MS) (*6,7*); however, studies reported in the literature have employed two separate columns (anionic and cationic). Using a single column to separate the six aforementioned arsenic species is difficult because AsC exists in cationic form at most pH levels, whereas the other five species can be made anionic by adjusting the pH. Consequently, many studies demonstrate only effective separation of five arsenic species in food and urine using an anion-exchange column, with AsB eluting at the void volume (*8–11*). Other studies report separation of six or more arsenic species on an anion-exchange column in food and biologic matrices but either report analysis time of more than 1 hr (*12*) or do not employ a rugged method useful for a larger number of samples (*13*). One study reported the use of a cation- and anion-exchange column in series (*14*), with sodium carbonate as the mobile phase. Our investigation of this buffer system resulted in good separation but poor ruggedness. It also resulted in low sample throughput because the analysis had to be stopped frequently to clean the heavy salt deposits on the ICP-MS sampling interface.

Tris acetate buffer was previously used in our laboratory (with ICP-MS detection) to resolve and to quantitate four arsenic species (AsIII, AsV, DMA, and MMA) in drinking water on an anion-exchange column (*15*). The method demonstrated acceptable linearity, precision, and accuracy and was successfully applied for analysis of hundreds of drinking water samples. The mobile phase resulted in negligible salt deposits on the ICP-MS interface, enabling the routine analysis of a large number of samples. For the urine studies, the Tris acetate buffer separation method was modified by adding a cation-exchange column in series to the anion-exchange column to separate cationic forms of arsenic species along with the anionic forms. The separation was compared with that obtained using ammonium carbonate buffer with the same two columns in series. The ammonium carbonate buffer system demonstrated a significant improvement over the Tris buffer system and was selected for use in the speciation of six arsenic species in urine. A total of 262 urine samples (251 samples + 11 control samples) were analyzed for the six arsenic species of interest using the ammonium carbonate buffer system. The same samples were also analyzed for their total arsenic content by flow injection analysis (FIA) coupled to ICP-MS, and the total and species data were compared to assess the mass balance.

Materials and Methods

Ion chromatography. A Waters 600S controller (Waters, Milford, MA) was used to operate a metal-free 626 high-performance liquid chromatographic (HPLC) gradient pump (Waters) as well as a 717 autosampler for the IC (Waters). The cation- and anion-exchange columns used were Hamilton PRP-X100 (10 µm, 4.1 × 250 mm i.d.) and PRP-X200 (10 µm, 4.6 × 150 mm) (Hamilton Co., Reno, NV), respectively.

Periodic regeneration of the columns was necessary (after every 75–100 samples) to ensure reproducible performance of the separation. The cation-exchange column was back-flushed with 0.1 M nitric acid (Fisher Scientific, Pittsburgh, PA) for approximately 1 hr followed by type I deionized water (Hydro Services, Research Triangle Park, NC). The anion-exchange column was back-flushed with 0.1 M sodium hydroxide (Fisher) for 1 hr, followed by deionized water for 1 hr. The regeneration was necessary to remove the heavy chloride buildup on the column over time (which may contribute to the formation of $^{40}Ar^{35}Cl$).

Inductively coupled plasma mass spectrometry. A VG Elemental PQ-XR ICP-MS instrument (VG Elemental, Winsford, UK) equipped with a Meinhard concentric

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nebulizer and a water-cooled, Scott double-pass spray chamber, which were purchased from CPI (Santa Rosa, CA) was used for this work. The forward power was 1,348 W, and the coolant and auxiliary and nebulizer gas flows were 16, 1.4, and 0.7 L/min, respectively. Time-resolved data acquisition software (PQ Vision version 4.0, VG Elemental) was used to simultaneously monitor arsenic species at m/z 75 and ArCl interference at m/z 77 as well as any elements used for internal standards (antimony, m/z 121; indium, m/z 115).

Buffer reagents. The Tris buffer was prepared by the addition of Tris (hydroxymethyl)aminomethane (Aldrich, Milwaukee, WI) to deionized water and adjusting the pH to 7 with glacial acetic acid (≥ 99.99%; Aldrich). The ammonium carbonate buffer was prepared by dissolving ammonium carbonate (J.T. Baker, Phillipsburg, NJ) in deionized water. HPLC-grade methanol (Fisher) was also used with the ammonium carbonate mobile phase (6% vol/vol of total mobile phase). Before use, all mobile phases were filtered through 0.45-µm filters (Alltech, Deerfield, IL). The gradient programs used for each buffer are summarized in the flow charts in Figure 1.

Standards/samples. Four arsenic standards were purchased, including sodium arsenate (AsV; Pfaltz & Bauer Inc., Waterbury, CT), arsenious oxide (AsIII; 99.999% purity; GFS Chemical Inc., Powell, OH), MMA (97% purity; Chem Service, West Chester, PA), and cacodylic acid (DMA; 99% purity; Pfaltz & Bauer, Inc.). William Cullen's group at the University of British Columbia synthesized AsB and AsC, which are not commercially available. A spectrometric-grade single-element standard containing AsV (High Purity Standards, Charleston, SC) was used for the preparation of standards for the determination of total arsenic by FIA-ICP-MS. Potassium hexahydroxy antimonate V (Aldrich) was used as the internal standard for speciation studies and indium standard (High Purity Standards) was used as the internal standard for the flow injection work.

A total of 262 samples (251 urine samples + 11 field control samples) were made available for this study. The samples were collected from 300 homes from different individuals as part of the National Human Exposure Assessment Survey study, conducted in U.S. Environmental Protection Agency Region V from July 1995 to May 1997 (*16–18*). Region V consists of the Great Lakes area (Minnesota, Wisconsin, Illinois, Indiana, Ohio, Michigan), in which the demographic characteristics of the population (e.g., races, ethnic groups, socioeconomic distribution) are similar to the national profile (*18*). The samples were collected in 50-mL polypropylene tubes and stored at 20°C.

Sample preparation. **Speciation.** Before taking a 1-mL aliquot for speciation analysis, samples were allowed to thaw to room temperature in a class 100 hood and centrifuged. The samples were then filtered using a 0.2-µL syringe filter into an autosampler vial, spiked with the internal standard (potassium hexahydroxy antimonate V) and diluted 1:10 using the mobile phase buffer before being injected on to the chromatographic system.

Total arsenic. A flow injection system with the same controller, chromatograph, and autosampler coupled to ICP-MS was used to determine total arsenic concentration in urine samples. Urine samples for this analysis were diluted 1:10 with 1% (vol/vol) nitric acid and spiked with 10 ng/mL indium before injection into a 1% (vol/vol) nitric acid carrier stream.

Quality control: arsenic speciation. Each day of sample analysis, the chromatographic system was equilibrated with the starting mobile phase, and deionized water was injected to establish the background and to verify that there was no arsenic carryover from previous injections. National Institute of Standards and Technology standard reference material (SRM 2670; normal level, toxic metals in freeze-dried urine) was evaluated for use as the matrix but was deemed unsuitable because it contained a high level of arsenic that was incompatible with the goal of extending the arsenic linearity to low levels (0.5 ng/mL). The concentration of arsenic in SRM 2670, normal level, was 60 ng/mL, which corresponds to 6 ng/mL with 1:10 dilution as defined in the method. The daily calibration standards (0.5, 1.0, 5.0, and 30 ng/mL) were prepared in a urine matrix collected from individuals who had not consumed seafood for 2–3 days before collection. The urine was screened for arsenic before its use as the control matrix, and the urine containing the lowest arsenic was used for the matrix. The samples were analyzed in batches ranging from 10 to 15 per day. A duplicate analysis of the same solution or duplicate preparation of a sample was included with each batch of samples. At the end of the day, a predetermined mid-level calibration standard (continuous calibration check) was reanalyzed to verify the calibration and the instrument performance.

Quality control: total arsenic analysis. Calibration standards (ranging from 0.5 to 10 ng/mL) were prepared in 1% (vol/vol) nitric acid. Each sample was injected 3 times into the 1% (vol/vol) nitric acid carrier stream, and the average of the three readings was used to calculate the concentration of arsenic in the sample. Duplicate injections and duplicate preparations were analyzed daily along with the continuous calibration check standard.

Data processing. The ICP-MS data for each injected chromatographic run were collected and then converted to ASCII format. The chromatographic results were processed using Grams/32 software (Galactic Industries Corporation, Salem, NH). Final sample concentrations were calculated using the regression equations of measured response (peak areas) versus concentration for each species of arsenic.

Results

Selection of a mobile phase for speciation. In our laboratory, a Tris acetate buffer gradient was developed and successfully applied for the speciation of four arsenic species (AsIII, AsV, DMA and MMA, and internal standard) in drinking water using IC-ICP-MS (*15*). Consequently, this buffer was evaluated for the separation of six species in urine. The separation was unsuccessful and resulted in coeluting peaks for AsB and AsIII as well as insufficient baseline separation of MMA and AsC (Figure 2).

An ammonium carbonate buffer mobile phase was evaluated next for arsenic speciation in urine, and a successful separation was achieved for the six arsenic species and the potassium hexahydroxy antimonate V internal standard (Figure 3).

A 100%*a* 3 min *Hold* 100%*a* 0.1 min *Step* 100%*b* 7 min *Ramp* 100%*c* 20 min *Hold* 100%*c* 0.1 min *Step* 100%*a*

B 100%*a* 7 min *Ramp* 100%*b* 13 min *Hold* 100%*b* 0.1 min *Ramp* 100%*a* 10 min *Hold* 100%*a*

Figure 1. Flow diagrams. (*A*) Tris acetate buffer gradient. (*B*) Ammonium carbonate buffer gradient. (*A*) *a*, 5 mM Tris acetate; *b*, 30 mM Tris acetate; *c*, 100 mM Tris acetate. Flow rate, 1.5 mL/min; injection volume, 200 µL; column temperature, 25°C. Detection, ICP-MS; column configuration; PRP-X200 cation exchange guard; PRP-X200 cation exchange column, PRP-X100 anion exchange column. (*B*) *a*, 94:6 (vol/vol) 10 mM $(NH_4)_2CO_3$:MeOH; *b*, 94:6 (vol/vol) 50 mM $(NH_4)_2CO_3$:MeOH. Flow rate, 1.0 mL/min; injection volume, 200 µL; column temperature, 25°C. Detection, ICP-MS; column configuration, PRP-X100 anion exchange guard, PRP-X200 cation exchange column, PRP-X100 anion exchange column.

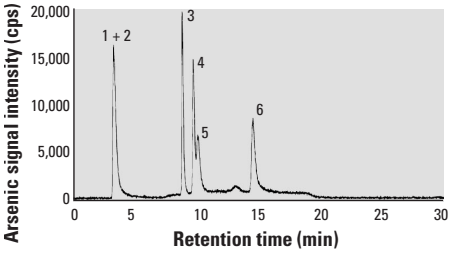

Figure 2. Chromatogram of a 10-ppb calibration standard. Peak 1, AsB; peak 2, AsIII; peak 3, DMA; peak 4, MMA; peak 5, AsC; peak 6, AsV. Chromatographic conditions: mobile phase, Tris acetate buffer at pH 7.

Method performance evaluation for arsenic speciation in urine. The ammonium carbonate buffer method performance was assessed by determining linearity, precision, accuracy, and detection limits for the six species in a urine matrix. The matrix used consisted of urine samples from several individuals who did not consume seafood 2–3 days before collection.

The linearity for the six species was demonstrated. Plots of normalized peak area versus arsenic concentration for concentrations ranging from 0.50 to 100 ng/mL were constructed using a $1/x^2$ weighted linear least-squares regression for each arsenic species. Method detection limits (MDLs) and method quantitation limits (MQLs) were calculated for each of the six species and are presented in Table 1 along with the correlation coefficients. The values for MDL were calculated as 3 times the standard deviation (SD), and MQL were calculated as 10 times SD of three replicate analyses of pooled urine spiked at 0.50 ng/mL for each of the six species. The short-term (4-hr) precision was demonstrated by the analysis of a urine matrix fortified with three concentrations (0.5, 2.0, and 5.0 ng/mL) of all six arsenic species (Table 2). The percent relative SD (%RSD) was greatest for AsB at 0.5 ng/mL, most likely because of the relatively high AsB concentration (0.5–1 ng/mL) found in the urine matrix. The long-term precision was demonstrated by a %RSD of ≤ 15% for replicate analyses of urine fortified with 2.0 ng/mL of each species over a 3-day period (Table 2). Accuracy was demonstrated by a percentage bias ≤ 18% for all species through the replicate analysis of urine fortified at three different concentrations (0.5, 1.0, and 10.0 ng/mL), with the exception of AsB at 0.5 ng/mL (Table 3). Because the MQL for AsB is 0.5 ng/mL, this may account for the 60% bias.

Urine samples. Results for the determination of arsenic species in urine are presented in Table 4. The field controls were deionized water samples subjected to the same sample preparation steps as the urine samples. DMA is the most commonly found arsenic species and exists in the highest concentration for the samples in this study.

After completion of sample analysis, eight samples that contained the highest measurable values of the five species (AsB, AsIII, DMA, MMA, and AsV) were selected for reanalysis to further evaluate the method. A second aliquot of each was removed from the original sample, prepared, and analyzed. Analysis periods varied as much as 3 months between the duplicate samples (as presented in Table 5).

Urine samples were also analyzed for total arsenic by FIA-ICP-MS. Because the urine matrix contains a significant level of chloride, a correction equation was employed to account for the contribution from $^{40}Ar^{35}Cl$ to the ^{75}As signal (*19*).

Discussion

The current reported method for determination of six arsenic species in urine using an ammonium carbonate buffer resulted in acceptable performance and significant improvement over the existing methods reported in the literature (*6–14*). The method uses cationic and anionic columns in series for complete baseline separation of six arsenic species and the internal standard, in a single chromatographic run of less than 30 min.

Method performance was demonstrated using the ammonium carbonate buffer to determine six arsenic species in urine. The calculated detection limits are comparable and in some cases superior to detection limits determined by other investigators (*20–22*). The use of ammonium carbonate as a buffer resulted in minimal salt deposit on the cones; thus, a high throughput of samples was achieved despite urine's high chloride concentration. The lack of salt deposition on the cones, as well as the infrequent column regeneration (every 75–100 samples), results in less instrument downtime and thus less cost per sample. The method is also rugged, as demonstrated through the duplicate sample analysis results obtained over several months. Finally, evaluation of species and total arsenic data suggests that mass balance was close and that the majority of arsenic species in the urine were accounted for (Figure 4).

Additional improvements could be made. Although the detection limits are similar or superior to those in other studies reported in the literature, further improvements in sensitivity could lower the detection limits. It may also be beneficial to employ a "mixed-bed" stationary-phase column containing both cationic and anionic elements instead of two columns to perform the separation. This would reduce the column regeneration time and possibly the species separation time. An evaluation of additional arsenic species other than the aforementioned six may improve the mass balance.

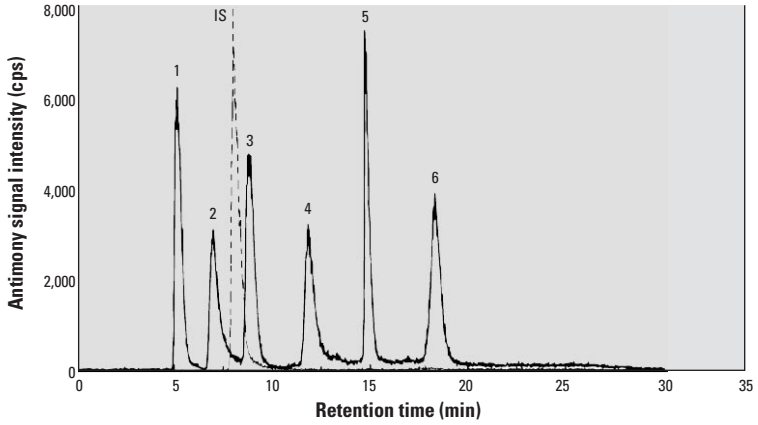

Figure 3. Chromatogram of a 10-ppb calibration standard. Peak 1, AsB; peak 2, AsIII; peak 3, DMA; peak 4, AsC; peak 5, MMA; peak 6, AsV; IS, internal standard (potassium hexahydroxy antimonate V). Chromatographic conditions: mobile phase $(NH_4)_2CO_3$ buffer at pH 9.

Table 3. Analysis results for the method accuracy.[a]

Arsenic species	Method accuracy (% bias) 0.50 ng/mL (*n* = 3)	1.0 ng/mL (*n* = 3)	10 ng/mL (*n* = 5)
AsB	60	11	–3.0
AsIII	0	2.0	4.1
DMA	4.0	–10	–1.8
AsC	–10	–4.0	–3.1
MMA	4.0	–5.0	1.3
AsV	18	–1.0	16

[a]Calculated as [(predicted concentration – nominal concentration)/(nominal concentration)] × 100.

Table 1. Detection limits and correlation coefficients.

Arsenic species	MDL (ng/mL)[a]	MQL (ng/mL)[a]	Correlation coefficient
AsB	1	4	0.9950
AsIII	0.2	0.8	0.9960
DMA	2	5	0.9940
AsC	1	5	0.9936
MMA	0.2	0.6	0.9970
AsV	2	5	0.9952

[a]Values based on species in undiluted urine.

Table 2. Short-term and long-term precision.

Arsenic species	Short-term (4-hr) precision (% RSD) 0.50 ng/mL	1.00 ng/mL	5.00 ng/mL	Long-term (3-day) precision (% RSD) 2.00 ng/mL
AsB	4	5	4	6
AsIII	8	2	3	4
DMA	5	5	5	5
AsC	18	12	5	12
MMA	33	10	4	11
AsV	45	14	8	15

Table 4. Summary of arsenic species present in urine samples.

Sample type	AsB (ng/mL)	AsIII (ng/mL)	DMA (ng/mL)	AsC (ng/mL)	MMA (ng/mL)	AsV (ng/mL)
Field controls	ND (*n* = 11)	ND (*n* = 11)	ND (*n* = 11)	ND (*n* = 11)	ND (*n* = 11)	ND (*n* = 11)
Field samples	≤ 1,300–≥ 1000 (*n* = 1)	≤ 15.0–≥ 10.0 (*n* = 3)	≤ 60.0–≥ 10.0 (*n* = 19)	≤ 10.0–≥ 5.00 (*n* = 2)	≤ 10.0– ≥ 5.00 (*n* = 7)	≤ 10.0–≥ 5.00 (*n* = 8)
	≤ 999–≥ 100 (*n* = 3)	≤ 9.99–≥ 2.00 (*n* = 3)	≤ 9.99–≥ 5.00 (*n* = 73)	≤ 4.99– ≥ 2.00 (*n* = 1)	≤ 4.99– ≥ 2.00 (*n* = 49)	≤ 4.99– ≥ 2.00 (*n* = 19)
	≤ 99.9–≥ 10.0 (*n* = 17)	≤ 1.99–≥ 1.00 (*n* = 23)	≤ 4.99– ≥ 1.00 (*n* = 105)	≤ 1.99– ≥ 1.00 (*n* = 5)	≤ 1.99– ≥ 1.00 (*n* = 42)	≤ 1.99– ≥ 1.00 (*n* = 28)
	≤ 9.99–≥ 0.01 (*n* = 63)	≤ 0.99– ≥ 0.02 (*n* = 11)	≤ 0.99– ≥ 0.01 (*n* = 17)		≤ 0.99– ≥ 0.01 (*n* = 24)	≤ 0.99– ≥ 0.01 (*n* = 29)
	ND (*n* = 167)	ND (*n* = 211)	ND (*n* = 37)	ND (*n* = 243)	ND (*n* = 129)	ND (*n* = 167)

Abbreviations: *n*, number of samples in the group; ND, not detected.

Table 5. Duplicate sample analysis results (ng/mL).

Sample	AsB	AsIII	DMA	AsC	MMA	AsV
UP0018	0.21, 0.25[a] (13)[b]	0.08, 0.15 (43)	1.04, 1.64 (32)	ND	0.41, 0.48 (12)	0.46, 0.33 (22)
UP0076	ND	ND	ND	ND	0.24, 0.23 (2.2)	
UP0169	ND	ND	ND	ND	0.52, 0.38 (23)	0.55, 0.44 (17)
UP0179	ND	ND	ND	ND	ND	0.56, 0.51 (6.3)
UP0188	ND	0.21, ND	ND	ND	ND	ND
UP0222	ND	0.25, 0.29 (12)	5.07, 6.72 (20)	ND	0.55, 1.65 (71)	ND
UP0225	128, 94.6 (21)	ND	5.48, 4.44 (15)	ND	0.29, 0.40 (23)	0.74, 0.23 (75)
UP0089	21.2, 19.2 (7.0)	ND	ND	ND	ND	ND

[a]Represents calculated concentrations for duplicate samples. [b]Percent RSD.

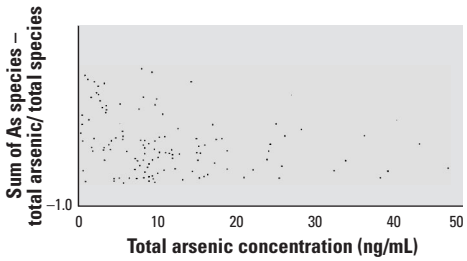

Figure 4. Comparison of speciated arsenic versus total arsenic.

Trivalent MMA and DMA species were not accounted for in this study. These species were observed in human urine when sodium 2,3-dimercapto-1-propane sulfonate was orally administered to humans before collection of their urine samples (*22,23*). Furthermore, these species are reported to be unstable in solution and are readily oxidized to their pentavalent forms (referred to as DMA and MMA in this study) (*22*).

In summary, this method is complementary to other speciation methods in the literature, with the primary advantage of being rugged, which is highly desirable in exposure, toxicology, and epidemiology studies that generate large numbers of samples over a long period of time. It is a viable choice for speciation of arsenic species in urine.

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