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Speciation of Arsenic Compounds Using High-Performance Liquid Chromatography at Elevated Temperature and Selective Hydride Generation Atomic Fluorescence Detection

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Understanding arsenic toxicity and metabolism requires quantitation of individual arsenic species. However, it has been difficult to separate many biochemically and environmentally important arsenic species on a single chromatography column. We have studied the separation of 11 arsenic compounds by using ion pair chromatography at 30, 50, and 70 °C column temperatures. The use of elevated column temperature improved separation efficiency and dramatically reduced chromatographic retention time for arsenobetaine, arsenocholine, tetramethylarsonium, and arsenosugars. On-line microwave derivatization combined with hydride generation and atomic fluorescence spectrometry (HGAFS) was used for detection, which was able to differentiate more toxic from less toxic arsenic species. The speciation technique was successfully applied to a study of metabolism of arseno sugars present in commercial seaweed products. Two uncharacterized arsenic-containing metabolites were detected in urine samples collected 20−33 h after the consumption of the seaweed product. These metabolites did not form hydride without microwave digestion. Up to 90 ng/mL of dimethylarsinic acid was detected in the urine samples collected 25−35 h after the consumption of seaweed, compared to a background level of less than 15 ng/mL in urine samples collected before the ingestion of seaweed. The combined high-temperature ion pair chromatography with selective arsenic detection provided a rapid approach to monitoring metabolism of arsenic compounds.

It has been widely recognized that toxicological behaviors and many biochemical functions of trace elements strongly depend on chemical forms (species) of the element (e.g., ref 1). Extensive studies on the toxicity and biochemistry of arsenic have clearly depicted the importance of chemical speciation of this element.[2−5] Arsenite, arsenate, monomethylarsonic acid (MMAA), and dimethylarsinic acid (DMAA), which are present in natural waters,[6−8] are much more toxic than arsenobetaine (AB), arsenocholine (AC), tetramethylarsonium ion (Me_4As^+), and arseno sugars, which are found in marine organisms.[4,5,9−12] Metabolic fates of these arsenic species also differ dramatically. While most arsenic species, such as arsenite and arsenate, are metabolized in experimental animals and human beings,[13−16] arsenobetaine, the major arsenic species in edible crustacean seafood is excreted rapidly into urine as its original form in the seafood.[16−18]

Much work on arsenic speciation has dealt with arsenite, arsenate, MMAA, and DMAA. However, great interest in studying the biochemical behavior of arsenic compounds and in understanding biogeochemical cycling of arsenic in marine environment also requires the speciation of other organoarsenic species.[3−5,7,8] Hyphenated techniques involving high-performance liquid chromatography (HPLC) and atomic spectrometry have been commonly used for the speciation of trace elements because these techniques combine the capability of HPLC separation with element-selective and sensitive atomic spectrometry detection. Ion

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exchange,[19−26] ion pair,[25−28] and gel permeation[29] chromatography have been studied and are usually successful for the separation of only four or five arsenic species. Most of these studies involve extensive optimization of several HPLC parameters, including the composition, pH, and the flow rate of the mobile phase, the stationary phase, and the gradient elution. No consideration was given to column temperature optimization.

HPLC column temperature is an important parameter for optimizing separation.[30−37] When column temperature increases, viscosity of 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 can reduce band broadening and increase efficiency.[30,31] A temperature increase can also accelerate the interactions involved in the separation process, thereby increasing efficiency. For these reasons, elevated temperature HPLC has been successfully applied to the separation of peptides and proteins,[34,35] and increasing column temperature has been considered essential for the enhancement of efficiency in the chromatography of large molecules.[31] Furthermore, a temperature change can have a marked effect on the selectivity of a chromatographic separation, especially when selectivity is based on shape discrimination. For example, optimization of column temperature has resulted in an enhancement in enantioselectivity of chiral separation[36] and in the separation of polycyclic aromatic hydrocarbon isomers.[37] Appropriate use, optimization, and programming of column temperature have also been shown useful in studying molecular conformation and isomerization.[38,39]

Despite the great success in using column temperature as a variable for improving the HPLC separation of large biomolecules, a similar strategy has not been explored for chemical speciation studies. Consequently, potential benefits of optimizing and controlling column temperature in chemical speciation studies of trace elements using HPLC have not been realized. If successful, controlled-temperature HPLC can be extremely useful for the separation of those biologically and environmentally important chemical species, which has been difficult to achieve by optimizing other HPLC parameters.

We present here our studies on the effect of column temperature on the HPLC separation of 11 arsenic compounds. The use of superambient temperature (e.g., 70 °C) enhances the separation efficiency and dramatically reduces the HPLC run time using ion pair chromatography. A combination of the elevated temperature HPLC separation with hydride generation and atomic fluorescence detection provides a convenient approach for rapid speciation of arsenic compounds. We demonstrate an application of the technique to a study of the metabolism and urinary excretion of arsenic following the consumption of commercial seaweed products, which contain arsenosugars as the major arsenic species.

EXPERIMENTAL SECTION

Standards and Reagents. Sodium arsenite, sodium arsenate, sodium monomethylarsonate, and dimethylarsinic acid were obtained from commercial sources (Aldrich, Milwaukee, WI). Arsenobetaine, arsenocholine, and tetramethylarsonium iodide were synthesized and characterized by literature methods.[9,40,41] Standard solutions of these arsenicals were prepared as described previously.[18,26,42]

All HPLC eluents, including sodium hexanesulfonate, tetrabutylammonium hydroxide, tetraethylammonium hydroxide, and malonic acid, were obtained from Aldrich, prepared in distilled deionized water, and filtered through a 0.45-μm membrane. The pH of these eluents was adjusted by using sodium hydroxide and nitric acid. An appropriate amount of methanol (HPLC grade, Fisher) was added prior to the pH adjustment and the filtration.

Sodium borohydride (Aldrich) solutions in 0.1 M sodium hydroxide (Fisher) and microwave digestion reagents containing 0.1 M potassium persulfate (Fisher) and 0.3 M sodium hydroxide were prepared fresh daily. All reagents used were of analytical grade unless stated otherwise.

Instrument. The HPLC system consisted of a Gilson (Middletone, WI) HPLC pump (Model 307) with a 5 mL/min stainless steel pump head, a Rheodyne 6-port sample injector (Model 7725i) with a 20-μL sample loop, and a reversed-phase HPLC column. The columns used were two 250- × 4.6-mm ODS(3) columns (100-Å pore diameter, 5-μm particles) and a 300- × 3.9-mm C18 (150-Å pore diameter, 10-μm particles), all from Phenomenex (Torrance, CA). The analytical column and a guard column (30 mm long) packed with the same material were 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, is able to provide a +0.1 °C temperature stability and +1 °C accuracy. Isocratic HPLC operation was performed under a 1 mL/min flow rate.

A hydride generation atomic fluorescence spectrometer (HGAFS, Model Excalibur 10.003, PS Analytical, Kent, UK)[43] was used as an HPLC detector. An on-line microwave digestion unit, similar to that described previously,[26] was incorporated into the

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HGAFS system, prior to the hydride generation step. A solution containing 0.1 M potassium persulfate and 0.3 M sodium hydroxide efficiently decomposed organoarsenicals to arsenate with the aid of microwave heating[26,42] and this mixture decomposition reagent was used in the present study. The decomposition reagent (4 mL/min) was introduced by using a peristaltic pump (Gilson) and met with the HPLC effluent (1 mL/min) at a T-joint. This solution mixture flowed through a polytetrafluoroethylene coil (5 m × 0.8 mm i.d.) located inside a continuously operating microwave oven (550 W, 2450 MHz, Danby Products, Ontario, Canada), where the decomposition took place. The solution from the microwave oven then met at two Teflon T-joints with continuous flows of hydrochloric acid (3 M, 3.4 mL/min) and sodium borohydride (0.64 M, 3.4 mL/min), introduced by using a multichannel peristaltic pump (Gilson Model Minipuls 3). Upon mixing of the microwave-digested HPLC effluent, hydrochloric acid, and sodium borohydride solution, hydride generation took place. Arsenic hydride, along with the byproduct hydrogen, generated from the reaction was carried by a continuous flow of argon carrier gas (350 mL/min) to the atomic fluorescence detector. Argon/hydrogen flame was used for atomization. An arsenic hollow cathode lamp was used for fluorescence excitation. Atomic fluorescence was collected at a right angle, filtered with a multireflectance filter, and detected with a solar blind photomultiplier tube. A Hewlett Packard (Boise, ID) 3390A integrator with both peak area and peak height measurement capability was used to record signals from the atomic fluorescence detector.

Alternatively, the HPLC effluent bypassed the microwave oven and directly met the continuous flows of hydrochloric acid and sodium borohydride at two T-joints. Arsines produced upon the hydride generation were introduced into the atomic fluorescence detector for measurement.

Seaweed Samples. The commercial seaweed products Yakinori were purchased from a local food store (Edmonton, Canada). They were products of Japan and were in the form of thin sheets. Several packages of Yakinori, approximately 200 g in total, were cut to small pieces and mixed, to provide representative samples for analysis and for ingestion studies.

Duplicate Yakinori samples (0.5−1 g dry weight) were extracted by using a procedure similar to that described by Shibata and Morita.[44] Each sample was weighed into a test tube (15 mL). To each tube was added 10 mL of a methanol/water mixture (1:1 v/v). The tube was sonicated for 10 min, and after centrifugation, the extract was removed and placed in a 100-mL beaker. The extraction process with the aid of sonication was repeated a further four times for each sample. 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 analyzed by using HPLC/HGAFS.

Ingestion of Seaweed and Collection of Urine Samples. A 35-year-old male volunteer refrained from eating any seafood for at least 72 h prior to commencing the seaweed ingestion experiment. The volunteer was instructed to collect 2−3 urine samples during the 12-h period prior to the consumption of seaweed. These samples were used to determine the background level of arsenic in the urine of the volunteer resulting from a regular diet that excluded any seafood.

The volunteer then consumed 10.0 g (dry weight) of the seaweed product Yakinori in one meal. The time of this meal was referred to as time zero. Six hours later, the volunteer ingested another portion of 10.0 g of the seaweed. Following the first consumption of the seaweed, urine samples were collected in separate 500-mL polyethylene containers for consecutive 4 days. No other seafood was eaten during the experiment period. All urine samples were stored at 4 °C and were analyzed within 48 h. The samples were then kept frozen at −20 °C for later use. No preservative was added.

Experimental details and possible health effects concerning seafood ingestion in this experiment were discussed with the volunteer prior to conducting the experiment. All procedures followed were in accordance with the ethical standards of the Research Ethics Board, Faculty of Medicine, University of Alberta.

Speciation of Arsenic Compounds in Urine Samples. Three HPLC systems were used, all performed at 70 °C. The first system involved the use of a 250- × 4.6-mm ODS(3) column, with 10 mM tetraethylammonium hydroxide, 4 mM malonic acid, and 0.1% methanol as mobile phase (pH 6.8). This was used primarily for the speciation of arseno sugars and their metabolites. The second system used another ODS(3) column, with 5 mM tetrabutylammonium hydroxide, 1 mM hexanesulfonate, 1 mM malonic acid, and 0.5% methanol as mobile phase (pH 5.5). The third system used a 300- × 3.9-mm C18 column, with 10 mM hexanesulfonate, 1 mM tetraethylammonium hydroxide, and 0.5% methanol as mobile phase (pH 4.0).

The HPLC columns were equilibrated with the appropriate eluent flowing at 1 mL/min for at least 2 h before sample analysis. Urine samples were filtered through 0.45-μm nylon filters (SPE Ltd., Ontario, Canada) to remove any suspended particulate before they were subjected to HPLC analysis. A 20-μL aliquot of a sample was injected for analysis. Arsenic species in the urine samples were identified by matching the retention times of the chromatographic peaks from the samples with those of standards spiked into the sample. Concentration of arsenic species in urine samples was obtained by calibrating against known concentrations of arsenic standards.

RESULTS AND DISCUSSION

The separation of arsenic species by HPLC is pH dependent. At neutral pH, arsenate ($pK_{a1} = 2.3$), MMAA ($pK_{a1} = 3.6$), and DMAA ($pK_a = 6.2$) are present as anions; arsenocholine ($(CH_3)_3As^+CH_2CH_2OH$) and the tetramethylarsonium ion ($(CH_3)_4As^+$) as cations; arsenobetaine as a zwitterion ($(CH_3)_3As^+CH_2COO^-$); and arsenious acid ($pK_{a1} = 9.3$) as an uncharged species. Thus, both cation exchange[19,20,22] and anion exchange[19−26,29] chromatography have been commonly used for the separation of ionic arsenic species. Reversed-phase ion pair HPLC has also been used[19,25−28] with appropriate counterions, e.g., tetramethylammonium cation or heptanesulfonate anion, in the mobile phase. The counterion forms an ion pair with oppositely charged analyte ions, thus introducing additional interactions for a better separation. Although many studies have dealt with HPLC separation of some arsenic species, few systems have been able to resolve all commonly encountered arsenic species.

Table 1 shows HPLC retention times of seven arsenic species on a reversed-phase C18 column under controlled temperatures of 30, 50, and 70 °C. Mobile phase (pH 3.5) contained 10 mM hexanesulfonate, an ion pair reagent, and 0.1% methanol. Although most arsenic species are well resolved from one another,

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Table 1. Effect of Column Temperature on Retention Time of Seven Arsenic Compounds

	retention time (min)						
temp (°C)	arsenate	arsenite	MMAA	DMAA	AB	AC	Me_4As^+
30	2.6	3.4	4.5	6.5	5.0	20.0	27.6
50	2.6	3.4	4.5	6.2	4.6	16.2	20.4
70	2.6	3.4	4.4	5.9	4.5	13.2	16.3

a major problem with this separation under ambient temperature is that arsenocholine and the tetramethylarsonium cation retain too strongly on the column, resulting in band broadening and lengthy analysis time. An increase of HPLC column temperature from 30 to 70 °C dramatically reduces the HPLC run time. The retention times for arsenocholine and tetramethylarsonium are reduced from 20.0 and 27.6 min at 30 °C, to 16.2 and 20.3 min at 50 °C, and to 13.2 and 16.3 min at 70 °C, respectively. Thus, the HPLC analysis time is reduced by 11 min simply by using a column temperature of 70 °C instead of ambient temperature.

The increase of column temperature has less effect on the retention of the early-eluting compounds than on the late-eluting ones. The increase of column temperature from 30 to 70 °C results in only small changes of retention time for arsenite, arsenate, MMAA, and DMAA, which elute within 7 min, as shown in Table 1.

The detection system combining on-line microwave digestion, hydride generation, and atomic fluorescence spectrometry (HGAFS) provides a unique approach to differentiating some arsenic compounds even when they are not well resolved upon HPLC separation. This is based on differences in reactivity of various arsenic compounds with sodium borohydride. Arsenite, arsenate, MMAA, and DMAA can readily form gaseous hydride upon reacting with sodium borohydride and, therefore, can be detected by hydride generation atomic fluorescence. Arsenobetaine, arsenocholine, and tetramethylarsonium, on the other hand, do not form hydride upon the same chemical treatment. These compounds require derivatization to arsenate by using potassium persulfate and sodium hydroxide with the aid of microwave heating before they can be detected by HGAFS. Therefore, these two groups of arsenic compounds can be differentiated using the microwave derivatization on-and-off options. In the present study, the detection system allows for the speciation of all the seven arsenic species shown on Table 1, even though MMAA and arsenobetaine are not baseline resolved under the given chromatographic conditions. Without microwave digestion, the analysis of a sample provides speciation information on arsenite, arsenate, MMAA, and DMAA. Arsenobetaine, arsenocholine, and tetramethylarsonium ion do not form hydride and, therefore, are not detected by HGAFS. Analysis of the same sample for the second time with the microwave digestion provides information on these arsenic species.

Further optimization of ion pair chromatography conditions in combination with the use of elevated column temperature results in a nearly baseline resolution of the above seven arsenicals (Figure 1). This was achieved by using elevated column temperature and mixed ion pair reagents, 10 mM sodium hexanesulfonate and 1 mM tetraethylammonium hydroxide. Multiple modes of separation mechanism, including ion-pairing, ion exchange, hydrophobic interaction with C18, and hydrophilic

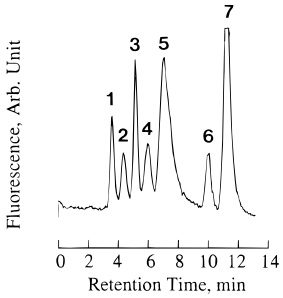

Figure 1. Chromatogram of seven arsenicals obtained on a reversed-phase C18 column (300 × 3.9 mm, 10 μm particles) with 10 mM hexanesulfonate, 1 mM tetraethylammonium hydroxide, and 0.5% methanol as eluent (pH 4.0). Microwave digestion combined with hydride generation and atomic fluorescence spectrometry was used for detection. Column temperature was 70 °C. Peak 1, arsenate (10 ng); 2, arsenite (5 ng); 3, arsenobetaine (15 ng); 4, MMAA (10 ng); 5, DMAA (40 ng); 6, arsenocholine (15 ng); and 7, tetramethylarsonium (40 ng).

interaction with silanol groups, for various arsenic species are probably responsible for the excellent separation.

While the speciation of the above arsenicals has drawn much attention, only a few studies have dealt with the speciation of arsenosugars.[5,29,45] Arseno sugars are major arsenic compounds found in most seaweeds[8,12,29,45] and are also present in marine bivalves.[44,45] They play an important role in completing the marine arsenic cycle. Understanding their presence and metabolism is essential to a reliable assessment of their health effects, as the consumption of seaweeds, oysters, mussels, and clams may result in the ingestion of substantial amount of arsenosugars.

Arsenosugars contain hydroxyl, phosphate, and/or sulfate groups (4, 5, 8, 12). They exist as uncharged or anionic species at neutral pH. Thus, tetraethylammonium cation was chosen for ion pair chromatographic separation of arsenosugars. Chromatographic analyses of a seaweed sample, performed at two column temperatures, 30 and 70 °C, are compared in Figure 2. It is clear that the use of 70 °C column temperature improves the separation and dramatically reduces the HPLC run time by 8 min. Two major arsenosugars[8,29,44,45] and several other minor arsenic compounds are found in the seaweed sample, which is consistent with literature report demonstrating arsenosugars as the major arsenic species present in seaweeds.[4,5,8,12,29,44,45]

These arsenosugars do not form stable volatile hydride upon treatment with sodium borohydride (Figure 2a). They require microwave digestion with potassium persulfate and sodium hydroxide in order to be detected by HGAFS (Figure 2b,c). This characteristic is similar to that of arsenobetaine.

While arsenobetaine is well know to possess little toxicity and to be rapidly excreted into urine following administration, much less is known about the metabolic fate of arsenosugars. Previous reports by others have assumed that arsenosugars were also excreted into urine in their unchanged forms.[46] But there was no evidence to support the claim. We decided to apply our technique, involving elevated temperature HPLC separation and HGAFS detection, to a study of the metabolism of arsenosugars. In general, urinary excretion is the major pathway for the elimination of arsenic compounds,[13−18] and thus we chose urine samples for our arsenic speciation study.

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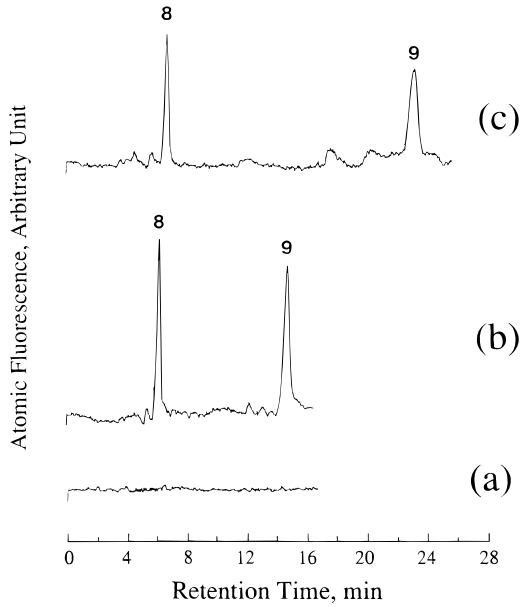

Figure 2. Chromatograms obtained from HPLC analyses of an extract of seaweed product Yakinori, using an ODS(3) column (250 × 4.6 mm, 5 μm particle size). Mobile phase consisted of 10 mM tetraethylammonium hydroxide, 4 mM malonic acid, and 0.1% methanol, pH 6.8. Peaks 8 and 9 are two arsenosugars.[29,44,45] (a) Without microwave digestion; column temperature, 70 °C. (b) With microwave digestion; column temperature, 70 °C. (c) With microwave digestion; column temperature, 30 °C.

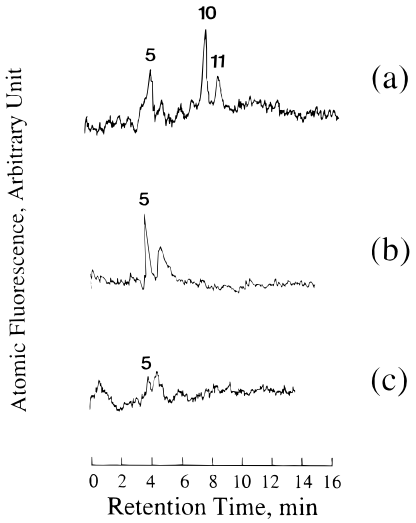

Figure 3. Chromatogram obtained from the analyses of urine samples collected 27 h after (a, b) and 2 h before (c) the consumption of seaweed product Yakinori. Hydride generation atomic fluorescence with (a, c) and without (b) microwave digestion was used for detection. Column temperature was 70 °C. Same column and mobile phase as in Figure 2 were used. Peak 5 is DMAA; peaks 10 and 11 are two new arsenic metabolites.

Figure 3 shows typical chromatograms obtained from the analysis of urine samples collected before and after the ingestion of seaweed. The arseno sugars ingested, as shown in Figure 2b (peaks 8 and 9) are not detected in the urine samples. Instead, two metabolites (peaks 10 and 11) are clearly observed in the urine samples collected 27 h after the ingestion of seaweed (Figure 3a). These are also present in samples collected between 20 and 33 h after the ingestion of seaweed. Their concentration in urine is 20−50 ng/mL, as calibrated against arsenite standards. They are not detected in samples collected before the ingestion of seaweed (Figure 3c), nor in samples collected 40 h after the ingestion. The detection limit (three times signal-to-noise ratio) for these metabolites is 10 ng/mL as arsenic.

The identity of the metabolites is not clear. However, they are arsenic-containing compounds because the HGAFS was tuned to selectively detect arsenic. The retention times of these metabolites do not correspond to those of a dozen arsenic standards currently available to us. The chromatographic behavior of the metabolites is also significantly different from that of the original arsenosugars present in the seaweed (Figure 2). Coinjection of the urine sample and the seaweed extract onto HPLC for analysis clearly showed that the metabolites are distinct from the original arsenosugars. This study confirms the metabolism of arsenosugars by humans, making the previous assumption that arsenosugars are excreted unmodified invalid.[46]

It is interesting to note that these metabolites do not form hydride without microwave digestion (Figure 3b). Although the exact chemical nature of the metabolite is not yet clear, the fact that all arsenicals that do not form hydride are much less toxic than those do suggests that the metabolites are also less toxic arsenic species. Further studies will be carried out to identify the metabolites and to investigate the toxicity of the metabolites. From animal testing, Shibata *et al.*[47] have found that arsenosugars are much less toxic than inorganic arsenic. The present technique, having microwave digestion on-and-off options, makes it possible to differentiate readily the more toxic from the less toxic species of arsenic.

It is also important to note the significant increase of DMAA concentration due to the consumption of seaweed. We found the concentration of DMAA to be 40−90 ng/mL in urine samples collected 25−35 h after the ingestion of seaweed, compared to less than 15 ng/mL in the samples collected before the ingestion. The seaweed product Yakinori we used does not contain high levels of these arsenicals (Figure 2). Our mass balance study also confirms that the amount of inorganic and methylated arsenic ingested due to the consumption of the seaweed cannot account for the increased amount of DMAA in the urine samples. The elevated concentration of DMAA in urine samples collected following the ingestion of seaweed is, therefore, also a result of the metabolism of arseno sugars.

Occupational exposure to arsenic often involves arsenite, arsenate, MMAA, and DMAA, released through activities such as mining, smelting, glass making, and pesticide manufacturing. Urinary excretion is the major pathway for the elimination of arsenic from the body.[13−18] Therefore, speciation of these arsenic species in workers' urine is useful for assessing occupational exposure to arsenic. However, it should be considered when arsenosugar-containing seafoods such as seaweeds are ingested that the arsenosugars are metabolized to species such as DMAA, which is also excreted into urine. Arsenic species from seafood sources are usually eliminated from the body within 3 days after ingestion.[16−18] For this reason, workers should not eat any seafood for at least 3 days before urine samples are taken for the assessment of occupational exposure to arsenic.

Inorganic arsenic and DMAA are more toxic forms of arsenic, their median lethal dose values (LD_{50}) in rats being arsenite 14, arsenate 20, and DMAA 700−2600 mg/kg, respectively.[7] The significant increase of urinary DMAA after the ingestion of seaweed suggests that health effects associated with the consump-

(47) Shibata, Y.; Jin, K.; Morita, M. *Appl. Organomet. Chem.* **1990**, *4*, 255−60.

tion of seaweed should be carefully evaluated. There is only one literature report[47] on the toxicity of arseno sugars, which suggested that arsenosugars have no cytotoxicity or mutagenecity to experimental rats. Most previous studies have assumed that arsenosugars are not metabolized and are simply excreted rapidly in their unchanged form into urine. However, the present study clearly demonstrates the metabolism of arsenosugars. Although the two unknown metabolites do not form hydride and perhaps are not very toxic, the significant increase of DMAA has important health implications with regard to the regular consumption of seaweed. Both metabolism and the nature of metabolites should be taken into consideration when assessing the overall toxicological effect of seaweed ingestion. The analytical techniques described here will be further applied to the assessment of toxicity, exposure, and health risk associated with the consumption of seaweed. Further studies will also deal with the characterization and identification of the metabolites.

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