Urinary Trivalent Methylated Arsenic Species in a Population Chronically Exposed to Inorganic Arsenic

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Key words- arsenic, trivalent arsenic, urine metabolites, metabolism, arsenic speciation, methylation, trivalent methylarsenic species, arsenic-skin lesions, hyperkeratosis; hypopigmentation.

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Abbreviations
iAs- inorganic arsenic; iAs$V$-arsenate; iAs$^{III}$-arsenite; MAs$^{III}$-monomethylarsonous acid; MAs$V$-monomethylarsonic acid; DMAs$^{III}$-dimethylarsinous acid; DMAs$^{V}$-dimethylarsinic acid-TMAs$^{V}$O-trimethylarsine oxide; TAs-total arsenic; SAM-Sulfo-adenosylmethionine; HG-AAS- hydride generation atomic absorption spectrophotometry; DMPS-2,3-dimercaptopropane-1-sulfonic acid; TWE-time weighted exposure; OR-odd ratio; CI-confidence interval
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Abstract

Chronic exposure to inorganic arsenic (iAs) has been associated with increased risk of various forms of cancer and of non-cancerous diseases. Metabolic conversions of iAs that yield highly toxic and genotoxic methylarsonate (MAs\textsuperscript{III}) and dimethylarsinite (DMAs\textsuperscript{III}) may play a significant role in determining the extent and character of toxic and cancer promoting effects of iAs exposure. This study examines the relationship between urinary profiles of MAs\textsuperscript{III} and DMAs\textsuperscript{III} and skin lesions markers of iAs toxicity in individuals exposed to iAs in drinking water. The study subjects were recruited among the residents of an endemic region of central Mexico. Drinking water reservoirs in this region are heavily contaminated with iAs. Previous studies carried out in the local populations have found an increased incidence of pathologies, primarily skin lesions, that are characteristic of arseniasis. The goal of this study was to investigate the urinary profiles for the trivalent and pentavalent As metabolites in both high and low iAs-exposed subjects. Notably, methylated trivalent arsenicals were detected in 98% of analyzed urine samples. In average the major metabolite, DMAs\textsuperscript{III}, represented 49% of total urinary As, followed by DMAs\textsuperscript{V} (23.7%), iAs\textsuperscript{V} (8.6%), iAs\textsuperscript{III} (8.5%), MAs\textsuperscript{III} (7.4%), and MAs\textsuperscript{V} (2.8%). More importantly, the average MAs\textsuperscript{III} concentration was significantly higher in the urine of exposed individuals with skin lesions as compared to those who drank iAs-contaminated water, but had no skin lesions. These data suggest that urinary levels of MAs\textsuperscript{III}, the most toxic species among identified metabolites of iAs may serve as an indicator for identify individuals with increased susceptibility to toxic and cancer promoting effects of arseniasis.
Introduction

Arsenic (As) is a ubiquitous element found in several forms in foods and environmental media such as soil, air, water; the predominant form in drinking water is inorganic arsenic (iAs), which is both highly toxic and readily bioavailable. iAs is recognizing carcinogen in humans (NRC 2001). Chronic ingestion of iAs-contaminated drinking water is therefore considered the major pathway behind the risk to human health. It has been estimated that 200 million people world-wide are at risk from health effects associated with high concentrations of arsenic in their drinking water (NRC 2001). Several other regions in the world are exposed to levels above the maximum permissible limit recommended by the World Health Organization (WHO 1993).

In humans, the chronic ingestion of iAs (> 500μgAs/day) has been associated with cardiovascular, nervous, hepatic, and renal alterations and diabetes mellitus as well as cancer of the skin, bladder, lung, liver and prostate (ATSDR 2000). Characteristic features of arseniasis include skin manifestations, such as hyperpigmentation, hypopigmentation and hyperkeratosis on the palms and soles, and skin cancer at later stages (Cebrian et al. 1983; Schwartz 1997; Tseng et al. 1968).

In humans, the mechanism by which iAs exerts its toxic effects is very complex because during their metabolism it involves at least 5 metabolites, that can exert toxic effects. The scheme for the stepwise conversion of arsenite (iAs\textsuperscript{III}) into mono-, di-, and trimethylated products is showns in [1]:

\[
\text{As}^{\text{III}}\text{O}_3^{\text{3-}} + \text{CH}_3^+ \rightarrow \text{CH}_3\text{As}^{\text{V}}\text{O}_3^{\text{2-}} + 2\text{e}^- \rightarrow \text{CH}_3\text{As}^{\text{III}}\text{O}_2^{\text{2-}} + \text{CH}_3^+ \rightarrow (\text{CH}_3)_2\text{As}^{\text{V}}\text{O}^- + 2\text{e}^- \\
\rightarrow (\text{CH}_3)_2\text{As}^{\text{III}}\text{O}^- + \text{CH}_3^+ \rightarrow (\text{CH}_3)_3\text{As}^{\text{V}}\text{O}^- 
\]

[1]
Briefly the metabolic process is carried out in two processes: a) the reactions of reduction that become the pentavalency species to trivalency and b) reactions of oxidative methylations where iAs is converted to mono di- and trimethyl arsenic forms (MAs, DMAs and TMAsO, respectively). Thus, both pentavalent and trivalent methylarsenic forms are intermediates or products of this pathway (Lin et al. 2002; Thomas et al. 2004).

Using S-adenosylmethionine (SAM) as a methyl group donor, the arsenic methyltransferase (cyt19, E.C.2.1.1.138) has been shown to catalyze reactions, reduction and oxidative methylation, in rodents and humans (Waters et al. 2004). In other studies it has been informed of the capacity of two mammalian proteins to reduce iAs$^{V}$, the glutathione S transferase omega (GSTΩ, E.C.2.5.1.18) (Zakharyan et al. 2001) and purine nucleoside phosphorylase (PNP, E.C. 2.4.2.1) (Nemeti et al. 2003; Radabaugh et al. 2002).

Urinary As is generally regarded as the most reliable indicator of recent exposure to iAs and is used as the main biomarker of exposure (Mushak and Crocetti 1995). In addition, the urinary profiles of iAs metabolites have frequently been used in epidemiological studies to assess the capacity of exposed individuals to methylate iAs. During almost 20 years the methylation of iAs has been generally evaluated using urinary measurement of iAs$^{(III+V)}$, MAs$^{(III+V)}$ and DMAs$^{(III+V)}$ in people exposed to arsenic. Nevertheless, the differentiation of the trivalent intermediaries of the As metabolism is important because the trivalent methylated arsenicals, MAs$^{III}$ and DMAs$^{III}$ are more potent than either iAs$^{III}$ or iAs$^{V}$ as cytotoxic (Styblo et al. 2000), genotoxic (Mass et al. 2001; Nesnow et al. 2002), inhibitors of enzymes with antioxidative functions (Lin et al. 2001; Styblo et al. 1997). Therefore, the formation of MAs$^{III}$ and DMAs$^{III}$ in the methylation
pathway for iAs may play a significant role in the induction of toxic effects associated with exposures to iAs.

The goal of this study was to assess the urinary pattern of arsenic methylation including trivalent methylated metabolites, in an arsenic-endemic population using freshly collected samples and analyzed as soon as possible to avoid the oxidation of MAs$^{\text{III}}$ and DMAs$^{\text{III}}$, even at temperatures below 0°C (Del Razo et al. 2001; Gong et al. 2001). Additionally, we compared the pattern of urinary trivalent methylated metabolites between persons with and without skin lesions associated to arsenicism in an endemic Mexican area.
Methods

**Site selection.** Study subjects were residents of Zimapan in the state of Hidalgo, this population is located in the central part of Mexico, to 220 km away of Mexico City. It has been a mining district since the 16th century. By 1810 there were 40 smelters operating in and around Zimapan (Garcia and Querol 1991). There have been no active smelters in Zimapan since the 1940s; however, tailing piles from the flotation process have accumulated in Zimapan for more than 60 years. Tailings are sediments resulting from settling of ore’s wastes. Some rocks from the Zimapan Valley have higher than world average iAs concentration, from 2,550 to 21,400 mg/kg were found in all the tailings, for the rock type. (Mendez and Armienta 2003). Most of the current exposure occurs outside the Zimapan basin, but old tailing near the edge of the town are still an iAs pollution source. Two major pathways contaminate groundwater with iAs: a) iAs dissolved from ore and other minerals in the mining district can be transported though fractures in the limestones (mainly arsenopyrite) to water source and b) rainwater leaching through mine tailing piles surrounded (Armienta et al. 1997a).

The National Water Commission of Mexico found high iAs concentrations in many of the wells in 1992. Water samples collected from springs, and drilled wells (about 180 m total depth) presented levels between 21 to 1,100 µgAs/L (CNA 1992).

In 1999, the municipality closed one of the wells connected to the municipal water system that contained the highest iAs concentration (1,100 µg/L). This action reduced the average iAs concentration in the municipal water from 580 to 350 µg/L. However, over 40% of the valley residents in this area are not connected to this municipal supply and rely on local springs and norias (bucket-wheel wells) for their potable water, and some of these sources are still heavily polluted with iAs.
Subject selection. A cross-sectional study was conducted with 104 participants; 76 Zimapan residents exposed to $\geq 50 \mu g$ iAs/L and 28 individuals exposed to $\leq 10 \mu g$ iAs/L in water of human consumption (named controls) in accordance with the regulations of the Ethical Committee of the Faculty of Medicine, Juarez University of Durango. Subjects were recruited through door-to-door contact. They had to be at least 15 years old and lived in the town for the previous 2 years. Before enrollment in the study each participant read and signed an informed consent form. Subjects were interviewed by trained interviewers regarding general characteristics with emphasis in personal habits, history and habits of water consumption, smoking habits, and medical, occupational, residential histories. They underwent physical examination looking for typical dermatological signs of arseniasis. The skin signs of arseniasis were evaluated by medical health care physicians. The physicians were blind to time and level of exposure for each subject at the time of physical examination. The physicians have been evaluating dermatological signs of arseniasis in Mexico for about 10 years. Participants were asked to exclude seafood from the diet for the preceding 4 days. Individuals who had received drugs with well-defined organ toxicity within the past 4 months or suffering chronic alcoholism were not included. Each family's drinking water was analyzed for total arsenic (TAs) concentration. The final decision on study eligibility was based on the measurement of TAs concentration in the household water source and looking approximately 50% of the iAs-high exposed group as presenting at least one skin sign of arseniasis, such as hypo/hyperpigmentation, palmpoplantar hyperkeratosis and ulcerative lesions as described by Yeh (1973).

Exposure assessment. The total liters of drinking water consumed per day by each subject were estimated from water drunk during the day. Daily estimates of As consumption were calculated as the product of the number of liters consumed per day and
the As concentration in the subject's drinking water source. Cumulative exposure to iAs or
time weighted iAs exposure (TWE) was calculated using the duration of exposure, the
number of liters consumed per day and the historical As concentration reported by
Mexican’s National Water Commission from 1992 to the present time (Garcia-Vargas et al.
1994).

**Chemicals.** Arsenic acid, disodium salt (Na$_2$HAs$^V$O$_4$), and sodium m-arsenite
(NaAs$^{III}$O$_2$), both better than 99% pure, were from Sigma Chemical Co. (St. Louis, MO).
Methylarsonic acid, disodium salt (MAs$^V$), CH$_3$As$^V$O(ONa)$_2$, 99% pure, was obtained from
Ventron (Danvers, MA), and dimethylarsinic acid (DMAs$^V$, (CH$_3$)$_2$As$^V$O(OH)), 98% pure,
was obtained from Strem (Newburyport, MA). The trivalent methylated arsenicals,
methylarsenic acid (MAs$^{III}$O, CH$_3$As$^{III}$O) and iododimethylarsine of DMAs$^{III}$ (DMAs$^{III}$I,
(CH$_3$)$_2$As$^{III}$I) were synthesized by Dr. William R. Cullen (University of British Columbia,
Vancouver, Canada) using previously described methods (Cullen et al. 1984; Styblo et al.
1997). Identity and purity of the synthesized arsenicals were confirmed using 1H NMR,
mass spectrometry, and hydride generation–atomic absorption spectrophotometry (HG–
AAS) as previously described (Hughes et al. 2000). In aqueous solutions, MAs$^{III}$O and
DMAs$^{III}$I are presumed to form MAs$^{III}$ and DMAs$^{III}$, respectively. Working standards of
these arsenicals that contained 1μg of As/mL were prepared daily from stock solutions.
Sodium borohydride (NaBH$_4$) was obtained from EM Science (Gibbstown NJ). Tris–
hydrochloride was purchased from J.T. Baker (Phillipsburg, NJ). Kits of creatinine was
purchased from Randox (San Diego CA). All other chemicals used were at least analytical
grade. Standard reference material water (SRM 1463c) and urine [SRM 2670; National
Institute of Standards and Technology (NIST), Gaithersburg, MD] were used for quality control of TAs in water and urine analysis, respectively.

**Drinking water collection and processing.** Tap water samples were collected in the homes of potential subject families using acid-washed containers transported to the site of the study at the same time as the investigators. A total of 91 water samples were collected from 80 households, (more than one sample was obtained from each household if participants used different source of water to drink and cook). Water samples were stored at -20°C until subsequent assay. Water samples were transported to the Cinvestav-IPN laboratories in Mexico City for TAs analysis. TAs was determined by HG-AAS, using Perkin Elmer 3100 (Norwalk, CT, USA), equipped with a FIAS-200 flow injection atomic spectroscopy system as reported previously (Del Razo et al. 1990). All measurements were made using an arsenic electrode less discharge lamp. Standard Reference Material SRM 1463c was used for quality control of TAs in water analysis. The certified TAs concentration in SRM 1463c is 82.1 ± 1.2 μg/L. Replicate analyses of this SRM using the method described above gave concentrations of 82.7 ± 1.7 μg/L, which is in good agreement with the certified value.

**Urine collection and processing.** After clinical exploration, all participants were scheduled for urine sample collection each third day over three weeks in groups of 10 to 12 individuals each time. Subjects were seen between 7 and 8 am at the local Health Center, where urine spot samples were collected with a minimum of contamination in 250-ml polypropylene containers given and labeled by us. Urine samples were immediately frozen in dry ice. To prevent oxidation of unstable trivalent methylated arsenicals, frozen urine samples were immediately transported to Cinvestav-IPN laboratory and analyzed within the 6 hours after collection.
A pH-specific HG-AAS has been optimized to permit simultaneous analysis of all known metabolites of iAs, including iAs\textsuperscript{III}, iAs\textsuperscript{V}, MAAs\textsuperscript{III}, MAAs\textsuperscript{V}, DMAs\textsuperscript{III}, DMAs\textsuperscript{V} in urine (Del Razo et al. 2001). This method is based on a pH specific generation of hydrides from tri- and pentavalent iAs, MAAs and DMAs with subsequent chromatography and determination of As contents in HG-AAS. The HG–AAS apparatus was based on the design of Crecelius et al. (1986). For hydride generation at pH 2 or lower, 1 ml of sample urine, 5 ml of deionized water, and 1 ml of 6M hydrochloric acid (HCl) were placed into the reaction vessel. This mixture had a final pH of 1–2. For hydride generation at pH 6, 1 ml of sample urine 5 ml of deionized water, and 1 ml of 2.5 M Tris–HCl, and 0.06 M NaOH buffer, pH 6, were placed into the reaction vessel. This mixture had a final pH of approximately 6. At either pH, thorough mixing of the contents of the reaction vessel was followed by injection into the reaction vessel of 1 ml of a 4% solution of NaBH\textsubscript{4} in 0.02 M NaOH. Cold-trapped arsines were released from the U tube by its removal from the liquid nitrogen and application of heat, for the later arsines separation for a gradient of temperature.

SRM 2670 was used to validate analysis of TAs; SRM 2670 consists of two bottles of urine-one containing an elevated concentration and one containing a normal concentration of As. The certified TAs concentration in the elevated urine is 480 ± 100 µg/L. Arsenic in normal urine is not certified; however, the NIST provides a reference value of 60 µg/L. Replicate analyses of these standard reference materials using the method described above give concentrations of 507 ± 17 µg/L and 64 ± 5 µg/L, respectively, which are in good agreement with the certified and reference values. The TAs concentration in urine samples reported in this paper is the sum of the concentrations of iAs\textsuperscript{III}, iAs\textsuperscript{V}, MAAs\textsuperscript{III}, MAAs\textsuperscript{V}, DMAs\textsuperscript{III} and DMAs\textsuperscript{V}.
*Creatinine in urine.* Urinary creatinine was measured by the Jaffe reaction using a Randox commercial kit. Arsenicals species concentrations in urine were corrected for creatinine concentration as indication of urine dilution.

*Statistical Methods.* Data analysis was carried out using statistical software Stata 8.0 (Stata, Corp. College Station Tx). Arsenical values were transformed to a log scale in order to calculate means and range, to perform statistical comparisons between groups and to evaluate potential confounding factors. Mann-Whitney tests were used to compare urinary arsenic metabolites among exposed group with and without lesions. Potential confounding risk factors evaluation includes age, gender, sunlight exposition and TWE.
Results

Eighty families with a total of 104 participants completed the sampling protocol (Table 1). Because of lack of good job opportunities in this area, most of young men emmigrate to out of the country; in consequence most of the subjects (90%) were female. The concentration of TAs in home drinking water of study participants ranged from 1 to 1,054 μg/L. In twenty-four homes (30%) the subjects drank bottled water in addition to municipal water.

Urine samples from both control and individuals chronically exposed to high iAs by consumption of drinking water containing this metalloid were analyzed to determine the concentrations of iAs\textsuperscript{III}, MAs\textsuperscript{III}, and DMAs\textsuperscript{III}. Arsenical values were adjusted by creatinine concentration. Urinary creatinine measurements ranged from 105 to 3,230 mg/L with averaging 595. Average urinary concentrations of trivalent and pentavalent arsenic species in total study group is shown in Table 2. Methylated trivalent arsenicals were detected in 98% of analyzed urine samples. In addition, trivalent arsenicals (iAs\textsuperscript{III}+MAs\textsuperscript{III}+DMAs\textsuperscript{III}) were predominant species in urine samples (65%). On average the major metabolite, DMAs\textsuperscript{III}, represented 49% of total urinary As, followed by DMAs\textsuperscript{V} (23.7%), iAs\textsuperscript{V} (8.6%), iAs\textsuperscript{III} (8.5%), MAs\textsuperscript{III} (7.4%), and MAs\textsuperscript{V} (2.8%).

Cutaneous signs of arsenicism were observed in fifty-five individuals from the group exposed to ≥ 50 μgAs/L in drinking water. The kind and proportion of cutaneous signs observed in participants of this study are shown in Table 3. Hyperkeratosis in palm or sole, were the most frequent skin signs of arsenicism (56.6%).

Table 4 summarizes the average arsenical concentrations according the level of As exposure and the presence of skin lesions. Interestingly, in the high As exposure group, subjects presenting cutaneous signs there were significant increases in the concentration of
MAs$^{\text{III}}$. In addition, the average of relative proportion of urinary MAs$^{\text{III}}$ was marginally higher in exposed individuals with skin lesions as compared to those who drank iAs-contaminated water, but had no skin lesions (Table 5).

The conditional logistic regression model was based on seventy-six subjects exposed to $\geq 50$ µg/L in drinking water, this model was adjusted by aged, gender and TWE. The risk of occurrence arsenisiasis related to both absolute and relative quantity of MAs$^{\text{III}}$ were significant (p $<$0.008; $<$0.004, respectively). The odds ratios (OR) for the subjects having hyperkeratosis plantar was estimated to be 1.06 [95% confidence intervals (CI), 1.03-1.19] for concentration of MAs$^{\text{III}}$ and 1.22 (95% CI, 1.07-1.44) for relative proportion of MAs$^{\text{III}}$. Even though DMA$^{\text{III}}$ was the main specie found in urine, neither its concentration nor its relative proportion was associated with the risk of arsenic-skin lesions (data not shown).

Other variable, independent of iAs metabolites that was significantly associated with the presence of hyperkeratosis plantar (p = 0.003) in the group who drank water with As $> 50$ µg/L, was the lifetime iAs-exposure, estimated as TWE (OR = 1.20; 95% CI = 1.06-1.35).
Discussion

**MAs\textsuperscript{III} and DMA\textsubscript{as}\textsuperscript{III} in urine**

The analysis of urinary trivalent methylated metabolites of iAs using freshly collected urines, within 6 hours of collection to reduce differences among samples in handling and to minimize the extent of oxidation of trivalent arsenicals before analysis allowed to detected the presence of MAs\textsuperscript{III} and DMA\textsubscript{as}\textsuperscript{III} in 98% of the urines collected, even in urine samples from subjects with low As exposure (< 10 μg/L in drinking water) (Table 4). The optimization of As speciation techniques has only recently permitted analysis of oxidation states of As in methylated metabolites. Initial studies using the optimized techniques detected small amounts of MAs\textsuperscript{III} and/or DMA\textsubscript{as}\textsuperscript{III} in urines from residents exposed to iAs in drinking water in several geographical regions, including Romania (Aposhian et al. 2000) Inner Mongolia (Le et al. 2000), Mexico (Del Razo et al. 2001), and West Bengal (Mandal et al. 2001). However, most of these studies analyzed urines that were stored for an extended time after collection. Because MAs\textsuperscript{III} and DMA\textsubscript{as}\textsuperscript{III} are rapidly oxidized even at temperatures below 0°C (Del Razo et al. 2001; Gong et al. 2001), these studies probably underestimated the concentrations of these metabolites. In contrast, analyses of urines freshly collected in this study showed that methylated trivalent arsenicals (MAs\textsuperscript{III} and DMA\textsubscript{as}\textsuperscript{III}) are in fact prevalent urinary metabolites of iAs (Table 2). DMA\textsuperscript{as}\textsuperscript{III} also was detected only when fresh void urine was collected in DMA\textsuperscript{V} fed rats (Cohen et al. 2002). It has been shown that intravenously injected iAs was excreted mainly as MAs\textsuperscript{III} (Csanaky and Gregus 2002) conjugated by glutathione [MAs\textsuperscript{III} (GS)\textsubscript{2}] in the rat bile (Suzuki et al. 2001).

Urinary detection of methylated and dimethylated arsenicals containing As in both oxidation states indicates that metabolism of As involves changes in the oxidation state of
As during methylation. It is likely that interactions of trivalent iAs metabolites with proteins and other cellular constituents are responsible for retention and toxic effects of arsenic in tissues of animals and humans exposed to iAs. In a study with the population chronically exposed to iAs from drinking water indicated that methylated arsenicals are also retained in tissues (Aposhian et al 1997, 2001). These As-exposed individuals were treated with an As chelator, 2,3-dimercaptopropane-1-sulfonic acid (DMPS), resulted in a massive release of MAs, including MAs III, in urine, suggesting that DMPS mobilized tissue depots of iAs.

**Skin lesions and trivalent methylated metabolites of iAs**

Skin keratosis and changes in skin pigmentation are two hallmark signs of arseniasis. Many residents of Zimapan area had skin lesions related to iAs exposure (Armienta et al. 1997b) (Table 3). According the historical values of As in drinking water, most of the families who participated in this study were exposed to extremely high As concentrations at least from 1992 to 1999. After this time, the level of concentration of As in water has been decreased because the municipality closed one of the wells connected to the municipal water system that contained the highest iAs concentration (1,100 μg/L). In addition, several residents used to drink bottled water with normal values of As instead of drinking water from the municipal. This fact can explain the great prevalence of dermatotoxicity, in spite of the moderate concentration of arsenicals in urine observed in the present study (Table 4).

The profile of iAs metabolites on dermatotoxicity was also assessed (Table 3). Previous studies in other regions such as Lagunera Region in Mexico (Del Razo et al. 1997) and Taiwan (Yu et al. 2000), showed that subjects with arseniasis were more likely to have a higher concentration of MAs in urine than exposed individuals who had did not have
arseniosis; at that time the speciated arsenical concentrations in urine were made without mentioning their oxidation state. Recent advent of techniques for speciation of As has helped establish the speciation of As according to oxidation states.

Importantly, trivalent methylated metabolites, especially DMAs\textsuperscript{III}, were not stable in human urine and oxidized quickly to yield pentavalent MAs\textsuperscript{V} and DMAs\textsuperscript{V} (Del Razo et al. 2001). As noted above, these samples were analyzed within 6 hours of collection to reduce differences among samples in handling and to minimize the extent of oxidation of As\textsuperscript{III} before analysis.

Interestingly in our study, the iAs-exposed individuals bearing skin signs of arsenicism had significantly higher urinary relative proportions and concentrations of MAs\textsuperscript{III}. These data suggest that a high output of MAs\textsuperscript{III} in urine may predict increased susceptibility to arseniosis.

MAs\textsuperscript{III} and DMAs\textsuperscript{III} have been reported to be highly toxic in mammalian cells (Styblo et al. 2000, 2002) and genotoxic (Mass et al. 2001; Nesnow et al. 2002). The reason for the high toxicity of methylated trivalent arsenicals has not been adequately explained, except that methylated trivalent arsenicals exert genotoxicity via reactive oxygen species (Nesnow et al. 2002). Although the comparative toxicity of MAs\textsuperscript{III} (GS\textsubscript{2}) was about 9 times higher than that of iAs\textsuperscript{III}, the accumulation rate of MAs\textsuperscript{III} (GS\textsubscript{2}) was 40 times higher than that of iAs\textsuperscript{III}. These results suggest that MAs\textsuperscript{III} (GS\textsubscript{2}) was more toxic than iAs\textsuperscript{III}, at least in part due to the more efficient accumulation of arsenic in cells (Hirano et al. 2004). Moreover, Vega et al. (2001) showed that normal human epidermal keratinocytes exposed to low doses (0.001 to 0.01 μM) of MAs\textsuperscript{III} stimulated expression of certain pro-inflammatory cytokines and growth factors that are critical to maintaining homeostasis and
barrier integrity in the skin suggesting that the overexpression of these products can lead the skin pathological processes.

Other possible mechanism for higher toxicity of trivalent arsenicals compared to the corresponding pentavalent forms is that trivalent species have higher affinity for thiol compounds (Shiobara et al. 2001).

In the light of these observations, biomethylation of iAs, a process yielding toxic trivalent methylated metabolites, appears to be a mechanism of activation of arsenic as a toxin and possibly as a carcinogen. Because of the adverse biological effects of these metabolites, the analysis of urinary MAs\textsuperscript{III} may serve as an effective tool for the evaluation of health risks associated with exposure to iAs.

There were no significant associations between confounding other risk factors, as duration of sunlight, and skin lesions (data not shown).

Other important factor associated with the presence of As-skin lesion was the lifetime exposure of As evaluated as TWE. Table 1 shows the significantly difference between this variable between exposed As subjects, demonstrating that the magnitude of exposure is directly related to the presence of skin lesions. In other words, the basic principle of dose-response relationship was fulfilled for the presence of arsenic-skin lesions.

The major limitations of this study are the small sample of population and the fact of most of the participants as females. Previous reports have indicated that age, dose, pregnancy and gender are among factors that affect the urinary profiles of iAs metabolites (Del Razo et al. 1997; Hughes et al. 2000; Yu et al. 2000). Results obtained in this study may not generalize to males or broad population.
This study provides novel data on the pattern of trivalent and pentavalent metabolites of iAs clearance in fresh urine from a population exposed to iAs in drinking water. Unlike other studies, in which urine samples were stored for several weeks before analysis, this study shows that MAs$^{\text{III}}$ and DMAs$^{\text{III}}$ in urine are predominant As species compared with their corresponding pentavalency arsenical.

MAs$^{\text{III}}$, the most potent toxicant in the entire metabolic pathway of iAs, could be mainly responsible for the toxic and carcinogen effects of iAs exposure and its detection and quantification in humans populations, can evaluated of risk assessment and could be the cause of arsenic carcinogenesis. Our finding support the view of the extent and character of adverse effects associated with iAs exposures are at least in part determined by the rate of the formation and by the composition of iAs metabolites.

Further research in other arseniasis areas populations is needed to confirm the potential relationship between the concentrations of MAs$^{\text{III}}$ in biological samples from human populations and the little-understood etiology of hyperkeratosis, skin-hypo-hyperpigmentation, and cancer that can result from chronic iAs exposure.
References


Table 1. Demographics of the Study Population.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Low iAs exposure</th>
<th>High iAs exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Without skin lesions</td>
</tr>
<tr>
<td>Number of subjects</td>
<td>28</td>
<td>21</td>
</tr>
<tr>
<td>Gender&lt;sup&gt;a&lt;/sup&gt; (Male)</td>
<td>2 (7.1%)</td>
<td>1 (4.8%)</td>
</tr>
<tr>
<td></td>
<td>26 (92.9%)</td>
<td>20 (95.2%)</td>
</tr>
<tr>
<td>Gender (Female)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age&lt;sup&gt;b&lt;/sup&gt; (years)</td>
<td>35 (18 - 50)</td>
<td>35 (21 - 49)</td>
</tr>
<tr>
<td>Sunlight exposure&lt;sup&gt;b&lt;/sup&gt; (hours)</td>
<td>2.3 (0 - 8)</td>
<td>2.2 (0 - 8)</td>
</tr>
<tr>
<td>TAs concentration in drinking water&lt;sup&gt;b&lt;/sup&gt; (µg/L)</td>
<td>1.6 (1 - 6)</td>
<td>117 (50 – 1,504)</td>
</tr>
<tr>
<td>Duration of iAs exposure&lt;sup&gt;b&lt;/sup&gt; (years)</td>
<td>26 (4 -50)</td>
<td>21 (4 - 49)</td>
</tr>
<tr>
<td>TWE&lt;sup&gt;b&lt;/sup&gt; (mg)</td>
<td>0.01 (0.01 – 0.06)</td>
<td>5.8 (0.02 – 16.3)</td>
</tr>
</tbody>
</table>

Values are mean and<sup>a</sup> proportion<sup>b</sup> range. <sup>*</sup> Duration of well water consumption. <sup>**</sup> Statistically significant difference (p < 0.05) between the exposed individuals with and without skin lesions.
Table 2. Concentration and relative proportion of arsenic species in residents from Zimapán Area (n = 104).

<table>
<thead>
<tr>
<th>iAs metabolites in Urine</th>
<th>Concentration (^a) (µg/g creatinine)</th>
<th>Relative Proportion (^b) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>iAs(^V)</td>
<td>5.84 (1 – 65.5)</td>
<td>8.6</td>
</tr>
<tr>
<td>iAs(^{III})</td>
<td>4.46 (0.1 – 172.3)</td>
<td>8.5</td>
</tr>
<tr>
<td>MA(^V)</td>
<td>1.45 (0.1 – 28.3)</td>
<td>2.8</td>
</tr>
<tr>
<td>MA(^{III})</td>
<td>4.93 (0.1 – 101.9)</td>
<td>7.4</td>
</tr>
<tr>
<td>DMA(^V)</td>
<td>14.56 (1 – 710)</td>
<td>23.7</td>
</tr>
<tr>
<td>DMA(^{III})</td>
<td>30.75 (0.1 – 506.3)</td>
<td>49</td>
</tr>
<tr>
<td>TAs</td>
<td>84.85 (9.1 – 1,398.1)</td>
<td>100</td>
</tr>
</tbody>
</table>

\(^a\) Concentration of metabolites of arsenic in urine are reported as geometric mean and range.


Table 3. Distribution of skin lesion in iAs exposed subjects (n=76).

<table>
<thead>
<tr>
<th>Skin lesions</th>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypopigmentation</td>
<td>36 (47.4 %)</td>
<td>40 (52.6 %)</td>
</tr>
<tr>
<td>Hyperpigmentation</td>
<td>29 (38.2 %)</td>
<td>47 (61.8 %)</td>
</tr>
<tr>
<td>Hypo-hyperpigmentation</td>
<td>9 (11.8 %)</td>
<td>67 (88.2 %)</td>
</tr>
<tr>
<td>Hyperkeratosis on the palms</td>
<td>33 (43.4 %)</td>
<td>43 (56.6 %)</td>
</tr>
<tr>
<td>Hyperkeratosis on the soles</td>
<td>30 (39.5 %)</td>
<td>46 (60.5 %)</td>
</tr>
<tr>
<td>Hyperkeratosis on the palms or soles</td>
<td>43 (56.6 %)</td>
<td>33 (43.4 %)</td>
</tr>
<tr>
<td>Keratosis on the trunk</td>
<td>17 (22.4 %)</td>
<td>59 (77.6 %)</td>
</tr>
<tr>
<td>Cutaneous horns</td>
<td>4 (5.3 %)</td>
<td>72 (94.7 %)</td>
</tr>
<tr>
<td>Bowen’s disease</td>
<td>1 (1.3 %)</td>
<td>75 (98.7 %)</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>1 (1.3 %)</td>
<td>75 (98.7 %)</td>
</tr>
</tbody>
</table>

* Proportion calculated for 76 subjects exposed to ≥ 50 µg As/L in drinking water.
Table 4. Urinary pattern of iAs species in humans exposed to arsenic through drinking water in Zimapán Area, according to level exposition and the presence of arsenic-skin lesions (n = 104)

<table>
<thead>
<tr>
<th>As Species</th>
<th>Metabolites of iAs in Urine (µg/g creatinine)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (n = 28)</td>
</tr>
<tr>
<td>iAs⁵</td>
<td>3.6 (1.0 – 10.5)</td>
</tr>
<tr>
<td>iAs₃</td>
<td>1.6 (0.1 – 9.7)</td>
</tr>
<tr>
<td>MAs⁵</td>
<td>0.6 (0.1 – 5.5)</td>
</tr>
<tr>
<td>MAs₃</td>
<td>2.2 (0.4 – 9.6)</td>
</tr>
<tr>
<td>DMAs⁵</td>
<td>7.4 (1.8 – 38.5)</td>
</tr>
<tr>
<td>DMAs₃</td>
<td>7.9 (0.1 – 65.4)</td>
</tr>
<tr>
<td>TAs</td>
<td>33.3 (9.1 - 106)</td>
</tr>
</tbody>
</table>

* Geometric mean and range are shown. **Statistically significant difference (p < 0.05) between the exposed individuals with and without skin lesions (by Mann-Whitney test).
Table 5. Comparison of relative proportion of arsenic species in urine among subjects As-exposed with and without skin-lesions.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Without Skin Lesions (n = 21)</th>
<th>With Skin Lesions (n = 55)</th>
</tr>
</thead>
<tbody>
<tr>
<td>% iAs&lt;sup&gt;V&lt;/sup&gt;</td>
<td>6.5</td>
<td>7.9</td>
</tr>
<tr>
<td>% iAs&lt;sup&gt;III&lt;/sup&gt;</td>
<td>10.9</td>
<td>8.0</td>
</tr>
<tr>
<td>% MAs&lt;sup&gt;V&lt;/sup&gt;</td>
<td>3.5</td>
<td>2.3</td>
</tr>
<tr>
<td>% MAs&lt;sup&gt;III&lt;/sup&gt;</td>
<td>5.9</td>
<td>*7.7</td>
</tr>
<tr>
<td>% DMAs&lt;sup&gt;V&lt;/sup&gt;</td>
<td>21.5</td>
<td>23.1</td>
</tr>
<tr>
<td>% DMAs&lt;sup&gt;III&lt;/sup&gt;</td>
<td>51.7</td>
<td>51.0</td>
</tr>
</tbody>
</table>

Values shown are mean. *Statistically marginal difference (p = 0.072) between the exposed individuals with and without skin lesions (by Mann-Whitney test). 