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Short Communication

Rapid and Effective Speciation Analysis of Arsenic Compounds in Human Urine using Anion-Exchange Columns in HPLC-ICP-MS

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Occupational arsenic exposure is mainly in the form of inorganic arsenic (iAs)[1, 2]. iAs is methylated to monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) in the human body as shown in Fig. 1[1–3]. The American Conference of Governmental and Industrial Hygienists (ACGIH)[1] and the Deutsche Forschungsgemeinschaft (DFG)[2] recommend the sum of iAs, MMA, and DMA as the biological exposure value for iAs exposure. However, seafood including seaweeds contains high levels of organoarsenic compounds, such as arsenobetaine (AsBe), DMA, arsenocholine (AsCho), and arsenosugars (AsSugs)[4, 5]. AsBe is only minimally metabolized in mammals[6]. AsCho is metabolized extensively to AsBe[7]. AsSugs are extensively metabolized to DMA[8]. These metabolic pathways are shown in Fig. 1. Therefore, large amounts of AsBe and DMA and small amounts of MMA and iAs are observed in the urine of people without occupational iAs exposure who ingest seafood[9].

Urinary speciation analysis of some of the above-mentioned organoarsenic compounds was performed by high-performance liquid chromatography (HPLC) with inductively coupled plasma mass spectrometry (ICP-MS). In our previous reports[9, 10], we analyzed arsenic compounds in the urine of Japanese and Bangladeshi people using a cation exchange column and detected some unknown peaks in the urine of Japanese, but not Bangladeshis. When we analyzed extracts from hijiki seaweed using a cation exchange column, oxo-arsenosugars were found at the same retention time as sodium arsenate (AsV), suggesting insufficient separation of the metabolites of AsSugs from iAs. Here, we compared speciation analysis of urinary arsenic in Japanese using anion and cation exchange columns.

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Materials and Methods

Subjects

The subjects were 172 healthy male workers with a mean age of 46.5 ± 13.6 yr (range 18–74 yr) who were working in Kita-Kyushu, Japan. They had no occupational arsenic exposure for at least six months. Urine sampling was performed in the afternoon in a regular medical examination or in a medical checkup conducted by the subjects' employer between August 2008 and October 2008. After urine sampling, the company's health supervisor erased the subjects' names from the samples, and we then analyzed them. This study was approved by the Ethics Committee of the Tokyo Rosai Hospital (approval number: 20–9).

Chemicals

Sodium arsenite (AsIII), sodium arsenate (AsV), MMA, and AsBe were purchased from Wako Pure Chemical Industries (Osaka, Japan). DMA was obtained from Tri Chemical Laboratory (Yamanashi, Japan). Nitric acid (HNO_3; Tamapure AA-100 (68%)), ammonia solution (Tamapure AA-10 (20%)) (Tama Chemicals, Tokyo, Japan), 2,6-pyridinedicarboxylic acid (Tokyo Kasei Industry, Tokyo, Japan), ammonium nitrate (NH_4NO_3; Wako Pure Chemical Industries), and ammonium bicarbonate (NH_4HCO_3; Fulka BioUltra) (Sigma-Aldrich, St. Louis, MO, USA) were used for the HPLC mobile phase. Germanium standard solution for atomic absorption spectrophotometry (Kanto Chemical, Tokyo, Japan) was used as an internal standard for ICP-MS detection. Ultrapure water for analysis was prepared using a Milli-Q-ICP/MS Ultrapure Water Purification System (Millipore, Tokyo, Japan).

Certified reference material, NIES CRM No. 18 (human urine), from the National Institute for Environmental Studies, Japan, was used to validate the analytical procedure.

Analytical conditions for HPLC and ICP-MS

Arsenic speciation analysis was performed using two instrumental systems. For the separation of arsenic compounds, we used anion and cation exchange columns as two separation modes.

In the anion mode experiment, an Agilent 1200 HPLC series and an Agilent 7500cx ICP-MS (Agilent Technologies, Santa Clara, CA, USA) were used to separate arsenic species and detect arsenic, respectively. The instrument conditions for ICP-MS were as follows:

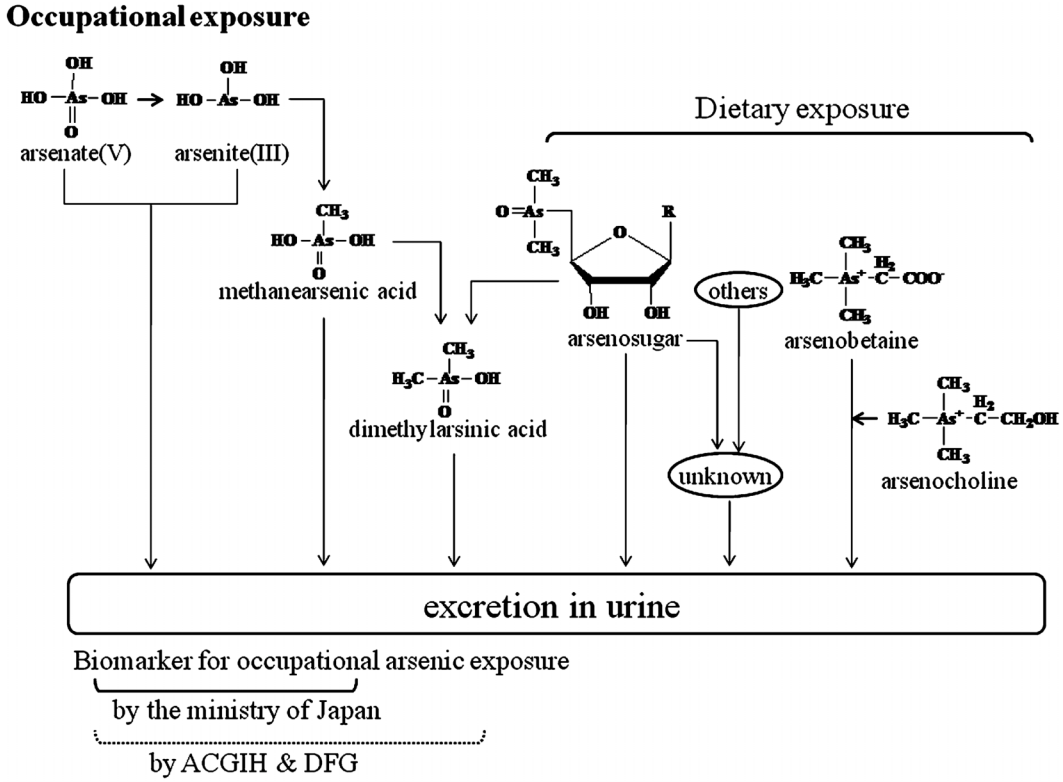

Fig. 1. Metabolic pathways of arsenic compounds from occupational and dietary exposure.

RF power, 1,500 W; plasma argon gas flow rate, 15 *l*/min; carrier argon gas flow rate, 0.9 *l*/min; make-up argon gas flow rate, 0.2 *l*/min; a coaxial-type nebulizer, nickel sampling, and skimmer cones were used. As an anion exchange column, we used a Dionex IonPac AS22 (250 × 4.0 mm i.d., Dionex, Sunnyvale, CA, USA) under the following conditions: mobile phase, 20 mM NH_4HCO_3 at pH 10.0 (adjusted with ammonia solution); flow rate, 1.2 m*l*/min; column temperature, 40°C; and injection volume, 50 μl.

In the cation mode experiment, a PU712 pump and a CO631A column oven (GL Science, Tokyo, Japan) and a Midas autosampler (Spark, Emmen, Netherlands) were used to separate the arsenic species, and an Elan DRCII ICP-MS (Perkin Elmer Sciex, Concord, Ontario, Canada) was used for arsenic detection. Instrumental conditions for ICP-MS were as follows: RF power, 1,300 W; plasma argon gas flow rate, 15 *l*/min, auxiliary argon gas flow rate, 1.2 *l*/min, and nebulizer argon gas flow rate, 1.0 *l*/min; a coaxial-type nebulizer was used; skimmer and sample cones were platinum. As a cation exchange column we used a Shodex RSpak NN-614 (150 × 6.0 mm i.d.; Showa Denko, Tokyo, Japan) under the following conditions: mobile phase, 5 mM HNO_3/6 mM NH_4NO_3/1.5 mM 2,6-pyridinedicarboxylic acid; flow rate, 1.0 m*l*/min; column temperature, 40°C; and injection volume, 50 μl.

Stock standard solutions of AsIII, AsV, MMA, DMA, and AsBe were prepared by dissolving each compound in ultrapure water at a concentration of 10 mgAs/*l*. Working standard solutions were prepared every month by diluting the stock standard solution to 0.1 mgAs/*l*. The final mixed standard solution (5 μgAs/*l*) was prepared from the working standard solution just before use. To obtain precise measurements, 0.1 mg/*l* of germanium solution was used as the internal standard for ICP-MS. ICP-MS detection mass was set as *m/z* 75 ($^{75}As^+$), *m/z* 35 ($^{35}Cl^+$), and *m/z* 72 ($^{72}Ge^+$). Limits of detection for AsIII, AsV, MMA, DMA, and AsBe were calculated according to the definition given by Gibbons[11] as 3.14 times the standard deviations of the concentrations corresponding to the peak area ratio obtained for 50 μl injection of standard solution containing these compounds at 5.0 μg/*l* each.

The accuracy of the analysis was confirmed using reference material, CRM No.18 (human urine), from the National Institute for Environmental Studies (NIES), Tsukuba, Japan.

Urine sample preparation

Urine samples were stored in sealed plastic tubes at –80°C in a refrigerator until analysis. Arsenic species

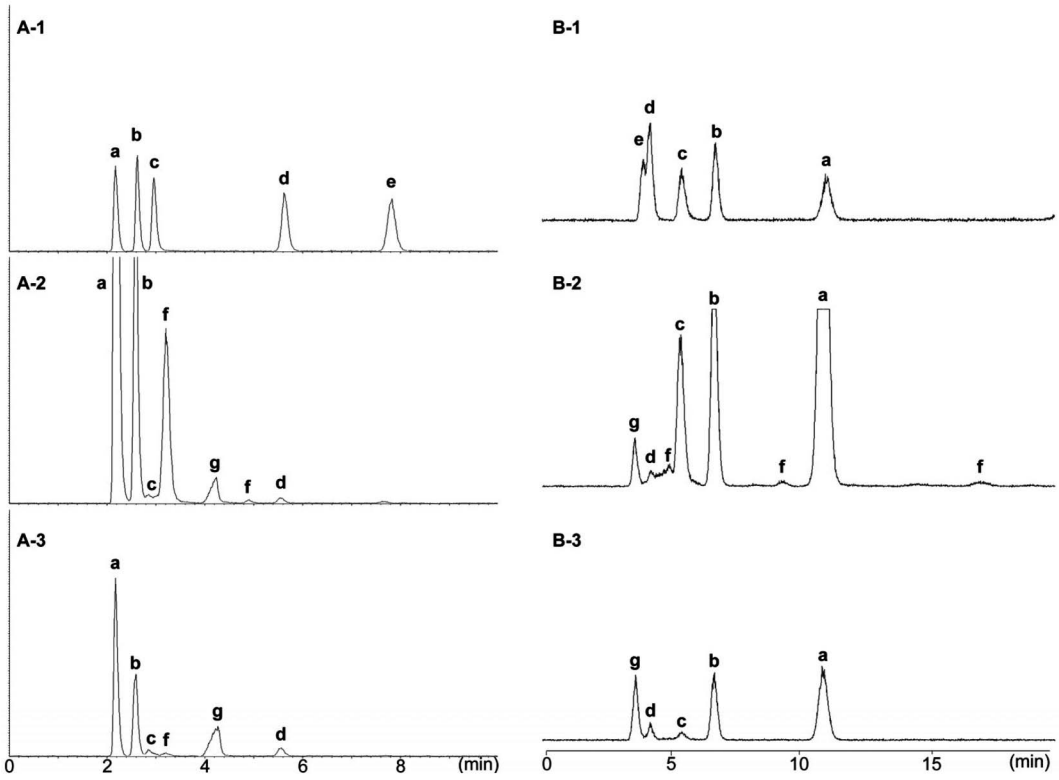

Fig. 2. Chromatograms obtained by HPLC-ICP-MS of a standard solution and two different urine samples. A, analysis with the anion column; B, analysis with the cation column (see Materials and Methods). 1, standard solution; 2, urine sample containing a high UK peak; 3, urine sample containing a very low UK peak. The peaks show: a, AsBe; b, DMA; c, AsIII; d, MMA; e, AsV; f, UK; g, ArCl. The total arsenic (the sum of arsenics detected) were 530.1 μg/*l* for A-2, 473.6 μg/*l* for B-2, 88.6 μg/*l* for A-3, and 79.3 for B-3, respectively.

in urine were stable under the conditions described above[12, 13]. The defrosted samples were diluted five-fold with ultrapure water, filtered through a 0.45-μm polyvinylidene fluoride membrane filter (Whatman 13 mm GD/X syringe filter; Whatman, Florham Park, NJ, USA), and analyzed by HPLC-ICP-MS as described above.

Creatinine in urine was analyzed photometrically using creatiAse and N-(3-sulfopropyl)-3-methoxy-5-methylaniline using a commercial kit (Pure Auto CRE-N; Daiichi Pure Chemicals, Tokyo, Japan).

Statistical analysis

Data collected using a questionnaire and by urinary determinations were analyzed by the means of the SPSS statistical package (SPSS version 11.5J for Windows, SPSS Japan, Tokyo, Japan).

Results and Discussion

Figure 2 shows the chromatograms obtained by HPLC-ICP-MS of a standard solution and two different urine samples. A high unknown (UK) peak (peak f) was detected at a retention time (Rt) of 3.2 min in the anion mode (Fig. 2, A-2), but not in the cation mode (Fig. 2, B-2). An argon chloride (ArCl) peak (peak g) was detected in the urine samples at Rt of 4.2 and 3.8 min in the anion (Fig. 2, A-2 and A-3) and cation (Fig. 2, B-2 and B-3) modes, respectively. In our laboratory, cation mode analysis has been used for the speciation analysis of five arsenic species in human urine using HPLC-ICP-MS[14], and we reported the median values (μgAs/*l*) of urinary arsenics for 210 Japanese male subjects without occupational exposure as 3.5 for AsIII, 0.1 for AsV, 3.1 for MMA, 42.6 for DMA, and 61.3 for AsBe[9]. However, since the separation of AsV and MMA was not sufficient, as shown in Fig. 2, B-1, a chromatographic condition for complete separation of the five arsenics was studied. The anionic condition using an IonPac AS22 column and a mobile phase of ammonium hydrogen carbonate buffer gave successful separation of the five arsenic species

Table 1. Urinary arsenic species concentrations of 172 workers without occupational iAs exposure

a) Analysis by anion column of IonPac AS22								
Item	AsV	AsIII	MMA	DMA	AsBe	Others	T-As	iAs + MMA
25%tile	ND	ND	1.3	21.1	31.3	1.9	70.9	2.9
	(ND)	(ND)	(1.3)	(19.4)	(28.8)	(1.5)	(63.9)	(2.6)
Median	ND	1.5	2.3	41.1	74.5	4.1	132.2	4.4
	(ND)	(1.1)	(1.8)	(30.8)	(52.8)	(3.3)	(97.5)	(3.5)
75%tile	0.9	2.7	3.7	62.6	120.7	8.6	200.7	7.1
	(0.8)	(1.7)	(2.4)	(42.8)	(92.2)	(6.5)	(148.3)	(4.9)
95%tile	1.7	5.4	6.2	109.2	243.7	23.7	368.4	12.6
	(2.3)	(2.9)	(3.6)	(87.9)	(217.6)	(21.8)	(314.6)	(8.2)
b) Analysis by cation column of RSpak NN-614								
Item	AsV	AsIII	MMA	DMA	AsBe	Others	T-As	iAs + MMA
25%tile	ND	2.6	2.3	23.3	27.9	ND	72.4	5.6
	(ND)	(2.1)	(2.2)	(22.0)	(26.7)	(ND)	(63.8)	(4.8)
Median	ND	5.3	4.3	44.0	65.0	ND	127.8	10.1
	(ND)	(3.9)	(2.9)	(32.6)	(47.1)	(ND)	(93.8)	(7.0)
75%tile	ND	10.1	6.2	66.1	100.8	2.6	191.0	16.2
	(ND)	(7.0)	(4.1)	(45.7)	(81.9)	(1.9)	(134.9)	(10.8)
95%tile	ND	22.2	10.6	116.4	218.7	8.1	338.4	30.6
	(ND)	(17.7)	(6.8)	(93.7)	(192.7)	(5.9)	(288.4)	(22.7)

Values are expressed as μg As/*l* and in parentheses as μgAs/g creatinine. T-As: total arsenic. ND: Lower than LOD.

within 10 min (Fig. 2, A-1). When we examined another anion exchange condition using a tandem joint of Gelpack GL-IC-A15 and an A13 column (150 × 4.6 mm i.d. and 100 × 4.6 mm i.d.; Hitachi High Technologies, Tokyo, Japan) with a phosphate buffer as a mobile phase, complete separation was obtained within 30 min. However, accumulation of phosphate salts on the sampling cone caused damage to the ICP-MS interface, and thus exchange of the cones was necessary every 60 analyses. The use of volatile ammonium hydrogen carbonate buffer decreased accumulation of salts on the cone to a minimum and resulted in less instrument downtime and a lower cost analysis. This analytical condition is suitable for routine analysis.

The limits of detection (LOD; μgAs/*l*) of the arsenic compounds in the anion and the cation modes were as follows: AsIII, 0.3 and 0.3; AsV, 0.2 and 0.4; MMA, 0.2 and 0.3; DMA, 0.3 and 0.2; AsBe, 0.4 and 0.3, respectively. The UK peak was measured using the DMA standard and the sum of UK was designated "Others." When no peak was detected at the corresponding retention time or the detected peak area was lower than the LOD, the concentration of the compound was treated as zero or was presented as the half of the LOD, respectively, to calculate total arsenic.

The AsBe, DMA, and total arsenic concentrations (μgAs/*l*) in the reference material of CRM No. 18 urine were determined to be 64.0 ± 1.5, 39.3 ± 0.5, and 137.3 ± 4.2 (n=5), respectively, using the anion exchange column, and 70.1 ± 1.0, 37.2 ± 0.5, and 131.5 ± 1.2 (n=5) using the cation exchange column, respectively. These values were within the ranges for the certified values of 69 ± 12, 36 ± 9, and 137 ± 11, respectively.

The results of speciation analysis of arsenic in the urine of 172 subjects are shown in Table 1. For both the anion and cation columns, the highest arsenic concentration in urine was that of AsBe, followed by that of DMA. DMA and AsBe concentrations were almost the same as measured by the two columns, indicating that these two peaks were well separated in both columns. The ratios of these two species increased with total arsenic concentration. AsIII and MMA concentrations measured using the anion column were lower than those measured using the cation column; the reverse was found for the concentration of Others. The sums of iAs and MMA measured using the anion column were much lower than those measured using the cation column. We then examined the relationship between the difference in the sum of iAs and MMA values obtained using the anion column and the cation column, and the difference in the value for Others using the anion column and the cation column. As shown in Fig. 3, the regression line clearly

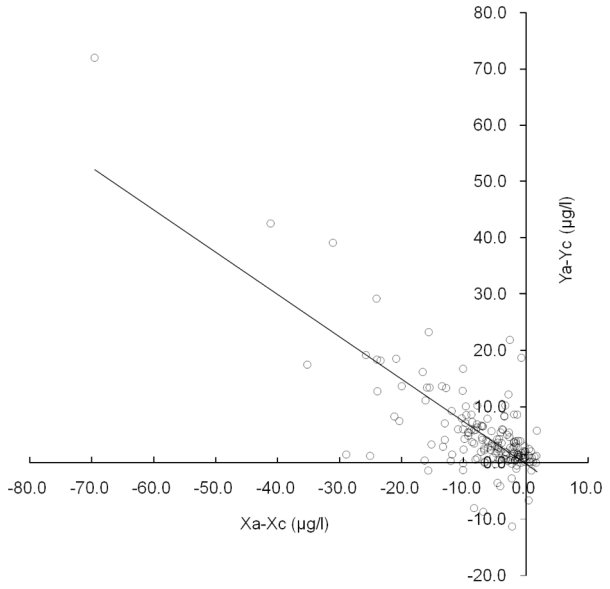

Fig. 3. Relationship between the differences in the sum of iA and MMA values with the anion column (Xa) and the cation column (Xc), and the differences in the Others value with the anion column (Ya) and the cation column (Yc). The horizontal axis shows X = Xa–Xc and the vertical axis Y=Ya–Yc. The regression formula is expressed as follows: y=–0.752x – 0.1541 (R^2=0.5898, p<0.001).

shows a negative linear correlation, indicating that the difference in the sum of iAs and MMA in the two analytical modes may be caused by false recognition of the UK compound; i.e., the UK peak in the anion column may be detected as AsIII or MMA in the cation column.

It is known that seafood and marine samples contain various organic arsenic compounds, such as AsBe, AsCho, DMA, and AsSug[15]. Using liquid chromatography coupled to tandem quadrupole mass spectrometry, we found six oxo-arsenosugars in extracts from hijiki seaweed sold in a Japanese market[16]. After seaweed ingestion, AsSugs are metabolized and their metabolites are excreted in urine[8, 17]. Although we did not identify the UK peaks detected using the anion column, they might be the metabolites of AsSugs or other organic arsenic compounds.

Recently, the consumption of seafood has been increasing throughout the world. Seafoods contain DMA; therefore, DFG recommends that only the inorganic arsenic fraction should be determined in future[2]. For biological monitoring of the occupational iAs exposure of workers who habitually consume seafood, the sum of iAs and MMA is reported to be more suitable than the sum of iAs, MMA, and DMA[9]. A Japanese government ministry also defines the sum of iAs and MMA in urine as the iAs exposure indicator[18].

Conclusion

We determined urinary arsenic concentrations in 172 subjects living in Japan without occupational exposure to arsenic for at least six months by HPLC-ICP-MS using anion and cation exchange columns. Use of an anion exchange column of Dionex IonPac AS22 with a volatile buffer can completely separate iAs and the metabolites within 10 min and prevents the accumulation of salts on the cone, resulting in less instrument downtime and less costly analysis. The ninety-fifth percentiles of AsV, AsIII, MMA, and the sum of iAs and MMA concentrations in the urine of 172 healthy subjects were 1.7, 5.4, 6.2, and 12.6 μgAs/l, respectively. We propose the 95th percentile of the sum of iAs and MMA concentrations measured by the anion-exchange column, 12.6 μgAs/l, as the background value for the biological index of occupational iAs exposure.

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