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HPLC-ICP-MS Speciation Analysis of Arsenic in Urine of Japanese Subjects without Occupational Exposure

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Abstract: HPLC-ICP-MS Speciation Analysis of Arsenic in Urine of Japanese Subjects without Occupational Exposure: Akihisa HATA, *et al*. Department of Preventive Medicine and Environmental Health, Graduate School of Medicine, Osaka City University—The toxicity and carcinogenicity of arsenic depend on its species. Individuals living in Japan consume much seafood that contains high levels of organoarsenics. Speciation analysis of urinary arsenic is required to clarify the health risks of arsenic intake. There has been no report of urinary arsenic analysis in Japan using high performance liquid chromatography with inductively coupled plasma mass spectrometry (HPLC-ICP-MS). We performed speciation analysis of urinary arsenic for 210 Japanese male subjects without occupational exposure using HPLC-ICP-MS. The median values of urinary arsenics were as follows: sodium arsenite (AsIII), 3.5; sodium arsenate (AsV), 0.1; monomethylarsonic acid (MMA), 3.1; dimethylarsinic acid (DMA), 42.6; arsenobetaine (AsBe), 61.3; arsenocholine, trimethylarsine oxide, and unidentified arsenics (others), 5.2; and total arsenic (total As), 141.3 μgAs/*l*. The median creatinine-adjusted values were as follows: AsIII, 3.0; AsV, 0.1; MMA, 2.6; DMA, 35.9; AsBe, 52.1; others 3.5; and total As, 114.9 μgAs/g creatinine. Our findings indicate that DMA and AsBe levels in Japan are much higher than those found in Italian and American studies. It appears that the high levels of DMA and AsBe observed in Japan may be due in part to seafood intake. ACGIH and DFG set the BEI and BAT values for occupational arsenic exposure as 35 μgAs/*l* and 50 μgAs/*l*, respectively, using the sum of inorganic arsenic (iAs), MMA, and DMA. In the general Japanese population, the sums of these were above 50 μgAs/*l* in 115 (55%) samples. We therefore recommend excluding DMA concentration in monitoring of iAs exposure.
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Key words: Arsenic, Urine, Speciation, HPLC-ICP-MS, Japanese

Arsenic is widely distributed in water, air, and soil[1]. There is significant occupational exposure to arsenic in several industries, such as non-ferrous smelting, electronics, and wood preservation[1]. Arsenic in air and drinking water is in the form of inorganic arsenic (iAs)[2, 3], whereas seafood contains high levels of organoarsenic compounds such as arsenobetaine (AsBe), dimethylarsinic acid (DMA), arsenocholine (AsCho), and arsenosugars (AsSugs)[4–7]. Notably, *hijiki* seaweed contains high levels of iAs[8]. iAs is methylated to monomethylarsonic acid (MMA), DMA, and trimethylarsine oxide (TMAO) in mammals[9–11]. AsSugs are extensively metabolized to DMA[12, 13]. AsBe is only minimally metabolized in mammals[11]. AsCho is extensively metabolized to AsBe[14].

Several epidemiological studies have indicated that not only occupational exposure but also long-term exposure to iAs in drinking water can increase risks of cancer of the skin, bladder, and kidney[2, 15, 16]. The toxicity and carcinogenicity of arsenic depend on its species[17–19]. DMA or its derivatives are believed to be ultimate carcinogens[20, 21], while AsBe and AsCho are reported to be neither toxic nor carcinogenic[1].

For the general population without exposure to arsenic through occupation or an arsenic-polluted environment,

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food is a much more significant source of arsenic than water[1]. In addition, individuals living in Japan consume much seafood that contains high levels of organoarsenics[22–24].

Speciation analysis is required to clarify the health risks of arsenic intake. Studies of this type have been performed by atomic absorbance spectrometry (AAS) analysis with pretreatment by alkaline digestion. However, in this method, AsSugs are measured as DMA, and TMAO, AsBe, and AsCho are measured as trimethylarsine (TMA)[25]. High performance liquid chromatography with inductively coupled plasma mass spectrometry (HPLC-ICP-MS) analysis can directly measure arsenic species without pretreatment. In order to obtain accurate biological indicators of arsenic exposure, it is essential to clearly determine the confounding concentrations of arsenicals present in food.

No reports are available of urinary arsenic analysis performed on healthy Japanese subjects using HPLC-ICP-MS. We therefore performed speciation analysis of arsenic in the urine of Japanese individuals without occupational exposure using HPLC-ICP-MS.

Materials and Methods

Subjects

The subjects were 210 Japanese males with a mean age of 43.7 yr (range 19–72 yr) who were living in Kita-Kyushu, Japan. The subjects were new employees and office workers of a chemical factory, and had no occupational arsenic exposure. Urine sampling was performed in the afternoon at the regular medical examination or medical checkup on employment conducted by the company from September 2004 to May 2006. Each subject was asked whether he preferred eating meat, seafood, vegetables, and other foods by a questionnaire. After urine sampling, the health supervisor in the factory blinded subjects' names on these samples, and we analyzed them. Urine samples were stored in sealed plastic tubes at –80°C in a freezer until analysis.

This study was approved by the Ethics Committees of the Graduate School of Medicine, Osaka City University (approval number 442).

Chemicals

Sodium arsenite (AsIII), sodium arsenate (AsV), MMA, and AsBe were purchased from Wako Pure Chemical (Osaka, Japan). DMA, TMAO, and AsCho were obtained from Tri Chemical Laboratory (Yamanashi, Japan). Germanium standard solution (Kanto Chemical, Tokyo, Japan) was used as an internal standard. HNO_3 (Tama Chemicals, Tokyo, Japan), NH_4NO_3 (Wako Pure Chemical, Osaka, Japan), and 2, 6-pyridinedicarboxylic acid (Tokyo Kasei Industry, Tokyo, Japan) were used for the HPLC mobile phase. Super-pure water was purified by Milli-Q-ICP-MS (Millipore Japan, Tokyo, Japan). A certified reference material, NIES CRM No. 18 (human urine), from the National Institute for Environmental Studies, Japan, was used to validate the procedure used for analysis.

High performance liquid chromatography with inductively coupled plasma mass spectrometry (HPLC-ICP-MS)

A Model HP4500 ICP-MS (Agilent, CA, USA) was used for arsenic detection. A Model HP1100 HPLC series (Agilent, CA, USA) was used to separate arsenic species. The cation mode was used for separation of arsenic compounds. A Shodex RSpak NN-614 packed with cation-exchange resin (150 × 4.6 mm i.d., Showadenko, Tokyo, Japan) was used 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 ml/min, temperature 40°C, and injection volume 50 μl.

The operating conditions for ICP-MS were established in accordance with those reported by Inoue *et al.*[26]. The limit of detection of As was 0.1 $\mu g/l$. If a measurement was below the limit of detection, it was assumed to be half the value of this limit.

The laboratory involved in analytical measurements adheres to current quality assurance procedures and participates in the external quality program organized by the Institute of Occupational Social and Environmental Medicine of the University of Erlangen, Nuremberg[8].

Urinary arsenic analysis

The samples were centrifuged at 3,000 rpm for 10 min, and the supernatants were used for analysis. Urine samples were diluted ten-fold with super-pure water and analyzed by HPLC-ICP-MS as described above.

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

Statistical analysis

Data collected from the questionnaire and urinary determinations were analyzed using the SPSS statistical package (SPSS version 14 for Windows).

Results

Figure 1 shows the chromatogram obtained from HPLC-ICP-MS of a standard solution. Determinations were performed by calculation of peaks on the chromatograms obtained by HPLC-ICP-MS analysis.

The accuracy of the present analytical procedure was tested by analysis of NIES CRM No. 18, which is certified for DMA, AsBe, and total arsenic (total As). The values were within the allowable errors for certified values, as shown in Table 1.

The results of speciation analysis of arsenic in urine

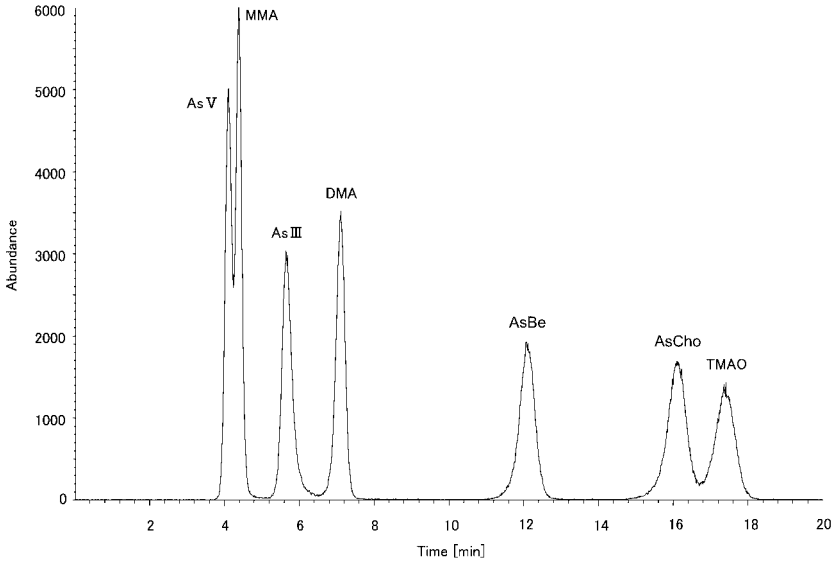

Fig. 1. HPLC-ICP-MS chromatogram of a standard solution containing seven arsenic compounds (10 μgAs/*l*). AsIII, arsenite; AsV, arsenate; MMA, monomethylarsonic acid; DMA, dimethylarsenic acid; AsBe, arsenobetaine; AsCho, arsenocholine; TMAO, trimethylarsine oxide.

Table 1. Arsenic analysis of reference urine (NIES CRM No.18)

	Total As	DMA	AsBe
Our results (n=5)			
mean ± SD (μg As/ *l*)	131.5 ± 1.2	37.2 ± 0.5	70.1 ± 1.0
Reference Value			
Tolerance range (μg As/ *l*)	137 ± 11	36 ± 9	69 ± 12

Analysis was performed by HPLC-ICP-MS.

are shown in Table 2. The AsCho peak overlapped with the tetramethylarsonium peak, and the TMAO concentration was at trace level (data not shown), with a peak overlapping those of unidentified arsenic species. The latter peaks were difficult to separate and identify. Therefore, peaks for AsCho, TMAO, and the unidentified peaks are together termed "others". The arsenic species with the highest concentration in urine was AsBe, followed by DMA. These two species accounted for about 40 and 30% of the median of total As, respectively, while iAs accounted for less than 2%. The median value of urinary creatinine was 1.3 g/*l*.

The median concentrations of urinary DMA and AsBe adjusted for creatinine were significantly different among the age groups as determined by the Kruskal-Wallis test (p<0.01, each case), though no difference was observed in actual urinary concentrations of DMA and AsBe or other arsenic species whether adjusted for creatinine or not.

The answer rate of the questionnaire on food preferences was 65%. We compared the concentration of urinary arsenic compounds between the subjects who preferred meat (n=49) and those who preferred seafood (n=81) by the Mann Whitney U-test. DMA (p< 0.01) and AsBe (p< 0.01) concentrations, adjusted for creatinine, were significantly higher in subjects who preferred seafood than in those who preferred meat. None of the other values differed significantly between the two groups.

Discussion

NIES CRM No. 18 was measured, and the results confirmed the accuracy of our method of HPLC-ICP-MS. The arsenic species in urine were stable under the conditions described above[27, 28].

Urinary concentrations are usually presented using actual urinary values and creatinine-adjusted values. However, there are no reports indicating which of the actual values or creatinine-adjusted values should be used. Therefore, in the present study, we used both actual

Table 2. Median values of arsenic species in urine of each age group, and 25, 75% tiles and medians for 210 male volunteers

Age	N	Total As		AsIII		AsV		MMA	
		μg As/*l*	μg As/g cre	μg As/*l*	μg As/g cre	μg As/*l*	μg As/g cre	μg As/*l*	μg As/g cre
<30	43	126.6	105.1	3.2	2.4	0.1	0.1	3.0	2.1
30–39	44	142.8	101.0	4.0	2.9	0.1	0.1	3.9	2.4
40–49	34	121.7	113.1	3.2	3.9	0.5	0.3	3.0	2.9
50–59	59	117.6	117.6	2.9	3.2	0.1	0.1	2.5	2.3
>59	30	204.9	193.7	5.4	4.6	0.1	0.1	3.6	3.3
25%tile	210	73.9	68.5	0.8	0.8	0.1	0.1	1.6	1.4
Median	210	141.3	114.9	3.5	3.0	0.1	0.1	3.1	2.6
75%tile	210	225.9	187.2	8.9	5.8	1.1	1.1	5.5	4.0

Age	N	DMA		AsBe		others	
		μg As/*l*	μg As/g cre	μg As/*l*	μg As/g cre	μg As/*l*	μg As/g cre
<30	43	40.0	27.6	50.4	32.7	5.7	2.8
30–39	44	52.7	32.1	62.5	45.4	5.6	2.4
40–49	34	35.6	28.7	74.1	65.8	4.7	3.6
50–59	59	41.0	37.8	55.4	62.3	4.8	3.9
>59	30	50.0	47.3	107.6	77.0	7.0	6.2
25%tile	210	27.1	23.2	31.7	26.8	1.3	0.9
Median	210	42.6	35.9	61.3	52.1	5.2	3.5
75%tile	210	65.9	47.9	131.8	88.5	11.2	8.5

Total As: total arsenic. AsIII: arsenite. AsV: arsenate. MMA: monomethylarsonic acid. DMA: dimethylarsenic acid. AsBe: arsenobetaine. μg As/*l*: actual measurement values. μg As/g cre: creatinine-adjusted values.

measured and creatinine-adjusted values.

In Europe and the United States, there are a few reports on urinary arsenic species associated with non-occupational arsenic exposure measured using HPLC-ICP-MS. In Italy, Apostoli *et al*.[29] reported urinary arsenic concentrations for 39 male subjects who consumed a diet poor in seafood. Their median arsenic concentrations were as follows: AsIII, 0.3; MMA, 0.9; DMA, 5.2; AsBe, 9.5; and total As, 10.6 μgAs/*l*. In the United States, Tsuji *et al*.[30] reported urinary arsenic concentrations for 439 subjects (206 males and 233 females) who were asked not to eat seafood for 3 days before measurement. Their mean arsenic concentrations were as follows: iAs, 0.78; MMA, 0.46; DMA, 2.5; and total As, 5.7 μgAs/*l*. Median values of arsenic in the present study are shown in Table 2. Mean values of arsenic were as follows: AsIII, 6.7; AsV, 0.6; MMA, 4.5; DMA, 54.0; AsBe, 97.0; others, 9.3; and total As, 173.3 μgAs/*l*. The DMA and AsBe levels found in the present study were much higher than those in the Italian and American studies. It is known that people living in Japan generally consume more seafood and seafood products than those living in Europe and the United States. The Food and Agriculture Organization[31] reported that the amounts of consumption of fish, seafood, and seafood products in Japan, Italy, and the United States were 182, 72, and 58 g/person/d, respectively. The Western Isles MINCH PROJECT[32] reported that the amounts of consumption of seaweed in Japan, North America, and the EU (excluding France) were 97,000, 240, and 70 t dry weight/yr, respectively. In the present study, urinary iAs level was about nine times those in the Italian and American studies, while MMA levels were about three and ten times those in the Italian and American studies, respectively. Individuals living in Japan consume much seaweed, and the findings for Japanese subjects might have been influenced by ingestion of *hijiki* seaweed in a side dish, which contains high levels of iAs[8]. Differences in food habits are thus probably reflected in urinary arsenic concentrations.

In the AAS method, dimethylarsenic species such as AsSugs are converted to DMA, and trimethylarsenic species such as TMAO, AsBe, and AsCho are converted to TMA, due to pretreatment with alkaline digestion and reductive reaction[25]. In the present study, species classified as others include TMAO, AsCho, and unidentified arsenic compounds. Together, their concentration was only about 5% of total As. Some researchers have reported that DMA and a number of dimethylarsenic compounds were detected in human urine

after ingestion of seaweeds or AsSugs, by the HPLC-ICP-MS method[12, 13, 33]. In the present study, unidentified arsenic compounds included dimethylarsenic species such as metabolites of AsSugs, although their concentrations were fairly low. It thus appears that most of the urinary DMA measured with the AAS method is probably DMA. We found that urinary concentrations of TMAO and AsCho were fairly low. Thus, most of the urinary TMA measured with the AAS method is probably AsBe. There are reports of measurement of urinary arsenic concentrations using the AAS method. In Italy, Buchet *et al.*[34] reported urinary arsenic concentrations for 22 male subjects who regularly consumed fish and shellfish. Their mean urinary arsenic concentrations were as follows: iAs, 3.0; MMA, 1.2; DMA, 6.6; and total As, 28.1 μgAs/*l*. The iAs, MMA and DMA level found in the present study are about two times, four times, and nine times, respectively, higher than those reported by Buchet *et al.* In Japan, Yamato[35] reported urinary arsenic concentrations of 102 subjects (94 males and 8 females) without dietary restrictions. Their mean concentrations were as follows: iAs, 12.7; MMA, 4.07; DMA, 38.5; TMA, 75.4; and total As, 131 μgAs/*l*. These values were consistent with our results.

The American Conference of Governmental Industrial Hygienists (ACGIH)[36] and Deutsche Forschungsgemeinschaft (DFG)[37] use the urinary excretion of the sum of iAs, MMA, and DMA for biological monitoring of occupational exposure to iAs. To clarify the confounding contributions of arsenic species in biological monitoring of occupational As exposure, the distributions of the sum of iAs, the sum of iAs and MMA, and the sum of iAs, MMA and DMA were compared in 210 male volunteers (Table 3). The ACGIH[36] reported that the sum of iAs, MMA and DMA concentrations in the general population is approximately 10 μgAs/*l* in Americans and 50 μgAs/*l* in Japanese, while the DFG[37] reported that the sum for the general European population was below 25 μgAs/*l*. In this study, the 75th percentile of this sum was 84.8 μgAs/*l*, and 99% of samples had more than 10 μgAs/*l*, 85% had more than 25 μgAs/*l*, and 55% had more than 50 μgAs/*l*. Since many urine samples had values higher than those reported by the ACGIH and DFG, both values appear unsuitable for use in evaluation of Japanese subjects. This difference in findings is attributable to the high urinary DMA concentration in Japanese subjects. Some researchers have reported that urinary DMA is increased by seafood intake[12, 33]. In the present study, the subjects who preferred seafood tended to have higher urinary DMA than the subjects who preferred meat. However, it would be difficult for Japanese individuals to decrease seafood intake to lower their urinary DMA levels to values similar to those of Americans and Europeans. Nakajima *et al.*[8] and Francesconi *et al.*[13] reported that the half-life of urinary DMA is above 20 h. To decrease the urinary DMA to the basal level, seafood intake must be avoided for more than 2 d. In Japan, seafoods that contain DMA are used on a daily basis. Intake of fish and shellfish also increases urinary DMA, though their DMA concentration is less than that of seaweed[38]. In Japan, not only seaweed itself but also seaweed as an ingredient of soup stock and processed foods is consumed[39]. It thus appears that Japanese dietary habits increase urinary DMA levels.

Table 3. Sum of inorganic and methylated arsenic concentrations in 210 male volunteers (μg As/ *l*)

	iAs	iAs+MMA	iAs+MMA+DMA
25%tile	1.1	4.8	33.1
Median	4.5	8.3	54.0
75%tile	9.6	16.1	84.8

iAs: inorganic arsenic. MMA: monomethylarsonic acid. DMA: dimethylarsenic acid.

Seaweed is consumed in some other Asian countries as well, such as China and Korea[32]. In East Asian countries, urinary DMA may thus be higher than those in Europe and the United States.

In the present study, high concentrations of organoarsenic compounds were detected. Thus, speciation analysis is necessary to clarify the health risks of arsenic exposure. It is also necessary to consider use of specific biomarkers for occupational arsenic exposure in areas where people consume seaweed. We recommend excluding DMA concentration from monitoring of occupational iAs exposure in these areas. Also, as mentioned above, intake of iAs from *hijiki* seaweed should be avoided.

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