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Cite This: Int. J. Toxicol. and Toxi. Assess, 2021, 1, 36-54

SciForce

International Journal of Toxicology and Toxicity Assessment

Journal homepage: www.sciforce.org

Stability of monomethylarsonous acid (MMA^{III}) and dimethylarsinous acid (DMA^{III}) in human urine and mouse tissues

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ARTICLE INFO

Article history:
Received 20230405
Received in revised form 20230405
Accepted 20230410
Available online 20230415

Keywords:
Stability;
Arsenic metabolites;
Monomethylarsonous acid (MMA^{III});
Dimethylarsinous acid (DMA^{III});
Urine;
Tissues;
HPLC-ICP-MS

ABSTRACT

Urine samples were collected from 75 subjects in the Lagunera area of Mexico. There were four groups, based on total arsenic concentrations in their drinking water (9-100µg/L). After collection, the samples were immediately put in a portable icebox containing dry ice, and they were kept frozen while being transported to the University of Arizona, Tucson where they were stored at -70°C before analysis. Arsenic metabolites, including MMA(III) and DMA(III), were measured in urine samples by using HPLC-ICP-MS.

The average percentage of MMA(III) in urine samples of arsenic exposed people in the Lagunera area of Mexico were 0.44%, 0.26%, N.D. (Not Detectable), and 0.20% of total arsenic for the groups 4, 1, 3, and 2 where arsenic concentrations in their drinking water were 9, 17.5, 52, and 100 (µg/L), respectively. This small percentage of MMA(III) were detected only in 14 urine samples (~18%) out of total 75 samples. DMA(III) was not measured in any of these urine samples (n=75) but measured DMA(V) in all samples. The highest percentage of arsenic metabolites was DMA(V) (61% to 74%) of the total arsenic in urine. It indicates that most of the MMA(III) methylated to DMA(V) in tissues, but less percentage or most of the DMA(V) may be reduced to DMA(III) and due to its instability, it could be immediately oxidized to DMA(V) in tissues again or in urine after collection. These findings suggest that the +5-oxidation state of arsenic metabolite, DMA(V), could be the most dominant methylated arsenic metabolite in humans' urine of arsenic exposed population.

In animal tissues, the +3-oxidation state of arsenic metabolites, MMA(III) and DMA(III), were measured in mice tissues after administering a single intra-muscular dose of sodium arsenate (4.16 mg As/kg body weight). Liver, kidneys, urinary bladder tissue, lungs, testes, and heart were removed at the following times, 0, 0.5, 1, 2, 4, 8, and 12 h. The tissues were homogenized at 4°C (cold room), and the homogenized samples were extracted immediately. After extraction, I measured the arsenic species as soon as possible by using HPLC-ICP-MS (Chowdhury, et al., 2006).

The concentration of the very toxic MMA(III) was significantly higher than that of MMA(V) in all of the tissues tested (Liver, kidneys, urinary bladder tissue, lungs, testes, and heart). At 2 h, after injection, the highest concentration of MMA(III) was in the kidneys. Compared with the other species of arsenic, MMA(V) concentrations were the lowest in all tissues examined.

On the other hand, the concentration of DMA(V) was higher than DMA(III) in the liver, kidneys, urinary bladder tissue, lungs, and testes at all times. DMA(V) accumulated at a higher concentration in the urinary bladder tissue and lungs, but the concentration of DMA(III) was the highest in the urinary bladder tissue, followed by the kidneys, lungs, heart, testes, and liver. In all of the tissues, both DMA(V) and DMA(III) were highest at 4

h for the WT (wildtype) mice. The concentration of DMA(III) was significantly higher in the urinary bladder tissue than in other tissues ($p<0.05$), except in the kidneys of the mice.

The results indicate that MMA(V) reduced to MMA(III) faster, comparing to DMA(V) to DMA(III). It could be that most of the MMA(V) reduced to MMA(III) and methylated to DMA(V), but less/most of the MMA(III) converted to DMA(V)↔ DMA(III), but DMA(III) may be very instable and oxidized to DMA(V) very quickly. This might have been because the DMA(III) would be more instable in the tissues, comparing to MMA(III), and oxidized faster, compared to MMA(III). Also, it could be suitable for the bonding of MMA(III) to tissue components or proteins, compared to DMA(III).

These results also indicate that due to the instability and faster oxidation of DMA(III), there was no detectable level of DMA(III), but low level of MMA(III) was measured in some urine samples due to its more stability and bonding capacity with proteins, compared to DMA(III). Both MMA(III) and DMA(III) were detected in tissues but not in urines, and these reports have suggested that tissue levels of MMA(III) and DMA(III) are both more relevant and less susceptible to oxidative artifacts than urine samples.

In conclusion, the distribution of arsenic metabolites in urine was supported with the distribution of these species in mouse tissues. The trivalent MMA(III) compound could be the most dominant, very toxic arsenic metabolite in humans' tissues for a short period of time (e.g., MMA(III) was highest at 2 h in mouse tissues after injection of sodium arsenate) but not in urine because it methylated to DMA(V) in tissues, and some percentage of MMA(III) could also be oxidized to MMA(V) again. On the other hand, DMA(V), instead of DMA(III), would be another dominant arsenic metabolite in tissues as well as in urine because we found the final metabolites of arsenic in urine where most of the arsenic metabolite was DMA(V). The stability/instability of MMA(III) and DMA(III) may depend on biological environment, genetic variability, and other factors. However, more experiments are needed to understand the mechanism of inorganic arsenic biotransformation and stability or instability of highly toxic arsenic metabolites, MMA(III) and DMA(III).

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Background

The mechanisms of the toxicity and carcinogenicity of inorganic arsenic at the molecular level in humans remain anenigma[1-8], even though there are millions of people in the world that are drinking water, containing carcinogenic concentrations of inorganic arsenic. For example, more than 25 million people in Bangladesh[9] and 6 million people in West Bengal, India[9], are drinking water that contains arsenic concentrations above 50 µg/L, even though the WHO recommends that arsenic in drinking water should not exceed 10 µg/L. An estimated 36 million people in the Bengal Delta are also at risk for arsenic-caused cancer[10]. Chronic exposure to inorganic arsenic has led to cancer of the skin, lungs, urinary bladder tissue, kidneys, and liver[11-14].

The chemical forms of arsenic determine the toxicity and bioavailability of arsenic compounds[7,15]. Although the tissue distributions of arsenic species with an oxidation state of +5 have been investigated extensively, this is not the case for the more reactive MMA(III) and DMA(III) found in human urine[16].

The studies have demonstrated that when humans have been chronically exposed to inorganic arsenic, they excreted in the urine arsenic bio-transformants in the +3 oxidation state[16,17] (Fig 1). These important studies have been confirmed by others[18,19]. The +3-oxidation state metabolites, MMA(III) and DMA(III), are more potent cytotoxins, genotoxins, and enzyme inhibitors than inorganic arsenate, arsenite, or the +5-oxidation state methylated arsenic metabolites, MMA(V), and DMA(V)[20-25]. High concentrations of MMA(III), monomethylarsine and DMA(III), dimethylarsine damaged DNA in vitro[26]. Therefore, there is an increasing interest in the determination of MMA(III) and DMA(III) in studies of arsenic metabolism and health effects.

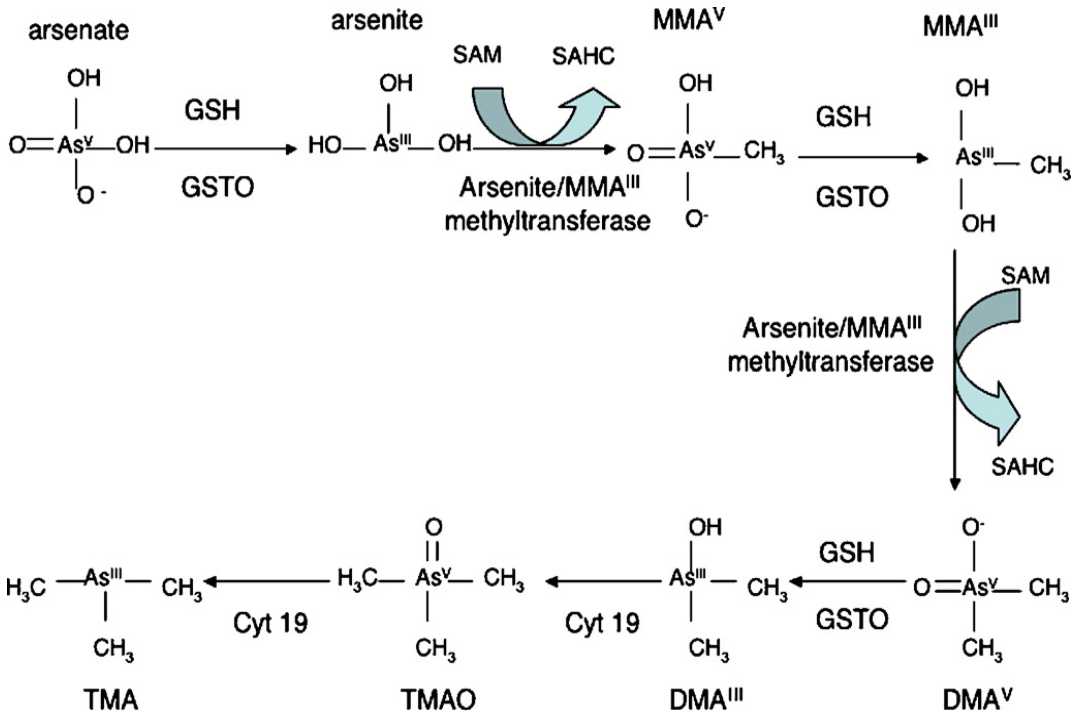


Figure 1. Proposed pathway for inorganic arsenic biotransformation. Abbreviations: SAM, S-adenosyl-L-methionine; SAHC, S-adenosyl-L-homocysteine; GSTO, glutathione-S-transferase-omega; GSH, glutathione.

The detection and quantification of urinary MMA(III) and DMA(III) in various populations have been reported over the past 20 years. Some reports suggest that in exposed populations, these trivalent compounds are major or even dominant components of urinary arsenic[18,19,27]. Other investigators suggest that there is little or no DMA(III), and very low levels of MMA(III) occur in populations with a range of arsenic exposures[28-30]. Based on these prior reports with highly variable findings, the usefulness of measuring urinary arsenic metabolites as a biomarker of exposure or susceptibility has been an open question of importance for planning future arsenic exposed population studies.

However, there are a few studies on the oxidative stability of MMA(III) and DMA(III) arsenic species. It is important to know whether and to what extent these trivalent metabolites are changed during sample storage and handling. The objective of this study was to find the distribution of arsenic metabolites, including MMA(III) and DMA(III), in human urine samples where the samples were immediately frozen after collection and kept frozen during transportation from Mexico to the University of Arizona, Tucson. These samples were immediately stored at -70°C after arrival until analysis. It is also very important to know the oxidative stability of the +3 oxidation state metabolites, MMA(III) and DMA(III), in tissues, and we studied to find the distribution of arsenic metabolites in mice tissues after administering a single intra-muscular dose of sodium arsenate. Information about the concentration and distribution of these +3 arsenic species in tissues might help in elucidating their biotransformation process, stability/instability in tissues, and distribution in urine.

Materials and Methods

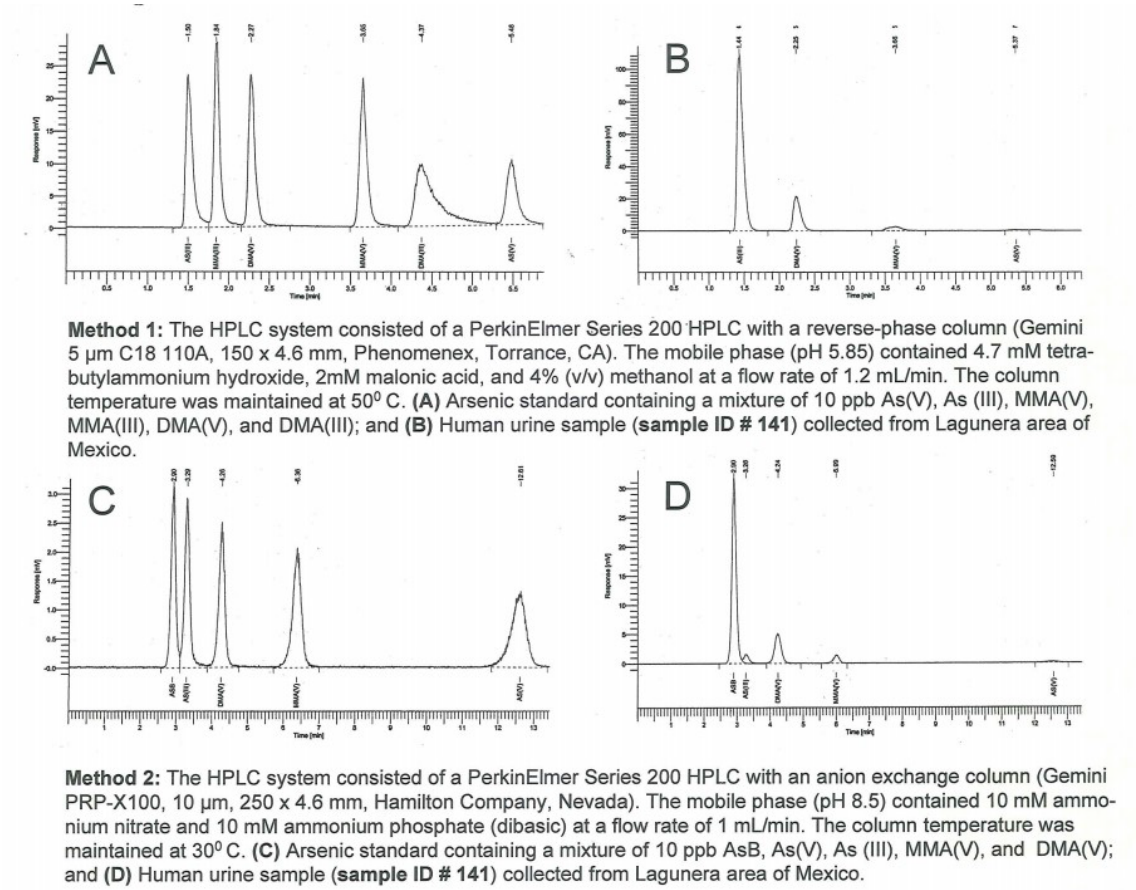
A. Arsenic metabolites in human urine of arsenic exposed people

Reagents. The chemicals used and their sources are as follows: Sodium arsenate (Na_3AsO_4; ACS reagent grade) from MCB Reagents (Cincinnati, OH); DMA(V), dimethylarsinic acid [$(NaCH_3)_2AsO_2$], ammonium phosphate (dibasic), and arsenobetaine from Sigma Chemical Co. (St. Louis, MO); sodium m-arsenite ($NaAsO_2$) and ammonium nitrate from Sigma-Aldrich Co. (St. Louis, MO); MMA(V), monomethylarsonic acid/disodium methylarsenate ($Na_2CH_3AsO_3$) from Chem Service, Inc. (West Chester, PA). The source of MMA(III), monomethylarsonous acid and DMA(III), dimethylarsinous acid were the solid oxide (CH_3AsO) and solid iodide [$(CH_3)_2AsI$], respectively. The stock solution (1000 ppm) of MMA(III) and DMA(III) were prepared as follows: MMA(III) in doubly deionized water, but DMA(III) in 10% methanol + doubly deionized water and stored both at -70°C. The arsenic standard solution was from SPEX Certiprep (Metuchen, NJ). All other chemicals were analytical reagent grade or the highest quality obtainable. Water was doubly deionized and distilled.

Subjects. Urine samples and blood samples were collected from 75 subjects in the Lagunera area of Mexico. There were four groups, based on total arsenic concentrations in their drinking water (9-100 µg/L).

Urine and blood collection. All collecting containers were soaked overnight in 2% nitric acid (Baker analyzed for trace metal analysis) (J. T. Baker, Inc. Phillipsburg, NJ) and rinsed with double distilled and deionized water. All plastic measuring and collecting equipment were similarly washed, sealed in bags, placed in locked footlockers, and transported by air to the site of the study at the same time as the investigators. After collection, the urine sample was immediately frozen in a portable icebox, containing dry ice. Blood was collected by venous puncture, into Vacutainers containing EDTA, transferred to a vial, and immediately frozen. The samples were kept frozen while being transported to the University of Arizona, Tucson where they were stored at -70°C before analysis.

Separation techniques of urinary arsenic metabolites. Arsenic concentration in drinking water is the reason for the elevated levels of arsenic in urine. Even arsenic from seafood (arsenobetaine, AsB) may be responsible for the elevated levels of arsenic in urine. Thus, to know the nature of arsenic contamination and the measurement of arsenic metabolites, HPLC-ICP-MS (High Performance Liquid Chromatography-Inductively Coupled Plasma-Mass Spectrometry) is the most advanced and reliable technique. The method of Gong et al. (2001)[31] could not separate AsB and AsB was overlapped with arsenite (Figs.2B & 2D). In this study, an HPLC-ICP-MS method[32] was modified by Chowdhury UK (2021)[33] for the measurement of arsenic metabolites, including AsB in urine (Figs. 2C & 2D). One of the urine samples contained very high levels of arsenic (697 µg/L urine) due to AsB (509 µg/L urine, i.e., 73% of total arsenic; Fig. 2D), and we excluded this sample from our results.



Method 1: The HPLC system consisted of a PerkinElmer Series 200 HPLC with a reverse-phase column (Gemini 5 µm C18 110A, 150 x 4.6 mm, Phenomenex, Torrance, CA). The mobile phase (pH 5.85) contained 4.7 mM tetra-butylammonium hydroxide, 2mM malonic acid, and 4% (v/v) methanol at a flow rate of 1.2 mL/min. The column temperature was maintained at 50^0 C. **(A)** Arsenic standard containing a mixture of 10 ppb As(V), As (III), MMA(V), MMA(III), DMA(V), and DMA(III); and **(B)** Human urine sample (**sample ID # 141**) collected from Lagunera area of Mexico.

Method 2: The HPLC system consisted of a PerkinElmer Series 200 HPLC with an anion exchange column (Gemini PRP-X100, 10 µm, 250 x 4.6 mm, Hamilton Company, Nevada). The mobile phase (pH 8.5) contained 10 mM ammonium nitrate and 10 mM ammonium phosphate (dibasic) at a flow rate of 1 mL/min. The column temperature was maintained at 30^0 C. **(C)** Arsenic standard containing a mixture of 10 ppb AsB, As(V), As (III), MMA(V), and DMA(V); and **(D)** Human urine sample (**sample ID # 141**) collected from Lagunera area of Mexico.

Fig 2: HPLC methods for separation of urinary arsenic metabolites. Method 1: The method of Gong et al. (2001)[31] and Method 2: The modified method[33] of Reuter et al. (2003)

Arsenic metabolites [AsB, As(V), As(III), MMA(V), and DMA(V)] analysis. Frozen urine samples were thawed at room temperature, filtered with a 0.45µm filter (Nanosep MF Centrifugal Devices, Pall Life Sciences, Ann Arbor, MI), and diluted 5-fold using Milli-Q water before injection. An HPLC-ICP-MS speciation method[32] was modified[33] for the measurement

of arsenic concentrations. The HPLC system consisted of a PerkinElmer Series 200 HPLC with an anion exchange column (Gemini PRP-X100, 10µm, 250 x 4.6mm, Hamilton Company, Nevada) (Fig 2 and Method 2). The mobile phase (pH 8.5) contained 10 mM ammonium nitrate and 10 mM ammonium phosphate (dibasic) at a flow rate of 1 mL/min. The column temperature was maintained at 30°C. An ELAN DRCe ICP-MS (Perkin-Elmer) with a cyclonic quartz spray chamber, and Mein hard nebulizer was used as a detector for the analysis of arsenic species [AsB, As(V), As(III), MMA(V), and DMA(V)] in urine at 4°C and sample injection volume 50 µL. The operating parameters were as follows: R_f power, 1400 W; plasma gas flow, 15 L/min; nebulizer gas flow, 0.82 L/min; auxiliary gas flow, 1.2 L/min; oxygen flow for DRC, 0.87 mL/min; and arsenic was measured at miz 91. The working detection limits were 0.80 - 1.75 µg/L for arsenic metabolites. Accuracy values were calculated by spiking standard compounds of all five species (5 µg/L) in urine samples. The recoveries of the added compounds were 98-103%. Standard samples (5 µg/L), containing all five arsenic species, were also analyzed after analysis of the urine samples each day. The values of mean ± SE for AsB, As(V), As(III), MMA(V), and DMA(V) were found to be 4.86 ± 0.08, 5.09 ± 0.11, 5.16 ± 0.11,5.02 ± 0.10, and 4.90 ± 0.05µg/L, respectively.

The +3 oxidation state metabolites, MMA(III) and DMA(III) analysis. Frozen urine samples were partially thawed at room temperature, then the samples were completely thawed on ice and immediately filtered with a 0.45 µm filter (Nanosep MF Centrifugal Devices, Pall Life Sciences, Ann Arbor, MI) in cold room (4ºC) before injection. An HPLC-ICP-MS speciation method[31] was used for the measurement of arsenic concentrations. The HPLC system consisted of a PerkinElmer Series 200 HPLC with a reverse-phase column (Gemini 5µm, C18, 110A, 150 x 4.6mm, Phenomenex, Torrance, CA). The mobile phase (pH 5.85) contained 4.7 mM tetra-butyl ammonium hydroxide, 2 mM malonic acid, and 4% (v/v) methanol at a flow rate of 1.2 ml/min (Fig 2 and Method 1). The column temperature was maintained at 50°C. An ELAN DRCe ICP-MS (Perkin-Elmer) with a cyclonic quartz spray chamber and Mein hard nebulizer was used as a detector for the analysis of arsenic species [MMA(III) and DMA(III)] in urine at 4°C and sample injection volume 50 µL. The operating parameters were as follows: R_f power, 1400 W; plasma gas flow, 15 L/min; nebulizer gas flow, 0.82 L/min; auxiliary gas flow, 1.2 L/min; oxygen flow for DRC, 0.87 mL/min; and arsenic was measured at miz 91. The detection limits for MMA(III) and DMA(III) were 0.09 and 0.16 µg/L, respectively. Accuracy values were calculated by spiking standard compounds of all six species [1µg/L; As(V)+As(III)+MMA(V)+MMA(III)+DMA(V)+DMA(III)] in urine samples. The recoveries of the added compounds of MMA(III) and DMA(III) were 90% and 97%, respectively. Standard samples (1µg/L) containing all six arsenic species were also analyzed after analysis of the urine samples every day. The values of average ± SD for MMA(III), and DMA(III) were found to be 0.90 ± 0.05 and 0.97 ± 0.27µg/L, respectively.

Total arsenic analysis in urine samples. Urine samples in acid washed polypropylene tubes were digested with nitric acid (5: 1) while a water bath for 40 min at 70°C. Freeze-dried urine reference material for toxic elements containing arsenic at a level of 220 ± 10 µg As/L was used for quality control and to validate the assay. After acid digestion, analysis of this standard by ICP-MS yielded a range of 216.0 - 236.0 µg As/L with a range of recoveries of 98.18 - 107.27%. We also analyzed the spiking standard compounds of all the arsenic species [AsB, As(V), As(III), MMA(V), and DMA(V)] at levels of 10 µg total As/L and 20 µg total As/L. The recoveries of the spiking samples were 104.20 % (10.42 ± 0.13 µg total As/L) and 97.70 % (19.54 ± 0.24 µg total As/L), respectively.

Statistical analysis: The mean and standard error (SE) were calculated. The unpaired t test (Graph Pad Software, Inc., 2005) was used to analyze the significance difference. The correlation coefficients for different variables were tested using the Spearman rank order correlation test (Richard Lowry, 1998, 2008). *P* values less than 0.05 (two-tailed) were considered significant.

B. Arsenic metabolites in mice tissues after administering a single intra-muscular dose of sodium arsenate[34].

Reagents[34]. TRIZMA (Reagent grade, minimum 99.95), bovine serum albumin, and glutathione (GSH) were purchased from Sigma Chemical Co. (St. Louis, MO). [^{14}C]-MMA(V) (0.55 µCi/nmol, 98% purity) was synthesized by Professor Eugene A. Mash, Jr., Department of Chemistry, The University of Arizona. Disodium methylarsenate was obtained from Chem Service, Inc. (West Chester, PA). Monoflow-3 scintillation cocktail was from National Diagnostics (Atlanta, GA). Sodium arsenate (ACS reagent grade) was purchased from MCB Reagents (Cincinnati, OH). Diethyldithiocarbamic acid diethylammonium salt (DDDC) and carbon tetrachloride (CCl_4, 99.5+% anhydrous) were analytical reagent grade and were purchased from Aldrich Chemical Co., Inc. All other chemicals were analytical reagent grade or of the highest quality obtainable. Water was doubly deionized and distilled.

Animals[34]. Male wild type (WT) mice (DBA/1LacJ), 8 weeks of age, were purchased from Jackson Laboratory, Bar Harbor, Maine, USA. Upon arrival, mice were acclimated for at least 1 week and maintained in an environmentally controlled animal facility operating on a 12-h dark/12-h light cycle and 22–24°C. They were provided with Teklad (Indianapolis, IN) NIH-31 Modified Mouse/Rat Sterilizable Diet #7013 and water, ad libitum.

Preparation of the homogenate for analysis of arsenic species[34]. Wildtype (WT male, average age 10.3 weeks, n=18) mice were administered a single intra-muscular dose of sodium arsenate (4.16 mg As/kg body weight) inside of the left hind leg. In order to observe the metabolites of arsenate within a short period of time after a single injection of arsenate, we chose im injection because this was a consistent and less error prone way to get the arsenate into the body. We wished to detect MMA(III) and DMA (III) as quickly as possible before they were further metabolized. At the following times, 0 (control), 0.5, 1, 2, 4, 8, and 12 h, mice were decapitated rapidly by guillotine. Liver, kidneys, urinary bladder tissue, lungs, testes, and heart were removed, blotted on tissue papers (Kimtech Science, Precision Wipes), and weighed. The mouse tissues were homogenized in 0.02 M Tris–HCl (pH 8.0) and 20 mM DDDC at 4°C (cold room), using a glass homogenizer and Teflon coated steel pestle, transferred to a 10-mL acid-washed polypropylene tube and extracted immediately. The samples were always kept on dry ice. Protein concentration was determined by the method of Bradford[36], using bovine serum albumin as a standard.

Extraction of arsenic species[34]. Arsenic species were extracted from homogenates with CCl_4 and 20 mM DDDC by continuous shaking for 30 min at room temperature (Fig. 3) as described previously[35]. After centrifugation (11,000×g for 15 min at 4°C), an organic phase, aqueous phase, and interphase were obtained and treated as follows:

The organic phase (trivalent species)[34] was transferred to another tube and back extracted two times into 200 µL of 0.1M NaOH by shaking for 30 min at room temperature; centrifuged (11,000×g at 4°C) for 15 min; and the NaOH phase (upper phase) transferred to another 5 mL acid-washed polypropylene tube.

The aqueous phase (pentavalent species)[34] was transferred to another acid-washed polypropylene tube.

The inter-phase (trivalent and pentavalent species)[34] was homogenized in 0.020 M Tris–HCl (pH 8.0) and 20 mM DDDC and extracted twice with CCl_4 and 20 mM DDDC. The NaOH phase, containing +3 oxidation state arsenicals, and the aqueous phase, containing +5 oxidation state arsenicals, were each oxidized by addition of H_2O_2 to a final concentration of 5%, mixed and incubated over night at room temperature. The following morning, each sample (aqueous or NaOH phase) was boiled for 10 min. The aqueous phase was centrifuged (11,000×g for 15 min, 4°C). Each supernatant of NaOH phase and aqueous phase was transferred to separate acid-washed Eppendorf tubes. The pH was adjusted (5.5 to 6.5) by using NaOH or HCl, and the tubes were centrifuged again (14,000×g for 10 min at 4°C). Finally, the supernatant was transferred to another Eppendorf tube and stored at −20°C for HPLC-ICP-MS analysis. Fig. 3 shows the diagram of this procedure.

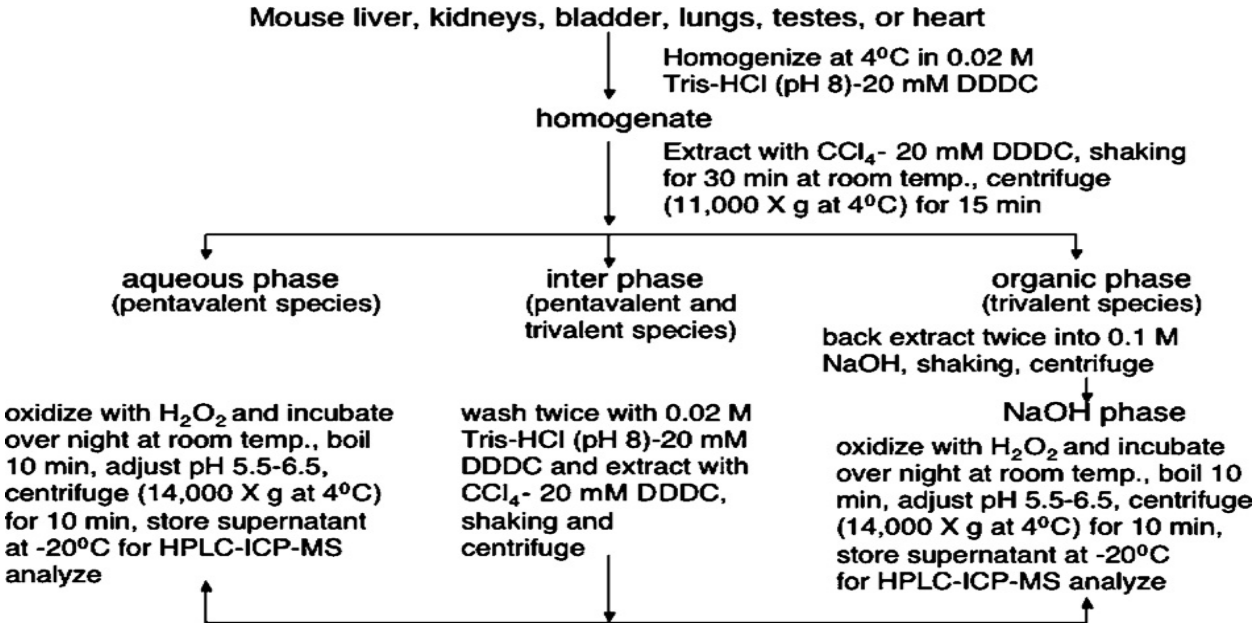


Fig 3. Extraction method for trivalent and pentavalent arsenicals of WT mice tissues[34]

HPLC-ICP-MS assay for arsenic species[34]. The HPLC system consisted of an Agilent 1100 HPLC (Agilent Technologies, Inc.) with a reverse-phase C18 column [Prodigy 3µ ODS, 150×4.60 mm, Phenomenex, Torrance, CA]. The mobile phase (pH 5.85) contained 4.7 mM tetrabutylammonium hydroxide, 2 mM malonic acid, and 4% (v/v) methanol at a flow rate of 1.2 mL/min. The column temperature was maintained at 50°C. This was essentially the method of Gong et al. (2001)[31], except ICP-MS instead of HG-AFS was used. An Agilent 7500a ICP-MS with a Babington nebulizer was used as the detector. The operating parameters were as follows: R_f power 1500 W, plasma gas flow 15 L/min, carrier flow 1.2 L/min, and arsenic was measured at 75 m/z. The validity of the procedure used for analysis has been published previously[34,35].

Results and Discussion

Arsenic metabolites including MMA^{III} and DMA^{III} in human urine. Urine and blood samples were collected from 75 subjects in the Lagunera area of Mexico. There were four groups, based on total arsenic concentrations in their drinking water (9-100µg/L). After collection, the urine samples were immediately frozen in a portable icebox, containing dry ice. The samples were kept frozen while being transported to the University of Arizona, Tucson where they were stored at -70°C before analysis.

Six different arsenic metabolites including MMA(III) and DMA(III) were measured in urines of four groups, based on total arsenic concentrations in their drinking water (9-100µg/L) from people (n=75) of Mexico. After acid digestion, total arsenic was also measured on the same urine samples by using ICP-MS, and the results show that the average concentration of total arsenic in urines (µg/L) of the corresponding groups were very close with the average sum of arsenic metabolites (µg/L) of the same urine samples (Table 1). These results show very little or no AsB (BDL) in these urine samples. The results also indicate that the people in our study may have drunk water from different sources because arsenic in drinking water were not strongly correlated with the total arsenic in their urines.

Table 1: Average concentration of total As (µg/L) and sum of arsenic metabolites in human urines of arsenic exposed people in the Lagunera area of Mexico

Group	No. of samples	As in drinking water (µg/L)	Average concentration of total As in human urine (µg/L)	
			Total As (µg/L)	Sum of As^{III}+As^{V}+MMA^{V}+DMA^{V} (µg/L)
4	19	9	38.45	41.92
1	19	17.5	30.45	28.28
3	20	52	38.21	36.75
2	17	100	59.15	55.11

The detection and quantification of urinary MMA(III) and DMA(III) in various populations have been reported over the past 20 years but with highly variable findings. Some reports suggest that in exposed populations, these trivalent compounds are major or even dominant components of urinary arsenic[18,19,27]. Other investigators suggest little or no DMA(III), and very low levels of MMA(III) occur in populations with a range of arsenic exposures[28-30]. In our study, we could not detect DMA(III), and very low levels of MMA(III) in a few urine samples occurred in populations of Mexico with a range of arsenic exposure from 9-100 µg/L (Table 2). Detectable MMA(III) was found in a total of 14 (~18%) out of 75 cases. Kalman, et.al. (2014)[37] was reported that they found detectable MMA(III) in a total of 41(~6%) of 643 cases.

The average concentration of MMA(III) in human urine of arsenic exposed people in the Lagunera area of Mexico were 0.17µg/L (n=6), 0.08 µg/L (n=2), and 0.12 µg/L (n=7) out of total 19 (group 4), 19 (group 1), and 17 (group 2) urine samples, respectively (Table 2). MMA(III) was not detectable in urines of group 3 people (n=20), but most of the metabolite was DMA(V) (~64% of total urinary As), where total As concentration in their drinking water was 52 µg/L. This means MMA(V) was reduced to MMA(III); then, MMA(III) methylated to DMA(V), even though there was no detectable MMA(III) in urines of group 3 people. There was also important information from these study groups that DMA(III) was not detectable in any of these urine samples (n=75). These results indicate that DMA(III) could be more instable compared to MMA(III), and reports also suggest that these trivalent compounds, MMA(III) and DMA(III), would not be the dominant components in urinary arsenic of this arsenic exposed people in Mexico.

Questions: Why was MMA(III) found in a few urine samples (n=14) out of total 75 samples? Why was DMA(III) not found/detected in any of these urine samples (n=75)? Does it oxidize during analyses? Does it depend on arsenic concentration in drinking water? Does it depend on the physiological/biological/environmental conditions of humans? Does it depend on the storage condition and duration of storage time? Does it depend on the genetic variability of humans? Does it depend on the reductase activities of humans? Does it oxidize before urine collection? What else?

Table 2: Average concentration of arsenic species in human urine (µg/L) of arsenic exposed people in the Lagunera area of Mexico

Group	No. of samples	As in drinking water (µg/L)	Average concentration of As species in human urine (µg/L)						Sum of As species in urine (µg/L)
			As(V)	As(III)	MMA(V)	MMA(III)	DMA(V)	DMA(III)	
4	19	9	2.3 (n=19)	5.38 (n=19)	5.55 (n=19)	0.17 (n=6)	28.62 (n=19)	ND (n=19)	42.02
1	19	17.5	1.33 (n=19)	3.66 (n=19)	4.31 (n=19)	0.08 (?) (n=2)	18.95 (n=19)	ND (n=19)	28.33
3	20	52	1.71 (n=20)	4.51 (n=20)	5.92 (n=20)	ND (n=20)	24.60 (n=20)	ND (n=20)	36.74
2	17	100	3.39 (n=17)	9.48 (n=17)	6.43 (n=17)	0.12 (n=7)	35.95 (n=17)	ND (n=17)	55.37

ND, not detectable

The average percentage of MMA(III) in urine samples of arsenic exposed people in the Lagunera area of Mexico were 0.44%, 0.26%, N.D. (Not Detectable), and 0.20% for the groups 4, 1, 3, and 2 where arsenic concentrations in their drinking water were 9, 17.5, 52, and 100 µg/L, respectively (Table 3). The percentage of MMA(III) was at very low levels compared with other arsenic metabolites detected or measured in these urine samples. The highest percentage of arsenic metabolite was DMA(V) (61% to 74% of the total arsenic in urine after acid digestion), and it indicates that most of the MMA(III) converted to DMA(V)↔ DMA(III), but DMA(III) may be very instable and oxidized to DMA(V) very quickly.

Table 3: Average percentage (%) of arsenic metabolites of total arsenic in urine of arsenic exposed people in the Lagunera area of Mexico

Group	No. of samples	As in drinking water (µg/L)	Average percentage (%) of As species in human urine					
			As(V)	As(III)	MMA(V)	MMA(III)	DMA(V)	DMA(III)
4	19	9	5.98	13.99	14.43	0.44	74.43	ND
1	19	17.5	4.37	12.02	14.15	0.26	62.23	ND
3	20	52	4.48	11.80	15.49	ND	64.38	ND
2	17	100	5.73	16.03	10.87	0.20	60.78	ND

ND, not detectable

Reductase activities in human blood

As(V) Reductase Assay. The reaction mixture (100 µL), containing 0.1 M MOPS buffer, pH 7.5, 1.0 mM EDTA, 10 mM inosine, 0.37-6 mM arsenate, 0.5 µCi of carrier-free [^{73}As] arsenate, 0.5-5 mM DHLP, and human blood arsenate reductase, was incubated for 30 min at 37°C. Arsenite, 0.1 M (100 µL) after incubation, was added rapidly, and the tubes were immersed in a boiling water bath for 2 min, cooled on ice for 5 min, and then centrifuged at 12,000 rpm for 6 min. A 100 µL sample of the supernatant was injected into a 4.9 mm x 250 mm Hamilton PRP-X100 anion exchange column, using 30 mM sodium phosphate (pH 5.0), with a flow rate of 1.45 mL min^{-1}. The arsenite and arsenate were quantified, using a post column in line Beckman 171 radioisotope detector. Monoflow III was used as the scintillation fluid, and a flow rate was used, and it was 3 times that of the mobile phase (Radabaugh et al. 2002)[38].

MMA(V) Reductase Assay. The reaction mixture contained 0.1M Tris HCL (pH 8.0), 5 mM GSH, 15 mM MMA(V), 8.1 x 109 cpm of [^{14}C]MMA(V), and human blood in a final volume of 250 µL and incubated for 60 min at 37°C. The MMA(III) that was produced was isolated by the extraction method previously described[34,39,40,41].

DMA(V) Reductase Assay. The reaction mixture (250 µL) was the same as in MMA(V) reductase assay, except that 8.2 x 109 cpm of [^{14}C]DMA(V), and 10 mM sodium DMA(V) replaced MMA(V). The product of the reaction that was formed was measured as previously described[34,39,40,41.]

Protein Assay. Protein concentrations were determined using bovine serum albumin as the standard[42].

(a) As(V) reductase activities

Human blood samples were collected from 75 subjects in the Lagunera area of Mexico. There were four groups, based on total arsenic concentrations in their drinking water (9-100µg/L). Blood was collected by venous puncture, into Vacutainers containing EDTA, transferred to the vial, and immediately frozen. The samples were kept frozen while being transported to the University of Arizona, Tucson where they were stored at -70°C before analysis.

The average As(V) reductase activities in bloods of our study population were 122.02±3.96, 115.44±4.51, 118.21±4.00, and 120.19±6.39 As(III) formed/ mg protein/h for groups 4, 1, 3, and 2, respectively (Table 4).The results show that As(V) reductase activities in bloods of different groups of humans were very similar, and these reductase activities were not dependent on the arsenic concentrations in their drinking water or urinary arsenic.

Table 4: Average As(III) formed/ mg protein/h in human blood of arsenic exposed people in the Lagunera area of Mexico

Group	No. of samples	As in drinking water (µg/L))	Formed As(III) (nmol/mg protein)		Average total As in urines (µg/L)
			Average	SE	Average
4	19	9	122.02	3.96	38.45
1	19	17.5	115.44	4.51	30.45
3	20	52	118.21	4.00	38.21
2	17	100	120.19	6.39	59.15

(b) MMA(V) and DMA(V) reductase activities[41,43]

Blood samples were collected from different exposure groups of 191 subjects (98 females and 93 males), aged 18-77 years in the Lagunera area of Mexico[43]. There were five groups, based on total arsenic concentrations (38-116 µg/L) in their drinking water (Table 5). The general characteristics of the study population have been previously described in detail[43]. Human blood samples were collected and immediately frozen. The samples were kept frozen while being transported to the University of Arizona, Tucson where they were stored at -70°C before analysis.

Table 5: The study population in Lagunera area of Mexico[43]

	Sex	Group 1 (Gp1)	Group 2 (Gp2)	Group 3 (Gp3)	Group 4 (Gp4)	Group 5 (Gp5)	All groups
As in drinking water (µg/L)		38.2	43.5	96.0	105.1	116.3	
As (mean±SE) in urine (µg/L)		34.52±5.44	39.51±6.78	72.49±9.43	116.09±18.65	123.28±13.15	
No. of participants	F	17	19	19	22	21	98
	M	21	14	20	18	20	93
Ages (mean±SE, years)	F	37.94±3.1	39.32±2.9	43.17±3.1	49.00±3.5	43.92±3.5	42.69±1.5
	M	40.00±3.6	46.92±5.0	41.44±4.6	42.06±2.8	39.00±3.3	41.47±1.7

The activities of MMA(V) reductase were significantly lower than DMA(V) reductase activities in the bloods of our study population ($p<0.01$; Mean values: 3.87±0.22 MMA(III) formed/ mg protein/h versus 6.34±0.22 DMA(III) formed/ mg protein/h, respectively). These reductase activities in blood between females and males of different arsenic exposure groups were almost similar, except for a few cases (Fig. 5). The results indicate that the activities of MMA(V) reductase and DMA(V) reductase in humans' blood don't depend on the arsenic level in their urinary arsenic/arsenic concentration in their drinking water (Figs. 4 and 5).

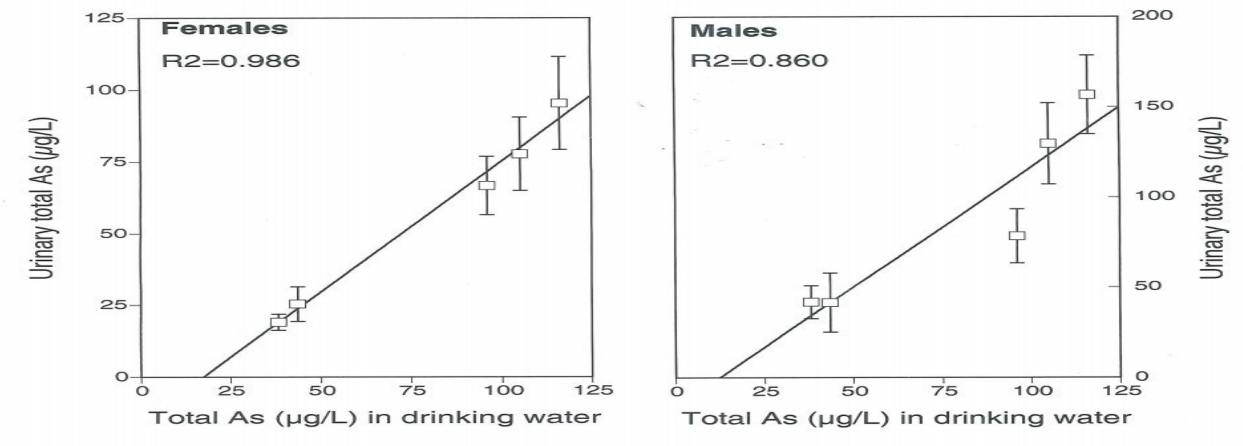


Fig. 4: Correlations between arsenic in drinking water of different arsenic exposed groups (38-116 µg/L) and total arsenic concentrations in their urine samples (µg/L) (after acid digestion) of females and males in the Lagunera area of Mexico[43].

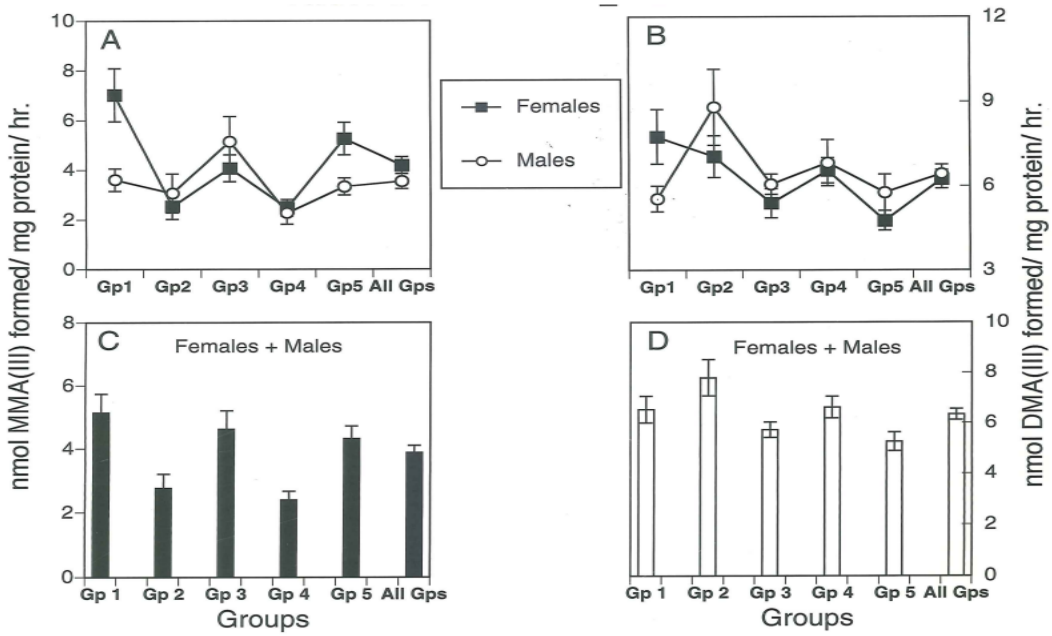


Fig. 5: Distribution of MMA(V) and DMA(V) reductase activities in bloods of females and males of different arsenic exposure groups (Gp1, 38 µg/L; Gp2, 43 µg/L; Gp3, 96 µg/L; Gp4, 105 µg/L; and Gp5, 116 µg/L). Values are the mean ± SE[41].

The correlations between MMA(V) reductase and DMA(V) reductase activities in blood of both females and males were very weak (r= +0.06 and r= -0.03, respectively). These correlations data indicate that there may be two different enzymes, and the mechanisms of reduction activities could be different. The activities of MMA(V) reductase and DMA(V) reductase were not depended on ages of these population (r= -0.05 and r= -0.11, respectively) too. A weak correlation found between MMA(V) reductase activities in blood and % MMA(V) in urine of males (r= -0.14, p=0.19) but not of females (r= -0.01, p=0.93). We also found a negative and statistically significant correlation between DMA(V) reductase activities in blood and %DMA(V) in urine of males (r= -0.23, p<0.05). This correlation was not statistically significant for females (r=-0.12, p=0.25).

Distribution of MMA^{III} and DMA^{III} in animal tissues[34]

The chemical forms of arsenic determine the toxicity and bioavailability of arsenic compounds[7,15]. MMA(III) and DMA(III) are more potent cytotoxins, genotoxins, and enzyme inhibitors than are inorganic arsenate, arsenite, or the +5-oxidation state methylated arsenic metabolites, MMA(V) and DMA(V)[20-25].

When mice were injected intramuscularly with Na arsenate (4.16 mg As/kg body weight); tissues removed at 0.5, 1, 2, 4, 8, and 12 h after arsenate injection; and the arsenic species measured by HPLC-ICP-MS. The results indicated that the highest concentration of the very toxic MMA(III), a key biotransformation, was in the kidneys, but DMA(III) was in the urinary bladder tissue of mice (Fig. 6)[34].

The distribution of arsenic metabolites, including the very toxic MMA(III) and DMA(III) in mice tissues, will be emphasized in the present discussion, and these results would help to know their distribution in urines. Sampayo-Reyes et al. (2000)[35] detected MMA(III) and DMA (III) in hamster liver 15 h after a single ip dose of arsenate. MMA(III) has also been reported in rat liver 2 h after iv injection of arsenate[44], although the use of the rat as a useful model for the human is of questionable and limited value[2].

The concentration of the very toxic MMA(III) was significantly higher than that of MMA(V) in all of the tissues tested (Liver, kidneys, urinary bladder tissue, lungs, testes, and heart). This is a validation of the extraction procedure used to measure the relatively unstable MMA(III). At 2 h after injection, the highest concentration of MMA(III) was in the kidneys, which was greater than the urinary bladder tissue, testes, heart ($p<0.001$), or liver and lungs ($p<0.01$) of mice[34]. Compared with the other species of arsenic, MMA(V) concentrations were the lowest in all tissues examined[34].

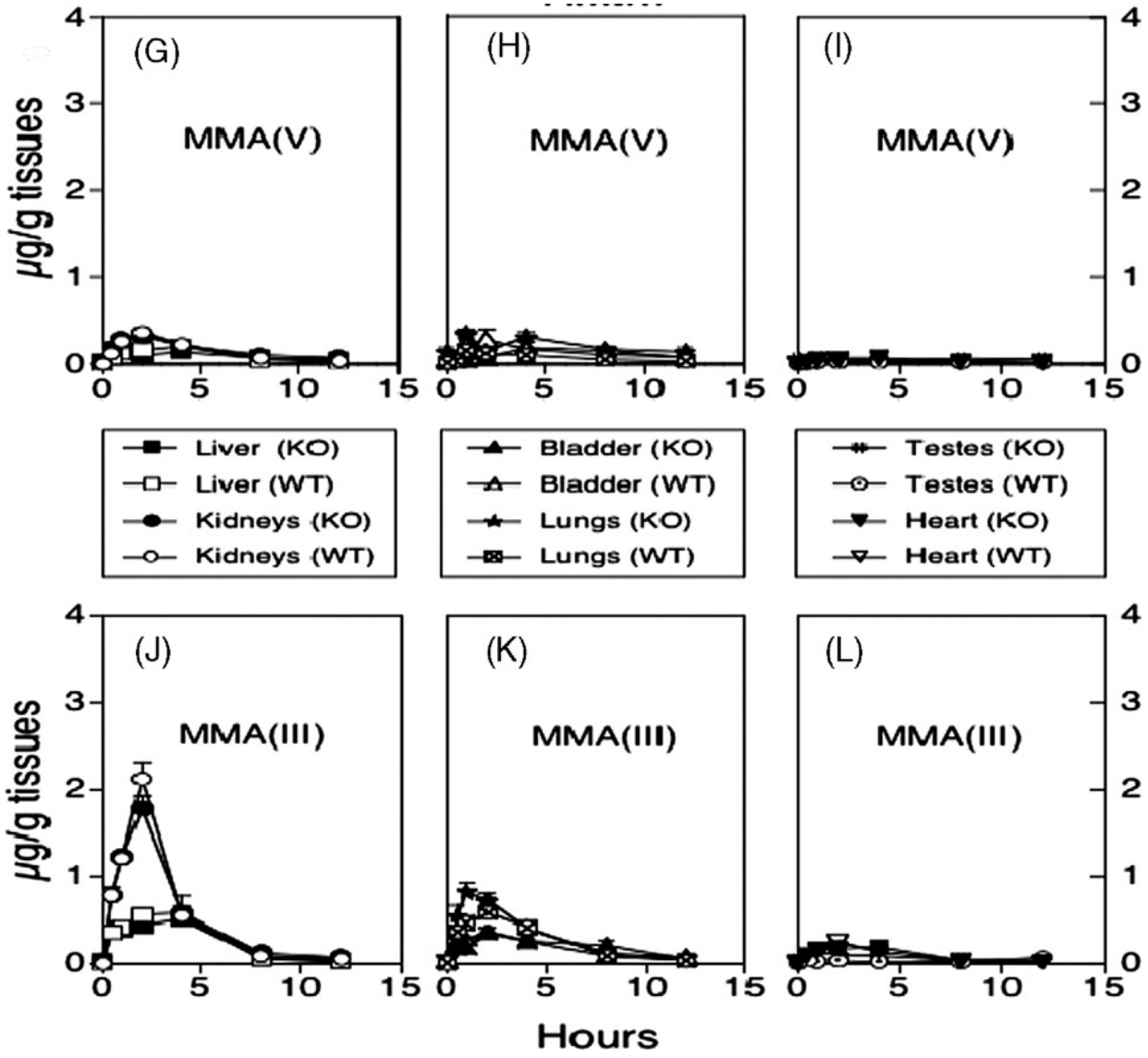


Fig. 6. Tissue concentrations of various arsenic metabolites in WT mice after a single intramuscular injection of sodium arsenate (4.16 mg As/kg body weight). The tissues were removed at 0 (control, injected doubly deionized and distilled water, and dedicated immediately), 0.5, 1, 2, 4, 8, and 12 h after arsenate injection. Values are the mean±SE (n=3)[34].

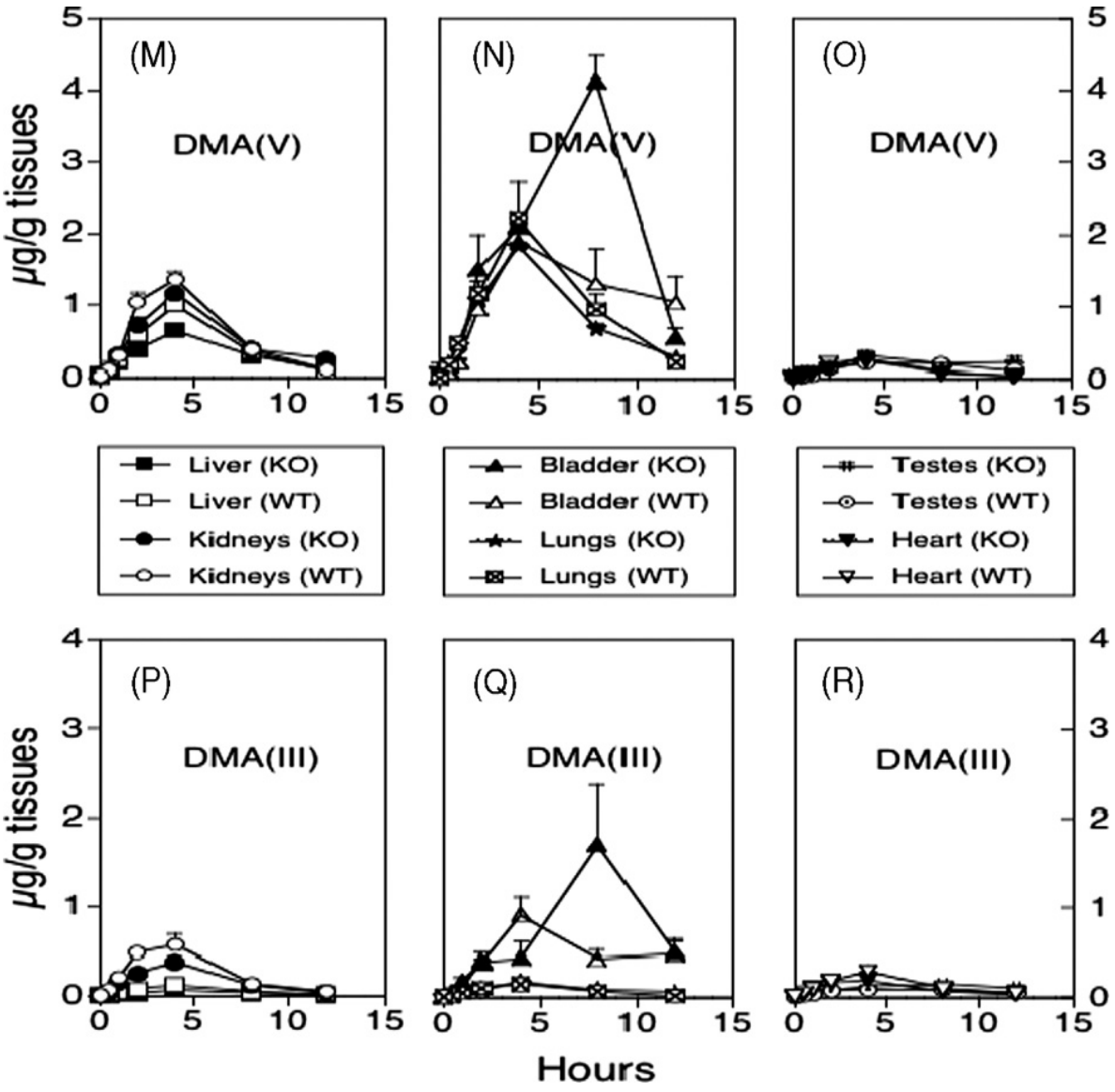


Fig. 6. (continued)[34].

On the other hand, the concentration of DMA(V) was higher than DMA(III) in the liver, kidneys, urinary bladder tissue, lungs, and testes at all times. DMA(V) accumulated at a higher concentration in the lungs and urinary bladder tissue, but the concentration of DMA(III) was highest in the urinary bladder tissue, followed by the kidneys, lungs, heart, testes, and liver. In all of the tissues, both DMA(III) and DMA(V) were the highest at 4 h for the WT mice. The concentration of DMA(III) was significantly higher in the urinary bladder tissue than in other tissues ($p<0.05$), except in the kidneys of the mice.

According to these results, the concentration of MMA(III) was significantly higher than that of MMA(V) in all of the tissues tested (Liver, kidneys, urinary bladder tissue, lungs, testes, and heart). But the concentration of DMA(V) was higher than DMA(III) in the liver, kidneys, urinary bladder tissue, lungs, and testes at all times. The results indicate that MMA(V) reduced to MMA(III) faster compared to DMA(V) to DMA(III) because we found significantly high amounts of MMA(III) in all the tissues, compared to MMA(V) at the same time (2 h), but DMA(V) was significantly high in the tissues, compared to DMA(III) at 4 h. It could be all or most of the MMA(V) reduced to MMA(III) and methylated to DMA(V), but less percentage of this DMA(V) reduced to DMA(III). It might be due to the fact that DMA(III) was more instable in the tissues, compared to MMA(III) and oxidized faster, compared to MMA(III). These results also indicate that due to the instability and faster oxidation of DMA(III), we could not measure/detect it, but we found low percentage of MMA(III) in urines of some arsenic exposed people due to its more stability and bonding capacity with tissues, comparing to DMA(III). May be due to genetic variability and other factors, we found MMA(III) in urines of some of them but not all in our study groups.

MMA(V) reducing activity of WT mice tissues[34]

The rate of MMA(V) reducing activity was determined in various tissues, including blood hemolysates of WT mice. The indicated results are based on the rate (limiting amounts) and not the limit (enzyme excess). For the wild-type mice, the urinary bladder tissue had the largest enzyme activity, and the blood hemolysates had the smallest[34]. The order of MMA(V) reductase activity to highest to lowest are bladder, heart, brain, lungs, spleen, skin, kidneys, liver, testes, and blood hemolysates. The mechanism of inorganic arsenic biotransformation and the stability or instability of more toxic MMA(III) and DMA(III) may depend on biological environment and genetic variability, but more experiments are needed to understand these mechanisms.

Are MMA(III) and DMA(III) oxidized before urine collection?

There was no detectable levels MMA(III), but it was 64% DMA(V) in urines of group 3 people (n=20) (Table 3). That means, MMA(V) was reduced to MMA(III) and then MMA(III) methylated to DMA(V), even though there was no detectable MMA(III) in urines of this group 3 people. Another important information of this study group is that DMA(III) was not measured in any of these urine samples (n=75). These results indicate that DMA(III) could be more instable and/or produced very less amount, comparing with MMA(III) and suggest that the +5-oxidation state of arsenic metabolite, DMA(V), could be the most dominant methylated arsenic metabolite in humans' urine of this arsenic exposed people.

The average percentages of MMA(III) were 0.44%, 0.26%, N.D. (Not Detectable), and 0.20%, but the percentages of DMA(V) were 74.43%, 62.23%, 64.38%, and 60.78% in urines for the population of the group's 4, 1, 3, and 2, where arsenic concentrations in their drinking water were 9, 17.5, 52, and 100 (µg/L), respectively. There was a small percentage of MMA(III), compared with other arsenic metabolites, in these urine samples, but there was no detectable DMA(III). The highest percentage of arsenic metabolite was DMA(V) (61% to 74%) of the total arsenic in urines, and it indicates that most of the MMA(III) converted to DMA(V) ↔ DMA(III), but DMA(III) may be very instable and oxidized to DMA(V), quickly.

In animal experiment, mice were injected intramuscularly with Na arsenate and the arsenic species in tissues measured by HPLC-ICP-MS. The results indicated that the highest concentration of very toxic MMA(III), a key biotransformant was in the kidneys, but DMA(III) was in the urinary bladder tissue of mice[34].

The concentration of the very toxic MMA(III) was significantly higher than that of MMA(V) in all of the tissues tested at all times after arsenate injection. At 2 h after injection, the highest concentration of MMA(III) was in the kidneys, which was greater than the urinary bladder tissue, testes, heart ($p<0.001$), or liver and lungs ($p<0.01$) of mice. Compared with the other metabolites of arsenic, MMA(V) concentrations were the lowest in all tissues examined[34], i.e., most of the MMA(V) were reduced to MMA(III) immediately and then it was methylated to DMA(V). However, some percentage of MMA(III) could also be oxidized to MMA(V) before methylated to DMA(V).

On the other hand, the concentration of DMA(V) was higher than DMA(III) in the liver, kidneys, urinary bladder tissue, lungs, and testes at all times. DMA(V) accumulated at a higher concentration in the urinary bladder tissue and lungs, but the concentration of DMA(III) was highest in the urinary bladder tissue followed by the kidneys, lungs, heart, testes, and liver. In all of the tissues, both DMA(III) and DMA(V) were highest at 4 h for the WT mice. The concentration of DMA(III) was significantly higher in the urinary bladder tissue than in other tissues ($p<0.05$), except in the kidneys of the mice. The results indicate that maybe the less percentage of DMA(V) was reduced to DMA(III) or after reduction it may be immediately oxidized to DMA(V) in tissues.

The highest concentration of As(V) was in the urinary bladder tissue (~30 µg/gm tissue) and the kidneys (~11 µg/gm tissue) at 0.5h[34]. But the concentration of As(III) and MMA(III) were highest in kidneys, compared to bladder tissue. Specially, MMA(III) was ~2 µg/gm tissue vs ~0.3 µg/gm tissue in kidneys vs. bladder tissues at the same time (2h), respectively. Even though MMA(III) was significantly low in bladder tissues compared to kidneys, the highest concentration of both DMA(V) and DMA(III) were measured in bladder tissues compared with all of the tissues tested (for example, ~2.2µg/gm tissue vs ~1.4 µg/gm tissue of DMA(V) and ~1µg/gm tissue vs ~0.6µg/gm tissue of DMA(III) for bladder tissue vs kidneys at 4h, respectively). The results indicate that it could be DMA(V) accumulated in the bladder, and DMA(V) would be the most dominated arsenic metabolite in the bladder tissue fluid.

According to the results, the concentration of MMA(III) was significantly higher than that of MMA(V) in all of the tissues tested (Liver, kidneys, urinary bladder tissue, lungs, testes, and heart). On the other hand, the concentration of DMA(V) was higher than DMA(III) in the liver, kidneys, urinary bladder tissue, lungs, and testes at all times. The results indicate that MMA(V) reduced to MMA(III) faster compared to DMA(V) to DMA(III) because we found significantly highest amount of MMA(III) in all the tissues, compared to MMA(V) at the same time (2 h), but DMA(V) was significantly highest in the tissues, compared to DMA(III) at 4 h. It could be that most of the MMA(V) reduced to MMA(III) and methylated to DMA(V), but less or more percentage of this DMA(V) reduced to DMA(III), but DMA(III)

may be very instable and oxidized to DMA(V), quickly and found higher percentage of DMA(V) compared to DMA(III) in tissues. It might be because DMA(III) was more instable in the tissues, compared to MMA(III), and oxidized faster, compared to MMA(III). It could also be suitable for binding of MMA(III) to tissue protein components, compared to DMA(III).

In our study groups, we could not detect DMA(III), and low levels of MMA(III) in a few urine samples occurred in populations of Mexico with a range of arsenic concentration in their drinking water from 9-100 µg/L. We may explain the distribution of MMA(III) and DMA(III) in urines of humans from the distribution of MMA(III) and DMA(III) in tissues of mice because DMA(III) could be more instable in the tissues and oxidized faster, compared to MMA(III). Therefore, due to the instability and faster oxidation of DMA(III), we could not detect DMA(III), but we found some percentage of MMA(III) in some urine samples of arsenic exposed people due to its more stability, compared to DMA(III). In tissues, the concentration of DMA(V) was higher than DMA(III) in the liver, kidneys, urinary bladder tissue, lungs, and testes at all times. Most of the MMA(III) was methylated to DMA(V), and DMA(V) was the dominated metabolite in tissues, starting at 4h, which was also true for human urine because DMA(V) was the dominated arsenic metabolite in there. We detected both MMA(III) and DMA(III) in tissues but not in urines, and the reports suggested that tissue levels of MMA(III) and DMA(III) are both more relevant and less susceptible to oxidative artifacts than are urine samples.

It has been reported that inorganic arsenic is commonly methylated in liver,[45,46] but in our mouse studies we found that the highest amount of MMA(III) was in kidney, followed by liver and bladder. On the other hand, both DMA(V) and DMA(III) were highest in bladder followed by kidney>liver[34]. The concentrations of MMA(V) were higher in kidney and bladder tissues than liver, and MMA(V) concentrations were the lowest, comparing with other arsenic metabolites in all tissues examined[34]. If liver is the main/common organ for arsenic methylation, then liver should contain more MMA(III) and DMA(III), but the results show different. These results indicate that maybe MMA(III) and DMA(III) bonding proteins/components in liver maybe less or weak, comparing to bladder, kidneys, etc., and migrated these toxic metabolites to other tissues, or the liver may not be the only most common organ of inorganic methylation/biotransformation process in vivo.

The urine flows from the kidneys through the ureters to the bladder. The urine is stored in the bladder until it leaves the bladder through the urethra. Urine is typically the same as a person's body temperature. On average, this is 98.6ºF (37ºC). Some people have normal temperature variations that may be slightly hotter or slightly cooler than this. Urine will usually maintain its temperature outside the body for about four minutes. If MMA(III) and DMA(III) metabolites could survive at body temperature (37ºC), then why aren't they stable at frozen or below frozen temperature (-20ºC, -70ºC, etc)? Also, the concentration of MMA(III) does not depend on the concentrations of total arsenic in urines. May be, most of them are oxidized before urine collection, and a small percentage of MMA(III) and DMA(III) are bound with some components/proteins in urine under biological environment, which are not suitable under invitro conditions, even though they are kept at frozen or below frozen.

Stability reports of MMA(III) and DMA(III) from other researchers

Potential problems in the determination of MMA(III) that have been noted in the past include storage instability and measurement artifacts[47,48]. Based on the reported storage instability of MMA(III) in urine samples at –20ºC, negative findings for several of these studies[49-54] might be due to oxidation during storage, while findings of high levels and high proportions of MMA(III) suggest other method artifacts. Laboratory studies of MMA(III) instability have led to conclusions that it might be more prevalent than reported in urine samples from population studies, due to losses in sample handling and measurement[55]. Lindberg and co-workers[49] have suggested that MMA(III) may be too reactive toward oxidation or binding to tissue components to be suitable for use as a urinary biomarker. More recent reports have suggested that tissue levels are both more relevant and less susceptible to oxidative artifacts than urine samples[56].

The wide variation in reports of the frequency, proportion, and level of MMA(III) and DMA(III) in human urine may indicate variability due to a variety of factors, summarized recently byTseng[57]. These include sample collection, preservation, and measurement; population-specific environmental or nutritional factors; or underlying genetic differences[58-60], related to the enzymes that control biotransformation. Resolving the questions regarding issues of sample collection/storage/analysis is therefore of prime importance in determining the biological significance of measured levels of trivalent methylated arsenic in urine.

Varying degrees of instability of MMA(III) in solution, and the role of sample matrix, temperature, and pH have been noted previously. Arsenic species (excluding trivalent methylated forms) in aqueous samples are reported to be less stable than either fresh or reconstituted freeze-dried urine[61], but several reports indicate that MMA(III) is more unstable in urine than in water[47,53,62]. Of equal significance, variable stability in different urine samples has been noted[62]. Using MMA(III)-fortified urine samples, Gong et al.[47] found 30% loss after 1 day, at both 4ºC and –20ºC. The losses of MMA(III) from urine over 1 to 3 days at 25ºC, reported by Gong and his co-workers[47], are particularly significant because that is the time scale and temperature for samples during the actual analysis.

Conclusion

Analysis of urinary arsenic species in samples (n=75), having total urinary arsenic of 30 to 59 µg/L, showed few cases (~18%) of detectable MMA(III). The finding from this study of mostly undetectable MMA(III), and there were no detectable levels of DMA(III) in these urine samples. The highest percentage of arsenic metabolite was DMA(V) (61% to 74%) of the total arsenic, and it indicates that most of the MMA(III) converted to DMA(V) and a part of this DMA(V) ↔ DMA(III), but it may be

very unstable and oxidized to DMA(V) again, quickly. It seems that maybe both MMA(III) and DMA(III) are unstable, but DMA(III) could be more unstable comparing MMA(III).

The results indicate that MMA(V) reduced to MMA(III) faster, compared to DMA(V) to DMA(III), because there was a significantly high amount of MMA(III) in all the tissues, compared to MMA(V) at the same time (2 h), but DMA(V) was higher in most of the tissues, compared to DMA(III), and it was highest at 4 h. The concentration of the very toxic MMA(III) was significantly higher than that of MMA(V) in all of the tissues tested (Liver, kidneys, urinary bladder tissue, lungs, testes, and heart) at 0.5, 1, 2, 4, 8, and 12h after arsenate injection. On the other hand, the concentration of DMA(V) was higher than DMA(III) in the liver, kidneys, urinary bladder tissue, lungs, and testes at all times. It could be all or most of the MMA(V) reduced to MMA(III) and methylated to DMA(V) but less percentage of DMA(V) reduced to DMA(III). Maybe due to the fact that DMA(III) was more unstable in the tissues comparing MMA(III) and oxidized faster compared to MMA(III). These results also indicate that due to the instability and faster oxidation of DMA(III) we could not measure/detect DMA(III), but due to comparing more stability of MMA(III), we found low levels of MMA(III) in urine of exposed people.

Finally, the trivalent MMA(III) compound could be the most dominant toxic arsenic metabolite in humans' tissues for a period of time but not in urine because arsenic metabolism/biotransformation process occurred in tissues, and urine contains the end products only. On the other hand, DMA(V), instead of DMA(III), would be another dominant arsenic metabolite in tissues as well as in urine. Their stability/instability may depend on biological environment, genetic variability, and other complex factors. More experiments are needed to understand the mechanism of inorganic arsenic biotransformation, and stability or instability of very toxic MMA(III) and DMA(III).

Acknowledgement

The Author wants to dedicate this paper to the memory of Dr. H. Vasken Aposhian and Mary M. Aposhian who passed away on September 6, 2019, and September 9, 2009, respectively. Prof. Vas Aposhian and Mary Aposhian had collected these urine and blood samples from people in the Lagunera area of Mexico. Dr. Aposhian was an Emeritus Professor in the Department of Molecular and Cellular Biology at the University of Arizona, and he was the author's supervisor.

The Author would like to thank Ms. Shreya Chowdhury for reading, reviewing, and editing this manuscript.

The Superfund Basic Research Program NIEHS Grant Number ES 04940 from the National Institute of Environmental Health Sciences and the Southwest Environmental Health Sciences Center P30-ES-06694 supported this work.

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