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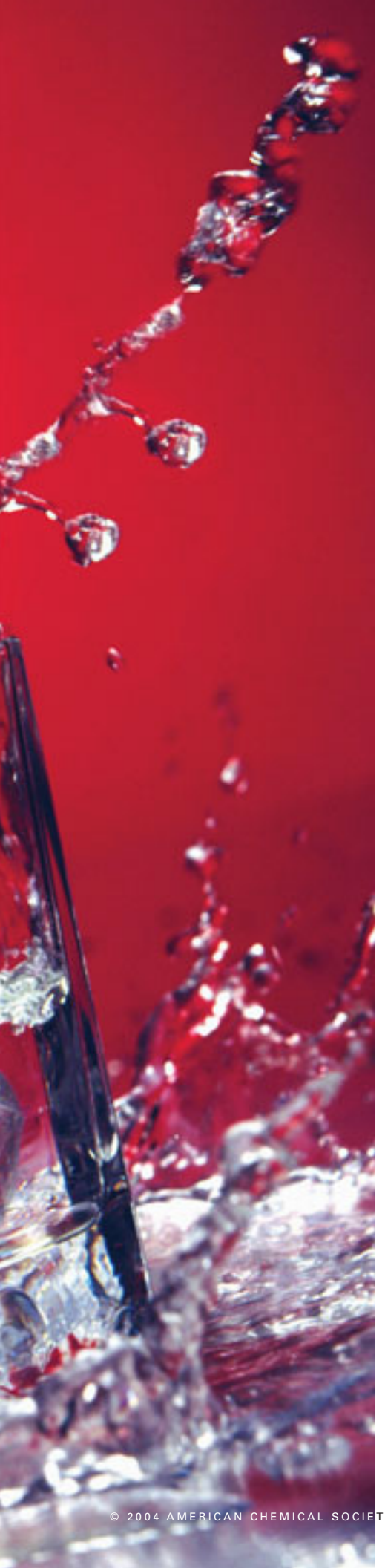

ARSENIC SPECIATION

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With more than 20 arsenic compounds present in the natural environment and biological systems, it is important to identify and quantify each individual chemical species of the element.

As_2O_3 is a water-soluble powder that produces a colorless, tasteless, and odorless solution and was a favorite poison during the Middle Ages. It is still used for that purpose today, although not to the same extent (*1*). Napoleon was allegedly poisoned by arsenic-tainted wine during his exile on the island of St. Helena. Rather than murder, what has commanded world attention today are the devastating effects of mass arsenic poisoning in many parts of the world. In Bangladesh and India alone, millions of people drink water containing high levels of arsenic (*2*). Millions of wells have been installed since the 1970s with the intention of providing clean water that is free of microbial pathogens. However, arsenic naturally contained in the bedrock has been released into the water, resulting in a concentration as high as several hundred micrograms, or in some cases, milligrams, per liter in some wells (*2*, *3*). Ingesting such high levels over several years can cause cancers of the skin, bladder, and lung as well as neurological and cardiovascular problems (*4*).

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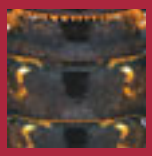
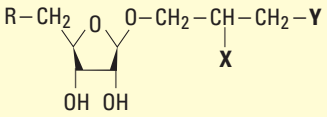



Table 1. Arsenic species in biological systems.

Name	Abbreviation	Chemical formula
Arsenite, arsenious acid	As^{III}	$As(OH)_3$
Arsenate, arsenic acid	As^{V}	$AsO(OH)_3$
Monomethylarsonic acid	MMA^{V}	$CH_3AsO(OH)_2$
Monomethylarsonous acid	MMA^{III}	$CH_3As(OH)_2$
Dimethylarsinic acid	DMA^{V}	$(CH_3)_2AsO(OH)$
Dimethylarsinous acid	DMA^{III}	$(CH_3)_2AsOH$
Trimethylarsine oxide	TMAO	$(CH_3)_3AsO$
Trimethylarsine	TMA^{III}	$(CH_3)_3As$
Arsenobetaine	AsB	$(CH_3)_3As^+CH_2COO–$
Arsenocholine	AsC	$(CH_3)_3As^+CH_2CH_2OH$
Tetramethylarsonium ion	Me_4As^+	$(CH_3)_4As^+$
Dimethylarsinoyl ethanol	DMAE	$(CH_3)_2AsOCH_2CH_2OH$

Table 2. Structure of arsenosugars.



	R	X	Y
X	$(CH_3)_2As(O)–$	–OH	–OH
XI	$(CH_3)_2As(O)–$	–OH	$–OPO_3HCH_2CH(OH)CH_2OH$
XII	$(CH_3)_2As(O)–$	–OH	$–SO_3H$
XIII	$(CH_3)_2As(O)–$	–OH	$–OSO_3H$
XIV	$(CH_3)_2As(O)–$	$–NH_2$	$–SO_3H$
XV	$(CH_3)_3As^+–$	–OH	$–OSO_3H$

The magnitude of the problem and the adverse health effects make arsenic the single most important environmental contaminant, with the consequence of increasingly stringent regulations. The World Health Organization guideline for arsenic in drinking water is 10 µg/L. The U.S. Environmental Protection Agency has recently reduced its maximum contaminant level from 50 to 10 µg/L. The Canadian interim maximum acceptable concentration is 25 µg/L and is currently under review by Health Canada. For individuals consuming 2 L of water daily containing 10 µg/L of arsenic, the daily intake of arsenic is 20 µg.

Arsenic is also abundant in seafood at concentrations as high as several hundred micrograms per gram (*5*). For example, the typical concentration in lobsters is 10 µg/g, so individuals consuming 200 g (about half a pound) could ingest 2000 µg of arsenic in a single meal. However, the high levels of arsenic in lobsters pose no known health concern because it is primarily in the form of the innocuous arsenobetaine (*5–7*). Arsenobetaine, the predominant species present in most crustaceans, is essentially nontoxic (*8*), unlike the highly toxic trivalent arsenic species, such as inorganic arsenite, monomethylarsonous acid, and dimethylarsinous acid (*9–13*). More than 20 arsenic compounds are present in the natural environment and biological systems (Table 1). Thus, assessments of environmental impact and risk to human health that are based strictly on the total element concentration are not reliable. It is important to identify and quantify the individual chemical species of the element. Speciation plays an essential role in understanding biogeochemical cycling, metabolism, and toxicological effects.

The concentrations of arsenic in the environment and biological systems are usually very low. For example, the total arsenic concentration in seawater is ~3 µg/L; in human urine and blood, it is on the order of 10–100 µg/L (*4*, *7*) and composed of several arsenic species. Arsenic species must be differentiated from one another and the sample matrix. Sub-microgram-per-liter detection limits are required to quantify individual arsenic species in these systems. Therefore, most speciation techniques involve a combination of chromatographic separation with atomic spectroscopy and MS detection (*14*). In this article, we will focus on how these techniques are used to study arsenic metabolism in humans and the interaction of arsenic with proteins. Microbial and plant metabolism of arsenic has recently been summarized (*15*, *16*).

Metabolism of arsenosugars and excretion of arsenobetaine

Arsenosugars are abundant in seaweed, mussels, oysters, and clams (*6*, *7*, *17*, *18*), but little is known about their toxicity (Table 2). Because arsenobetaine is rapidly excreted into urine without metabolic change, an incorrect perception exists that all organoarsenicals from seafood are excreted unchanged. Speciation studies of seaweed, mussels, and human urine samples collected before and after ingestion of arsenosugars show that arsenosugars undergo metabolism (*17–20*).

HPLC/ICPMS was the primary analytical technique used in these studies. HPLC provides excellent separation of various arsenosugars and arsenic metabolites; ICPMS offers element-specific detection with high sensitivity. Figure 1 shows chromatograms obtained from HPLC/ICPMS analyses of a mussel extract and urine samples before and after 250 g of mussels were ingested (*18*). The mussels contained arsenobetaine and two arsenosugars (X and XI; Figure 1a). Approximately 95 µg/L of arsenobetaine were detected in the urine sample collected 2 h after the mussels were eaten, demonstrating rapid excretion (Figure 1c). The arsenosugars were completely metabolized—no trace of the original arsenosugars was detected in any of the urine samples. Instead, several new arsenic compounds were present in the urine samples collected 17 h and 42.5 h after the mus-

sels were eaten (Figures 1d and e), which was the result of metabolism of the arsenosugars. Metabolism of arsenosugars has been confirmed by human ingestion of individual synthetic arsenosugar (*19*) and by sheep that consume seaweed as their primary diet (*20*).

Although ICPMS offers specific detection of arsenic, it cannot provide molecular (structural) information, because all the arsenic species are broken apart in the high-temperature ICP. Furthermore, HPLC/ICPMS alone cannot identify new arsenic metabolites that have no standards for chromatographic comparison. Electrospray (ES) MS techniques have been applied to the speciation of arsenosugars in extracts from various samples, primarily algae (*21–24*). Because of the complex sample matrix, the extracts usually need to be fractionated by using chromatography. On-line coupling of HPLC with ES MS further expands the capacity to characterize and quantitate individual arsenosugar species (*25*).

In the metabolism of synthetic arsenosugar experiments, ~80% of the total ingested arsenosugar was excreted in the urine within four days of ingestion (*19*). The major metabolite was identified as dimethylarsinic acid (DMA^V), which accounted for 67% of the total arsenicals excreted; dimethylarsinoyl ethanol, 5%; and trimethylarsine oxide, 0.5%. More than 20% of the total arsenicals excreted consisted of nine other arsenic species yet to be identified; these species were detected by HPLC/ICPMS but could not be positively identified, in part because of their very low levels. Further concentration and purification of these arsenicals may be required for their characterization by ES MS, NMR, and X-ray crystallography.

DMA^V (Figures 1d and e), a major metabolite of arsenosugars, consistently and substantially increases in urine after seaweed, mussels, and a synthetic arsenosugar are consumed (*17–19*). Urinary DMA^V is a common indicator for assessing human exposure to inorganic arsenic because it is also a major metabolite of inorganic arsenic metabolism. The increase in DMA^V in urine samples caused by metabolism of ingested arsenosugars from the diet affects the reliability of this indicator, invalidating its use as a biomarker of exposure to inorganic arsenic. One way to deal with this problem is to refrain from eating any arsenosugar-containing foods for at least three days before sample collection (*17–19*, *26*). Furthermore, unique arsenosugar metabolites may be used to indicate

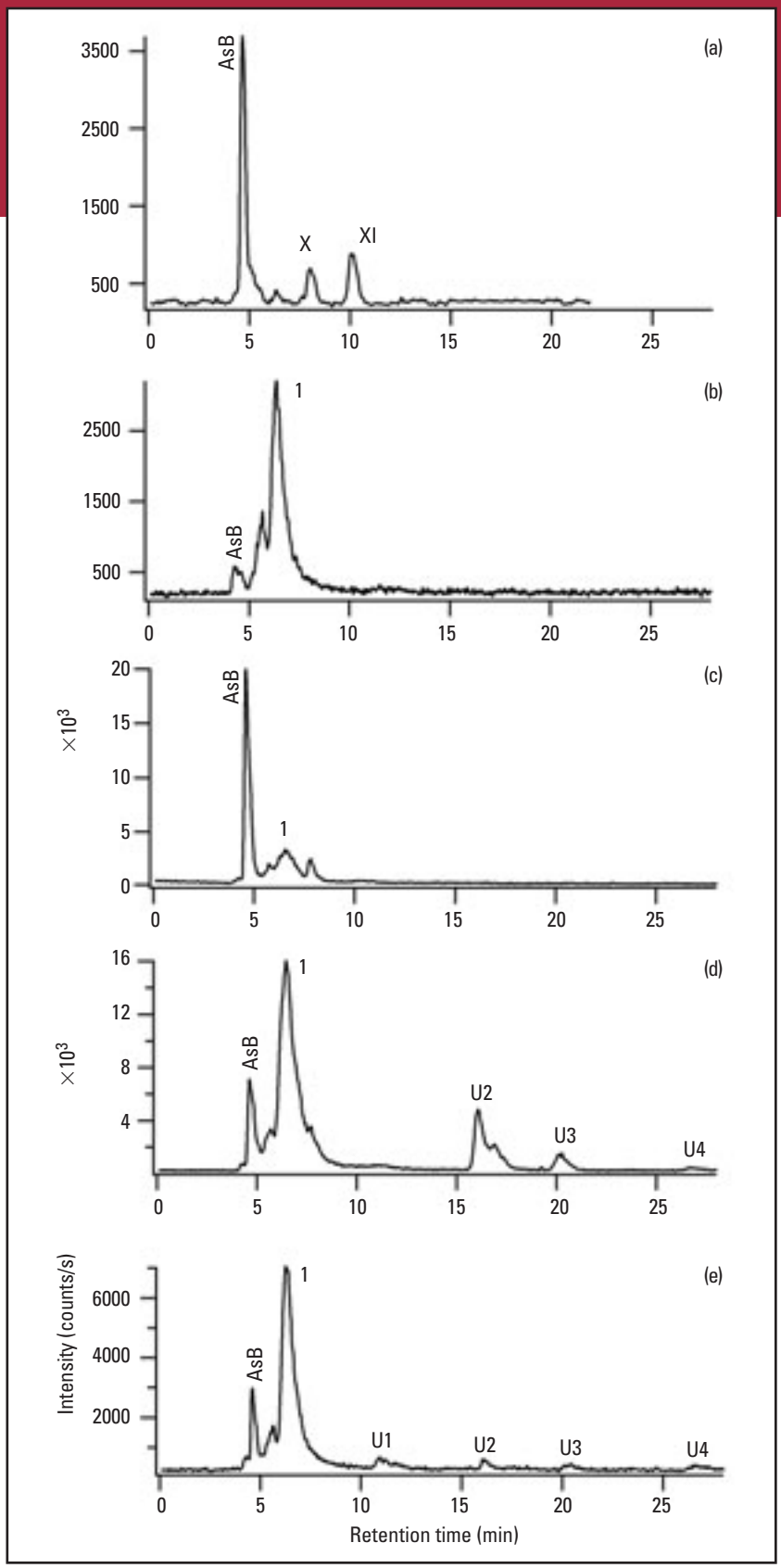


FIGURE 1. Arsenic in mussels.

(a) HPLC/ICPMS of arsenic speciation of a mussel extract. (b) Urine samples collected 0.5 h before and (c) 2 h, (d) 17 h, and (e) 42.5 h after ingestion of 250 g of mussels. Peak 1 represents DMA^V. U1, U2, U3, and U4 are four unidentified arsenic metabolites. X and XI are two arsenosugars. The separation was performed on a 250 × 4.6 mm ODS-2 column. The mobile phase was 10 mM tetraethylammonium hydroxide, 4.5 mM malonic acid, and 0.1% methanol at pH 6.8 at a flow rate of 0.8 mL/min. (Adapted from Ref. 18.)

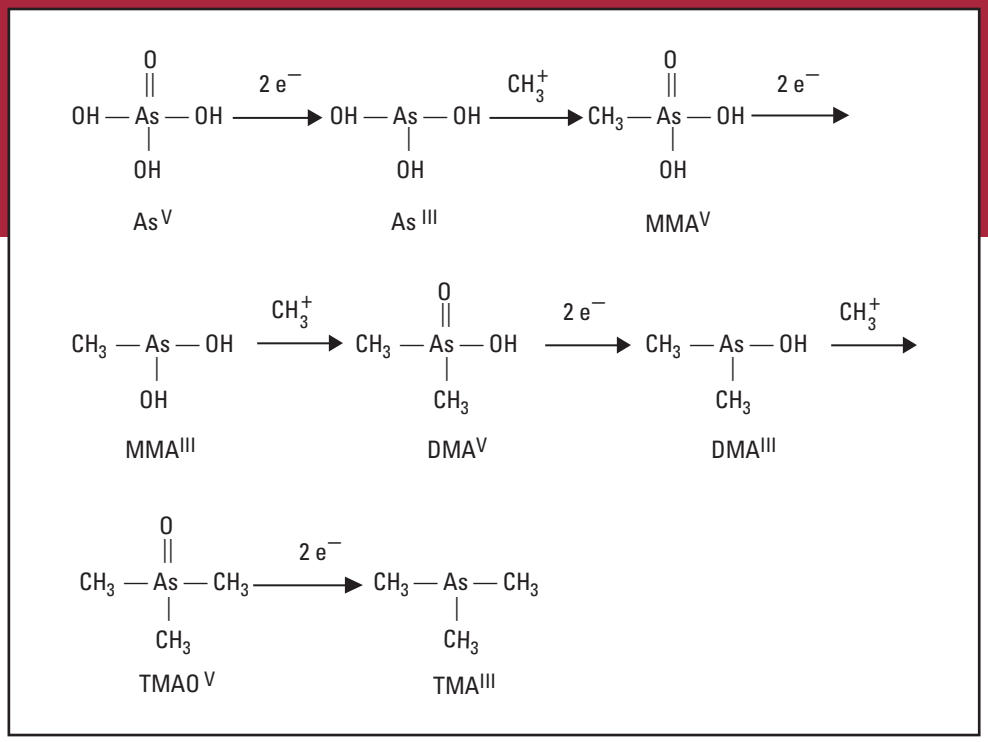


FIGURE 2. Biomethylation of inorganic arsenic involving alternate reduction of pentavalent arsenic to trivalent arsenic species followed by oxidative addition of a methyl group. (Adapted from Ref. 32.)

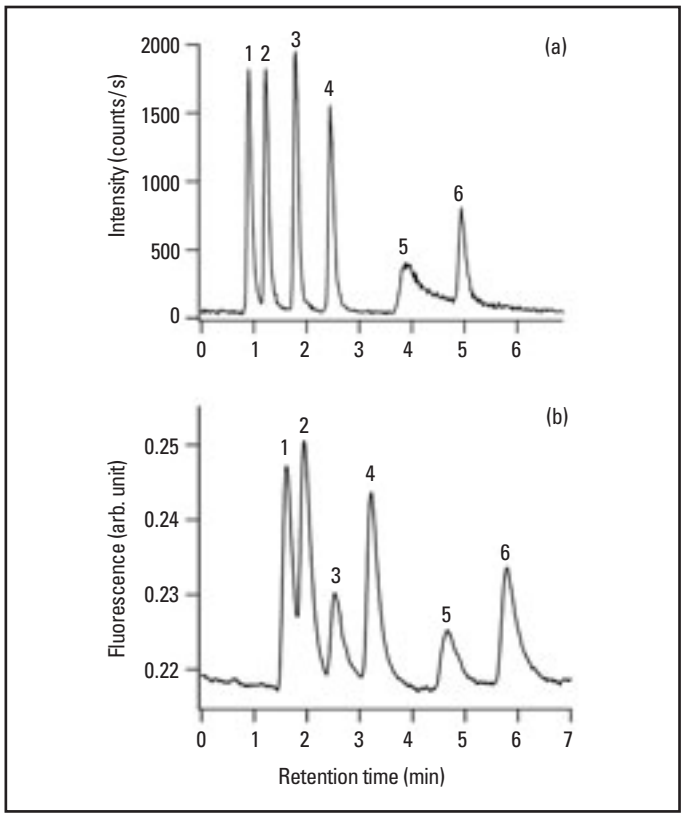


FIGURE 3. (a) ICPMS vs (b) HGAFS.

Chromatograms showing speciation analyses of As^{III}, MMA^{III}, DMA^{V}, MMA^{V}, DMA^{III}, and As^{V} in deionized water. Separation was performed on a 15 cm × 4.6 mm, 3-µm ODS-3 column maintained at 50 °C. The mobile phase contained 5 mM tetrabutylammonium hydroxide, 3 mM malonic acid, and 5% methanol at pH 5.9 at a flow rate of 1.2 mL/min. Peaks: 1, As^{III}; 2, MMA^{III}; 3, DMA^{V}; 4, MMA^{V}; 5, DMA^{III}; and 6, As^{V}. The concentration of each arsenic species was 10 µg/L, and the injection volume was 20 µL. (Adapted from Ref. 32.)

arsenosugar ingestion, which is helpful for interpreting exposure data.

Inorganic arsenic

The arsenic species ingested from drinking water are mainly inorganic arsenate (As^{V}) and arsenite (As^{III}). Biomethylation is the major metabolic process for inorganic arsenic (*27–30*). The stepwise methylation process is believed to involve a sequence of a two-electron reduction of arsenic followed by the oxidative addition of a methyl group (*31*). It is essential to determine all the metabolites to understand the entire methylation process (Figure 2). Separation of inorganic arsenicals and their metabolites can be accomplished with ion-pairing chromatography. By using the ion-pairing agent tetrabutylammonium hydroxide and a malonic acid buffer at pH 5.9, the six target arsenic species (As^{V} through DMA^{III}) can be resolved on a C_{18} column (Figure 3). Whereas anion exchange can separate As^{III}, DMA^{V}, monomethylarsonic acid (MMA^{V}), and As^{V}, ion-pairing chromatography enables further separation of two additional trivalent methylation metabolites, MMA^{III} and DMA^{III} (*32*).

Figure 3 shows that both ICPMS and hydride generation-atomic fluorescence spectroscopy (HG-AFS) provide excellent detection for the six species involved in metabolism of inorganic arsenic. Detection limits are 0.5–2 µg/L, which are sufficient for speciation in urine samples from the general population (*4*, *7*). Although HPLC/ICPMS is the best available technique for trace element speciation, HPLC/HG-AFS is an inexpensive alternative for arsenicals that are amenable to HG. AFS normally suffers from high spectral background when a liquid sample is directly sprayed to the instrument. By using HG, arsenic species in the solution are converted to gaseous hydrides, thereby dramatically reducing spectral interference. The efficiency of sample introduction is also increased. By using liquid nebulization, efficiency is typically 3–5%, and for HG, it is almost 100%, which results in an improvement in the detection limit by 2 orders of magnitude. For the six arsenicals that readily form hydride upon reacting with borohydride, both HG-AFS and ICPMS offer comparable detection limits.

HPLC/HG-AFS and HPLC/ICPMS studies discovered MMA^{III} and DMA^{III} in samples from people who were exposed to high levels of arsenic in drinking water (*33*). Subsequently, two complementary techniques making use of HPLC/ICPMS and selective HG-GC as well as atomic absorption also confirmed the presence of MMA^{III} and DMA^{III} in human urine collected from individuals in India (*34*) and Mexico (*35*) who were ex-

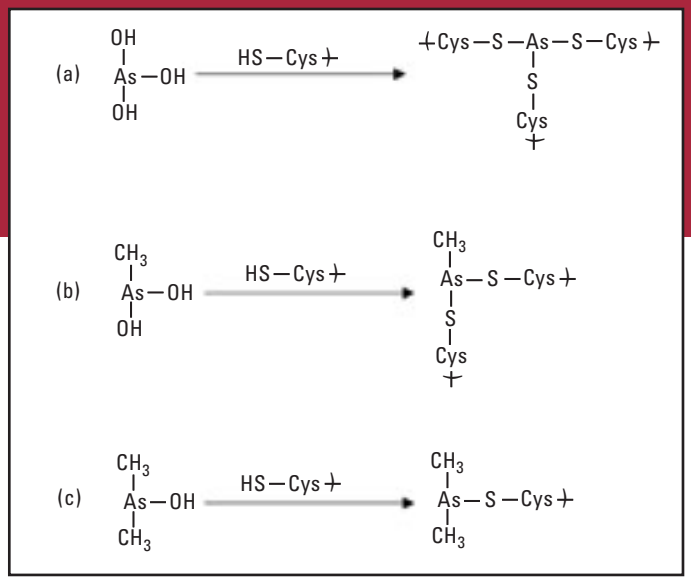


FIGURE 4. The binding stoichiometry between MT and (a) As^{III}, (b) MMA^{III}, and (c) DMA^{III}. HS–Cys+ represents a cysteine residue in MT. (Adapted from Ref. 39.)

posed to high levels of arsenic in drinking water. DMA^{III} was also detected in urine from rats administered with DMA^{V} (*36*).

Previously, only As^{III}, As^{V}, MMA^{V}, and DMA^{V} were commonly determined because of a lack of analytical techniques to measure MMA^{III} and DMA^{III}. Finding MMA^{III} and DMA^{III} provides direct evidence in support of the arsenic biomethylation pathway (Figure 2). Because MMA^{V} and DMA^{V} are less toxic than inorganic arsenic, methylation was previously believed to be a detoxification process. However, this notion was based on an earlier, incomplete understanding that did not take into account the intermediate trivalent metabolites, MMA^{III} and DMA^{III}. Recent studies of human cytotoxicity of As^{III}, MMA^{III}, and DMA^{III} (*9*); acute toxicity in hamsters (*10*); enzyme inhibition (*10–12*); and potent ability to cause DNA damage (*13*) have shown that MMA^{III} and DMA^{III} are at least as toxic as, if not more so than, inorganic arsenic species. These new findings challenge the conventional wisdom of arsenic toxicity with regard to biomethylation, which may actually be an activation process.

Understanding arsenic's health effects

Although arsenic in drinking water affects millions of people worldwide, the physiological mechanisms are not known (*4*, *30*, *37*, *38*). Understanding arsenic binding to proteins may provide useful insight. HPLC/ICPMS of reaction mixtures of trivalent arsenicals and metallothionein (MT) demonstrated the formation of complexes. Analysis of the complexes using ES quadrupole TOF tandem MS revealed the detailed binding stoichiometry between arsenic and the 20 cysteine residues in the MT molecule (*39*). Inorganic As^{III} is able to bind to three thiol groups, forming $As(–Cys)_3$. Therefore, up to six As^{III} could be bound to a single MT. MMA^{III} $[CH_3As(OH)_2]$ could bind with two thiols. DMA^{III} $[(CH_3)_2AsOH]$ could bind to only one thiol group. Therefore, up to six As^{III}, 10 MMA^{III}, or 20 DMA^{III} could bind to each MT when As^{III}, MMA^{III}, and DMA^{III} are in excess (Figure 4).

Figure 5 shows typical ES mass spectra of reaction mixtures that contain a constant concentration of MT (7 μM) and increasing concentrations of As^{III} (0.35–140 μM). The numbers on the peaks represent the number of arsenic atoms bound to a single MT. At a low concentration of As^{III} relative to MT (Figure 5a), the unbound apo-MT ions, *m/z* 1226.2 carrying a charge of 5^+ and *m/z* 1532.5 carrying a charge of 4^+, are the dominant species. A complex peak corresponding to the apo-MT bound with one arsenic (peak 1) (*m/z* 1240.7, 5^+ and *m/z* 1550.8, 4^+) is also observed. When the As^{III} concentration is equal to the concentration of MT, the MT-As (peak 1), MT-As_2 (peak 2), and MT-As_3 (peak 3) complexes become the major species (Figure 5b). In the presence of a 5-fold excess of As^{III} over MT, MT-As_5 and MT-As_6 complexes are formed (Figure 5c). Increasing As^{III} to an As:MT of 20:1 results in the formation of the MT-As_6 complex as the dominant species (Figure 5d). The maximum number of arsenic atoms bound to MT is six.

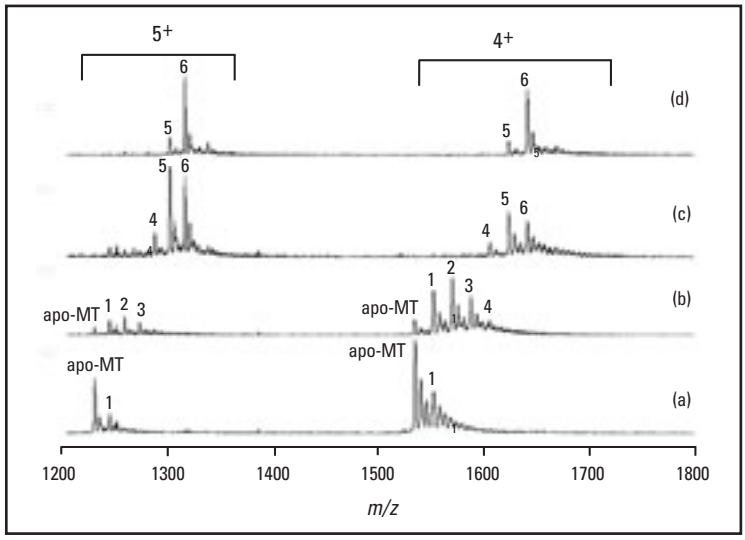


FIGURE 5. ES mass spectra of solutions containing 7 μM MT and As^{III} (0.35, 7, 35, and 140 μM).

MT and As^{III} in deionized water were incubated at room temperature for 2 h. The solution was diluted with 50% methanol and acidified with formic acid to pH 2.0 immediately prior to analysis. The peaks labeled with numbers are complexes of MT and As^{III}; the numbers on the peaks represent the number of As^{III} bound to the MT molecule. For example, peak 6 represents MT-As_6. The ratios of As^{III} to MT are (a) 1:5, (b) 1:1, (c) 5:1, and (d) 20:1. (Adapted from Ref. 39.)

A further increase of As^{III} to an As:MT of 100:1 did not show the formation of an MT complex with more than six As^{III} moieties. MT contains 20 cysteine residues. Because each As^{III} is able to bind with three cysteines, the maximum number of As^{III} that can be bound on a MT molecule is six. Results in Figure 5 show the expected binding stoichiometry, consistent with arsenic coordination chemistry (Figure 4).

Figure 6 shows ES mass spectra of MMA^{III} binding with MT, confirming the arsenic-binding stoichiometry. At a

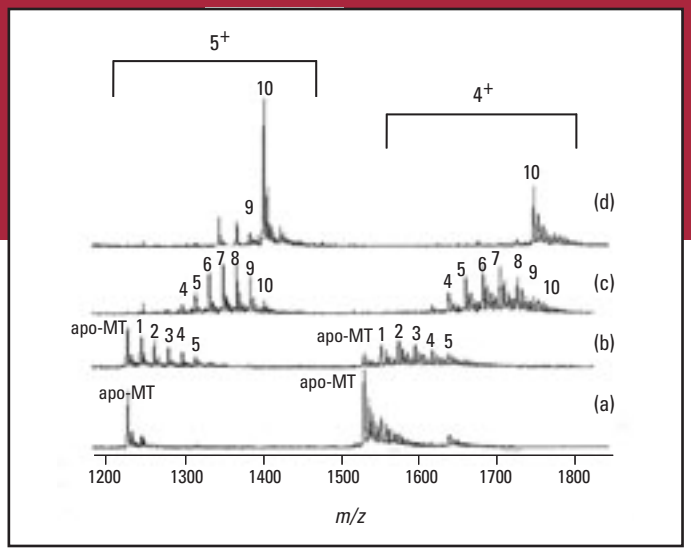


FIGURE 6. ES mass spectra of solutions containing 7 μM MT and MMA^{III} (0.35, 7, 35, and 350 μM).

MT and MMA^{III} in deionized water were incubated at room temperature for 2 h. The solution was diluted with 50% methanol and acidified with formic acid to pH 2.0 immediately prior to analysis. The peaks labeled with numbers are complexes of MT and MMA^{III}; the numbers represent the number of MMA^{III} bound to the MT molecule. For example, peak 6 represents MT-$(AsCH_3)_6$. The ratios of MMA^{III} to MT are (a) 1:5, (b) 1:1, (c) 5:1, and (d) 50:1. (Adapted from Ref. 39.)

MMA^{III}:MT of 1:1, MT-$AsCH_3$, MT-$(AsCH_3)_2$, MT-$(AsCH_3)_3$, MT-$(AsCH_3)_4$, and MT-$(AsCH_3)_5$ are observed (Figure 6b). Increasing the MMA^{III} concentration to a 5-fold excess over MT leads to the binding of a maximum of 10 MMA^{III} moieties on the MT, MT-$(AsCH_3)_{10}$ (Figure 6c). With a 50-fold excess of MMA^{III} over MT, the dominant complex species is MT-$(AsCH_3)_{10}$ (Figure 6d). The binding of a maximum of 10 MMA^{III} with MT is the expected binding stoichiometry in the presence of excess MMA^{III} (Figure 4). Similarly, the binding of DMA^{III} with MT resulted in complex peaks corresponding to MT-$[As(CH_3)_2]_n$, where n is 1–20. The maximum number of $As(CH_3)_2$ binding with MT was 20 in the presence of excess DMA^{III} over MT. This end result is understandable because each $As(CH_3)_2OH$ is able to bind with 1 cysteine, and there are 20 cysteines in the MT.

HPLC/ICPMS and ES MS enabled the characterization of As^{III}, MMA^{III}, and DMA^{III} binding with sulfhydryl groups in MT. Data from accurate mass measurements of the species and analysis of fragment ions of MT-As species using tandem MS supported the assignments of the mass spectral peaks. Arsenic inhibits several enzymes, such as glutathione reductase (*11*), thioredoxin reductase (*12*), and pyruvate dehydrogenase (*10*). Arsenic binding with proteins via sulfhydryl groups may be responsible for the observed effects of arsenic on these proteins.

The interaction of arsenic with MT may serve as a model for the interaction of arsenic with other cellular proteins, including DNA repair proteins. Some evidence shows that the trivalent arsenic species cause oxidative damage to DNA (*13*, *40*). Electron spin resonance with spin trapping has detected free radicals produced in cells incubated with As^{III} (*41*). Effects of certain arsenic species on the DNA repair process have been reported (*42*, *43*), and the trivalent arsenic species may alter the conformation and function of DNA repair proteins. Arsenic effects on cell proliferation and gene expression have also been examined (*15*, *16*, *30*, *38*, *44*–*47*).

Although As^{III} is widely known as a poison and a cancer-causing agent, it has also been successfully used as a chemotherapeutic agent to treat acute promyelocytic leukemia (APL) (*47*). This treatment typically involves repeated injections of a saline solution of ~7.4 mg arsenic (10 mg As_2O_3)/day for about a month. It is now used worldwide to treat APL patients who have relapsed since undergoing other primary therapy. Although As_2O_3 is usually named as the therapeutic agent, the actual arsenic species injected is probably arsenious acid (As^{III}) because of the dissolution of As_2O_3 in water. Researchers are currently conducting more than 20 clinical trials in the United States to evaluate the use of As^{III} for the treatment of other types of cancers. Because MMA^{III} and DMA^{III} are more toxic than As^{III}, determining whether they would be more effective chemotherapeutics than As^{III} is a topic of interest. However, the development of alternative trivalent arsenic compounds would be necessary because MMA^{III} and DMA^{III} are unstable in aqueous solutions (*48*).

Challenges to be met

Stability of chemical species during sample handling and storage is a prerequisite to obtaining reliable results, especially for speciation analysis. Contamination must be prevented, and loss of trace analytes must be minimized. Traditionally, samples are acidified to reduce potential adsorption of trace elements onto the sample container surface; however, acidification can alter arsenic speciation. Another problem is that the trivalent arsenic methylation metabolites, MMA^{III} and DMA^{III}, are readily oxidized (*48*). Although using preservatives and low temperatures may extend the stability of these species, developing on-site and in situ analytical methods would be most useful.

Standard reference materials (SRMs) are necessary for the accuracy and reliability of analytical results (*49*). However, most SRMs for trace element analysis are only certified for the total concentration of the element. Very limited information is available on the identity and concentration of specific chemical species in only a few SRMs. Standards organizations need to develop SRMs for speciation analyses. Species stability during sample preparation, storage, and distribution should be a priority to further assess interconversion of toxic arsenic species.

Analysis of arsenic speciation in solid samples, such as biological tissues, generally requires a quantitative extraction into solution without altering the original speciation. Sample dissolution using acid digestion is not suitable because it changes arsenic speciation. Milder extraction by physical shaking or sonication using water/methanol mixtures usually does not alter speciation, but in many samples, not all the arsenic is extracted. Extraction in combination with enzyme digestion using various proteases, lipases, and cellulases has shown some promise. Sequential, accelerated solvent, and microwave-assisted extraction techniques have also improved extraction efficiency (*50*). However, extraction efficiency for arsenic in some materials, such as




seaweed, remains low and is not quantitative (*50*). A need still exists to develop methods for extracting arsenic species from food, soil, and biological tissues.

Although the mechanisms of action responsible for various toxicities of arsenic are not clear, trivalent arsenicals probably exert some toxic effects by binding with proteins. However, no specific or unique arsenic-binding or arsenic-containing protein has been characterized. Studies of arsenic interaction with proteins in vitro and speciation of arsenic-binding proteins in vivo will certainly provide new insights to arsenic toxicology.

Arsenic species also interact with chemical species of other trace elements, such as selenium. Arsenic and selenium have been found to counteract the toxicity of each other, although the mechanisms for this antagonistic effect are not clear. Possible changes of arsenic and selenium species and the processes responsible for such changes are crucial to a better understanding of the metabolism and interactions. Techniques are needed for the simultaneous speciation of arsenic and selenium, particularly for identifying co-binding with other biomolecules.

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