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Short-Column Liquid Chromatography with Hydride Generation Atomic Fluorescence Detection for the Speciation of Arsenic

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Increasing concerns over human exposure to arsenic and more stringent environmental regulations require rapid determination of trace levels of individual arsenic species, which presents an analytical challenge. We describe a method that is capable of speciating nanogram-per-milliliter levels of arsenite (As(III)), arsenate (As(V)), monomethylarsonic acid (MMAA), and dimethylarsinic acid (DMAA) within 3 min. Speciation of two common inorganic species in drinking water, As(III) and As(V), is complete in 1.5 min. The method is based on a combination of fast high-performance liquid chromatography (HPLC) separation of arsenic species on 3-cm HPLC guard columns and the sensitive detection of arsenic hydride by atomic fluorescence spectrometry. Detection limits for the four arsenic species in urine samples are 0.4−0.8 ng/mL. This simple method allows for the direct speciation of arsenic present in natural water samples and in human urine samples from the general population, with no need of any sample pretreatment. Our results from the determination of arsenic species in urine and water standard reference materials are in good agreement with the certified values of total arsenic concentration. The method has been successfully applied to speciation studies of metabolism of arsenosugars following the consumption of arsenosugar-containing mussels by human volunteers. Speciation of arsenic in urine samples collected from four volunteers after the ingestion of mussels reveals significant increases of DMAA concentration, resulting from the metabolism of arsenosugars. These results suggest that the commonly used biomarkers for assessing human exposure to inorganic arsenic, which are based on the determination of urinary arsenite, arsenate, MMAA, and DMAA, are not reliable when arsenosugar-containing seafood is ingested.

Chemical speciation of arsenic compounds is a topic under extensive study, because of the very rich chemistry of arsenic, the diverse toxicity of various arsenic compounds, the dramatic differences in metabolism of various arsenic species, and the as-yet not well understood roles of arsenic in biological systems. The need for studying trace levels of individual arsenic species in biological and environmental systems has driven the development of new analytical methods for chemical speciation. In turn, the powerful speciation techniques developed during the past few years have played essential roles in providing information for understanding the distribution and fates of arsenic in biological and environmental systems.[1−7]

The combination of chromatographic separation with element-specific spectrometric detection has proven to be most useful for the speciation of trace levels of arsenic compounds. In particular, high-performance liquid chromatographic (HPLC) separation with inductively coupled plasma mass spectrometry (ICPMS),[7−15] chemical derivatization atomic spectrometry,[14−18] and electrospray mass spectrometry (ESMS) detection[19,20] have played important roles in chemical speciation studies of arsenic. With good separation by HPLC and sensitive detection by atomic and mass spectrometry, these methods have been applied to health science and environmental science studies with respect to the absorption, distribution, metabolism, excretion, and environmental arsenic cycles. Much research continues on improving our understanding of the health effects of arsenic species.[4,6,21,22]

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While a dose−response relationship between the exposure to high levels of inorganic arsenic and the prevalence of cancers has been established,[23−35] estimates of cancer risk resulting from the chronic exposure to low levels of arsenic are under debate.[26−28] Epidemiological surveys to establish such dose−response relationships at the relevant levels of environmental exposure require the speciation of arsenic in a large number of human urine, blood, and diet samples. Analytical methods required must be simple, rapid, and inexpensive. With respect to the exposure to inorganic arsenic from drinking water, the species of interest are arsenite, arsenate, monomethylarsonic acid (MMAA), and dimethylarsinic acid (DMAA), because the inorganic arsenic species can be metabolized to MMAA and DMAA in the human body prior to excretion into the urine. The current maximum allowable limit of arsenic in drinking water is 25 ng/mL in Canada[29] and 50 ng/mL in the United States.[30] The U.S. Environmental Protection Agency (EPA) is considering reducing the guideline limit from the current 50 ng/mL to as low as 2 ng/mL.[6,21]

To comply with the anticipated more stringent regulation on arsenic in drinking water, it is essential to speciate arsenite, arsenate, MMAA, and DMAA in such samples as human urine and drinking water. The method should be able to quantify these species at nanogram-per-milliliter levels. They should be rapid, inexpensive, and simple, so that they can be applied to large numbers of samples in routine analysis. Although some techniques, such as HPLC/ICPMS, are able to speciate nanogram-per-millilter levels of arsenic, they are expensive and often time-consuming.

We have previously reported the use of elevated column temperature for the HPLC separation of 11 arsenic compounds.[18] The use of elevated column temperature (e.g., 70 °C) improved separation efficiency and dramatically reduced chromatographic retention time for several arsenic species. When the column temperature increases, the viscosity of the mobile phase decreases, and diffusion rates increase, resulting in an enhancement of the mass-transfer rate between mobile phase and stationary phase. A higher mass-transfer rate reduces band broadening and increases efficiency.[31,32] The improved separation efficiency leads us to study the separation of arsenic species using short columns, thereby dramatically reducing the analysis time.

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We report in this study the development and application of a method for the rapid speciation of arsenite, arsenate, MMAA, and DMAA. The method is based on the rapid separation of the four target arsenic species on one or two 3-cm HPLC guard columns, followed by very sensitive hydride generation atomic fluorescence detection. We have achieved almost baseline resolution of the four arsenic species, within 2 min, using ion pair chromatography on a 3-cm-long C18 guard column. Conventional HPLC methods for the separation of these arsenic species using typical 25-cm columns take more than 8 min. We also demonstrate an application of the new method to a study of the metabolism of arsenosugars following the consumption of arsenosugar-containing mussels by human volunteers. We further examine the validity of the commonly used biomarkers for assessing human exposure to inorganic arsenic, which are based on the determination of urinary arsenite, arsenate, MMAA, and DMAA.

EXPERIMENTAL SECTION

Instrument. We used a HPLC system consisting of a Gilson (Middletone, WI) HPLC pump (model 307), a Rheodyne six-port sample injector (model 7725i) with a 20-μL sample loop, and an appropriate column. The column was mounted inside a column heater (Model CH-30, Eppendorf) which was controlled by a temperature controller (Model TC-50, Eppendorf). Mobile phase was preheated to the temperature of the column by using a precolumn coil of 50-cm stainless steel capillary tubing, which was also placed inside the column heater. The temperature controller, according to the manufacturer, was able to provide a ±0.1 °C temperature stability and ±1 °C accuracy. Isocratic HPLC operation was performed under a 0.9−1.5 mL/min flow rate.

In the first approach, reversed-phase C18 guard columns (30 mm × 4.6 mm, 5 μm particle size) (Phenomenex, Torrance, CA) were used for the separation. For comparison, a conventional C18 analytical column (250 mm × 4.6 mm), which has the same packing material as in the guard columns, was also used. The second approach employed C18 columns with 3-μm particle size packing materials (150 mm × 4.6 mm) for rapid separation. In the interlaboratory comparison study, anion exchange separation was performed on a Hamilton (Reno, NV) PRP X-100 anion exchange column (250 mm × 4.1 mm). The detailed HPLC separation conditions are summarized in Table 1.

A commercial hydride generation atomic fluorescence detector (HGAFD) (model Excalibur 10.003, P.S. Analytical, Kent, UK) was used for the detection of arsenic. The combination of HPLC and HGAFD has been described previously.[18] Briefly, effluent from the HPLC column directly meets at two T-joints, with continuous flows of hydrochloric acid and sodium borohydride introduced by using a peristaltic pump. Upon mixing the HPLC effluent, acid, and borohydride solutions, hydride generation takes place. Hydride generated from the reaction is separated from liquid waste in a gas/liquid separator apparatus and carried by a continuous flow of argon carrier gas to the atomic fluorescence detector. A Pentium computer with Varian (Victoria, Australia) Star Workstation software and ADC board was used to acquire and process signals from the atomic fluorescence detector. A Hewlett-Packard (Boise, ID) 3390A integrator with both peak area and peak height measurement capability was also used to record chromatograms.

Table 1. HPLC Separation Conditions Used in the Present Study

method	HPLC column	column temp (°C)	mobile phase	detector
1	Phenomenex ODS(3) 30 mm × 4.6 mm 5-μm particle size	30	10 mM tetrabutylammonium hydroxide 1 mM malonic acid, 5% methanol pH 6.0, flow rate 1.0 mL/min	HGAFD HGAAS
2	two guard columns Phenomenex ODS(3) 30 mm × 4.6 mm	30	5 mM tetrabutylammonium hydroxide 2 mM malonic acid, 5% methanol pH 5.9, flow rate 0.9 mL/min	HGAFD
3	Phenomenex ODS(3) 250 mm × 4.6 mm 5-μm particle size	70	5 mM tetrabutylammonium hydroxide 4 mM malonic acid, 5% methanol pH 6.0, flow rate 1.0 mL/min	HGAFD
4	Phenomenex ODS(3) 150 mm × 4.6 mm 3-μm particle size	70	5 mM tetrabutylammonium hydroxide 4 mM malonic acid, 5% methanol pH 5.8, flow rate 1.5 mL/min	HGAFD
5	Hamilton PRP-X100 250 mm × 4.1 mm 5-μm particle size	ambient	50 mM $H_2PO_4^-/HPO_4^{2-}$ buffer pH 6.0 adjusted with NH_4OH flow rate 1.0 mL/min	ICPMS
6	GL Science Inertsil ODS(2) 250 mm × 4.6 mm 5-μm particle size	ambient	10 mM tetraethylammonium hydroxide 4.5 mM malonic acid, 0.1% methanol pH 6.8, flow rate 0.8 mL/min	ICPMS

The ICPMS detection system used was the same as described elsewhere.[7,14,15] An atomic absorption spectrophotometer (Varian, model SpectrAA-5) was also used in one experiment. An arsenic ultra lamp (193.7 nm) was operated at 10 mA using an external control module (Varian). The spectral bandwidth was 0.5 nm. A T-shaped quartz absorption tube (Varian) heated to 925 °C with a temperature controller module (Varian, model ETC-60) was used as the atomization cell. Hydride generation atomic absorption spectrometry (HGAAS) coupled with HPLC was carried out using procedures similar to those described previously.[15]

Reagents. Deionized water from a Maxima ultrapure water system (Elga) was used for the preparation and dilution of all reagents, samples, and standards. Standard solutions of arsenite, arsenate, MMAA and DMAA were prepared by appropriate dilution with deionized water from 1000 mg/L stock solutions, as described previously.[7,15,18] Standard solutions containing above 1 μg of As/mL were stable for several months. Standard solutions containing less than 10 ng of As/mL were prepared fresh daily by serial dilution with deionized water from 1 μg of As/mL arsenic standard solutions. Arsenic concentration in the stock solutions was standardized against an atomic absorption arsenic standard solution containing 1000.0 mg of As/L (Aldrich, Milwaukee, WI) using both ICPMS and flame atomic absorption spectrometry.

The reagents used in HPLC mobile phases, including tetrabutylammonium hydroxide, tetraethylammonium hydroxide, malonic acid, NaH_2PO_4, and Na_2HPO_4, were obtained from Aldrich. HPLC grade methanol was from Fisher (Pittsburgh, PA). These mobile-phase solutions were prepared in deionized water and filtered through a 0.45-μm membrane prior to use. Sodium borohydride (Aldrich) solutions (1.3%) in 0.1 M sodium hydroxide (Fisher) were prepared fresh daily. All reagents used were of analytical grade or better.

A Standard Reference Material, Toxic Metals in Freeze-Dried Urine SRM 2670, was obtained from National Institute of Standards and Technology (NIST, Gaithersburg, MD). The freeze-dried urine was reconstituted by the addition of 20.0 mL of deionized water, as recommended by the supplier. The certified value for total arsenic concentration is 480 ± 100 ng/mL in two bottles containing elevated levels of toxic metals. In the other two bottles, containing normal levels of toxic metals, the concentration of arsenic is not certified, and a reference value of 60 ng/mL has been provided. No arsenic speciation information was given for the SRM.

Mussels and Urine Samples. Mussels were purchased from a local supermarket in Edmonton, Canada. A subsample of the mussel meat (20 g wet weight) was extracted by using a procedure similar to that described by Shibata and Morita.[33] The homoginized sample was weighed into a test tube, to which was added 20 mL of a methanol/water mixture (1:1 v/v). The tube was sonicated for 20 min. After centrifugation, the extract was removed and placed in a 150-mL beaker. The extraction process with the aid of sonication was repeated a further four times. The extracts were combined in the beaker and evaporated to dryness, and the residue was dissolved in 10 mL of deionized water. After filtration through a 0.45-μm nylon membrane, the sample was subjected to HPLC/HGAFD analyses.

Four male volunteers (33, 34, 35, and 41 years old) refrained from eating any seafood for at least 72 h prior to commencing the mussel ingestion experiment. The volunteers collected 2−3 urine samples during the 12-h period prior to the consumption of mussels. These samples were used to determine the background level of arsenic species in the urine resulting from a regular diet that excluded any seafood.

The volunteers then consumed approximately 200 g (wet weight) of cooked mussels in one meal. The time of this meal was referred to as time zero. All urine was completely collected in separate 500-mL polyethylene containers for consecutive 3 days. The volunteers did not eat any other seafood during the experiment period. The urine samples were stored at 4 °C and were analyzed for arsenic speciation within 1 week.

The volunteers were aware of the experimental details and possible health effects concerning the ingestion of mussels in this experiment. All procedures followed were in accordance with the ethical guidelines of the Research Ethics Board, Faculty of Medicine, University of Alberta.

RESULTS AND DISCUSSION

Speciation of Four Arsenic Compounds Using 3-cm Columns. Figure 1 shows two chromatograms for the separation of As(III), As(V), MMAA, and DMAA using a conventional 25-

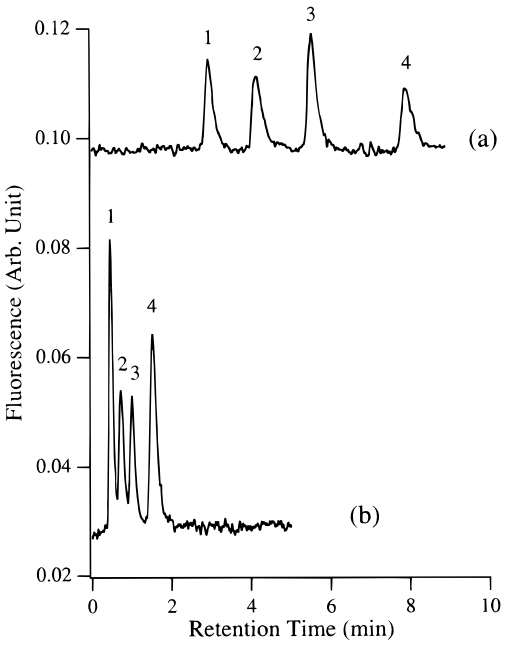

Figure 1. Chromatograms obtained from the analyses of As(III), As(V), MMAA, and DMAA in standard solutions. (Top) Conventional HPLC separation with HGAFD detection. A Phenomenex ODS(3) reversed-phase column (250 mm × 4.6 mm, 5-μm particle size) was used, and its temperature was maintained at 70 °C. Mobile phase contained 5 mM tetrabutylammonium hydroxide, 4 mM malonic acid, and 5% methanol (pH 6.0), and its flow rate was 1.0 mL/min (method 3). (Bottom) A guard column (30 mm × 4.6 mm) was used for separation, and hydride generation atomic absorption spectrometry (HGAAS) was used for detection. Mobile phase contained 10 mM tetrabutylammonium hydroxide, 1 mM malonic acid, and 5% methanol (pH 6.0), and its flow rate was 1.0 mL/min (method 1). Peaks 1: As(III); 2, DMAA; 3, MMAA; and 4, As(V).

cm-long and 4.6-mm-diameter reversed-phase C18 column (Figure 1a) and a much shorter 3-cm column of the same kind (Figure 1b). The ion pair chromatographic mode was chosen because it enabled us to separate over a dozen common arsenic species, including anionic, cationic, and neutral species.[14,18] Using the conventional 25-cm analytical column, we have obtained baseline resolution for the four arsenic species (Figure 1a). This chromatographic technique, coupled with hydride generation atomic fluorescence detection (HGAFD), has been successfully used to speciate these arsenic compounds in urine samples.[18,34] A chromatographic separation time of 8 min is typical for these four arsenic species and is comparable to those reported in the literature, whether ion exchange[35−37] or ion pair chromatography[10,38,39] has been used.

In an effort to reduce the analysis time for routine analysis of a large number of samples, we have studied the application of both a shorter HPLC column and columns packed with smaller particle size packing materials. Figure 1b shows a chromatogram of the four target arsenic species, separated on a 3-cm-long and 4.6-mm-diameter guard column. Resolutions between the adjacent peaks are 1.08 (As(III) and DMAA), 1.06 (DMAA and MMAA), and 1.58 (MMAA and As(V)), respectively. The entire chromatographic separation takes less than 2 min. This is the shortest time ever reported for the chromatographic separation of these four arsenic species. The dramatic reduction of the chromatographic retention time makes this HPLC technique especially promising for the rapid speciation of arsenic compounds.

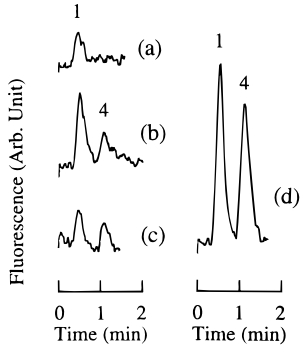

Figure 2. Chromatograms obtained from the HPLC/HGAFD analyses of (a) an untreated water sample, (b) the water sample spiked with 2 ng/mL of As(III) and As(V), (c) 2 ng/mL of As(III) and As(V) in standard solution, and (d) 10 ng/mL of As(III) and As(V) in standard solution. A short column (30 mm × 4.6 mm) was used for separation and HGAFD was used for detection. Mobile phase contained 5 mM tetrabutylammonium hydroxide, 4 mM malonic acid, and 5% methanol (pH 5.9), and its flow rate was 1.0 mL/min. The untreated water sample was obtained from a drinking water plant in Saskatchewan, Canada. No preservative was added to the sample. Peaks 1, As(III); and 4, As(V).

The retention time can be further reduced when only inorganic As(III) and As(V) are to be speciated. We achieve this by increasing the concentration of buffer (malonic acid) and thereby increasing the ionic strength of the mobile phase. As a result, the separation of As(III) and As(V) can be accomplished within 1.5 min (Figure 2).

Inorganic As(III) and As(V) are the major arsenic species in drinking water.[40] Although biological activities can result in the methylation of inorganic arsenic to MMAA and DMAA, the presence of the methylated arsenic species in drinking water is usually negligible compared to that of inorganic As(III) and As(V). Therefore, speciation of only As(III) and As(V) in drinking water is often adequate for water utilities.

To demonstrate an application of the method to water analysis, we obtained several water samples from a drinking water plant in Saskatchewan, Canada. No preservative was added to the samples. The samples were kept in 100-mL polypropylene bottles and shipped by courier, in ice. They were analyzed for arsenic species within 3 days following sample collection.

Figure 2 shows chromatograms obtained from the analyses of As(III) and As(V) in a raw (untreated) water sample and the standard solutions. Each HPLC/HGAFD analysis takes only 1.5 min, offering speciation information on both As(III) and As(V). The analysis of the water sample reveals the presence of approximately 2 ng/mL of As(III) (Figure 2a). When an additional 2 ng/mL each of As(III) and As(V) is spiked into the sample, a quantitative recovery of both arsenic species is achieved (Figure

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Table 2. Reproducibility of Retention Time Obtained from Four Replicate HPLC Analyses of a Standard Solution Containing 5 ng/mL Each of As(III), As(V), MMAA, and DMAA

species	t_R (min)	SD (min)	RSD (%)
As(III)	0.975	0.006	0.6
DMAA	1.43	0.01	0.7
MMAA	2.14	0.01	0.5
As(V)	3.07	0.01	0.3

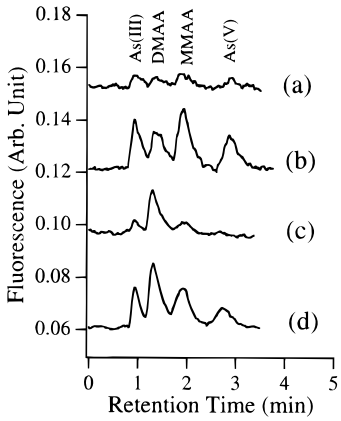

Figure 3. Chromatograms obtained from HPLC/HGAFD analyses of (a) 1 ng/mL each of As(III), As(V), MMAA, and DMAA in standard solution, (b) 5 ng/mL of the four arsenic species in standard solution, (c) a urine sample, and (d) the urine sample spiked with 5 ng/mL of the four arsenic species. Two guard columns (30 mm × 4.6 mm) in tandem were used for separation, and HGAFD was used for detection. Mobile phase contained 5 mM tetrabutylammonium hydroxide, 2 mM malonic acid, and 5% methanol (pH 5.9), and its flow rate was 0.9 mL/min (method 2).

2b), indicating that there is no interference with the determination. Standard addition curves and the calibration curves (0−50 ng/mL) are also parallel.

We have attempted to use the single guard column for the speciation of As(III), As(V), MMAA, and DMAA in human urine samples. However, the presence of complex matrix in urine samples results in a variation of chromatographic retention time. This may reflect a low capacity of the short column used (3 cm). To solve this problem, we have used two short columns in tandem: both are 3 cm long. In analogy to the conventional HPLC approach, one of the short columns acts as an analytical column and the other as a guard column. With this modification, we have obtained good stability and reproducibility and are able to separate the four arsenic species within 3.5 min. Retention time and its standard deviation from four replicate HPLC analyses are summarized in Table 2.

Figure 3 shows chromatograms obtained from the analyses of standards containing the four arsenicals (Figure 3 a,b), a urine sample (Figure 3c), and the sample spiked with 5 ng/mL of the four arsenic species (Figure 3d). The recovery of the four arsenicals spiked into the urine sample is in the range of 85−100%. Eight replicate analyses of the urine sample give reproducible chromatograms, with relative standard deviation of the peak retention time being 0.3−1%. The retention times for the arsenic species in the urine sample are identical to those in the standard solution. These results demonstrate that there is no interference from the urine sample matrix in the speciation of the four arsenic compounds. The urine sample is from a volunteer who has not exposed to excess arsenic. DMAA (5 ng/mL), MMAA (∼1 ng/mL), and As(III) (∼1 ng/mL) are detected in the urine sample. The concentrations of these arsenic species in the urine sample represent low background levels of arsenic in urine from the general population. Detection limits calculated from 3 times the signal-to-noise ratio of arsenic species in the urine samples are 0.5, 0.7, 0.4, and 0.8 ng/mL for As(III), DMAA, MMAA, and As(V), respectively. The rapid and sensitive method is suitable for surveying arsenic species in a large number of urine samples from the general population. The method is simple, and there is no need for sample pretreatment procedures.

We have validated the method using both urine standard reference material (SRM 2670) and interlaboratory comparison studies. DMAA (49 ± 5 ng/mL) and MMAA (11 ± 3 ng/mL) are determined in the urine containing a normal level of arsenic by using the two-guard-column HPLC/HGAFD method. The sum of these two arsenic species is in excellent agreement with the reference value of total arsenic concentration (60 ng/mL). Similar concentrations of DMAA (46 ± 5 ng/mL) and MMAA (11 ± 3 ng/mL) are found in the urine SRM containing elevated level of arsenic, but an elevated amount of As(V) (461 ± 31 ng/mL) is also present in this sample. The sum of MMAA, DMAA, and As(V) concentration, as determined by using the developed method (517 ± 29 ng/mL), is again in good agreement with the certified value of total arsenic concentration (480 ± 100 ng/mL).

There is no certified value available on speciation information from any standard reference materials. Therefore, we have carried out a blind interlaboratory comparison study. Another laboratory, which has prior experience with arsenic speciation, used anion-exchange chromatography with ICPMS detection. Two chromatograms from the HPLC/HGAFD analysis (Figure 4a) and the HPLC/ICPMS analysis (Figure 4b) of the same standard reference material (SRM 2670), containing the elevated level of arsenic, are shown in Figure 4. Similar chromatographic patterns are observed. A difference is that arsenobetaine is detected by using the HPLC/ICPMS method (Figure 4b) but not by using the HPLC/HGAFD method (Figure 4a). This is because hydride generation procedures are unable to derivatize arsenobetaine to a volatile arsenic hydride,[7,16] and, therefore, arsenobetaine cannot be directly detected by the HGAFD method. Arsenobetaine can be decomposed using potassium persulfate and sodium hydroxide with the aid of microwave digestion[15,18] or UV irradiation[37,41] and subsequently be amenable for hydride generation detection. In the case of HPLC/ICPMS method, there is no need for the chemical derivatization process, and all arsenic species can be detected.

Another dramatic difference between the two chromatograms (Figure 4 a,b) is the time required for the analysis. Much shorter chromatographic time (3.5 min) is needed when using the present short-column HPLC method compared to the conventional HPLC method (8 min). As a consequence, sample throughput is improved by more than 2-fold.

Arsenic speciation results from eight replicate analyses of the SRM samples are shown in Table 3. For comparison, results obtained from the interlaboratory study using HPLC/ICPMS are

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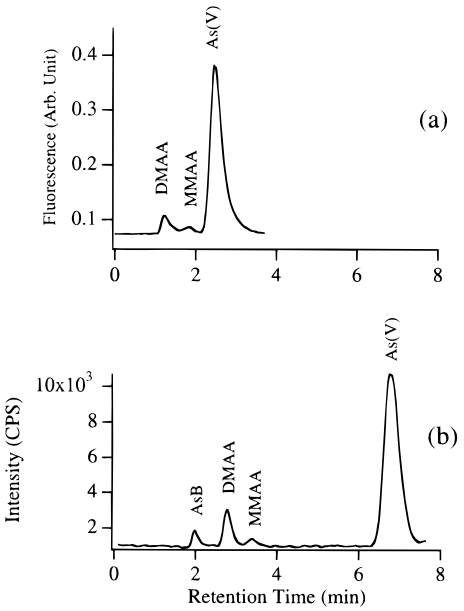

Figure 4. Comparison of chromatograms obtained from the HPLC/HGAFD analyses (a) and the HPLC/ICPMS analysis (b) of the Standard Reference Material SRM 2670 elevated level urine. For HPLC/HGAFD, the same conditions as shown in Figure 3 were used (method 2). For HPLC/ICPMS, a Hamilton PRP X-100 (250 mm × 4.1 mm) anion-exchange column was used for separation. The mobile phase contained 50 mM $H_2PO_4^-$/HPO_4^{2-} buffer (pH 6.0 adjusted with NH_4OH), and its flow rate was 1.0 mL/min (method 5). ICPMS was used to monitor arsenic, *m*/*z* 75. AsB stands for arsenobetaine.

Table 3. Arsenic Species in Urine Standard Reference Material (SRM 2670) (ng/mL)

urinary arsenic	this method	HPLC/ICMPS	certified/reference value
	Normal Level		
DMAA	49 ± 5	–[a]	–
MMAA	11 ± 3	–	–
total	60 ± 7	–	60[b]
	Elevated Level		
arsenobetaine	–	15 ± 3	–
DMAA	46 ± 5	49 ± 3	–
MMAA	11 ± 3	7 ± 2	–
As(V)	460 ± 25	443 ± 20	–
total	517 ± 29	514 ± 23	480 ± 100[c]

[a] Not available. [b] Reference value. [c] Certified value.

also summarized in Table 3. These results show good agreement between the two methods and with the certified (or reference) values.

A recently published study[42] on intercomparison of arsenic speciation results obtained by seven laboratories reports the following arsenic concentrations in the elevated level SRM 2670: DMAA, 38.7–69.9 ng/mL; MMAA, 9.3–22.7 ng/mL; inorganic As(III) and As(V), 362–509 ng/mL; and total arsenic, 432–570 ng/mL. Only one laboratory was able to differentiate inorganic As(III) from As(V) and found that inorganic As(V) was the major arsenic species in the urine SRM. We report here the speciation information on each of the four arsenic species. Our results are within the range reported by these laboratories.[42]

Separation of Arsenic Species on 3-μm Particle Size Columns. In principle, the HPLC column efficiency is inversely proportional to the square of the packing material diameter.[32,43] Therefore, separation can be improved by using columns packed with smaller particles. Conventionally, a 5–10-μm particle size is commonly used in HPLC columns. We have investigated the use of columns packed with 3-μm particles, and the improvement in separation efficiency enabled us to use shorter columns for the speciation of arsenic.

Furthermore, the optimum flow rate of mobile phase is higher when a smaller size particle is used in HPLC column packing. This is governed by the Van Deemter equation.[32,43] A higher flow rate results in a faster HPLC separation. Therefore, by combining the use of a shorter HPLC column (15 cm) packed with smaller particles (3 μm) and a faster flow rate, we obtained baseline resolution of the four arsenic species with a dramatically reduced separation time (4 min) (Figure 6a). Chromatographic separation of these arsenic species reported by others generally required a minimum of 8 min.[10,35–39]

Urinary Arsenic Species Following the Ingestion of Arsenosugars in Mussels. We demonstrate the applicability of the HPLC/HGAFD method to urinary arsenic speciation through a study on human metabolism of arsenosugars following the ingestion of arsenosugar-containing mussels. Urinary arsenite, arsenate, MMAA, and DMAA are common biomakers which have been widely used to assess human exposure to inorganic arsenic. We have previously observed[7,18,34] that the ingestion of seaweed can result in the urinary excretion of arsenic metabolites, including DMAA. The present study demonstrates that the consumption of mussels also contributes to the increase of DMAA, making the conventional biomarkers of exposure to inorganic arsenic unreliable.

HPLC/ICPMS analysis of the mussel extract shows that the mussels contain arsenobetaine and two arsenosugars as the major arsenic species (Figure 5a). The presence of arsenobetaine and arsenosugars in mussels, oysters, and clams has been previously reported.[7,33,44] However, the effects of the ingestion of these foods on the metabolism and urinary excretion of arsenic species have not been reported.

Figure 5b–e shows chromatograms from arsenic speciation in urine samples collected from a volunteer (34 years old, male) before and after the ingestion of approximately 250 g of mussels. The volunteer refrained from eating any seafood and did not have excess exposure to arsenic for at least 3 days prior to the mussel ingestion experiment. The arsenic species (23 ng/mL of DMAA, 5 ng/mL of MMAA, and 4 ng/mL of arsenite) in the urine samples collected 30 min before the ingestion of the mussels (Figure 5b) represents the background levels of arsenic in his urine. Following the ingestion of mussels, arsenobetaine is rapidly excreted into the urine (Figure 5c). Approximately 95 ng/mL of arsenobetaine is detected in the urine sample collected 2 h after the ingestion of mussels (Figure 5c). Arsenobetaine is a very stable arsenic compound, and it is essentially nontoxic.[1,2,45] Several groups have reported that arsenobetaine is directly excreted from the human body without metabolism.[7,14,46–49]

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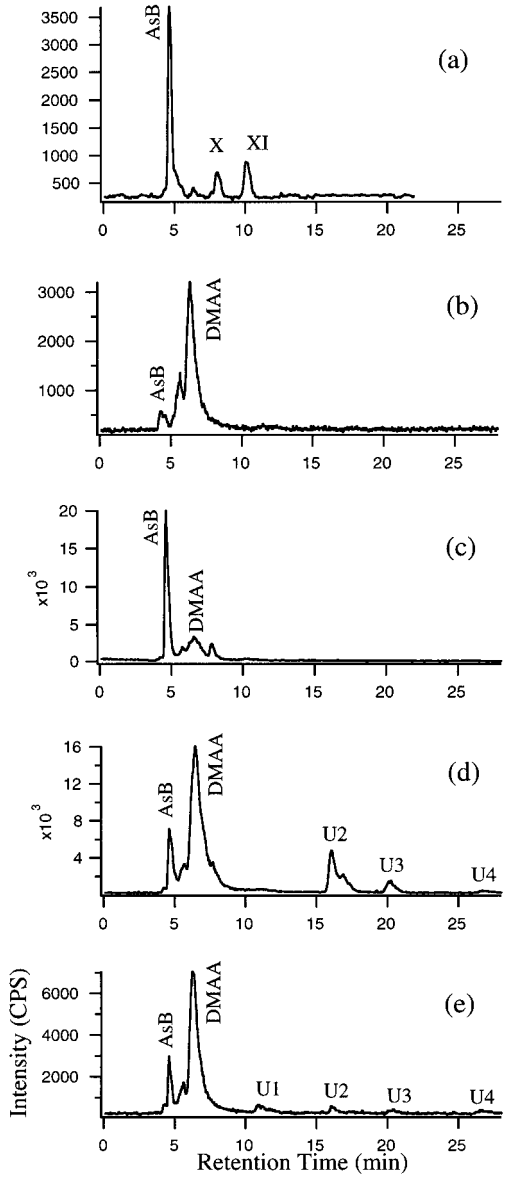
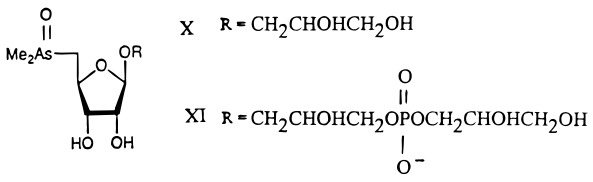


Figure 5. HPLC/ICPMS analyses for arsenic speciation of a mussel extract (a) and urine samples collected 0.5 h before (b) and 2 (c), 17 (d), and 42.5 h (e) after the ingestion of 250 g of mussels. HPLC separation conditions are shown in Table 1, method 6. U1, U2, U3, and U4 are four unidentified arsenic species. X and XI are two arsenosugars:



The arsenosugars ingested from the mussels are metabolized, and no trace of the original arsenosugars is detected in any of the urine samples (Figure 5c−e). The metabolism of the arsenosugars results in several as yet uncharacterized arsenic-containing metabolites. The retention times of these metabolites are different from those of more than a dozen known arsenic species available to us. They appear to be new arsenic species. Because of the trace levels of these metabolites in the urine samples (1−30 ng/mL), we have not been able to characterize these species. Further development of analytical techniques, including preconcentration techniques combined with HPLC, electrospray mass spectrometry, and nuclear magnetic resonance spectroscopy, will be useful for the characterization of the new metabolites.

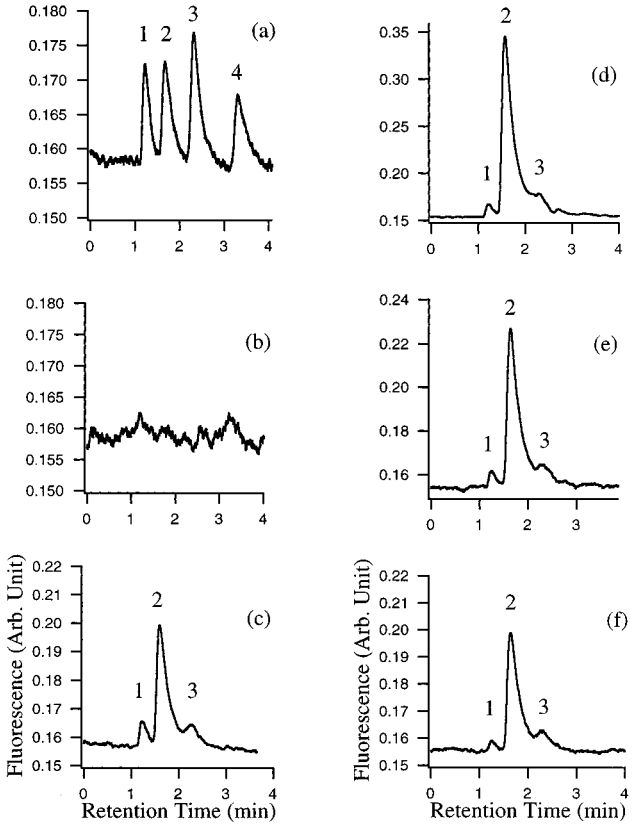


Figure 6. HPLC/HGAFD analyses of As(III), As(V), MMAA and DMAA in a standard solution (a), mussel extract (b), and urine samples collected 0.5 h before (c) and 17 (d), 28 (e), and 42.5 h (f) after the ingestion of mussels. HPLC separation conditions are shown in Table 1, method 4.

Figure 5 also shows the substantial increase of DMAA in urine samples collected after the consumption of mussels (Figure 5 d,e). Urinary DMAA is a commonly used indicator for the assessment of environmental and occupational exposure to inorganic arsenic. The increase of DMAA in urine samples as a result of arsenosugar metabolism (seafood ingestion) would affect the reliability (validity) of this indicator. Therefore, we decided to further investigate the effect of arsenosugar ingestion on the urinary excretion of inorganic arsenic, MMAA, and DMAA, and to examine the confounding factors when using the traditional biomarkers of exposure to arsenic. For this reason, we emphasize the speciation of only the four target arsenic compounds. We chose the present fast HPLC/HGAFD technique for monitoring these four arsenic species.

Figure 6 shows chromatograms obtained from HPLC/HGAFD analyses of the four target arsenic standards (a), the mussel

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(49) Vahter, M. *Appl. Organomet. Chem.* **1994**, *8*, 175−182.

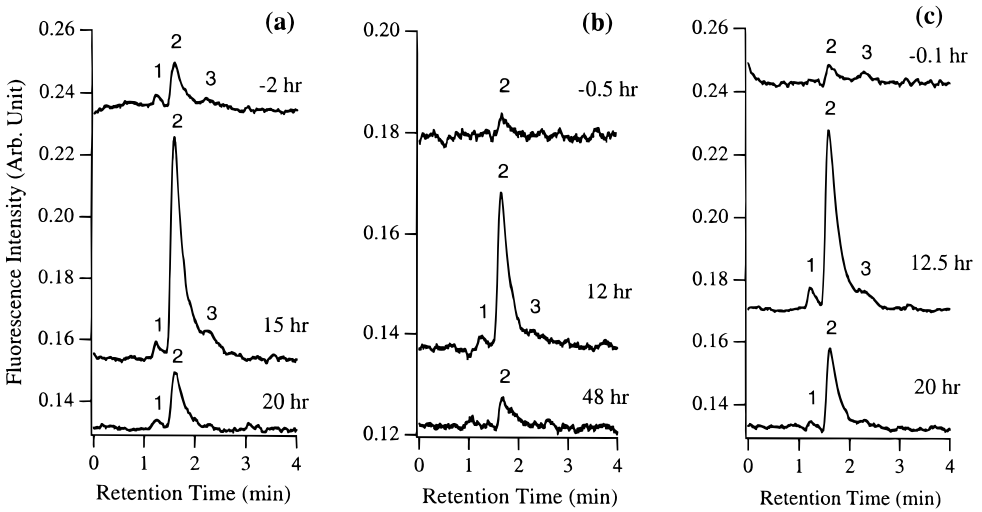

Figure 7. HPLC/HGAFD analyses of selected urine samples from three male volunteers: (a) 33, (b) 35, and (c) 41 years old. Sampling time with respect to the ingestion of approximately 200 g of mussels is shown in the figure. The same experimental conditions as shown in Figure 6 were used.

extract (b), and the urine samples collected before (c) and after (d−f) the ingestion of mussels. Arsenobetaine and arsenosugars, although present in the mussels as the dominant arsenic species, do not form hydride by using the $NaBH_4$ treatment and do not interfere with the determination of the four target arsenic species. Also, the unknown metabolites evident in Figure 5d,e do not form hydride with the $NaBH_4$ treatment and are not detected.

The chromatograms in Figure 6 clearly show the substantial increases of DMAA in urine samples collected from the volunteer after the consumption of mussels (Figure 6 d,e). The concentrations of DMAA are 23, 111, 38, and 26 ng/mL in urine samples collected 0.5 h before and 17, 28, and 42.5 h after the ingestion of mussels, respectively. The mussels do not contain significant levels of DMAA or inorganic arsenic (Figure 6b). The increase of DMAA in urine is due to the metabolism of arsenosugars. The metabolism of arsenosugars also produces other uncharacterized metabolites, which do not form hydride and are not detected by using HPLC/HGAFD.

Urine samples from three other male volunteers (33, 35, and 41 years old, respectively) before and after they ingested approximately 200 g of mussels were analyzed using the fast HPLC/HGAFD method, and selected chromatograms are shown in Figure 7. Relatively lower background levels of arsenic species (3−15 ng/mL) are present in the urine samples from these volunteers compared with that in the −0.5-h urine sample from volunteer 1 (32 ng/mL). These differences are probably due to the variation in the amount of arsenic ingested from their regular diet. Nonetheless, the increases of DMAA concentration in their urine samples following the ingestion of mussels are clearly demonstrated for all the volunteers. The urinary arsenic speciation results from the four volunteers confirm that arsenosugars ingested from the mussels can be metabolized in the human body and that the metabolites including DMAA can be excreted into the urine.

Arsenosugars are present in seaweeds, oysters, mussels, and clams,[3,7,33,44] which are common for human consumption. A recent study also identified an arsenosugar in a commercial algal product of terrestrial origin.[50] The presence of these arsenosugar-containing foods in the human diet will invalidate the use of the conventional biomarkers of exposure to inorganic arsenic. One approach to deal with this problem is to have the subjects refrain from eating any seafood for at least 3 days before urine samples are taken for the assessment of exposure to inorganic arsenic, because organoarsenicals from seafood sources are usually eliminated from the body within 3 days after ingestion.[14,46−49] Also, the unique arsenosugar metabolites may be used as an indicator of arsenosugar ingestion, which assists the interpretation of exposure data. Thus, one should take appropriate caution when attempting to establish any correlation between environmental and occupational exposure to inorganic arsenic and the elevated urinary arsenic levels. Possible contribution to urinary arsenic species from dietary sources should be considered.

The methods described in this study are simple, rapid, and inexpensive and are suitable for the speciation of trace levels of arsenic in urine samples from the general population and in drinking water samples. A recent pilot dose−response study (data not shown) involving the speciation of arsenic in more than 600 urine samples has shown that the methods are suitable for routine analysis of arsenic species.

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