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Origins, fate, and actions of methylated trivalent metabolites of inorganic arsenic: progress and prospects

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Abstract

The toxic metalloid inorganic arsenic (iAs) is widely distributed in the environment. Chronic exposure to iAs from environmental sources has been linked to a variety of human diseases. Methylation of iAs is the primary pathway for metabolism of iAs. In humans, methylation of iAs is catalyzed by arsenic (+ 3 oxidation state) methyltransferase (AS3MT). Conversion of iAs to mono- and di-methylated species (MAs and DMAs) detoxifies iAs by increasing the rate of whole body clearance of arsenic. Interindividual differences in iAs metabolism play key roles in pathogenesis of and susceptibility to a range of disease outcomes associated with iAs exposure. These adverse health effects are in part associated with the production of methylated trivalent arsenic species, methylarsonous acid (MAs^{III}) and dimethylarsinous acid (DMAs^{III}), during AS3MT-catalyzed methylation of iAs. The formation of these metabolites activates iAs to unique forms that cause disease initiation and progression. Taken together, the current evidence suggests that methylation of iAs is a pathway for detoxification and for activation of the metalloid. Beyond this general understanding of the consequences of iAs methylation, many questions remain unanswered. Our knowledge of metabolic targets for MAs^{III} and DMAs^{III} in human cells and mechanisms for interactions between these arsenicals and targets is incomplete. Development of novel analytical methods for quantitation of MAs^{III} and DMAs^{III} in biological samples promises to address some of these gaps. Here, we summarize current knowledge of the enzymatic basis of MAs^{III} and DMAs^{III} formation, the toxic actions of these metabolites, and methods available for their detection and quantification in biomatrices. Major knowledge gaps and future research directions are also discussed.

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Keywords

Arsenic; Methylarsonous acid; Dimethylarsinous acid; Metabolism; Toxicity; Analysis

Introduction

As organisms evolved in the iAs-rich environment of the primordial sea, enzymatically catalyzed methylation of inorganic arsenic (iAs) developed as a mechanism to cope with the presence of this toxic metalloid (Zhu et al. 2014). Although other mechanisms mediate iAs detoxification in other branches of the tree of life, iAs methylation plays a dominant role in detoxification of iAs in complex organisms, including mammals. Methylation of iAs produces monomethylated (MAs), dimethylated (DMAs) and trimethylated (TMAs) arsenicals that contain either pentavalent arsenic (As^{V}) or trivalent arsenic (As^{III}). Thus, in humans and many other mammals, exposure to iAs is associated with excretion of iAs and methylated arsenicals in urine (Thomas et al. 2001, 2007). Based on studies in microorganisms, a pathway for production of pentavalent and trivalent methylated As species during iAs methylation was first postulated by Challenger (Challenger 1947). Since then, the presence of MAs^{III} , MAs^{V} , DMAs^{III} , DMAs^{V} , TMA^{III} , and trinethylarsine oxide (TMA^{VO}) has been confirmed in in vitro and animal models used in laboratory studies, as well as in urine and tissue samples collected in human cohorts.

The classification of iAs methylation as a process for detoxification of iAs is based on the fact that the methylated metabolites of iAs are rapidly excreted from the body and that deficiencies in mechanisms responsible for the formation of these metabolites are associated with the accumulation of iAs in tissues and with enhanced toxicity (Chen et al. 2011; Douillet et al. 2017; Drobna et al. 2009; Huang et al. 2018a, 2018b; Thomas et al. 2001; Vahter and Concha 2001; Yokohira et al. 2010, 2011). In addition, the methylated pentavalent arsenicals (MAs^{V} , DMAs^{V} , and TMA^{VO}) are less acutely toxic than iAs species that are typically associated with environmental exposures, trivalent arsenite (iAs^{III}) and pentavalent arsenate (iAs^{V}) (Sarkar and Paul 2016; Vahter 2002; Vahter and Concha 2001). However, many laboratory studies show that methylated metabolites of iAs containing As^{III} , specifically MAs^{III} and DMAs^{III} , are significantly more toxic than their pentavalent counterparts and are also more toxic than either iAs^{V} or iAs^{III} . The first side-by-side comparison of the toxicities of trivalent and pentavalent iAs and methylated arsenicals in primary human and animal cells and cell lines derived from tissues that are targets for iAs exposure found that MAs^{III} to be the most toxic arsenical in all cell types (Stýblo et al. 2000). Furthermore, DMAs^{III} is as toxic as iAs^{III} in some of these cell types. Additional work from this and other laboratories has identified MAs^{III} and DMAs^{III} as the most toxic and reactive metabolites in the pathway for iAs methylation, suggesting that, although methylation promotes clearance of As from the body, the formation of these metabolites also contributes to the adverse effects associated with iAs exposure.

Laboratory research of the metabolism and biological effects of the methylated trivalent arsenicals has been historically hampered by limited availability of pure and stable MAs^{III} and DMAs^{III} . Until recently, these compounds were chemically synthesized only in a few

specialized academic laboratories. Among these, the laboratories of Dr. William Cullen (University of British Columbia) and Dr. Chris Le (University of Alberta) have been the main sources of MAs^{III} and DMAs^{III} for research laboratories worldwide. These laboratories have played a key role in supporting the toxicological research of MAs^{III} and DMAs^{III} . The supply of pure methylated trivalent arsenicals from these laboratories also supported development of novel methods for chemical analysis of MAs^{III} and DMAs^{III} . These methods allow for differential, oxidation state specific analysis of iAs and its methylated metabolites in many biological matrices. However, the rapid oxidation of MAs^{III} and DMAs^{III} to MAs^{V} and DMAs^{V} in oxic conditions remains a major challenge in determination of arsenic valency in samples collected in laboratory or field studies.

The goals of this review are to summarize information on mechanisms and functions of the enzymatic methylation of iAs, to examine evidence on formation and toxic effects of MAs^{III} and DMAs^{III} in biological systems, and to evaluate methods for the analysis of these arsenicals in biological matrices. Knowledge gaps and future research directions are also discussed.

Origins and mechanisms of enzymatically catalyzed methylation of iAs

Enzymatically catalyzed methylation of iAs is one of several adaptations that allows cells to thrive in environments that contain iAs. The role of iAs methylation is best understood in the context of all processes involved in arsenic metabolism at the cellular level. Figure 1 is a conceptual model for cellular metabolism of arsenic that includes pathways for entry and exit of this metalloid from the cell and pathways for chemical transformations of arsenic atoms that occur during their residence in the cell (Thomas 2007). Over the past several decades, research has characterized the molecular machinery involved in these processes and identified the genes that encode relevant transporters and enzymes. Thus, it is now possible to relate steps in cellular arsenic metabolism to functions of specific gene products and to explore phylogenetic relations among these genes as they appear in organism in all branches of the tree of life. As part of our consideration of the roles for arsenic methyltransferases, we begin with an overview of the biochemical processes that contribute to the cellular metabolism of arsenic.

Origins of iAs methylation

Nearly four billion years ago, primitive organisms in the primordial ocean adapted to the presence of iAs by acquisition of an array of genes that encode: (a) transmembrane transporters that facilitated arsenic fluxes, (b) oxidases and reductases that catalyzed reactions that altered the valence state of arsenic, and (c) methyltransferases that catalyzed reactions that coupled methyl groups to an arsenic atom (Zhu et al. 2014). High water temperature, low pH, and anoxic conditions produced higher levels of iAs^{III} in the primordial ocean. Primitive cells limited accumulation of iAs through development of transmembrane proteins that facilitated iAs^{III} efflux. Rising atmospheric oxygen levels during the Great Oxidation Event starting about 2.4 billion years ago oxidized iAs^{III} in the primordial ocean and increased levels of iAs^{V} . Because transmembrane transporters were selective for iAs^{III} efflux, cells acquired iAs^{V} reductases that catalyzed reduction of

iAs^V to iAs^{III} . The integration of existing pathways for detoxification of trivalent arsenicals with newly developed processes such as arsenate reduction provided additional protection of organisms against arsenic under a wider array of environmental conditions (Chen et al. 2020). Similarly, the transfer of genes encoding methyltransferases that catalyzed addition of methyl groups to an arsenic atom has been interpreted as a critical step in the adaptation of organisms to the presence of arsenic in the environment that facilitated the spread of organisms into arsenic-rich environments during the late Archean Eon and Proterozoic Eon (Chen et al. 2017a, b). In a broader context, repeated independent horizontal gene transfers among bacteria and from bacteria to eukaryotes provided organisms with the molecular machinery needed for tolerance to iAs present in the environment (Palmgren et al. 2017).

A global survey of soil microbiomes found a high frequency of occurrence of genes encoding an arsenic methyltransferase, suggesting an important role for arsenic methylation in the biogeochemical cycle for arsenic (Dunivin et al. 2019). Methylation of iAs by soil microbes likely fulfills multiple roles in the adaptation of life to environments contaminated with this toxic metalloid. For some microorganisms, production of monomethylarsonic acid (MAs^V) may act as an antibiotic and confer a selective advantage against competing microbial species (Li et al. 2016; Chen et al. 2019; Garbinski et al. 2020). In other species, formation of trimethylarsine (TMA_s^{III}), a volatile species that is released into the environment, not only reduces the cellular burden of arsenic but also contributes to global cycling of arsenic (Huang et al. 2016; Chen et al. 2014). The widespread occurrence of the capacity to form methylated metabolites of iAs in higher organisms, including humans, makes understanding the molecular basis of arsenic methylation central to understanding the risk associated with long-term exposure to iAs .

Enzymatic basis of iAs methylation

Studies in microorganisms in the nineteenth century suggested that methylated arsenicals were products of iAs metabolism (Cullen 2008). Continuing work on iAs methylation in microorganisms by Frederick Challenger and his associates led to a postulated reaction scheme in which reduction of pentavalent arsenic to trivalency is followed by an oxidative methylation reaction that produces a methylated product containing pentavalent arsenic atom (Challenger 1947, 1951). As shown in Fig. 2, the Challenger scheme is a sequence of reactions in which reduction of arsenic from pentavalency to trivalency alternates with oxidation methylation of an arsenic atom.

The Challenger scheme for iAs methylation significantly influenced the study of biomethylation processes because it made specific predictions about the formation of intermediates that contained either As^V or As^{III} and focused attention on development of analytical methods that used the oxidation state of arsenic as a discriminating factor in the separation and quantitation of arsenicals in environmental and biological materials. The postulated existence of methylated intermediates containing arsenic in either valence state also led to extensive studies of the effect of arsenic's valency in methylated arsenicals on the toxic potency of these compounds. Finally, the Challenger scheme also served as a framework for interpretation of data on putative mechanisms by which arsenic methyltransferase catalyzes reactions that methylate arsenic atoms. Evaluating putative

mechanisms for enzymatic catalysis in the context of the Challenger scheme also helped to identify chemically plausible pathways for methylation (Cullen 2014). As discussed below, recent evidence suggests that a pathway for methylation that does not involve obligatory oxidation of arsenic may be sufficient to describe the sequence of reactions catalyzed by arsenic methyltransferases.

Isolation and characterization of an arsenic methyltransferase from a mammalian source originates in early work that demonstrated that methylation of iAs in rat liver cytosol in an in vitro system is supported by an addition of glutathione (GSH) and S-adenosyl-L-methionine (AdoMet) (Buchet and Lauwerys 1985; Hirata et al. 1989). Later studies of iAs methylation in rat liver cytosol in an in vitro system characterized concentration dependencies for GSH and AdoMet in arsenic methylation reactions and showed that inhibitors of other non-DNA methyltransferases did not affect iAs methylation in this in vitro system, suggesting the reaction was catalyzed by a novel methyltransferase (Styblo et al. 1996). Because a simple in vitro system containing rat liver cytosol rapidly converts iAs to methylated species, purification of arsenic methyltransferase used rat liver as the starting material (Lin et al. 2002). This effort resulted in the identification of a ~43 kDa protein that catalyzed AdoMet-dependent methylation of substrates containing trivalent arsenic (iAs^{III} or MAs^{III}). This methyltransferase was initially identified as the product of the previously annotated *cyt19* gene. Following its cloning, the gene was annotated as Arsenic (+ 3 oxidation state) MethylTransferase (*AS3MT*) (Walton et al. 2003; <https://www.genenames.org>). Characterization of rat liver *As3mt* led to identification of genes encoding closely related proteins in genomes of higher organisms ranging from sea squirts to humans (Thomas et al. 2007). Notably, *As3mt* orthologs in many higher organisms share a suite of conserved cysteine residues and contain sequence motifs that are common in methyltransferases that catalyze reactions that methylate proteins or small molecules. Early work showed that *ArsM* genes in microorganisms are orthologs of *As3mt* gene found in higher organisms and that expression of *ArsM* genes in these organisms is associated with capacity to form methylated products from iAs and confers resistance to iAs-induced cytotoxicity (Qin et al. 2006).

Common features of arsenic methyltransferases

Our understanding of molecular aspects of enzyme-catalyzed iAs methylation derives from studies with products of both the mammalian *As3mt* and *ArsM* genes that encode a large family of iAs^{III} methyltransferases in archaea, bacteria, fungi, algae, and plants. From studies with these proteins, it has been possible to identify residues critical for catalysis, to elucidate the mechanisms used in methylation, and to explore the role of oxidation–reduction reactions in arsenic methylation.

Function of conserved cysteinyl residues

The predicted amino acid sequences of arsenic methyltransferases from *As3mt* and *ArsM* gene families contain motifs commonly found in non-DNA methyltransferase (Thomas and Rosen 2013). In particular, locations of cysteinyl residues in these protein sequences are strongly conserved. The role of the conserved cysteinyl residues in catalytic function

has been evaluated by their replacement with other amino acids. In recombinant rat As3mt, replacement of the conserved cysteinyl at position 156 (C156) with serine (C156S) eliminates its capacity to catalyze iAs^{III} methylation (Li et al. 2005). Systematic replacement of four conserved cysteinyl residues in human AS3MT (C150, C206, C226, C250) with serine affects structure and function of the modified proteins (Song et al. 2009). The secondary structures of C156S, C206S, and C250S mutant proteins differ from that of wild-type protein and are associated with a loss of capacity to catalyze iAs^{III} methylation. In contrast, the secondary structure and catalytic capacity of the C226S mutant do not differ from those of the wild-type protein. Functions of other cysteinyl residues in human AS3MT have been examined (Song et al. 2011). The C72S mutant protein display structural changes and do not catalyze iAs^{III} methylation. Structural changes in C334S and C360S mutant proteins lead slower turnover rates, suggesting that structural modifications affect efficiency of methylation reactions. Other cysteine to serine mutants (C32S and C61S) that do not catalyze iAs^{III} methylation could methylate MAs^{III} (Li et al. 2013a, b). By comparison, replacement of C156 or C206 lead to a complete loss of catalytic activity. Altered catalysis by these mutants provides evidence on the location and number of cysteinyl residues that bind iAs^{III} and MAs^{III} acid to the enzyme. Formation of critical disulfide bonds in human AS3MT affects its catalytic function (Wang et al. 2014). Reduction of a C250–C32 disulfide bond before iAs^{III} methylation alters the enzyme's secondary structure. Thus, formation and cleavage of disulfide bonds between critical cysteinyl residues affects the binding of arsenicals to the active site and changes the secondary structure around the active site.

Additional insights into the role of cysteinyl residues in the structure and function of arsenic methyltransferases come from studies with ArsM. In particular, determination of the 3D structure of CmArsM from the thermophilic eukaryotic alga *Cyanidioschyzon merolae* sp. 5508 (CmArsM) informs our understanding of the relation between enzyme structure and function for both ArsM and As3mt arsenic methyltransferases (Ajees et al. 2012). Replacement of any of the conserved cysteinyl residues (C72, C174, and C224) in CrArsM abolishes capacity to methylate iAs^{III}; however, the replacement of C72 yields an enzyme that can methylate MAs^{III} (Marapakala et al. 2012). Studies of the binding of aromatic organic arsenicals phenylarsenite and reduced roxarsone that contain trivalent arsenic to CrArsM show that both bound to thiol moieties in C174 and C224, suggesting that MAs^{III} would also be held in a bidentate structure with these residues. The structure of the enzyme-organic arsenical complex includes a C44 as a conserved cysteinyl residue that forms a disulfide bond with C72 (Marapakala et al. 2015). As discussed below, formation of the C44–C72 disulfide bond may play a crucial role in binding of substrates to the enzyme.

The relation between conservation of cysteinyl residues and substrate specificity in arsenic methyltransferases has been examined using ArsM from the fungus *Aspergillus fumigatus* (Chen et al. 2017a, b). Although ArsM genes from most organisms encode four conserved cysteinyl residues into an enzyme that accepts either iAs^{III} or MAs^{III} as a substrate, the four *arsM* genes in the *Aspergillus fumigatus* genome encode ArsMs that conserves only the second, third and fourth cysteinyl residues. This loss affects substrate specificity of the gene products. For example, MAs^{III} but not iAs^{III} is a substrate for methylation by the fungal ArsM1. Existence of arsenic methyltransferases that discriminates among substrates could reflect different selective pressures that fine-tune substrate specificity and the central role

of cysteinyl residues in control of enzyme function. The search for the minimal number of cysteinyl residues required for its function as an arsenic methyltransferase has been extended using BIArSM from *Bacillus* sp. CX-1, an arsenic methyltransferase with low sequence similarities (39%) to other ArsMs (Huang et al. 2018). Only two cysteinyl residues in this enzyme must be conserved for catalytic function; replacement of four of the six cysteinyl residues produces an enzyme that uses either iAs^{III} or MAs^{III} as substrates. The availability of these structurally diverse enzymes with similar catalytic functions should aid in understanding the role of cysteinyl residues in arsenic methyltransferases.

Mechanisms for arsenic methylation

Evaluating the role of cysteinyl residues in the catalytic function of arsenic methyltransferases provides insights into molecular processes involved in binding of reactants to the enzyme and transfer of a methyl group from AdoMet to an arsenic atom. Early work showed that endogenous reductants (e.g., thioredoxin/thioredoxin reductase/NADPH) support the activity of recombinant rat As3mt and that addition of GSH to reaction mixtures increases the rate of iAs^{III} methylation (Waters et al. 2004). The same effect of GSH on the rate of iAs^{III} methylation is observed in studies with recombinant human AS3MT and the common polymorph AS3MT(M287T) (Ding et al. 2012). Additional support for the role of GSH in the AS3MT-catalyzed methylation of iAs^{III} comes from a report that a complex of iAs^{III} with GSH (i.e., iAs^{III} -triglutathione) is the preferred substrate for this enzyme (Hayakawa et al. 2005). Notably, studies of protein fluorescence found that rates of binding of iAs^{III} or MAs^{III} to the active site of CmArSM increases in the presence of GSH (Marapakala et al. 2012). A role for arsenic-GSH complexes as substrates for methylation reactions is consistent with the presence of higher levels of GSH in cellular environments and the facile in vivo formation of arsenic-GSH complexes (Delnomdedieu et al. 1995).

Analysis of the order of binding of iAs^{III} and AdoMet to arsenic methyltransferases found that AdoMet is the substrate first bound to human AS3MT; this binding is associated with conformational changes (Wang et al. 2012). In CmArSM, AdoMet binding also changes protein conformation and positions this methyl donor for transfer to arsenic (Ajees et al. 2012). Mutational analysis of residues in human AS3MT demonstrates that a hydrogen bond network around the AdoMet binding site is required for catalytic activity (Li et al. 2013a).

Enzymatic catalysis and oxidation–reduction cycling

Information about the role of critical residues in the binding of substrates, changes in protein conformation, and structural data can be integrated to develop a model for enzyme-catalyzed methyl group transfer from AdoMet to arsenic. The current model builds on a scheme proposed less than a decade ago (Dheeman et al. 2014) that has been extended by studies with enzymes from diverse sources. Analysis of CmArSM identifies a structure in which iAs^{III} is 3-coordinately bound to conserved cysteinyl residues and the AdoMet binding site is occupied (Packianathan et al. 2018a). Here, AdoMet-induced conformational changes bring C44 close to C174 and C224 and permits 3-coordinate binding of iAs^{III} to cysteinyl residues. In this state, iAs^{III} is bound in proximity to AdoMet's methyl group; this conformation facilitates transfer of the methyl group to the trivalent arsenic atom. Notably,

the bound product of the first methylation step is MAs^{III} which can be the substrate for addition of another methyl group to the trivalent arsenic atom. Kinetic analysis shows that the relatively slower rate of the second methylation step reflects the slow reorientation of the AdoMet binding site to its initial position (Packianathan et al. 2018b). Upon reorientation, binding of AdoMet to this site starts the sequence steps that convert MAs^{III} to DMAs^{III} .

The alternate scheme for enzymatically catalyzed methylation of arsenic shown in Fig. 3 differs materially from the Challenger scheme. It lacks an obligatory cycle of oxidation and reduction of arsenic atoms during addition of methyl groups. Current kinetic and structural studies with arsenic methyltransferases provide evidence that these enzymes avidly bind As^{III} and that the addition of a methyl group to bound arsenic does not result in its oxidation. Rather, use of a disulfide bond cascade using conserved cysteinyl residues functions to maintain bound arsenic as a trivalent species. In this model, the appearance of methylated arsenicals containing As^{V} is a secondary process, requiring the oxidation of the As^{III} in methylated species that are the intermediates in enzymatically catalyzed methylation. Thus, the current model is sufficient to account for products containing As^{III} or As^{V} without redox cycling of arsenic required by the Challenger scheme. The lack of redox cycling of arsenic during the methylation process eliminates the need for an additional reduction step in the pathway. The reduction of As^{V} had been posited to depend on endogenous reductants (e.g., GSH) or unique reductases that catalyze this step (Delnomdedieu et al. 1995; Zakharyan and Aposhian 1999; Zakharyan et al. 2001). If catalysis by arsenic methyltransferases does not generate intermediates that contain As^{V} , it is likely that the presence of these species is a result of spontaneous oxidation of the trivalent intermediates formed during the enzymatic reaction. Post synthesis oxidation of trivalent arsenic present in methylation products could account for the occurrence of methylated arsenicals containing either trivalent or pentavalent arsenic in tissues and excreta (Currier et al. 2011a, b).

Roles of enzymatic methylation in metabolism and toxicity of iAs

There is a dichotomy at the center at any discussion of the significance of iAs methylation. The dichotomy is supported by the well-documented facts that: (a) conversion of iAs into methylated metabolites accelerates the rate of whole body clearance of arsenic, and (b) the methylated trivalent metabolites, specifically MAs^{III} and DMAs^{III} , are more reactive and toxic than inorganic arsenic. Thus, promotion of arsenic excretion through formation of methylated metabolites can be termed a detoxification process. Conversely, formation of more toxic species, MAs^{III} and DMAs^{III} , through methylation can be termed an activation or toxication process. In the following paragraphs, the dichotomous roles of iAs methylation are reviewed.

iAs methylation as a detoxification process

Studies in humans and other species illustrate the role of iAs methylation in the clearance of arsenic. Early studies in volunteers and workers who ingested or inhaled iAs found that mono- and di-methylated arsenical were quickly excreted in urine (Crecelius 1977; Smith et al. 1977). In volunteers who ingested 500 μg of arsenic as iAs^{III} , MAs^{V} , or DMAs^{V} , the urinary excretion rate for arsenic and the cumulative amount of arsenic excreted in urine

is affected by the ingested form (MAs > DMAs > iAs) (Buchet et al. 1981). The form of arsenic ingested also affects the extent of metabolism. About 75% of arsenic ingested as iAs^{III} is excreted in urine as MAs or DMAs; about 13% of ingested MAs^V is excreted as DMAs. In contrast, ingested DMAs^V is excreted in urine without additional methylation.

Further insights into the role of arsenic methylation in the clearance of arsenic come from studies that examined patterns for urinary excretion of arsenic-containing species after oral administration of mono- and di-methylated species containing either trivalent or pentavalent arsenic to female B6C3F1 mice. Orally administered MAs^V is rapidly cleared in urine with less than 10% of the administered arsenic converted to a dimethylated metabolite before excretion (Hughes et al. 2005). In contrast, MAs^{III} is highly retained in tissues; more than 90% of arsenic excreted in urine is a dimethylated metabolite. Related studies in female B6C3F1 mice that received single oral doses of DMAs^V or DMAs^{III} found that the oxidation state of arsenic affects patterns of metabolism and rates of urinary clearance (Hughes et al. 2008). After DMAs^V treatment, a rapid rise and fall in arsenic concentrations in tissues accompanies appearance of arsenic in urine. Compared to DMAs^V, treatment with DMAs^{III} produces higher levels of arsenic in tissues. Notably, treatment with either dimethylated arsenical results in the presence of TMAs^{VO} in urine, suggesting that either compound was a substrate for addition of a third methyl group. These studies demonstrated that the oxidation state of arsenic in methylated arsenicals affects the rate of whole body clearance with faster whole body clearance of arsenic after administration of MAs^V or DMAs^V. In addition, the oxidation state of arsenic in a methylated arsenical affects the likelihood that it will serve as a substrate for additional methylation reactions. For example, in mice MAs^{III} is readily converted to dimethylated product while MAs^V is not extensively metabolized. Because the increased rate of clearance of methylated arsenical species, especially those containing As^V, has a salutary effect by promoting the excretion of arsenic, methylation of iAs can be regarded as a detoxification process. Additional evidence for the detoxification function of iAs methylation has been obtained in studies using *As3mt* knockout mice. The metabolism of iAs in these mice is characterized by slow whole body clearance and accumulation of iAs in tissues (Drobna et al. 2009; Hughes et al. 2010). The *As3mt*^{-/-} mice are also more susceptible to As-induced toxicity than mice wild-type mice (Yokohira et al. 2011; Dodmane et al. 2013).

iAs methylation as an activation process

The Challenger scheme for iAs methylation that posits formation of methylated trivalent and pentavalent arsenicals has inspired many laboratory studies that compared effects of these intermediates on a wide range of biological end-points, using both in vitro and in vivo models. Attempts have also been made to assess roles of the methylated arsenicals containing As^{III} or As^V as potential mediators of adverse effects associated with iAs exposure in human populations. The following sections summarizes reports of these studies.

Cytotoxicity of methylated trivalent arsenicals

Studies of the comparative cytotoxicity of methylated arsenicals are based in part on studies of the lethality of inorganic and methylated arsenicals in rodents. In acute lethality

assays (LD₅₀), both MAs^V and DMAs^V are less toxic than iAs^V and iAs^{III} (Yamauchi and Fowler 1994). Cytotoxicity studies affirm that pentavalent methylated arsenicals, MAs^V and DMAs^V, are much less toxic for cultured cells than inorganic arsenicals. In addition, early work showed that oxidation state of arsenic affects toxicity of inorganic arsenicals; iAs^{III} was more cytotoxic than iAs^V (Fischer et al. 1985, 1989; Bertolero et al. 1987). One of the first side-by-side comparisons of the cytotoxicity of arsenicals containing As^{III} or As^V used primary and transformed cells derived from liver, a major site for iAs methylation, and from skin, lung, and urinary bladder that are targets for arsenic-induced tumors (Stýblo et al 2000). This study found that the methylated trivalent arsenicals (MAs^{III} and DMAs^{III}) are more cytotoxic than their pentavalent counterparts and that MAs^{III} is more cytotoxic than iAs^{III} or iAs^V. In addition, cytotoxicity of DMAs^{III} approaches or exceeds those of iAs^{III} and iAs^V. Other studies with these cell types also found MAs^{III} to be more cytotoxic than iAs^{III} (Dodmane et al. 2013; Hirano et al. 2004; Petrick et al. 2000; Vega et al. 2001).

The cytotoxicity of MAs^{III} has been examined in other cell types, including leukemia NB4, Jurkat, and Namalwa cells (Chen et al. 2003), differentiated 3T3-L1 adipocytes (Walton et al. 2004), rat insulinoma (INS-1) cells (Dover et al. 2018), isolated murine pancreatic islets (Douillet et al. 2013), cultured human neurons, astrocytes, microglia, and brain microvascular endothelial cells (Yoshinaga-Sakurai et al. 2020), JEG-3 placental cells (Meakin et al. 2020), human monoblastoid (U937), osteosarcoma (HOS) and neuroblastoma (SK-N-SH) cells, Chinese hamster lung (V79-4) cells (Stýblo et al. 2002). Without exception, these studies found MAs^{III} to be more cytotoxic than iAs^{III}. Notably, some of these studies found DMAs^{III} to be more cytotoxic than iAs^{III}.

Genotoxicity of methylated trivalent arsenicals

The genotoxic potential of trivalent methylated arsenicals has been assessed using in vitro systems containing naked viral (Phi 174 RF1) DNA and human peripheral lymphocytes (Mass et al. 2001). These investigators found that incubation with relatively high concentrations of MAs^{III} and DMAs^{III} caused nicks in viral DNA. In contrast, DNA nicking does not occur after incubation with iAs^{III}, iAs^V or the methylated pentavalent arsenicals. Because DNA nicking does not require addition of chemical or enzymatic activation system, MAs^{III} and DMAs^{III} likely interacts directly with DNA. Both MAs^{III} and DMAs^{III} are tenfold more potent than iAs^{III} in causing DNA damage in cultured human lymphocytes. Damage to DNA by MAs^{III} and/or DMAs^{III} has also been reported in other studies. Although micromolar concentrations of MAs^{III} or DMAs^{III} induce oxidative DNA damage in cultured HeLa cells, DMAs^{III} alone causes DNA breaks in supercoiled and circular PM2 DNA (Schwerdtle et al. 2003). MAs^{III} also significantly increases micronuclei frequency in human peripheral lymphocytes, suggesting aneuploidogenic properties (Colognato et al. 2007).

The potential of methylated trivalent arsenicals to induce chromosomal aberrations, mutagenicity, sister chromatid exchange (SCE), and prophage inductions has also been examined (Kligerman et al. 2003). Both MAs^{III} and DMAs^{III} induce chromosomal aberrations and are potent clastogens in human lymphocytes and mutagens in mouse lymphoma cells. Neither MAs^{III} nor DMAs^{III} are potent SCE or prophage inducer. These

findings suggest that DMAs^{III} and MAs^{III} act primarily as clastogens and are candidates for the ultimate genotoxic form of arsenic. In Chinese Hamster Ovary (CHO-9) cells, DMAs^{III} and MAs^{III} are also more genotoxic than DMAs^V, MAs^V, or iAs (Dopp et al. 2004). In this study, the high genotoxic potency of DMAs^{III} and MAs^{III} has been linked to a higher uptake and retention of DMAs^{III} and MAs^{III} compared to the methylated pentavalent and iAs species.

Cell transformation and carcinogenesis

The potential of methylated arsenicals to induce cell transformation and carcinogenesis has also been evaluated. UROtsa cells, a non-tumorigenic cell line derived from human urothelial cells, acquire the hallmarks of cancer cells when exposed to a low level of MAs^{III} (0.05 μ M) for up to 52 weeks (Bredfeldt et al. 2006). MAs^{III}-induced changes in DNA methylation patterns may contribute to development of the tumorigenic phenotype (Wnek et al. 2010). In SV-HUC-1 cells, an immortalized human uroepithelial cell line, treatment with iAs^{III} or MAs^{III} produces changes in gene expression patterns that may be linked cellular transformation (Su et al. 2006). Notably, the concentration for iAs^{III} that induces these changes in gene expression are several-fold higher than the concentration of MAs^{III} that alter gene expression.

Only a few studies have evaluated the carcinogenicity of methylated trivalent arsenicals in animals. The transplacental carcinogenicity of MAs^{III} has been demonstrated in pregnant CD-1 mice that drank water-containing MAs^{III} at 0 (control), 12.5, or 25 parts per million (ppm) from gestational days 8 to 18 (Tokar et al. 2012). Mice so exposed in utero were evaluated for tumor occurrence up to two years of age. In female offspring, dose-related increases in total epithelial uterine tumors, oviduct hyperplasia, adrenal cortical adenoma, and total epithelial ovarian tumors were found. Male offspring showed dose-related increases in hepatocellular carcinoma, adrenal adenoma, and lung adenocarcinoma. Notably, patterns of proliferative lesions in mice exposed to MAs^{III} during gestation resemble those found in mice exposed to iAs^{III} during gestation (Waalkes et al. 2007). Notably, 2-year feeding studies with a MAs^{III}-amended diet identified intestinal tract alterations as a common response in male and female Fischer F344 rats and B6C3F1 mice (Arnold et al. 2003). However, at the dosage levels tested, neither species show evidence of treatment-related neoplasia. The low toxic and carcinogenic potency of MAs^{III} in mice may be related to its rapid whole body clearance.

Additional, albeit indirect, evidence on the role of the valence state of arsenic on the toxicity of methylated arsenicals comes from studies of toxic and carcinogenic effects of DMAs^V in the urinary bladder of the rat. High dose levels studies in rats show that DMAs^V treatment produces a spectrum of changes in the uroepithelium that culminates in carcinogenic transformation in this organ (Arnold et al. 2006). Notably, analysis of urine from rats treated with dimethylthioarsinic acid identified DMAs^{III} and TMAs^{VO} as metabolites (Cohen et al. 2002; Adair et al. 2007). These findings suggested production of DMAs^{III}, a toxic and reactive species, caused requisite tissue injury needed for cellular transformation.

Enzyme inhibition

Early work showed that MAs^{III} or DMAs^{III} were more potent inhibitors of the purified yeast GSH reductase than iAs^{III} (Stýblo et al. 1997). Similarly, MAs^{III} acid is a more potent inhibitor than iAs^{III} of the DTNB reductase activity of purified thioredoxin reductase or in cultured rat hepatocytes (Lin et al. 1999, 2001). Notably, after 3-h incubation, MAs^{III} is methylated by rat hepatocytes to DMAs and thioredoxin reductase activity is almost completely recovered, suggesting that the dimethylated product does not inhibit this enzyme. iAs^{III} and MAs^{III} also inhibit activities of purified bovine glutathione peroxidase and equine glutathione S-transferase (Chouchane and Snow 2001). MAs^{III} is a more potent inhibitor of the activity of purified porcine pyruvate dehydrogenase than iAs^{III} ; parenterally administered MAs^{III} also inhibits pyruvate dehydrogenase activity in hamster kidneys (Petrick et al. 2001). When the co-factor is reduced, activities of pyruvate dehydrogenase and α -ketoglutarate dehydrogenase are inhibited by trivalent arsenicals (Bergquist et al. 2009). Here, the order of potency of these compounds is $\text{MAs}^{\text{III}} > \text{iAs}^{\text{III}} >> \text{DMAs}^{\text{III}}$. iAs^{III} inhibits complexes I and II in the inner membrane of isolated mitochondria; this inhibits electron transport chain, decreases mitochondrial ATP content, and increases ROS formation (Hosseini et al. 2013). A recent study found that MAs^{III} is more potent than iAs^{III} as an inhibitor of oxygen consumption rate and maximal respiration in INS-1 832/13 β -cell, suggesting that MAs^{III} also inhibits mitochondrial electron transport (Dover et al. 2018).

Disruption of cellular processes and signaling pathways

There is evidence for roles of MAs^{III} and DMAs^{III} in disruption of mechanisms involved in regulation of cellular processes, including inhibition of key signaling pathways. In a human urothelial cells line, trivalent arsenicals have been shown to affect activation of AP-1, one of the major transcription factors in mammalian cells (Drobna et al. 2003). MAs^{III} and DMAs^{III} are more potent than iAs^{III} in activating AP-1 and inducing its binding to DNA. MAs^{III} is also more potent than iAs^{III} in inducing the AP-1-dependent gene transcription in cells transiently transfected with an AP-1-dependent promoter-reporter construct. Because both MAs^{III} and DMAs^{III} induce phosphorylation of c-Jun, a component of the AP-1-DNA binding complex, and of extracellular signal-regulated kinase (ERK), the authors hypothesize that activation of an ERK-dependent signal transduction pathway is responsible for AP-1 activation in these cells.

The methylated arsenicals containing trivalent arsenic also disrupt pathways that control insulin release and action. A 48-h exposure to low concentrations of either MAs^{III} or DMAs^{III} (IC_{50} 100 nM) inhibits glucose-stimulated insulin release by isolated mouse pancreatic islets (Douillet et al. 2013). MAs^{III} also inhibits insulin secretion by cultured rat insulinoma INS-1 832/13 β cells that express human proinsulin (Dover et al. 2018). In both cell types, iAs^{III} is less potent than either MAs^{III} or DMAs^{III} as an inhibitor of insulin release. ATP production in mitochondria plays a key role in stimulation of glucose-dependent insulin secretion from β cells (Fu et al 2013). Studies examining mitochondrial metabolism in cultured INS-1 832/13 β cells exposed to iAs^{III} , MAs^{III} or DMAs^{III} found that oxygen consumption rate, a surrogate for mitochondrial function, is inhibited by exposure to 2 μM iAs^{III} or 0.375–0.5 μM MAs^{III} . These results suggest that these arsenicals inhibit insulin secretion by disrupting mitochondrial metabolism in β cells. Other studies

using isolated pancreatic islets show that trivalent arsenicals also inhibit glucose stimulated Ca^{2+} influx, a process that triggers assembly and exocytosis of insulin-containing vesicles (Huang et al 2019). Not surprisingly, MAs^{III} or DMAs^{III} are more potent inhibitors of Ca^{2+} influx than iAs^{III} .

In related work, the effects of iAs^{III} and MAs^{III} acid on mitochondrial function have been examined in cultured rat aortic smooth muscle cells (Pace et al. 2016). In these cells, MAs^{III} acid is more cytotoxic than iAs^{III} . Treatment of cells with MAs^{III} decreases basal and maximal oxygen consumption rates and increases compensatory extracellular acidification rates, a proxy for glycolysis.

The methylated trivalent arsenicals have also been shown to disrupt insulin signaling and insulin-dependent processes in cultured cells from tissues that play essential roles in glucose metabolism. In cultured mouse hepatocytes stimulated with insulin, short-term exposure to iAs^{III} (0.5 μM) or MAs^{III} (0.2 μM) decreases cellular glycogen concentrations by inducing glycogen phosphorylase activity and inhibits activation of glycogen synthase (Zhang et al. 2017). Actions of both arsenicals are mediated through inhibition of insulin-dependent phosphorylation of protein kinase B/Akt, a factor involved in insulin-dependent regulation of glycogen phosphorylase and glycogen synthase activities. An earlier study found inhibition of insulin-dependent phosphorylation of Akt by iAs^{III} and MAs^{III} in cultured differentiated 3T3-L1 adipocytes (Paul et al. 2007a). Here, inhibition of insulin signaling is associated with reduction of the insulin-stimulated glucose uptake. DMAs^{III} also inhibits insulin-dependent glucose uptake in adipocytes but does not affect Akt phosphorylation or any other upstream step in the insulin-activated signaling cascade.

In sum, these studies suggest that methylated arsenicals containing trivalent arsenic are potent disruptors of cellular signaling and secretion processes. Disruption of energy metabolism through effects on mitochondrial metabolism may be central to these effects of arsenic exposure.

Immunotoxicity

Effects of methylated trivalent arsenicals on components of the immune system have been reported in several published studies. Exposure to low concentrations of iAs^{III} , MAs^{III} or DMAs^{III} stimulates secretion of the growth-promoting and proinflammatory cytokines in cultures of normal human keratinocytes (Vega et al 2001). Similarly, in cultured human bronchial epithelial cells, secretion of cytokines (e.g., IL-6, IL-8) induced by bacterial challenge is increased by concurrent exposure to MAs^{III} (Notch et al. 2015). MAs^{III} has also been shown to affect the pre-lymphoid development of cells in bone marrow (Ezeh et al. 2016). Addition of 50 nM MAs^{III} to medium of cultured mouse bone marrow pre-B cells inhibits phosphorylation of the transcription factor Signal Transducer and Activator of Transcription 5 and thereby blocks activation of the IL-7 signaling pathway required for pre-B cell maturation. Notably, a tenfold higher concentration of iAs^{III} in medium is needed to attain equivalent inhibition of cell maturation.

The effects of iAs^{III} , MAs^{III} , and DMAs^{III} exposures on inflammatory responses in Caco-2 cells, a human intestinal epithelial cell line, have also been examined (Calatayud et al.

2014). Here, levels of biomarkers of inflammation are measured in cells and media after exposure for 2, 4, 6, and 24 h to trivalent arsenicals in presence or absence of lipopolysaccharide (LPS). Exposure to each trivalent arsenical alone decreases TNF- α mRNA levels and TNF- α levels in media and increases IL-8 mRNA levels and IL-8 levels in media. Here the potency of DMAs^{III} exceeds iAs^{III} and MAs^{III}. Co-exposure to LPS further enhances effects induced by trivalent arsenicals. A follow-up study has examined effects of trivalent arsenicals in co-cultures of Caco-2 cells and peripheral blood mononuclear cells with or without LPS treatment (Calatayud et al. 2015). In this co-culture, trivalent arsenicals increase IL-6, IL-8, and TNF- α secretion. LPS co-exposure potentiates the proinflammatory response compared to that produced by arsenicals alone. Co-exposure to DMAs^{III} or iAs^{III} and LPS also increases the permeability of intestinal monolayer.

Dysregulation of gene transcription

A large body of evidence generated by laboratory and population studies suggests that iAs exposure is associated with a widespread dysregulation of gene transcription. The affected genes are linked to a variety of adverse mechanisms and diseases, e.g., oxidative stress (Lantz and Hays 2006), cancer (Bailey et al. 2012; Huang et al. 2004; Valko et al. 2006; Yang and Frenkel 2002), metabolic disease (Martin et al. 2017; Venkatratnam et al. 2021), or inflammation (States et al. 2012). The effects of iAs exposure on gene expression have been linked, in part, to dysregulation of epigenetic mechanisms that regulate gene expression, e.g., DNA methylation (Bailey et al. 2013; Bailey and Fry 2014; Smeester et al. 2011), histone modification (Barr et al. 2009; Costa 2019; Eckstein et al. 2017), or changes in chromatin structure (Riedmann et al. 2015). However, the extent to which the methylated trivalent arsenicals contribute to these outcomes remains unclear. Design of most of the published studies carried out in in vitro or in vivo systems in which iAs is methylated, including human studies, cannot differentiate between effects of the parent compound and effects of its methylated metabolites.

Only few studies have been able to link dysregulation of gene transcription to specific arsenic species. Distinctive gene expression profiles have been reported in immortalized human uroepithelial cells SV-HUC-1 cells exposed to iAs^{III} or the methylated trivalent arsenicals (Su et al 2006). Gene expression profiles in iAs^{III} or MAs^{III}-exposed cells differ significantly from those in DMAs^{III}-exposed cells. Notably, profiles in iAs^{III} or MAs^{III}-exposed cells resemble gene expression profiles in a tumorigenic uroepithelial cell line. These arsenicals appear to have distinctive effects on different epigenetic mechanisms. MAs^{III} has been found to be more potent than iAs^{III} in suppressing gene expression and protein level of Xeroderma pigmentosum complementation group C, a DNA damage recognition protein, in normal human skin fibroblast (Nollen et al. 2009). Suppression of phosphatase and tensin homolog protein (PTEN) in MAs^{III}-exposed normal human urothelial (UROtsa) cells may be a potential mechanism for malignant transformation of cells by this arsenical (Oliva-González et al. 2017). MAs^{III} also downregulates expression and activity of cytochrome P450 1A1 in human hepatocellular carcinoma (HepG2) cells (Elshenawy et al. 2017) which methylate arsenic (Drobna et al. 2006). Finally, exposure of K6/ODC mice to MAs^{III} in drinking water alters expression of multiple genes in the mouse skin; notably, an increased transcript abundance of Fos11, Myc, and Rac1 oncogenes was

detected (Delker et al. 2009). However, it is unclear if methylation of MAs^{III} contributes to gene transcription dysregulation in this mouse model.

Expression of microRNAs, short, non-coding RNAs that negatively regulate gene expression at the post-transcriptional level, can be altered by exposure to iAs (Beck et al. 2017, 2018; Cardoso et al. 2018; Ferragut Cardoso et al. 2020; Sollome et al. 2016). Inhibition of glucose-stimulated insulin secretion in iAs^{III}-exposed INS-1 832/13 cells alters expression of several microRNAs, including microRNA-146a, which is involved in regulation of beta-cell function (Beck et al. 2019). Interestingly, MAs^{III} which is more potent than iAs^{III} as inhibitor of insulin secretion by INS-1 832/13 cells (Dover et al. 2018), does not alter microRNA-146a expression.

Methylated trivalent arsenicals as indicators of disease risk in humans cohorts

The efficiency of iAs methylation is one of the key factors that affect susceptibility to adverse effects of iAs exposure (Gamboa-Loira et al. 2017; Kuo et al. 2017; Tseng 2007). In humans exposed to iAs, risks of developing iAs-associated diseases have been linked to proportions of iAs and its methylated metabolites in urine, which are thought to reflect the efficiency of iAs methylation. The evaluation of the roles of MAs^{III} and DMAs^{III} in disease initiation and progression is difficult. These species can be easily oxidized to MAs^{V} and DMAs^{V} (Gong et al. 2001). Thus, it is unclear whether measurement of stable arsenicals in urine (iAs^{III} , iAs^{V} , MAs^{V} and DMAs^{V}) provides germane information about levels of MAs^{III} and DMAs^{III} in tissues or excreta. As a consequence, most population-based studies report total MAs and DMAs levels in urine and do not attempt to determine the oxidation state of As in methylated metabolites.

MAs^{III} and DMAs^{III} levels have been determined in some studies that analyzed freshly collected urines. Results from these studies has made it possible to examine associations between levels of these arsenicals in urine and disease phenotypes. In 2005, Valenzuela and associates reported presence of MAs^{III} and DMAs^{III} in urine of residents of Zimapan, Hidalgo state, Mexico, who were chronically exposed to iAs (Valenzuela et al. 2005). In this cohort, AS3MT (Met287Thr) variant was associated with a higher risk of premalignant skin lesions, a common indicator of chronic iAs toxicity (Valenzuela et al. 2009). Notably, the fraction of urinary As present as MAs is higher in individuals carrying this polymorphism than in other participants. In affected individuals, MAs^{III} accounts for a significant portion of urinary MAs, suggesting that this trivalent arsenical contributes to the cancer risk associated with iAs exposure. Later studies in Zimapan and Lagunera link urinary DMAs^{III} concentration to prevalence of diabetes mellitus (Drobna et al. 2013), another disease associated with chronic exposure to iAs (Kuo et al. 2017; Maull et al. 2012). In this cohort, carriers of AS3MT (Met287Thr) variant had higher concentrations of DMAs^{III} in urine and a higher likelihood of a diagnosis of diabetes (Drobna et al. 2013). Presence of MAs^{III} and/or DMAs^{III} in human urine has also been reported in several other studies (Le et al. 2000a, b; Aposhian et al. 2000). To date, only one population-based study has examined associations between a disease risk and concentrations of tri- and pentavalent arsenicals in cells originated from a tissue targeted by iAs exposure, specifically urinary bladder. This study found positive associations between risk of diabetes and concentrations

of trivalent, but not pentavalent, arsenicals in exfoliated urothelial cells isolated from urine of the individuals exposed to iAs in drinking water (Currier et al. 2014).

Oxidation state-specific analysis of arsenic species in biological matrices

Understanding dose–response relations for toxic actions of iAs and its methylated metabolites depends upon the ability to accurately measure the levels of these compounds in complex biological matrices. Methods used for analysis of arsenic species in biological matrices have been reviewed (Francesconi and Kuehnelt 2004; Gong et al. 2002a; Hsu et al. 2011; Reid et al. 2020). This section focuses on oxidation-state specific methods designed to differentiate among inorganic and methylated arsenic species that contain As^{III} or As^V. In broad terms, these methods involve two steps: (a) separation of As species based on arsenic oxidation-state and methylation status and (b) quantification of elemental arsenic in the separated fractions using established optical spectroscopic techniques or mass spectrometry. Differences in the approaches used to separate arsenic species influence the sensitivity and specificity of these methods and the requirement for sample processing and/or extraction before analysis. Two widely used analytical approaches are: (a) liquid chromatographic (LC) separation of arsenic species on the basis of charge or size of analytes and (b) hydride generation (HG) in which arsenicals are reduced to volatile arsines, cryogenically trapped, and separated by boiling points. For both approaches, separated arsenicals can be detected by various mass-dependent or spectrophotometric methods. The following paragraphs describe advantages and limitations associated with LC- and HG-based techniques in context of studies in which these techniques were used.

HG-based techniques

Analysis of arsenic species using HG is carried out in several steps. In the first step, samples are treated with a strong reductant, typically sodium borohydride. This treatment converts iAs and mono-, di- and tri-methylated arsenic species to their respective volatile arsines which are characterized by different boiling points (b.p.): arsine (AsH₃, b.p. – 55 °C), methylarsine (CH₃AsH₂, b.p. 2 °C), dimethylarsine [(CH₃)₂AsH, b.p. 36 °C] and trimethylarsine [(CH₃)₃As, b.p. 52 °C]. The HG reaction mixture is passed through a gas–liquid separator and the gaseous phase containing arsines is carried in a stream of an inert gas (e.g., helium) into a cryogenic trap (CT), typically a U-tube filled with a chromatographic sorbent, that is submerged in liquid nitrogen. Cold-trapped arsines are sequentially volatilized from the U-tube by controlled heating. Released arsines are then carried as separate fractions to an appropriate detector (Crecelius 1977). As summarized in Table 1, both optical and mass spectrometers have been used to detect and quantify elemental arsenic present in the arsines produced by HG.

The original method for As speciation by HG used a strongly acidic solution (pH ~ 1) to generate arsines from both tri- and pentavalent iAs, MAs, and DMAs (Ng et al. 1998). Two methods are currently used for oxidation-state specific analysis. Both methods require two aliquots of a single sample for complete analysis. In the first method, arsines from both tri- and pentavalent arsenicals are generated from one sample aliquot at pH 1. The second aliquot undergoes HG at pH 6. Under these conditions, arsines are generated only

from trivalent arsenicals. Concentrations of pentavalent arsenicals are then determined as the difference between the first and second measurement (Del Razo et al. 2001; Devesa et al. 2004). In the second method, trivalent arsenicals are measured in one sample aliquot after HG at pH 6. The second aliquot is treated with 2% cysteine prior to HG at pH 6. Cysteine reduces pentavalent arsenicals to trivalency. The concentrations of pentavalent arsenicals are again calculated as the differences between the concentrations measured in the cysteine-treated and in the untreated samples (Matoušek et al. 2008). The latter method is more convenient because the calibration is carried out using one HG condition (pH 6) and because the HG efficiencies for iAs^{III} , MAs^{III} and $DMAs^{III}$ are almost identical, allowing calibration with a single arsenic standard, typically iAs^{III} (Matoušek et al. 2008). Although TMA^{VO} can be measured by this method, lower efficiency for hydride generation for this species may require a separate calibration using an authentic standard.

Advantages of the HG-based techniques

HG-CT methods were developed specifically for detection of iAs and the methylated arsenicals formed during enzymatically catalyzed iAs methylation. Although published methods are typically designed for analysis of samples with volumes between 0.1 and 0.5 ml, the HG procedure can easily be scaled-up to use much larger sample volumes. In addition, the HG-CT step can be repeated before release of arsines to a detector, which allows quantification of arsenicals present at very low concentrations. In particular, very low detection limits have been achieved by methods that coupled the HG-CT steps with arsenic detection by inductively coupled plasma-mass spectrometry (ICP-MS) (Matoušek et al. 2013). Notably, the HG-CT-based techniques can be used for quantitative analysis of As^{III} and As^V -containing species in complex biological matrices without sample extraction. Elimination of sample processing before analysis minimizes the chance of conversion of As^{III} to As^V during extraction. HG-CT techniques, specifically HG-CT-atomic absorption spectrometry (AAS) or HG-CT-atomic fluorescence spectrometry (AFS), provide nearly complete recoveries of As when used for analysis of complex matrices (tissue homogenates or cell lysates) (Currier et al. 2011a, b, 2013, 2014, 2016; Matoušek et al. 2013; Musil et al. 2014; Paul et al. 2007b, 2008). Finally, the relatively low cost of instrumentation needed for As speciation analysis by HG-CT as well as very low detection limits that can be attained makes this technique an attractive alternative to more costly LC-based techniques for As speciation.

Limitations of the HG-based techniques

A major limitation is the lack of commercial automated HG-CT systems that directly interface with existing detectors. Thus, a typical setup requires assembly of an online system consisting of individual, often custom-made, parts that support HG reaction, separation of gas phase from the reaction mixture, delivery of the gas phase into a cryotrap, heating the cryotrap, and delivery of the volatile arsine fractions into a detector. The HG and CT steps, including controlled heating of the U-tube, are typically carried out on-line and are fully automated. The flow of the reagents and timing of each step are controlled by a programmable flow injection system. Although automated release of arsines from the cryotrap has been attempted (Matoušek et al. 2013), application of this method typically requires manual operations, including sample injection and removing the U-tube from liquid nitrogen prior to

heating. Because all critical steps cannot be easily automated, autosamplers cannot be used and presence of an operator is required during the entire procedure. Analysis of a sample takes approximately 6 min, using a well-tuned-up system.

The HG-CT techniques can be used only for analysis of arsenic species that form hydrides. Some arsenicals that are commonly found in food and biological systems cannot be detected by these techniques. Published studies have shown that arsenobetaine and arsenocholine, two arsenicals found in urine of individuals-consuming seafood, do not generate arsines under conditions typically used for HG-CT-based analyses of arsenic species (Currier et al. 2013). Similarly, generation of dimethyl arsine from dimethylthioarsinic acid, a product of arsenic metabolism by microbiota, is much less efficient than from DMAs^V or DMAs^{III} (Hernández-Zavala et al. 2008). Generation of hydrides from arsenosugars, arsenic species found in seaweed and some marine organisms, have been achieved only in a modified, strongly acidic HG reaction mixture (Marschner et al. 2018). Although viewed as a major limitation, the inability of HG-CT-based methods to detect arsenicals that do not form hydrides represents an advantage for analyses focusing on iAs and products of its methylation by AS3MT. It limits the possibility of false-positive signals caused by other arsenic species present in analyzed samples.

LC-based techniques

The LC techniques have been widely used for separation of arsenic species in aqueous and biological matrices. The most common methods involved separation of arsenic species by high-performance liquid chromatography (HPLC) coupled with an arsenic detector, typically ICP-MS. Several types of HPLC techniques have been used for separation of arsenicals, including anion exchange, cation exchange, reversed-phase, ion pair, or size exclusion chromatography (Francesconi and Kuehnelt 2004; Reid et al. 2020). In earlier studies focusing on iAs methylation, HPLC techniques were applied for analysis of iAs^{III}, iAs^V, and the pentavalent methylated arsenicals. After recent optimizations, these techniques have also been utilized for analysis of MAs^{III} and DMAs^{III} (Gong et al. 2002a) (Table 2). These methods are discussed in the following sections.

Advantages of the LC-based techniques

The instruments required for LC-based speciation analysis of arsenic, including HPLC instruments and chromatographic columns, as well as spectrometers, are commercially available and can easily be configured for a high-throughput analysis. Software for operating these instruments and for data analysis is also available. For optimized HPLC techniques, all tri- and pentavalent arsenic species formed in the AS3MT-catalyzed reactions can be quickly separated on a single column (Reid et al. 2020; Currier et al. 2013). Separation of other biologically relevant arsenicals can also be achieved on a single column or by combining several LC techniques (Reid et al. 2020). In principle, negatively charged arsenic compounds can be separated using anion-exchange chromatography while positively charged arsenicals are best separated using cation-exchange chromatography (Francesconi and Kuehnelt 2004; Reid et al. 2020). In addition, using ICP-MS in tandem with an electrospray ionization mass spectrometer (ESI-MS) can provide information about molecular structures of arsenic

species in chromatographic fractions (Reid et al. 2020). This is particularly important during analysis of biological samples in which arsenicals may be present in complexes with endogenous compounds, e.g., GSH (Raab et al. 2004). ICP-MS in tandem with ESI-MS can be also used to identify multiple arsenic species that are eluted with the same retention times (Reid et al. 2020). Thus, the LC-based techniques are suitable for routine analysis of known arsenic species, as well as for discovery of previously unidentified arsenic species.

Limitations of the LC-based techniques

Minimizing oxidation of unstable MAs^{III} and DMAs^{III} during sample preparation and chromatographic separation is the major challenge for LC-based techniques (Feldman et al. 1999; Gong et al. 2001). For example, bulk filtration of urine samples to remove particulates before sample injection to a chromatographic column or use of an in-line guard column between injector and the analytical column may promote the oxidation of MAs^{III} or DMAs^{III} . Analysis of more complex biological matrices requires liquid-phase extraction of arsenic species. A variety of solvents have been used with varied extraction efficiencies (Francesconi and Kuehnelt 2004; Luvonga et al. 2020). Sample extraction can lead to analyte dilution and adversely affect detection or quantification after LC separation. More importantly, MAs^{III} and/or DMAs^{III} may oxidize during extraction if an oxidizing solvent is used or if prolonged exposure to oxygen occurs. Notably, effects of the extraction by different solvents on oxidation states of arsenic in biological matrices have never been systematically studied.

Another challenge is associated with a potential loss of MAs^{III} and DMAs^{III} due to interaction with a mobile phase or a stationary phase of the column. A loss of DMAs^{III} on a Phenomenex Prodigy column with a tetrabutylammonium hydroxide-malonic acid-methanol mobile phase has been documented (Currier et al. 2013). However, no systematic research of the interaction between methylated trivalent arsenical and sorbents or solvents commonly used in LC techniques has been published. Thus, the oxidation state-specific analysis of arsenic species using LC techniques requires careful consideration and testing of chromatographic columns and mobile phases to minimize the oxidation of MAs^{III} and DMAs^{III} and to ensure a complete recovery of arsenic species during chromatography.

Knowledge gaps and future directions

Transport of arsenic species

The formation of methylated metabolites that contain either As^{III} or As^{V} in the course of iAs metabolism is supported by results of the oxidation-specific analyses of arsenic species in a variety of biological systems. Overwhelming evidence also suggests that AS3MT is the primary catalyst for arsenic methylation in cells. Recent studies on the protein structure of AS3MT and the molecular processes involved in AS3MT-catalyzed reactions support the production of MAs^{III} and DMAs^{III} at the catalytic center of this enzyme (Marapakala et al. 2012). DMAs^{V} and MAs^{V} are likely formed by a rapid oxidation of the unstable trivalent species in oxygen-rich environment. The efficiency of iAs metabolism has been linked to susceptibility to iAs toxicity in human population studies using the proportions of arsenic metabolites in urine as indicators of the capacity to convert iAs to MAs and MAs to

DMAs. However, it is reasonable to assume that the capacity to methylate iAs is not the only factor that determines the profiles of arsenic species in urine and the overall efficiency of iAs metabolism. For example, arsenic transporters that carry arsenic species from tissues to blood, bile or urine likely play a role. Several transporters have been proposed to participate in these processes, e.g., aquaglyceroporins, glucose (GLUT) transporters, organic anion transporters, or ATP-cassette transporters (Drobna et al. 2010; Garbinski et al. 2019; Leslie 2012; Liu 2010; Maciaszczyk-Dziubinska et al. 2012; Roggenbeck et al. 2016; Rosen and Liu 2009; Zangi and Filella 2012). However, a little is known about the specificity and affinity of these transporters for methylated arsenic species, specifically MAs^{III} and DMAs^{III} , and about contributions of these transporters to the whole-body metabolism and detoxification of iAs.

Protein binding of arsenicals

Another understudied issue is the binding of iAs and its metabolites to proteins in various cellular compartments. Binding to protein targets affects intracellular retention and distribution of arsenicals and the availability of these arsenicals for AS3MT-catalyzed reactions and for membrane transport. Although several studies have described subcellular distribution of arsenicals in mammalian cells (Dopp et al. 2008, 2010) and examined interactions between common protein motifs and arsenic (Kitchin and Wallace 2008; Lu et al. 2004; Shen et al. 2013; Yan et al. 2009, 2016; Zhang et al. 2015), these data are not sufficient to develop a comprehensive model for the role of protein–As interactions.

Data on binding of arsenicals, specifically MAs^{III} and DMAs^{III} , to cellular components would provide insights into the mechanistic basis of adverse effects associated with iAs exposure. Although reactions between iAs and protein and peptide thiol groups have been well-documented, our understanding of thiol interactions with MAs^{III} and DMAs^{III} are less complete.

Interactions between MAs^{III} and Zn finger motifs in the DNA-binding domain of the glucocorticoid receptor suggest a basis for some adverse effects of exposure to this arsenical (Spuches and Wilcox 2008). Similarly, identification of cysteinyl residues in alpha chain of rat hemoglobin as a high-affinity and high-capacity target for DMAs^{III} binding provides a molecular basis for the anomalous retention of DMAs^{III} in rat erythrocytes (Lu et al. 2007). Some studies have provided an indirect evidence for binding of methylated trivalent arsenicals to specific proteins. For example, MAs^{III} -dependent inhibition of insulin-stimulated phosphorylation of Akt by phosphoinositide-dependent protein kinase-1 (PDK-1) (Paul et al. 2007a; b) may involve an interaction with two closely spaced cysteines (Cys21 and Cys23) in the N-terminus of PDK1 (Alessi et al. 1997; Dong et al. 1999). The availability of MAs^{III} and DMAs^{III} should permit additional studies to identify proteins that bind these arsenicals and to elucidate the functions of these protein-arsenical complexes.

Analysis of MAs^{III} and DMAs^{III} in samples collected in human studies

The ultimate evidence linking the formation of MAs^{III} and DMAs^{III} to adverse health effects associated with iAs exposure will likely come from human population-based or clinical studies. A major obstacle to obtaining such evidence is associated with the low stability of

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MA^{III} and DMA^{III}, as well as with limitations associated with non-invasive collection of biological samples from human cohorts. In addition, many of the current analytical methods are not optimized or validated for the oxidation-state specific analysis of arsenic species in biological matrices. Attempts have been made to overcome these obstacles. One study measured tri- and pentavalent arsenicals in human urine within 1 to 6 h after collection; these urine samples were frozen in dry ice immediately after collection (Del Razo et al. 2011). Using this approach, only small fraction of DMA^{III} spiked into the urine samples was oxidized to DMA^V. Another approach to stabilize trivalent arsenicals in urine involves treatment of the donor with an arsenic chelator, 2,3-dimercapto-1-propane sulfonate (DMPS) (Gong et al. 2002b). Individuals in Romania who were exposed to high levels of iAs in their water supply received single oral doses of DMPS and urine was collected before and for up to 8 h post DMPS treatment. The presence of a MA^{III}-DMPS complex in urines collected after DMPS treatment suggests that it is possible to use chelation to stabilize the oxidation state of As. However, it is probably not feasible to use this approach in population-based studies. In addition, treatment with a chelator is likely to modify the pattern of iAs metabolism, thus confounding evaluation of the associations between iAs metabolism and disease risk. Direct addition of an As^{III}-complexing agent to freshly collected urine may be an alternative approach for stabilization of arsenic oxidation state before analysis. However, the capacity of some dithiols to reduce pentavalent arsenicals to trivalency may be an important caveat for this approach (Delnomdedieu et al. 1993, 1994).

Analysis of arsenic species in samples other than urine represents a conceptually different and challenging problem. Concentrations of tri- and pentavalent arsenicals in exfoliated urothelial cells isolated from urine of individuals exposed to iAs in drinking water have been measured (Currier et al. 2014). In this study, concentrations of iAs^{III}, MA^{III} and DMA^{III} in exfoliated cells positively correlated with indicators of the diabetogenic effects of iAs exposure. A similar approach could be used to analyze tri- and pentavalent arsenic species in other types of samples that can be collected using non-invasive procedures, e.g., buccal cells or nasal epithelial cells. In addition to providing information about the exposure to iAs and the disposition of arsenic species in tissues, this type of analyses may also help link the presence of individual arsenicals in tissues to adverse events related to iAs exposure.

Summary/conclusion

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Although research has greatly expanded our knowledge of arsenic metabolism at the molecular, cellular, and organismic level, there are still lacunae to be explored. In particular, our understanding of linkages among genotypic variation in AS3MT, interindividual variation in capacity for arsenic methylation, and interindividual variation in susceptibility to arsenic-induced diseases is incomplete. As discussed above, current evidence suggests that polymorphisms in the human AS3MT gene affects the pattern of methylated arsenicals in urine and that these changes in absolute and relative contributions of MAs or DMAs to the yield of arsenic metabolites are linked to increased incidences of a variety of diseases associated with exposure to iAs. However, it is unclear how relatively modest changes in the excretion of MAs and DMAs related to AS3MT genotype can account for differences in disease incidence. It is possible that these differences reflect genotype-dependent changes in relative proportions of MA^{III} and MA^V produced during AS3MT-catalyzed methylation.

Notably, other factors unrelated to AS3MT polymorphism, e.g., sex or diet have also been shown to modulate urinary profiles of iAs and its methylated metabolites with possible links to disease risks. The roles of these factors in formation of the methylated trivalent metabolites remains unclear. Development and application of oxidation state-selective analytical methods may permit detailed study of formation of tri- and pentavalent MAs and DMAs in cellular systems and provide a fuller understanding of the role of MAs^{III} and DMAs^{III} in disease initiation and progression.

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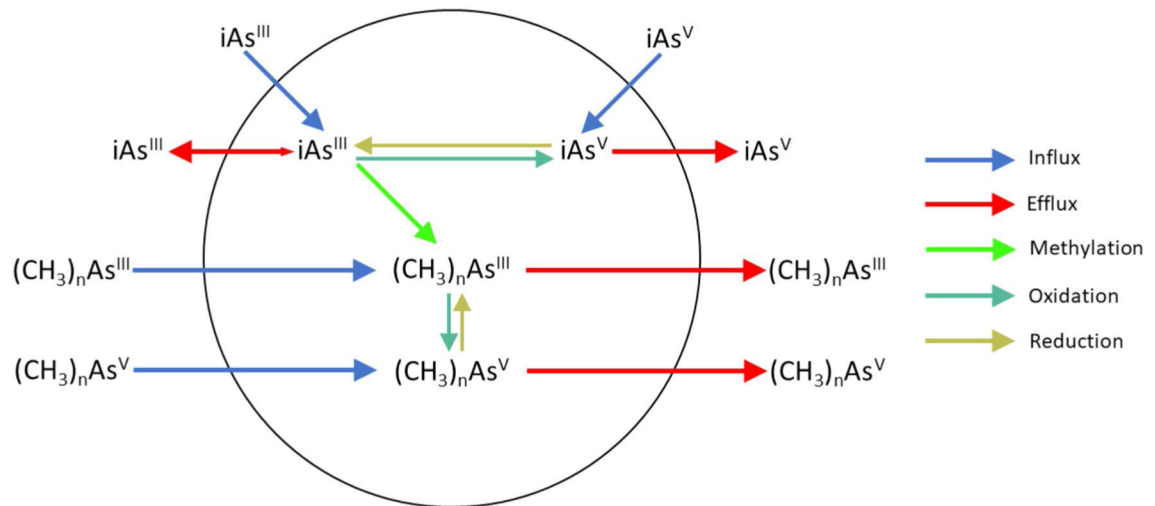


Fig. 1.

A conceptual model for the cellular metabolism of arsenic, including formation of the methylated trivalent and pentavalent arsenic metabolites. This model posits methylation without a change in oxidation state of arsenic and the ready interconversion of arsenic oxidation state in the cellular environment

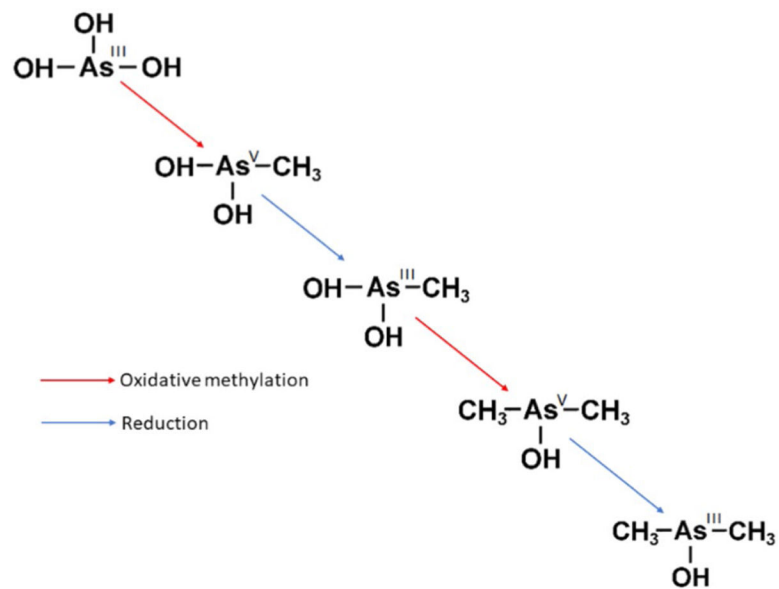


Fig. 2.
The Challenger scheme for the methylation of arsenic

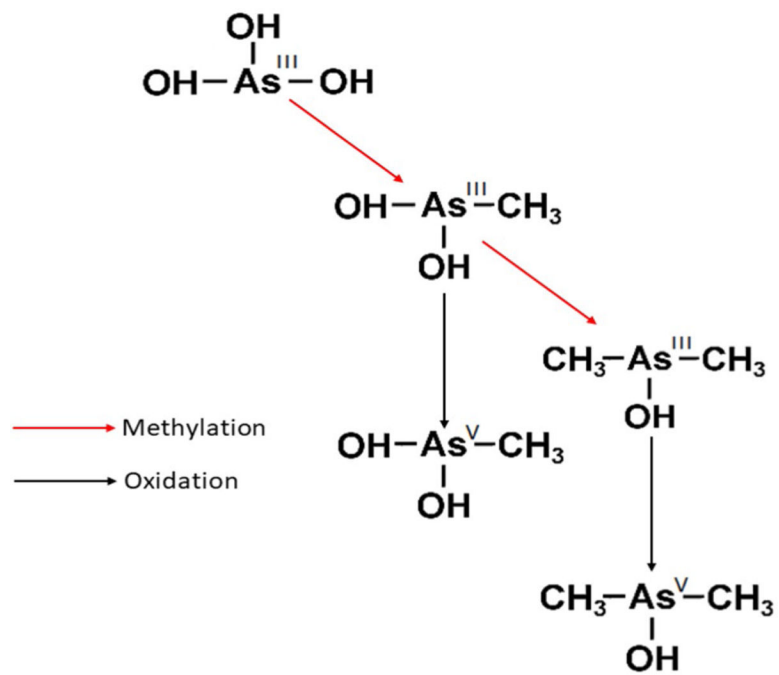


Fig. 3.
Alternate scheme for methylation of arsenic

Table 1

Examples of studies in which HG-CT-based techniques were used for detection and quantification of MAs^{III} and DMAs^{III} in biological samples

Technique	Sample pre-treatment	pH ^a	Sample	As species	References
HG-CT-AAS	None	pH 2 and pH 6	Urine	iAs^{III} , iAs^{V} , MAS^{V} , MAS^{III} , DMAS^{V} , DMAS^{III}	García-Montalvo et al. (2011)
	None	pH 2 and pH 6	Urine, hepatoma (HepG2) cells	iAs^{III} , iAs^{V} , MAS^{V} , MAS^{III} , DMAS^{V} , DMAS^{III}	Del Razo et al. (2001)
	None	pH 2 and pH 6	Urine	iAs^{III} , iAs^{V} , MAS^{V} , MAS^{III} , DMAS^{V} , DMAS^{III}	Valenzuela et al. (2005)
	None	pH 1 and pH 6	In vitro methylation system, primary hepatocytes, urine	iAs^{III} , iAs^{V} , MAS^{V} , MAS^{III} , DMAS^{V} , DMAS^{III} , TMA ₂ O	Devesa et al. (2004)
	± 2% Cysteine	pH 6	In vitro methylation system, cell culture	iAs^{III} , iAs^{V} , MAS^{V} , MAS^{III} , DMAS^{V} , DMAS^{III}	Hernández-Zavala et al. (2008)
	± 2% Cysteine	pH 6	In vitro methylation system, cell culture	iAs^{III} , iAs^{V} , MAS^{V} , MAS^{III} , DMAS^{V} , DMAS^{III}	Currier et al. (2013)
	± 2% Cysteine	pH 6	Homogenates from multiple tissues, plasma, blood cells	iAs^{III} , iAs^{V} , MAS^{V} , MAS^{III} , DMAS^{V} , DMAS^{III} , TMA ₂ O	Currier et al. (2016)
	± 2% Cysteine	pH 6	Urine	iAs^{III} , iAs^{V} , MAS^{V} , MAS^{III} , DMAS^{V} , DMAS^{III}	Matoušek et al. (2008)
	± 2% Cysteine	pH 6	Liver homogenate	iAs^{III} , iAs^{V} , MAS^{V} , MAS^{III} , DMAS^{V} , DMAS^{III}	Currier et al. (2011a)
	± 2% Cysteine	pH 6	UROtsa/F35, liver homogenate	iAs^{III} , iAs^{V} , MAS^{V} , MAS^{III} , DMAS^{V} , DMAS^{III}	Currier et al. (2011b)
	± 2% Cysteine	pH 6	Urine	iAs^{III} , iAs^{V} , MAS^{V} , MAS^{III} , DMAS^{V} , DMAS^{III}	Del Razo et al. (2011)
HG-CT-ICP-MS	± 2% Cysteine	pH 6	River water (CRM SLRS-4), exfoliated bladder epithelial cells	iAs^{III} , iAs^{V} , MAS^{V} , MAS^{III} , DMAS^{V} , DMAS^{III}	Matoušek et al. (2013)
	± 2% Cysteine	pH 6	Exfoliated urothelial cells	iAs^{III} , iAs^{V} , MAS^{V} , MAS^{III} , DMAS^{V} , DMAS^{III}	Currier et al. (2014)

Reports that described development of techniques for analysis of MAs^{III} and DMAs^{III} using mixtures in water or a solvent but did not validate these methods in biological matrices are not included

^a pH of the hydride generation mixture

Table 2

Examples of studies in which HPLC-based techniques were used for detection and quantification of MA_s^{III} and DMA_s^{III} (including MA_s^{III} and DMA_s^{III} complexes with glutathione) in biological samples

Separation	Column	Mobile phase	Detector	Sample	As species	References
Reversed phase/ion-pair	Phenomenex ODS(3)	5 mM TBAH, 3 mM malonic acid, 5% methanol, pH 5.85	HG-AFS	Urine	iAs^{III} , iAs^V , MA_s^V , MA_s^{III} , DMA_s^V	Le et al. (2000a), Aposhian et al. (2000)
	Phenomenex ODS(3)	5 mM TBAH, 3 mM malonic acid, 5% methanol, pH 5.85	HG-AFS	Urine	iAs^{III} , iAs^V , MA_s^V , MA_s^{III} , DMA_s^V , DMA_s^{III}	Le et al. (2000b)
	Phenomenex ODS(3)	4.7 mM TBAH, 2 mM malonic acid, 4% methanol, pH 5.85 or 5.9	HG-AFS	Urine	iAs^{III} , iAs^V , MA_s^V , MA_s^{III} , DMA_s^V , $DMPS-MA_s^{III}$	Gong et al. (2001)
	Phenomenex ODS(3)	5 mM TBAH, 3 mM malonic acid, 5% methanol, pH 5.85	HG-AFS	Red blood cells	iAs^{III} , iAs^V , MA_s^V , MA_s^{III} , DMA_s^V , DMA_s^{III}	Lu et al. (2007)
	Phenomenex ODS(3)	5 mM TBAH, 3 mM malonic acid, 5% methanol, pH 6.0	ICP-MS	Urine	iAs^{III} , iAs^V , MA_s^V , MA_s^{III} , DMA_s^V , DMA_s^{III}	Wang et al. (2004)
	Zorbax XDB	10 mM TMAH, pH 9.0	ICP-MS	SHIME	MA_s^{III} , DMA_s^{III}	Alava et al. (2012, 2013)
	Phenomenex ODS(3)	4.7 mM TBAH, 2 mM malonic acid, 4% methanol, pH 5.85	ICP-MS	In vitro methylation mixture	iAs^{III} , iAs^V , MA_s^V , MA_s^{III} , DMA_s^V , DMA_s^{III} , $DMMTA$	Currier et al. (2013)
	Phenomenex ODS(3)	5 mM TBAH, 3 mM malonic acid, 5% methanol, pH 5.65	ICP-MS	Hair, fingernails, toenails	iAs^{III} , iAs^V , MA_s^V , MA_s^{III} , DMA_s^V , $MMMTA$, $DMMTA$	Chen et al. (2018)
	Phenomenex ODS(3)	5 mM TBAH, 3 mM malonic acid, 5% methanol, pH 5.65	ICP-MS	Saliva	iAs^{III} , iAs^V , MA_s^V , MA_s^{III} , DMA_s^V , $MMMTA$	Chen et al. (2013)
	Phenomenex Jupiter CIS 300A	5 mM TBAH, 3 mM malonic acid, 5% methanol, pH 5.95	ICP-MS	<i>Cyano-bacterium</i> <i>Novstrac</i> sp. PCC 7120	iAs^{III} , iAs^V , MA_s^V , MA_s^{III} , DMA_s^V	Yan et al. (2015, 2017)
	Phenomenex Jupiter CIS 300A	5 mM TBAH, 3 mM malonic acid, 5% methanol, pH 5.95	ICP-MS	SHIME	iAs^{III} , iAs^V , MA_s^V , MA_s^{III} , DMA_s^V , $MMMTA$	Chi et al. (2020)
	Phenomenex Luna CIS	6 mM TBAH, 2 mM malonic acid, 5% methanol, pH 6.0	ICP-MS	Hepatoma (HepG2) cells	MA_s^V , MA_s^{III} , DMA_s^V	Hippler et al. (2011)
	Phenomenex Aqua CIS	0.1% formic acid (pH 2.6), acetonitrile gradient (0–40%)	ICP-MS	Urine	$iAs^{III}(SG)_3$, $MA_s^{III}(SG)_2$, $DMA_s(GS)$	Kala et al. (2004)
	Phenomenex Prodigy ODS-3	4.7 mM TBAH, 2 mM malonic acid, 4% methanol, pH 5.95 (adjusted with nitric acid)	ICP-MS	Urine	iAs^{III} , iAs^V , MA_s^V , MA_s^{III} , DMA_s^V , AsB	Rabieh et al. (2008)
	Waters Spherisorb C8 (at 10 °C)	0.05% trifluoroacetate, acetonitrile gradient	ICP-MS	8226/S Multiple myeloma cells	$DMMTA$, $DMMTA(GS)$, DMA^{III} , $DMA_s^{III}(GS)$	Stice et al. (2016)

Separation	Column	Mobile phase	Detector	Sample	As species	References
	Excepak ICS-A13 column	3 mM NaH ₂ PO ₄ at pH 6.0 with 1.0 mol l ⁻¹ NaOH	ICP-MS	Urine	MA ₅ ^{III} , DMA ₅ ^{III} , TMA ₅ ^V after extraction to DDDC in CCl ₄ and back-extraction to NaOH)	Okina et al. (2004)
Ion exchange	Phenomenex Phenosphere	20 mM phosphate, 5% methanol, pH 7.0	HG-AFS following on-line decomposition with 0.1 M NaOH	Urine	iAs ^{III} , iAs ^V , MA ₅ ^V , MA ₅ ^{III} , DMA ₅ ^V , DMPS-MA ₅ ^{III}	Gong et al. (2002b)
	Phenomenex Phenosphere SAX 80R	20 mM Phosphate, 5% methanol (pH 5.8) (pretreated with DMPS)	HG-AFS	Urine	iAs ^{III} , iAs ^V , MA ₅ ^V , MA ₅ ^{III} , DMA ₅ ^V , DMA ₅ ^{III}	Lu et al. (2003)
	Shodex Asahipak ES-502N 7C anion exchange	15 mM citric acid, 10% HNO ₃ , pH 2.0	ICP-MS	Urine	iAs ^{III} , iAs ^V , MA ₅ ^V , MA ₅ ^{III} , DMA ₅ ^V , DMA ₅ ^{III} , AsB, AsC	Mandal et al. (2001)
	Shodex Asahipak ES-502N 7C anion exchange	15 mM citric acid, 10% HNO ₃ , pH 2.0	ICP-MS	Hair, fingernails	iAs ^{III} , iAs ^V , MA ₅ ^V , DMA ₅ ^V , DMA ₅ ^{III}	Mandal et al. (2003)
	Shodex Asahipak ES-502N 7C anion exchange	10–40 mM citric acid, pH 2.0–3.5	ICP-MS	Urine, plasma, red blood cells, hair, fingernails	iAs ^{III} , iAs ^V , MA ₅ ^V , MA ₅ ^{III} , DMA ₅ ^V , DMA ₅ ^{III} , AsB	Mandal et al. (2004)
	Shodex Asahipak ES-502N 7C anion exchange	15 mM citric acid, 10% HNO ₃ , pH 2.0	ICP-MS	Urine, plasma, red blood cells	iAs ^{III} , iAs ^V , MA ₅ ^V , MA ₅ ^{III} , DMA ₅ ^V , DMA ₅ ^{III} , AsB	Mandal et al. (2007)
	Shodex Asahipak ES-502N 7C anion exchange	15 mM citric acid buffer, pH 2.0	ICP-MS	Bile	iAs ^{III} , MA ₅ ^{III} , iAs ^{III} (SG) ₃ , MA ₅ ^{III} (SG) ₂	Bu et al. (2011)
	Shodex Rspak NN-614 cation exchange	36 mM formic acid, 2 mM ammonium formate, pH 2.81	ICP-MS	Red blood cells	DMA ₅ ^V , DMA ₅ ^{III}	Shiobara et al. (2001)
	Shodex Rspak NN-614 cation exchange	5 mM HNO ₃ , 6 mM NH ₄ NO ₃	ICP-MS	Urine	MA ₅ ^{III} , DMA ₅ ^{III} (after extraction to DDDC in CCl ₄ and back-extraction to NaOH)	Okina et al. (2004)
	Shodex Asahipak ES-502N 7C anion exchange	15 mM citric acid (pH 2.0 adjusted with nitric acid)	ICP-MS	Bile	iAs ^{III} , MA ₅ ^V , iAs ^{III} (SG) ₃ , MA ₅ ^{III} (SG) ₂	Suzuki et al. (2001)
	Hamilton PRP-X100 anion exchange	20 mM phosphate-ammonia buffer, pH 6.0	ICPQQ-MS	<i>Elaphomyces spp.</i> (deer truffles)	iAs ^V , MA ₅ ^V , MA ₅ ^{III} , DMA ₅ ^V	Braeuer et al. (2018)
	Hamilton PRP-X100 anion exchange	98% 10 mM ammonium phosphate (pH 8.25) and 2% methanol	ICP-MS	Blood, multiple tissues	DMA ₅ ^V , DMA ₅ ^{III}	Twaddle et al. (2018a)
	Hamilton PRP-X100 anion exchange	98% 10 mM ammonium phosphate (pH 8.25) and 2% methanol	ICP-MS	Blood, multiple tissues	iAs ^{III} , iAs ^V , MA ₅ ^V , DMA ₅ ^V , MA ₅ ^{III} + DMA ₅ ^{III}	Twaddle et al. (2018b)
	Hamilton PRP-X100 anion exchange	98% 10 mM ammonium phosphate (pH 8.25) and 2% methanol	ICP-MS	Blood, multiple tissues	DMA ₅ ^V , DMA ₅ ^{III}	Twaddle et al. (2019)

Separation	Column	Mobile phase	Detector	Sample	As species	References
	Hamilton PRP-X100 anion exchange	10 mM ammonium dihydrogen phosphate, 10 mM ammonium nitrate, pH6.2	ICP-MS	<i>E. Coli</i>	iAs ^{III} , iAs ^V , MA ^S ^V , MA ^S ^{III} , DMA ^S ^V , TMA ^S O	Yan et al. (2017)
	Hamilton PRP-X100 anion exchange	10 mM ammonium dihydrogen phosphate, 10 mM ammonium nitrate, pH6.2	ICP-MS	SHIME	iAs ^{III} , iAs ^V , MA ^S ^V , MA ^S ^{III} , DMA ^S ^V , MMTA	Chi et al. (2020)
	Dionex AS11 and AG11 anion exchange	Water with 50 mM NaOH gradient (0–80%)	ICP-MS	Urine	iAs ^{III} , iAs ^V , MA ^S ^V , DMA ^S ^V , MA ^S ^{III} + DMA ^S ^{III} + AsB	Xie et al. (2006)
	Dionex PCX-500 cation exchange	70 mM nitric acid	ICP-MS	Urine	iAs ^{III} + iAs ^V + MA ^S ^V + DMA ^S ^V , MA ^S ^{III} , DMA ^S ^{III} , AsB	Xie et al. (2006)
Size exclusion (Gel filtration)	Shodex Asahipak GS-220 HQ	50 mM ammonium acetate buffer, pH 6.5, or 15 mM citric acid buffer, pH 2.0	ICP-MS	Liver	MA ^S ^V , DMA ^S ^V , DMA ^S ^{III} , DMTA ^V , DMTA ^{III} , AsB	Suzuki et al. (2004)

Reports that described development of techniques for analysis of MA^S^{III} and DMA^S^{III} using mixtures of standards in water or a solvent but did not validate these methods in biological matrices are not included

DDDC diethylammonium diethyldithiocarbamate, *TBAH* tetrabutylammoniumhydroxide, *TMAH* trimethylammoniumhydroxide, *SHIME* simulator of the human intestinal microbial ecosystem