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Comparison of methylation capacity among the people from the arsenic-affected areas of West Bengal, India

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ABSTRACT

Inorganic arsenic (iAs) is an established human carcinogen. Although methylation of iAs was considered as a detoxification mechanism, recently it is considered as an intoxification pathway in mammals. Our study population consisted of four groups A-D with drinking water iAs concentrations 33 ± 7, 148 ± 34, 210 ± 2.6, and 248 ± 59 µg As/l (mean ± SE), respec tively in West Bengal, India. The ratios (monomethylated arsenicals)/(inorganic As metabolites - arsenate) = (MMA + DMA)/(iAs Met - iAs^V), (dimethylated As)/(mono- and dimethylated As) = (DMA)/(MMA + DMA), and (dimethylated As)/(inorganic As metabolites - arsenate) = (DMA)/(iAs Met - iAs^V) were used to assess methylation efficiency in the present study. High performance liquid chromatography-inductively coupled argon plasma mass spectrometry (HPLC–ICP MS) was used to determine As species in spot urine samples. The detection limit of As compounds was 0.14-0.33 µg As/l. All trivalent arsenicals were stable for up to 2 months when arsenic spiked urine samples were stored at –28° C without any preservatives. Although females appeared to be better methylators than males and children (considering first and total methylations), they were statistically not significant ($p>0.05$). Only second methylation capacity was statistically significant between different age groups ($p<0.05$). Although methylation capacity is not statistically conclusive in the present study, this is the first study, which documents the results on reduction capacity of the As-affected population in the endemic areas. Another outcome of this study suggests that researchers must consider the intake route of As via food-chain during the preparation of toxicity model of As.

Key words: Arsenic speciation, methylation capacity, reduction capacity, genetic polymorphism, food chain, dimethylarsinous and monomethylarsonous acids.

INTRODUCTION

Ingestion of inorganic arsenic (iAs) might cause skin, urinary bladder, kidney, lung, and liver cancer as well as disorders of the circulatory and nervous systems [1]. The methylation of iAs

was considered a detoxification mechanism, since methylated arsenic (As) compounds were less actively toxic [2], less reactive with tissue components [3], and excreted faster in urine than iAs [4-6]. Occurrence of monomethylarsonous (MMA^{III}) and dimethylarsinous acids (DMA^{III}) in human urine [7], being more toxic than all other metabolites [8-10] forced us to rethink this hypothesis seriously.

Experimental studies indicated that the methylation of As was influenced by the dose level, route of administration, chemical forms of As administered, and nutritional status of the subject [11-12]. Possible reasons for such differences included age, gender, smoking, ethnicity, individuality, concurrent exposures to other agents or environmental factors, and genetic polymorphism.

A slight increase in the proportion of dimethylated arsenical (DMA) in urine with age was reported in adults [13]. Smoking ten cigarettes a day resulted in an increase of a few points in the percentage of monomethylated arsenical (MMA) and a corresponding decrease in the percentage of DMA [14-15]. In a study of the people exposed to iAs via the drinking water in northern Chile, women had approximately 3 % more DMA and less MMA in the urine than men [14]. Similar gender differences were found in northeastern Taiwan [16], but not in other studies [13, 17].

The relative proportions of urinary iAs, MMA, and DMA were used as indicators of methylation capacity [18-19]. It was proposed that saturation of the methylation capacity might lead to a threshold for the carcinogenecity of ingested iAs [20-21]. Under this hypothesis, as exposure increased, one would expect to see an increase in the proportion of iAs, with a corresponding decrease in MMA and DMA (the less toxic metabolites). However, data on iAs metabolism in humans were sparse and more studies were needed, especially involving low and moderate As doses. Genetic polymorphism was suggested to account for differences in the metabolism of As in humans [22].

Various sampling and storage protocols were reported in the literature, including refrigeration, freezing, centrifugation, and acidification (removal of air). Larsen et al. [23] observed that the concentrations of DMA, MMA, and arsenobetaine (AsB) were relatively constant, but rapid oxidation of arsenite (iAs^{III}) to arsenate (iAs^{V}) was observed. Palacios et al. [24] found that iAs^{V}, MMA, DMA, and AsB (200 µg As/l each) in urine were stable for the entire testing period of 67 days at 4^{o} C. Feldmann et al. [25] reported that iAs^{III}, iAs^{V}, MMA, DMA, and AsB concentrations in urine samples were stable for up to 2 months when standards injected urine samples were stored at 4^{o} C and - 20^{o} C. For longer period of storage (4 and 8 months), the stability of As species was dependent on urine matrices. Whereas the As speciation in some urine samples was stable for the entire 8 months at both 4^{o} C and -20^{o} C, other urine samples stored under identical conditions showed substantial changes in the concentrations of iAs^{III}, iAs^{V}, MMA, and DMA. Untreated samples maintained their concentrations of As species and additives had no particular benefit. Strong acidification was not appropriate for speciation analysis and partly converted iAs^{V} to iAs^{III}.

In the present study, both tri- and pentavalent As and its metabolites were used for the estimation of methylation capacity. There was little information available in the literature on the stability/preservation of human urine samples when assessing the oxidation states of As and its metabolites present naturally. Only Le et al. [26] recently reported that approximately 60 % of MMA^{III} and 95 % of DMA^{III} were oxidized to MMA^{V} and DMA^{V}, respectively, after 1 µM MMA^{III} and DMA^{III} was separately spiked into a urine sample and the sample was stored at 4^{o} C for 2 weeks, while Del Razo et al. [27] tested the stability of the oxidation state of As in

arsenicals in aqueous solution at 4° C for up to 2 months and found iAs^{III} was stable during the first 24 h, but about 7 % $MMA^{III}O$ and 60 % $DMA^{III}GS$ were oxidized over this time interval. During the 2-month period, 20 % iAs^{III}, 43 % $MMA^{III}O$, and more than 90 % $DMA^{III}GS$ were oxidized to pentavalent species. Simultaneously, urine samples of healthy people were spiked with 10 ng of iAs^{III}, 10 ng of $MMA^{III}O$, and 20 ng of $DMA^{III}GS$/ml and were stored at 4° C for up to 2 months and found that iAs^{III} (21 - 32 %), $MMA^{III}O$ (30 – 100 %), and $DMA^{III}GS$ (42 – 100 %) were oxidized at different rates in each samples [27]. Also, Gong et al., [28] studied the oxidative stability of these trivalent As species in water and urine samples and low-temperature conditions (4 and – 20° C) were recommended for the improved stability of these As species over the room temperature. The oxidation of MMA^{III} (to MMA^{V}) and DMA^{III} (to DMA^{V}) was found to be matrix-dependent [27-28]. Very recently, Le et al. [29] suggested that the use of diethylammonium diethyldithiocarbamate (DDDC) as preservative to make stable them in urine for long time.

In the present study, the relative proportion of the urinary As species concentration and the ratios between putative products and putative substrates of the As metabolic pathway were used as indicators of metabolic efficiency. The data was processed to assess the As methylation threshold hypothesis in human. Also the influence of gender and age on methylation capacity was discussed.

EXPERIMENTAL SECTION

Reagents. All reagents were of analytical grade. Milli-Q SP water (Millipore, Bedford, MA, USA) was used throughout. Stock solutions of As were prepared using the following standard compounds. Sodium arsenite ($NaAsO_2$), sodium arsenate, dibasic ($Na_2HAsO_4.7H_2O$) and dimethylarsinic acid [$(CH_3)_2AsO(OH)$] were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Monomethylarsonic acid [$CH_3AsO(OH)_2$] was purchased from Tori Chemicals Ltd. (Yamanashi, Japan). Trizma® HCl was purchased from Sigma (St. Louis, MO, USA). Arsenocholine (AsC) and Standard Reference Material Toxic Metals in freeze-dried urine SRM-2670 (National Institute of Standards and Technology, Gaithersburg, MO) were gifted by Dr. N. Chandra (National Institute of Health Sciences, Tokyo, Japan) and arsenobetaine (AsB) was gifted by Dr. T. Kaise (Tokyo University of Pharmacy and Life Sciences, Tokyo). Sodium disulfite, sodium thiosulfate and other analytical reagents were purchased from Wako Pure Chemical Industries Ltd. Stock solutions of all As compounds (1000 mg As/l) were prepared from respective standard compounds. All stock solutions were stored in the dark at 4° C. Dilute standard solutions for analysis were prepared daily prior to use. The mobile phase of HPLC was prepared by dissolving an appropriate amount of citric acid monohydrate in Milli-Q water to get the required concentration (15 mM/l). The pH of the mobile phase was adjusted to 2.0 by drop-wise addition of 10 % nitric acid. Before use, the mobile phases were filtered through 0.45 μm filter.

Instruments. The HPLC system consisted of a liquid chromatograph solvent delivery pump (PU-610, GL Sciences Co., Tokyo), ERC-3120 degasser (ERMA Optical Works, Tokyo) and a polymer-based anion exchange column (ion exchange capacity: 0.55 ± 0.02 meq/g, and diethylaminoethyl functional group) (Shodex Asahipak ES-502N 7C, 100 × 7.6 mm i.d., Showa Denko, Tokyo). All the tubings in contact with the mobile phase were made of inert PEEK material. An ICP MS (HP 4500, Yokogawa Analytical Systems Co., Musashino, Japan) was used as a chromatographic detector. The outlet of the HPLC system was coupled directly (with 30 cm × 0.25 mm i.d. long PEEK tubings) to the inlet of the ICP nebulizer. Signals at m/z of 75 and 77

were monitored for As, the signal at m/z of 77 being used to monitor the isobaric interference with $ArCl^+$. Off line data of ICP MS were processed with software developed in house.

Protocol. The subjects of this study were from four As affected blocks of three As affected districts in West Bengal, India (for review, see Refs. [30-31]). We selected those places where seafoods were not available. They were also asked not to take seafoods within three days of our sample collection. Our study consisted of four groups. Details of these groups were summarized in Table 1. One point should be noted here that all subjects of group C at present were drinking safe water (<3.0 µg As/l) for a period of nearly one and half years living at the same place, but all groups A-D could not avoid As contamination through the food chain. So, groups A, B, and D were the current-users of As contaminated water, whereas group C was the ex-users of As contaminated water. Our study did not get evidence of liver or kidney diseases in these subjects. We also analyzed 35 control urine samples from a group of people who never drank As contaminated water and lived at a place far from As contaminated regions of West Bengal, India (details in Table 1).

One of our authors collected water from each of the wells used by the subjects. Water was analyzed by our developed method [7]. The same method was used to analyze urine samples. To remove particles and minimize matrix effects, all urine were diluted 5-fold using Milli-Q water and filtered by Millex®-HV (0.45 µm syringe driven filter unit, Millipore, Bedford, MA, USA) prior to injection.

Urine collection. Spot urine samples were collected from all studied groups in pre-washed (with distilled water) new polyethylene bottles (for review, see Refs. [31-32]). The urine samples were collected without any chemical treatment from one study group in a day. Immediately after collection, the samples were stored in a salt-ice mixture and the sampleswere kept frozen during the return to home. The frozen urine samples were transferred by air in a cool-fisher to Chiba University, Japan, where they were stored at -28^oC for about two months. All urine samples were transported in salt-ice mixture near about 72 h.

Experimental procedure. Analyses were carried out using our developed HPLC–ICP MS technique [7]. The methods used to analyze for As species, detection limit, quality control,precision, and sensitivity of these analytical measurements and validation of the procedure were described in details by Mandal et al. [7]. The HPLC column used for the present study of eight As species: arsenocholine (AsC), arsenobetaine (AsB), dimethylarsinic acid (DMA^V), dimethylarsinous acid (DMA^{III}), monomethylarsonic acid (MMA^V), monomethylarsonous acid (MMA^{III}), arsenite (iAs^{III}), and arsenate (iAs^V) was a polymer-based anion exchange column. The detection limit of arsenic compounds was 0.14-0.33 µg As/l.

For the stability/preservation of As and its metabolites in human urine samples, some systematic experiments were done with both naturally and spiked arsenicals in urine in the present study. Replicate aliquots of 17 urine samples [containing AsB (0.62-3.54), iAs^V (11.5-21.5), iAs^{III} (13.7-43.5), MMA^V (3.8-124.4), MMA^{III} (5.3-28.2), DMA^V (65.7-1264), and DMA^{III} (21.6-70.3 µg/l)] were placed in separate polyethylene bottles and stored in the dark for up to 1 year at -28^o C (deep freezer), 8 months at 4^o C, and 3 months at 37^o C. The concentrations of arsenicals at the beginning were considered as 100% and other percentages at several time intervals were calculated with respect to the respective initial concentrations (Table 2). These urine samples were collected 2 months before and kept at -28^o C. Initially we did not conduct these types of stability experiments. Fortunately we analyzed only these 17 samples after 2 months of their first analyses for cross checking during data processing. We had no more analysis results after 4

months of their collection. Also, trivalent arsenicals (100, 400, 600, and 800 µg/l each) were spiked in 12 control urine samples and kept at 4 and –28^{o} C for two months.

Table 1. Demographic details of the studied groups in West Bengal, India

Particulars	Control	Group A	Group B	Group C	Group D
Location	Jhilimili, Ranibandh, Bankura	Bibipur, Baduria, 24 PGS (N)[a]	Sahadiarh, Domkol, Murshidabad	Kolsur, Deganga, 24 PGS (N)	Hogalberia, Karimpur I, Nadia
No of subjects	35	105	120	156	47
No of men	20	44	66	67	27
No of women	15	61	54	89	20
No of children (upto 10 yr)	5	40	39	52	15
No of patients[b]	-	-	13	29	17
Duration[c] (yr)	-	<1.0	3-10	1.5[f]	3-10
No of water samples analyzed	10	21	11	32	14
iAs^{V} in water[e]	ND[d]	25 ± 6.5	102 ± 23	<3 (111 ± 14)[f]	138 ± 32
iAs^{III} in water[e]	ND	7 ± 1.8	46 ± 13	-(98 ± 13)[f] <3	111 ± 27
Sum of iAs^{V} and iAs^{III} in water[e]	ND	33 ± 7	148 ± 34	(210 ± 26)[f]	248 ± 59
Ratio of iAs^{III}/ (iAs^{III} + iAs^{V})	-	0.23 ± 0.05	0.29 ± 0.02	- (0.46 ± 0.02)[f]	0.45 ± 0.01

[a]North 24 Parganas; [b]Arsenical dermatological patients; [c]Duration of As contaminated tube-well water intake; [d]Not detectable; [f]They were drinking this As contaminated water for a period of 5-20 years before one and half years of our sample collection; [e]Mean ±S.E., µg/l.

As contents analyzed at various time intervals were presented as percentages of the initial concentrations (µg/l) of the arsenicals considering 100% as shown in Table 2. Samples stored at –28^{o} C were thawed at room temperature before analysis. All samples were diluted 4-fold with double de-ionized water and filtered through 0.45 µm Millipore before injection onto the HPLC column for analysis. To study the effect of ascorbic acid and trichloroacetic acid (TCA) on urine stability, appropriate volumes of ascorbic acid (1 mg/l urine) and TCA (at a final concentration of 5 %) were added to urine samples separately and stored under the same conditions as mentioned above.

In our study, the concentrations of As and its metabolites were presented in absolute units (µg As/l). Because recently Sim et al. [33] suggested that 'creatinine adjustment for As and its metabolites in spot urine samples was not a method of choice in population-based studies due to a little higher iAs concentration than that measured in 24 h specimens, but 24 h urine samples with creatinine adjustment, where feasible, remained the method of choice in population-bases studies'. Moreover, creatinine excretion may vary with many factors such as diurnal variation, body mass, age, gender, health, diuresis (i.e., urine flow), drug and alcohol use, diet, and exercise [34]. Very recently, Hinwood et al. [35] suggested that spot samples may be adequate for measuring short-term exposure, using iAs as the outcome variable; however, additional work on a larger data set is required. Creatinine adjustment of urinary iAs concentrations may not be required in population studies investigating environmental exposure. In the present study, collection, storage, and transportation of 24 h urine samples were practically not feasible.

Statistical analysis. A detailed statistical analysis to substantiate statements regarding trends or differences between groups was done thoroughly by SAS/Stat General Linear Model (GLM) (*SAS*, USA). Type III sums of squares were preferred in testing effects in unbalanced cases because they test a function of the underlying parameters that is independent of the number of observations per treatment combination. Bivariate scattergrams were plotted between proportional urinary arsenicals, different As species ratios with total urinary As (µg As/l). The associations of the absolute, and relative urinary excretion of the As species with As exposure and other factors such as gender, age, and patients were tested using *Scheffe's* test. Student's *t* test was used to test the null hypothesis that the means of the two groups are the same and a significant p value ($p<0.05$) means they are not the same. Statistical significant values were considered in all cases as $p<0.05$.

RESULTS

78 Tube-well water and 428 human urine samples collected from four groups A-D in four As affected blocks of three As-affected districts in West Bengal, India were analyzed using our developed HPLC-ICP MS method [7]. Demographic details of these studied groups were presented in Table 1. Groups A-D consisted of 105 subjects (men 44, women 61, including children 40), 120 (men 66, women 54, including children 39), 156 (men 67, women 89, including children 52), and 47 (men 27, women 20, including children 15), respectively. Their drinking water contained 33 ± 7, 148 ± 34, 210 ± 2.6, and 248 ± 59 (µg As/l, mean ± SE), respectively, and the iAs^{III}/iAs ratios were 0.23 ± 0.05, 0.29 ± 0.02, 0.46 ± 0.02, and 0.45 ± 0.01 (mean ± SE), respectively. We also analyzed 35 control urine samples from a group of people who never drank As contaminated water and lived at a place far from As contaminated regions of West Bengal, India (details in Table 1). Only trace amount of MMA^{V} (2.4 ± 0.4 µg As/l, n = 21) and DMA^{V} (6.1 ± 1.2 µg As/l, n = 35) were found in the urine samples of the control group (8.1 ± 0.7 µg As/l).

The concentrations of the seven As species in the untreated urines (Table 2) were mostly unchanged for 4 months after collection, with recoveries ranging from 92.3 to 103.3 % stored at –28° C. But after 12 months the recoveries of AsB, DMA^{V}, DMA^{III}, MMA^{V}, MMA^{III}, iAs^{V}, and iAs^{III} in urine samples (Table 2) were 96.7 to 99.2 %, 511 to 870 %, 1.97 to 26.9 %, 176 to 264 %, 3.2 to 27.5 %, 154 to 179 %, and 4.4 to 70.7 %, respectively during storage at –28° C. All trivalent arsenicals were converted to their pentavalent forms stored at 37° C for 80 days after 10 months of their collection (Data not shown). Another important point was that one urine sample (Chromatogram not shown) was stored at 4° C after 4-fold dilution and ultra-filtration for 8 months after 4 months of its collection and it had 70 % DMA^{III} and 80 % MMA^{III} whereas original urine did not contain DMA^{III} and MMA^{III} after the same interval. These might suggest that the stability of As and its metabolites depended upon the urine matrices as well as individuals. All trivalent arsenicals were converted to their pentavalent forms with TCA (Chromatogram not shown), but 10 to 15 % DMA^{III} and no MMA^{III} were found with ascorbic acid (Chromatogram not shown). The systematic analysis of trivalent arsenicals spiked in control urine kept at –28° C for 66 days (Table 2) showed that the recoveries were 97.6-99.8, 97.8-99.1, and 97.9-99.1% for iAs^{III}, MMA^{III}, and DMA^{III}, respectively. But trivalent arsenicals spiked in control urine samples kept at 4° C for 66 days (Data not shown) showed that their recoveries were 92-95%. This might be due to frequent air exposure, vortex mixing and temperature variation. But recoveries of samples (n = 6) stored at 4 and –28° C in nitrogen medium showed that more than 99% arsenicals were intact after 2 months (Data not shown).

Table 2. Descriptive statistical data on stability of arsenicals in urine

Parameters	Stability data on arsenicals in urine keeping at – 28° C (n = 17)				Stability data on trivalent arsenicals kept at – 28° C after spiking in control urine (n = 12)							
Time	2M[a]	4M[a]	8M[a]	12M[a]	0[b]	10[b]	17[b]	25[b]	35[b]	48[b]	59[b]	66[b]
AsB:												
Mean (%)[c]	100	99.7	98.9*	97.2*								
Std Dev	0	1.1	0.66	0.56								
Minimum	100	97.8	96.2	96.7								
Maximum	100	102	99.7	99.2								
iAs^{V}:												
Mean (%)[c]	100	100	144*	166*								
Std Dev	0	0.77	17.4	10.0								
Minimum	100	98.6	123	154								
Maximum	100	102	176	178								
iAs^{III}:												
Mean (%)[c]	100	99.9	58.2*	38.3*	100	99.8	99.6	99.4	99.3	99.5	99.4*	98.9*
Std Dev	0	0.87	10.2	22.1	0	0.96	0.42	0.31	0.50	1.09	0.39	0.91
Minimum	100	99.2	44.8	4.36	100	99.3	99.1	98.9	98.4	98.3	98.8	97.6
Maximum	100	101	71.3	70.7	100	101	101	102	100	101	99.9	99.8
MMA^{V}:												
Mean (%)[c]	100	100	230*	205*								
Std Dev	0	1.16	19.7	28.0								
Minimum	100	99.1	206	176								
Maximum	100	102	254	264								
MMA^{III}:												
Mean (%)[c]	100	99.4	18.1*	15.6*	100	99.7	99.4	99.9	99.5	99.6	99.1*	98.5*
Std Dev	0	0.54	11.3	11.3	0	0.55	0.26	1.46	0.23	1.02	0.53	0.45
Minimum	100	98.4	5.47	3.19	100	99.1	98.9	98.5	99.3	98.6	98.5	97.8
Maximum	100	99.9	32.4	27.5	100	100	101	102	101	101	99.8	99.1
DMA^{V}:												
Mean (%)[c]	100	100	635*	691*								
Std Dev	0	0.76	134	123								
Minimum	100	99.3	498	511								
Maximum	100	101	837	870								
DMA^{III}:												
Mean (%)[c]	100	100	14.9*	13.1*	100	99.9	99.5	99.8	99.6	99.6	99.3*	98.4*
Std Dev	0	1.65	10.6	10.8	0	1.53	0.59	0.99	0.24	1.01	0.29	0.39
Minimum	100	98.8	4.06	1.97	100	98.3	98.7	98.8	99.3	98.5	98.8	97.9
Maximum	100	103	32.3	26.9	100	102	100	101	102	101	99.6	99.1

*[a]Month; [b]Day; [c]All percentages were calculated with respect to initial concentrations (at 2M or 0 day) of the arsenicals present in urine which were considered as 100%; *Significantly different relative to the starting value ($p<0.05$ (Student's t test)).*

This suggested /recommended that if possible to carry nitrogen cylinder to the field and samples were kept in nitrogen medium, all arsenicals in urine might be stable for a long time. Although in our applied method for urine samples collection trivalent arsenicals were 97.6-99.8% stable during the analysis after their spiking in control human urines, which were kept at –28° C and analyzed after 66 days in the present study i.e. it varied 2-3% from our recommended best

method for urine sample collection in epidemiological studies of As, our analysis results could be applicable for methylation and reduction capacity prediction ignoring this small/negligible variation.

The absolute concentrations of all urinary As species among groups, sex, age, and patients were given in Table 3 (current-users), and in Table 4 (ex-users), respectively. iAs^{V}, DMA^{V}, and tAs (µg As/l) were statistically significant among groups ($p<0.05$)(Table 3). iAs^{III}, MMA^{V}, DMA^{V}, and tAs (µg As/l) were statistically significant among males and females ($p<0.05$). All arsenical were statistically significant among different age groups ($p<0.05$). Except tAs (µg As/l), others were statistically not significant among people with and without arsenism ($p>0.05$)(Table 3). Table 4 showed that only tAs (µg As/l) was statistically significant among males-females and people with and without arsenism ($p<0.05$). In all studied groups AsB was 1.61 – 5.24 µg As/l, but AsC was not detected in any urine sample, which indicated minimum contamination through the seafoods. The efficiency of the methylation process was assessed by the ratios between urinary As concentrations of putative products and putative substrates of the proposed As metabolic pathway [7].

Table 3. Concentrations of urinary arsenic species (µg/l) in various subgroups among current users of arsenic contaminated water.

Factors	Sub-groups	AsB		iAs^{V}		iAs^{III}		MMA^{V}		MMA^{III}		DMA^{V}		DMA^{III}		tAs	
		Mean (n^{a})	CV^{b}	Mean (n^{a})	CV^{b}	Mean (n^{a})	CV^{b}	Mean (n^{a})	CV^{b}	Mean (n^{a})	CV^{b}	Mean (n^{a})	CV^{b}	Mean (n^{a})	CV^{b}	Mean (n^{a})	CV^{b}
Control	Child	-	-	-	-	-	-	2.241 (5)	1.00	-	-	13.24 (5)	1.33	-	-	15.24 (5)	-
	Adult	-	-	-	-	-	-	3.125 (30)	1.00	-	-	4.971 (30)	0.81	-	-	8.096 (30)	-
Group	Group A	2.75 (63)	170**	6.95 (48)	67.7**	19.16 (67)	84.7**	3.95 (72)	59.1**	3.11 (6)	-	23.58 (78)	78.4**	7.41 (33)	93.0	53.64 (78)	0**
	Group B	1.61 (28)	60.1	23.54 (118)	95.6*	27.43 (119)	86.3**	18.74 (120)	58.7**	6.35 (68)	71.9	106.4 (120)	42.1**	18.41 (95)	147*	194.9 (120)	0**
	Group D	5.24 (42)	61.9**	30.48 (41)	125*	134.7 (44)	57.8**	188.8 (44)	55.9**	30.88 (16)	54.3*	1215 (44)	29.1**	65.75 (28)	142*	1550	0**
	P value[#]	0.1288		0.0304		0.0956		0.3322		0.6009		0.0010		0.1774		0.0001	
Sex	Male	3.87 (64)	94.6**	23.54 (106)	108**	54.00 (118)	89.2**	56.39 (119)	92.3**	11.94 (47)	117**	352.8 (120)	29.8**	23.97 (82)	233*	504.1 (120)	0**
	Female	2.77 (69)	178	18.47 (101)	133**	36.65 (112)	80.0**	35.33 (117)	111**	8.92 (43)	104**	211.1 (122)	94.1**	25.25 (74)	235*	289.3 (122)	0**
	P value[#]	0.1478		0.1642		0.0013		0.0010		0.2619		0.0001		0.8925		0.0001	
Age (yrs)	Child (upto 10 yr)	4.02 (50)	147	21.33 (71)	98.1**	32.39 (81)	118**	31.42 (84)	66.6**	10.91 (32)	135*	275.5 (86)	31.2**	23.45 (55)	265*	373.4 (86)	0**
	Adult-males	3.32 (41)	76.2*	22.13 (70)	115	65.63 (73)	51.1**	68.09 (74)	84.2**	8.89 (32)	78.4*	367.3 (75)	23.2**	20.13 (50)	171*	538.2 (75)	0**
	Adult-females	2.52 (42)	139	19.73 (67)	139**	41.35 (76)	65.9**	42.03 (79)	97.1**	11.95 (26)	73.3**	218.1 (81)	108**	30.16 (51)	228*	301.1 (81)	0**
	P value[#]	0.0009		0.0001		0.0001		0.0001		0.0001		0.0001		0.0008		0.0001	
Patient	No	3.05 (115)	144*	19.87 (182)	108**	35.68 (203)	94.7**	30.49 (209)	90.9**	10.49 (77)	120**	212.5 (215)	42.8**	24.41 (136)	246**	312.7 (215)	0**
	Yes	4.87 (18)	71.7	29.83 (25)	129*	119.7 (27)	43.1**	165.6 (27)	67.5**	10.53 (13)	73.5*	829.9 (27)	46.7**	25.70 (20)	171	1057 (27)	0**
	P value[#]	0.1005		0.6147		0.8856		0.8314		0.9906		0.6775		0.9268		0.0001	

*[a]No of observations; [b]Coefficient of variation; [#]SAS/Stat General Linear Model, *Significant (p<0.05); **Significant (p<0.001).*

Table 4. Concentrations of urinary arsenic species (µg/l) in various subgroups among ex-users of arsenic contaminated water.

Factors	Subgroups	AsB		iAs^V		iAs^{III}		MMA^V		MMA^{III}		DMA^V		DMA^{III}		tAs	
		Mean (n^a)	CV^b	Mean (n^a)	CV^b	Mean (n^a)	CV^b	Mean (n^a)	CV^b	Mean (n^a)	CV^b	Mean (n^a)	CV^b	Mean (n^a)	CV^b	Mean (n^a)	CV^b
Group	Group C	3.32 (111)	157*	14.60 (122)	81.3**	48.12 (118)	194*	28.31 (120)	40.8**	10.53 (91)	78.5**	132.8 (122)	54.5**	43.91 (114)	125**	265.5 (122)	0**
Sex	Male	3.203 (45)	192	15.19 (70)	88.6*	56.35 (68)	216*	27.97 (69)	34.5**	9.943 (56)	70.2*	133.5 (70)	52.6**	46.45 (66)	117*	271.7 (70)	0**
	Female	3.396 (66)	138	13.81 (52)	68.1*	36.93 (50)	52.8**	28.77 (51)	43.7**	11.49 (35)	79.7*	131.7 (52)	59.4**	40.43 (48)	127**	257.1 (52)	0**
	P value[#]	0.8489		0.5260		0.2678		0.7097		0.3879		0.8921		0.5664		0.0001	
Patient	No	3.545 (86)	160*	15.55 (93)	81.1*	51.8 (92)	202*	28.32 (93)	42.4**	11.29 (75)	73.3**	132.1 (93)	50.7**	47.22 (90)	119**	274.5 (93)	0**
	Yes	2.630 (24)	65.3	11.75 (28)	61.3*	36.20 (25)	96.4*	29.19 (26)	33.4**	7.199 (15)	109	139.3 (28)	62.8**	32.54 (23)	158	243.4 (28)	0**
	P value[#]	0.3692		0.5018		0.8806		0.8799		0.7442		0.6217		0.7988		0.001	

*[a]No of observations; [b]Coefficient of variation; [#]SAS/Stat General Linear Model, *Significant ($p<0.05$); **Significant ($p<0.001$).*

The values of these ratios were presented in Table 5 for current-users and in Table 6 for ex-users of As contaminated water. The metabolic pathway used was as

$$iAs^V \rightarrow iAs^{III} \rightarrow MMA^V \rightarrow MMA^{III} \rightarrow DMA^V \rightarrow DMA^{III}$$

In the present study, methylation capacity was defined as sum of the substrates that had been methylated divided by sum of the substrates to be methylated. The ratios (MMA + DMA)/(iAs Met - iAs^V) were used for the measurement of first methylation capacity, (DMA)/(MMA + DMA) for second methylation capacity, and (DMA)/(iAs Met - iAs^V)for total methylation capacity in the present study.

Similarly, the ratios (iAs Met - iAs^V)/(iAs Met) were used for the measurement of first reduction capacity, (MMA^{III} + DMA)/(MMA + DMA) for second reduction capacity, and DMA^{III}/(DMA for third reduction capacity in the present study.

The values of these ratios were presented in Table 5 for current-users and in Table 6 for ex-users of As contaminated water. The metabolic pathway used was as

$$iAs^V \rightarrow iAs^{III} \rightarrow MMA^V \rightarrow MMA^{III} \rightarrow DMA^V \rightarrow DMA^{III}$$

In the present study, methylation capacity was defined as sum of the substrates that had been methylated divided by sum of the substrates to be methylated. The ratios (MMA + DMA)/(iAs Met - iAs^V) were used for the measurement of first methylation capacity, (DMA)/(MMA + DMA) for second methylation capacity, and (DMA)/(iAs Met - iAs^V)for total methylation capacity in the present study. Similarly, the ratios (iAs Met - iAs^V)/(iAs Met) were used for the measurement of first reduction capacity, (MMA^{III} + DMA)/(MMA + DMA) for second reduction capacity, and DMA^{III}/(DMA for third reduction capacity in the present study.

Table 5 suggested that except (DMA)/(MMA + DMA), all other ratios (MMA + DMA)/(iAs Met - iAs^V), (DMA)/(iAs Met - iAs^V), (iAs Met - iAs^V)/(iAs Met), (MMA^{III} + DMA)/(MMA + DMA), and DMA^{III}/DMA were statistically significant among groups ($p<0.05$). Table 6 indicated that no ratios were statistically significant between gender, and people with and without arsenism ($p>0.05$), but second methylation and all reduction capacities were satistically significant ($p<0.05$) among the subjects of the ex-users.

The behavior of the proportion of As species in a wider range of total As concentrations in urine was studied by performing a bivariate analysis pooling results (children and adults independently) from all groups except group C, because changes in exposure might alter excretion kinetics. The bivariate scattergrams between urinary As species (µg As/l) and As in drinking water (µg As/l) of the current users of As contaminated water showed that all As metabolites in human urine were dose dependent (Figure not shown). Similarly, the bivariate scattergrams between urinary As species (%) with urinary tAs (µg As/l), and ratios for methylation and reduction capacities with urinary iAs Met (µg As/l) of the child and adult current-users of As contaminated water indicated that the relative proportions of DMA^V and MMA^V to tAs in urine increased with As exposure and same trend was observed in case of first, second, and total methylations; first and second reduction capacities (Table 7).

A comparison of the ratios for methylation and reduction capacities between children, adult males, and adult females from current users showed that females appeared to be better methylators than child and males (considering first and total methylations), but statistically not significant ($p>0.05$) (Table 5).

Table 5. Results of methylation (MMA + DMA/iAs Met, MMA + DMA/iAs Met - iAs^V,and DMA/MMA + DMA, DMA/iAs Met - iAs^V) and reduction (iAs Met - iAs^V/iAs Met, MMA^{III} + DMA/MMA + DMA, and DMA^{III}/DMA) capacities in various subgroups among current users of arsenic contaminated water.

Factors	Subgroups	MMA+DMA/ iAs Met		MMA + DMA/ iAs Met - iAs^V		DMA/ MMA + DMA		DMA/ iAs Met - iAs^V		iAs Met - iAs^V / iAs Met		MMA^{III} + DMA/ MMA + DMA		DMA^{III}/DMA	
		Mean (n^a)	CV^b	Mean (n^a)	CV^b	Mean (n^a)	CV^b	Mean (n^a)	CV^b	Mean (n^a)	CV^b	Mean (n^a)	CV^b	Mean (n^a)	CV^b
Groups	Group A	0.686 (38)	67.1	0.495 (47)	44.9*	0.832 (71)	11.7	0.394 (48)	52.6	0.868 (48)	8.26	1.152 (7)	2.06*	0.384 (33)	60.9
	Group B	0.670 (58)	25.9*	0.799 (118)	19.5*	0.803 (120)	12.3*	0.644 (118)	22.7**	0.835 (118)	15.8**	0.851 (68)	9.35*	0.218 (95)	99.4
	Group C	0.903 (25)	4.18*	1.724 (41)	235*	0.857 (44)	9.61*	1.483 (41)	235*	0.965 (41)	9.18*	0.861 (16)	14.5	0.060 (28)	161*
	P value#	0.0001		0.0226		0.3476		0.0225		0.0042		0.0063		0.0010	
Sex	Male	0.701 (57)	26.9*	0.782 (106)	22.6*	0.853 (47)	7.28*	0.636 (106)	26.3**	0.863 (106)	15.7*	0.853 (47)	7.28*	0.195 (82)	110*
	Female	0.746 (64)	54.5	1.054 (100)	243**	0.899 (44)	38.1	0.874 (101)	251**	0.874 (101)	14.2*	0.899 (53)	38.1	0.257 (74)	89.0*
	P value#	0.3395		0.3289		0.4645		0.3173		0.5278		0.3705		0.0869	
Age (yrs)	Child (upto 10 yrs)	0.688 (40)	37.5	0.753 (71)	27.1**	0.830 (84)	12.7	0.625 (71)	30.9**	0.861 (71)	15.5*	0.875 (32)	7.59	0.246 (55)	94.4
	Adult-males	0.659 (32)	27.4	0.777 (70)	22.8*	0.807 (74)	12.1	0.629 (70)	26.2*	0.872 (70)	13.5**	0.841 (32)	7.72*	0.173 (50)	115*
	Adult-females	0.714 (44)	36.7*	1.233 (66)	247**	0.828 (78)	11.9	1.017 (67)	257**	0.875 (67)	14.9	0.954 (27)	65.1	0.252 (51)	91.5*
	P value	0.3579		0.3084		0.4534		0.2989		0.1356		0.6316		0.8091	
Patient	No	0.706 (110)	37.4*	0.739 (181)	28.0**	0.823 (208)	12.3*	0.604 (182)	31.9**	0.864 (182)	14.3**	0.869 (78)	18.3	0.245 (136)	95.7*
	Yes	0.799 (11)	16.2	2.177 (27)	227*	0.814 (27)	12.3	1.836 (25)	232*	0.898 (25)	14.9*	0.839 (13)	15.3	0.083 (20)	146
	P value#	0.5105		0.7173		0.6153		0.6997		0.3199		0.5200		0.0030	

[a]No of observations; [b]Coefficient of variation; [#]SAS/Stat General Linear Model, *Significant (p<0.05); **Significant (p<0.001).

A detailed statistical analysis to substantiate statements regarding trends or differences between groups was done. GLM procedure was done using the ratios MMA + DMA/iAs Met - iAs^{V}, DMA/MMA + DMA, DMA/iAs Met - iAs^{V}, iAs Met - iAs^{V}/iAs Met, MMA^{III} + DMA/MMA + DMA, and DMA^{III}/DMA as the dependent variables for current users (Table 8), ex-users (Table 9), current-users vs. ex-users (Table 10) of As contaminated water, and child, adult-males and adult-females of current users (Table 11), respectively. Table 8 showed that first and total methylation was statistically significant among groups and people with and without arsenism ($p<0.05$), but not among age and gender ($p>0.05$). Only second methylation was statistically significant among ages ($p<0.05$). All reduction capacities were statistically significant among groups, but first methylation capacity was significant with total urinary arsenicals ($p<0.01$).

In case of ex-users, no statistical significant methylation capacities were observed among groups, sex, age, and people with and without arsenism ($p>0.05$), but second and third reduction capacities were significant with sex, first and third reduction capacities were significant with total urinary arsenicals, and second reduction capacity was significant with age ($p<0.05$)(Table 9). Table 10 showed that both first and third methylation and reduction capacities were statistically significant ($p<0.0001$) between current and ex-users of arsenic contaminated water. Table 11 showed that no methylation and reduction capacities were significant between child, adult-males and adult-females from current users of arsenic contaminated water ($p>0.05$).

DISCUSSION

This is the first epidemiological study to investigate the methylation capacity among the people in terms of the ratios of the putative products and the putative substrates of the As metabolic pathway as suggested by Mandal et al. [7] considering all As metabolic species (iAs^{V}, iAs^{III}, MMA^{V}, MMA^{III}, DMA^{V}, and DMA^{III}). But in all other studies, all authors used inorganic As (iAs), monomethylated As (MMA), and dimethylated As (DMA) for the prediction of methylation capacity of the studied populations.

All other published reports on urinary As metabolites in human subjects (including populations in Europe, America, and Asia exposed to iAs in the general environment, experimentally, and occupationally) consistently showed average values of 10 – 30 % iAs, 10 – 20 % MMA, and 60 – 80 % DMA [19, 22]. In the present study, our results showed that on an average, urine consisted of approximately 3.47 – 16.5 % iAs^{V}, 9.58 – 14.9 % iAs^{III}, 7.83 – 14.3 % MMA^{V}, 2.09 – 4.41 % MMA^{III}, 44.1 – 80.4 % DMA^{V}, and 4.23 – 17.1 % DMA^{III} (Data not shown, but raw data in Table 3). This was very similar to the previously reported results [19, 22].

Table 6. Results of methylation (MMA + DMA/iAs Met, MMA + DMA/iAs Met - iAs^V, DMA/MMA + DMA, and DMA/iAs Met - iAs^V) and reduction (iAs Met - iAs^V/iAs Met, MMA^{III} + DMA/MMA + DMA, and DMA^{III}/DMA) capacities in various subgroups among ex-users of arsenic contaminated water.

Factors	Subgroups	MMA+DMA/ iAs Met		MMA + DMA/ iAs Met - iAs^V		DMA/ MMA + DMA		DMA/ iAs Met - iAs^V		iAs Met - iAs^V/ iAs Met		MMA^{III} + DMA/ MMA + DMA		DMA^{III}/DMA	
		Mean (n^a)	CV^b	Mean (n^a)	CV^b	Mean (n^a)	CV^b	Mean (n^a)	CV^b	Mean (n^a)	CV^b	Mean (n^a)	CV^b	Mean (n^a)	CV^b
Group	Group C	0.785 (58)	15.0	0.848 (120)	11.3	0.812 (120)	12.0*	0.698 (122)	18.8	0.925 (122)	5.51**	0.857 (91)	6.51*	0.343 (114)	72.1*
Sex	Male	0.763 (28)	10.3	0.858 (51)	7.46	0.808 (51)	11.6*	0.702 (52)	16.3*	0.925 (52)	5.15*	0.845 (35)	6.65*	0.293 (48)	83.5
	Female	0.796 (30)	18.4	0.841 (69)	13.4	0.814 (69)	12.1	0.696 (70)	20.6	0.925 (70)	5.84*	0.865 (56)	6.06	0.379 (66)	66.4
	P value[#]	0.4233		0.3376		0.7534		0.8332		0.9153		0.0903		0.0690	
Patient	No	0.789 (45)	9.48*	0.851 (93)	8.08	0.811 (93)	10.4*	0.692 (93)	15.2*	0.926 (93)	5.38**	0.857 (75)	5.79*	0.349 (90)	71.5*
	Yes	0.773 (15)	30.6	0.841 (26)	17.8	0.816 (26)	15.6	0.724 (28)	24.1	0.926 (28)	6.04	0.858 (15)	9.95	0.318 (23)	86.4
	P value[#]	0.5877		0.6604		0.5383		0.4554		0.3933		0.9311		0.8573	

[a]No of observations; [b]Coefficient of variation; [#]SAS/Stat General Linear Model, *Significant ($p<0.05$); **Significant ($p<0.001$).

These results clearly indicated the inter-individual variation in methylation ability among the people exposed to iAs. Concha et al. [36] reported the metabolism of iAs in children in three villages in northern Argentina: San Antonio de los Cobres and Taco Pozo, each with about 200 µg As/l in the drinking water, and Rosario de Lerma, with 0.65 µg As/l. This report mentioned an average urinary As metabolites as 323 and 303 µg As/l in San Antonio de los Cobres, 440 and 386 µg As/l in Taco Pozo, and 13 and 7.6 µg As/l in Rosario de Lerma for children and women respectively.

Table 7. Equations showing trends of urinary arsenicals (%) with urinary tAs (µg/l) and ratios for methylation and reduction capacities with urinary iAs Met (µg/l) of the adult and child current users of arsenic contaminated water.

	Adult current users			Child current users		
Parameters (Y Variables)	**Slope**	**Intercept**	**R^2**	**Slope**	**Intercept**	**R^{2c}**
iAs^V/tAs (%)	0.007	15.39	0.144	-0.004	15.425	0.082
iAs^{III}/tAs (%)	-0.008	24.339	0.081	-0.006	22.257	0.072
MMA^V/tAs (%)	0.002	9.305	0.058	-2.175E-4	8.776	0.002
MMA^{III}/tAs (%)	-0.001	4.389	0.113	-0.001	4.969	0.074
DMA^V/tAs (%)	0.013	46.002	0.111	0.012	47.805	0.111
DMA^{III}/tAs (%)	-0.005	14.422	0.064	-0.003	12.99	0.052
1^{st} MC^{ad}	1.15E-4	0.7	0.012	9.321	0.662	0.092
1^{st} MC^a	1.052E-4	0.697	0.109	8.65E-5	0.712	0.106
2^{nd} MC^a	1.324E-5	0.816	0.008	2.955E-5	0.819	0.048
3^{rd} MC^a	1.046E-4	0.556	0.122	1.456E-5	0.134	0.03
1^{st} RC^b	6.268E-5	0.861	0.102	4.472E-5	0.846	0.082
2^{nd} RC^b	3.254E-5	0.826	0.039	1.598E-5	0.858	0.03
3^{rd} RC^b	-1.042E-4	0.27	0.108	-7.7.483E-5	0.284	0.101

[a]Methylation capacity; [b]Reduction capacity; [c]How much of the variance is accounted for by the model, [d]Not considering oxidation states of arsenic.

Another report from the study at Region Lagunera, Mexico [37] reported that the exposed group had an average of 408 µg As/l of total As in their drinking water, whereas control individuals had 31 µg As/l. This report mentioned 561 µg As/g creatinine as total urinary As in the exposed group and 20.6 µg As/g creatinine as total urinary As in the control group. Kurttio et al. [13] reported the metabolism of As in the current and ex-users of As contaminated water (Averages 170 µg As/l for current, 292 µg As/l for ex-users, and <1 µg As/l for controls, respectively) from some drilled wells in Orivesi Village of southwestern Finland. This group informed the geometric means of the concentrations of total As in urine were 58 µg As/l for current users, 17 µg As/l for ex-users, and 5 µg As/l for controls. Hopenhayn-Rich et al. [14] reported the methylation study of a population consisted of 122 people in a town with As water levels around 600 µg As/l and 98 participants in a neighboring town with As levels in water of about 15 µg As/l located in the high Atacama Desert of northern Chile. They reported the corresponding mean urinary As levels as 580 µg As/l and 60 µg As/l, respectively.

In the present study, our study population consisted of four groups A-D with drinking water iAs concentrations 33 ± 7, 148 ± 34, 210 ± 2.6, and 248 ± 59 µg As/l (mean ± SE), respectively in West Bengal, India. The populations of group C (ex-users) were drinking safe water (<3.0 µg As/l) for 18 months before our collection. The corresponding mean urinary As levels were 54.5 and 57.9 µg As/l for adults and children in group A, 211.8 and 164.5 µg As/l for adults and children in group B, and 1571 and 1679 µg As/l for adults and children in Group D, and 266.8 and 235.3 µg As/l for adults and children in group C, respectively (Data not shown). The above discussion implied that the Finnish populations excreted very low percentage of urinary As compared to the Argentinean, Mexican, Chilean, and Indian studies. In case of ex-users of As contaminated water, the Finnish populations showed lower urinary As excretion than that of our study populations. The present study suggested that the excretion pattern of urinary As in group C was mostly similar to that of group B after drinking of As safe water for a duration of 18 months, but that of group D was induced (Table 3 and 4). This also suggested that people of Group C could not avoid the intake of As via their food and incorporation of As in the food-chain should be considered earnestly in the future course of As studies. Only trace amount of MMA^V (2.4 ± 0.4 µg As/l, n = 21) and DMA^V (6.1 ± 1.2 µg As/l, n = 35) were found in the urine samples of the control group (8.1 ± 0.7 µg As/l).

In the present study, DMA^V/tAsi (%) and MMA^V/tAsi (%) had an increasing trend in the adult current-users of As contaminated water (Table 7), whereas DMA^V/tAsi (%) had an increasing trend in the children current-users of As contaminated water (Table 7). But, some studies indicated a slight decrease in the relative amount of DMA in urine and a corresponding increase in the relative amount of MMA with increasing exposure to iAs via drinking water [11, 14, 16-17, 37-38].

In the present study, the concentrations of DMA^{III} and MMA^{III} were proportional to exposure level in the current-users (Figure not shown), but DMA^{III}/tAs (%) and MMA^{III}/tAs (%) had a decreasing trend with exposure (Table 7). Same trend was observed for the ex-users (figure not shown). This might be due to higher rate of increase of tAs with exposure than that of DMA^{III} and MMA^{III} with exposure.

At the higher exposure levels, the proportion of iAs in urine tended to be lower and dimethylated As (DMA) higher, which might reflect a more extensive methylation capacity in individuals exposed to higher levels [13]. Our results corroborated these findings, since iAs^{III}/tAs (%) decreased and DMA^V/tAs (%) increased with exposure (Table 7). Another study [39] compared two groups of people in Mexico according to the levels of iAs in their drinking water (390 µg As/l vs. 60 µg As/l) and reported that 20 - 30 % of the urinary As of both groups was in the form of iAs, even though the total urinary levels were 10 times higher in the high exposure group. But in the present study, lower percentage of iAs (approx. 9 %) and higher percentage of DMA^V (approx. 75 %) were found in the highest exposure group D (data not shown). The gender difference in the relative proportions of DMA and MMA was reported in the recent study in Mexico [37]. Their finding was that women appeared to be better As methylators than men. An *in vitro* study in the arsenical area of Mexico found that impaired proliferation was greater in lymphocytes from As exposed women than from exposed men [40].

Table 8. GLM using the ratios MMA + DMA/iAs Met, MMA + DMA/iAs Met - iAs^V, DMA/MMA + DMA, DMA/iAs Met - iAs^V, iAs Met - iAs^V/iAs Met, MMA^{III} + DMA/MMA + DMA, and DMA^{III}/DMA as the dependent variables for current users of arsenic contaminated water

Predictor variables	Df[a]	Type III sum of squares	Mean square	F-value
MMA + DMA/iAs Met (n = 236)				
Groups	2	1.543	0.771	2..81*
Sex (male vs. female)	1	0.070	0.070	0.26
Age (years)	1	0.078	0.078	0.28
Patient (yes vs. no)	1	0.251	0.251	0.91
tAs	1	1.863	1.863	6.78*
MMA + DMA/iAs Met - iAs^V (n = 206)				
Groups	2	29.72	14.86	3.86*
Sex (male vs. female)	1	2.964	2.964	0.77
Age (years)	1	0.255	0.255	0.07
Patient (yes vs. no)	1	39.41	36.41	10.24*
tAs	1	7.639	7.639	1.99
DMA/MMA + DMA (n = 235)				
Groups	2	0.021	0.011	1.06
Sex (male vs. female)	1	0.003	0.003	0.31
Age (years)	1	0.010	0.010	1.29*
Patient (yes vs. no)	1	0.014	0.015	1.47
tAs	1	0.013	0.013	1.29
DMA/iAs Met - iAs^V (n = 207)				
Groups	2	21.98	10.99	3.87*
Sex (male vs. female)	1	1.882	1.882	0.66
Age (years)	1	0.121	0.121	0.04
Patient (yes vs. no)	1	28.01	28.01	9.86*
tAs	1	5.271	5.271	1.85
iAs Met - iAs^V/iAs Met (n = 207)				
Groups	2	0.183	0.092	5.64*
Sex (male vs. female)	1	0.031	0.031	1.90
Age (years)	1	0.019	0.019	1.15
Patient (yes vs. no)	1	0.003	0.003	0.17
tAs	1	0.087	0.087	5.38*
MMA^{III} + DMA/MMA + DMA (n = 91)				
Groups	2	0.588	0.294	5.39*
Sex (male vs. female)	1	0.016	0.016	0.30
Age (years)	1	0.164	0.164	3.01
Patient (yes vs. no)	1	0.023	0.023	0.42
tAs	1	0.037	0.037	0.68
DMA^{III}/DMA (n = 156)				
Groups	2	0.682	0.341	7.29**
Sex (male vs. female)	1	0.019	0.019	0.42
Age (years)	1	0.012	0.012	0.26
Patient (yes vs. no)	1	0.022	0.022	0.47
tAs	1	0.062	0.062	1.32

[a]*Degrees of freedom; *Significant (p<0.05); **Significant (p<0.001).*

Table 9. GLM using the ratios MMA + DMA/iAs Met, MMA + DMA/iAs Met - iAs^V, DMA/MMA + DMA, DMA/iAs Met - iAs^V, iAs Met - iAs^V/iAs Met, MMA^{III} + DMA/MMA + DMA, and DMA^{III}/DMA as the dependent variables for ex-users of As contaminated water

Predictor variables	Df[a]	Type III sum of squares	Mean square	F-value
MMA + DMA/iAs Met (n = 120)				
Sex (male vs. female)	1	0.003	0.003	0.29
Age (years)	1	0.005	0.005	0.48
Patient (yes vs. no)	1	0.005	0.005	0.46
tAs	1	0.016	0.016	1.51
MMA + DMA/iAs Met - iAs^V (n = 120)				
Sex (male vs. female)	1	0.007	0.007	0.80
Age (years)	1	0.0001	0.0001	0.01
Patient (yes vs. no)	1	0.005	0.005	0.60
tAs	1	0.002	0.002	0.25
DMA/MMA + DMA (n = 120)				
Sex (male vs. female)	1	0.002	0.002	0.22
Age (years)	1	0.0002	0.0002	2.22
Patient (yes vs. no)	1	0.014	0.014	0.02
tAs	1	0.013	0.013	2.53
DMA/iAs Met - iAs^V (n = 122)				
Sex (male vs. female)	1	0.001	0.001	0.07
Age (years)	1	0.037	0.037	2.11
Patient (yes vs. no)	1	0.006	0.006	0.37
tAs	1	0.001	0.001	0.05
iAs Met - iAs^V/iAs Met (n = 122)				
Sex (male vs. female)	1	0.001	0.001	0.32
Age (years)	1	0.006	0.006	2.47
Patient (yes vs. no)	1	0.0001	0.0001	0.02
tAs	1	0.042	0.042	16.36*
MMA^{III} + DMA/MMA + DMA (n = 91)				
Sex (male vs. female)	1	0.013	0.013	4.16*
Age (years)	1	0.015	0.015	4.95*
Patient (yes vs. no)	1	0.001	0.001	0.35
tAs	1	0.000	0.000	0.00
DMA^{III}/DMA (n = 114)				
Sex (male vs. female)	1	0.324	0.324	5.28*
Age (years)	1	0.014	0.014	0.23
Patient (yes vs. no)	1	0.106	0.106	1.73
tAs	1	0.437	0.437	7.12*

[a]*Degrees of freedom; *Significant (p<0.05); **Significant (p<0.001).*

Similar gender differences were found in northeastern Taiwan [16] and Chile [14], but not in other studies [13, 17]. Another study in northern Chile reported that women had approximately 3 % more DMA and less MMA in the urine than men [14]. In the present study, although females appeared to be better methylators than child and males (considering first and total

methylations)(Table 5) no conclusion was drawn on methylation capacities among gender (Tables 8, 9 and 11).

Buchet et al. [41] reported average values of 12 % iAs, 28 % MMA, and 60 % DMA in children in Belgium (n = 14); Kalman et al. [42] reported average values of 13 % iAs, 16 % MMA, and 71 % DMA in children in the United States (n = 158), and Concha et al. [36] reported average values of 45.5 % iAs, 3.5 % MMA, and 45.5 % DMA (n = 34) in the Argentina. In the present study, we found average 13.9 % iAs^{V} (n = 71), 19.3 % iAs^{III} (n = 81), 8.67 % MMA^{V} (n = 86), 4.41 % MMA^{III} (n = 32), 51.6 % DMA^{V} (n = 86), and 11.6 % DMA^{III} (n = 55) in children in West Bengal, India for the current-users (Data not shown). Our results were very similar to both Buchet et al. [41-42], but not to Concha et al. [36]. Concha et al. [36] reported the increasing percentage of urinary DMA, and the corresponding decreasing percentage of iAs, with increasing urinary As in the children, but not in the women. Our findings differed from it and both children and females (figures not shown) had an increasing percentage of DMA^{V} and decreasing percentage of iAs with increasing As metabolites in their urines.

In the few studies that looked at methylation patterns in children, percentages of metabolites excreted in urine were similar to adults [41-42]. However, in both these latter studies As exposure was relatively low as indicated by total concentrations of As metabolites excreted in urine (i.e. <20 µg As/l). Vahter et al. [43] reported a unique pattern of urinary methylated metabolite excretion in a population of healthy native women in northwestern Argentina consuming an apparently protein-adequate diet and As contaminated drinking water (200 µg As/l). They suggested that the higher urinary DMA excretion in women in the village with the highest As in drinking water (200 µg As/l) compared to women in the village with lower As in drinking water (2.5 to 31 µg As/l) indicating induction of DMA excretion (with unusually low fractions of urinary MMA (~2%) as compared to levels found elsewhere). Concha et al. [36, 44] reported significant increases in the percentage DMA excreted in urine in Argentinean women during pregnancy, a possible reason for gender differences reported in some studies. Another study from Taiwan found that genetic polymorphisms of the detoxification enzymes, glutathione S-transferases M1 and T1 were associated with varying arsenic methylation patterns [17]. The researchers found that those with the null GSTM1 genotype had a higher percentage of % iAs, and those with the null GSTT1 genotype had increased % DMA in the urine. Some studies reported [45-46] that iAs metabolism was affected by liver disease. Patients with cirrhotic liver disease shifted the proportion of MMA and DMA excretion in the urine. The percentage of As excreted as MMA was decreased in liver disease compared to controls (6.1 ± 0.7 vs. 12.8 ± 0.7), while DMA was increased (40.7 ± 1.9 vs. 24.3 ± 1.6) [45].

Del Razo et al. [37] and Kurttio et al. [13] estimated the As metabolism via the ratios of MMA/iAs (first methylation reaction), DMA/MMA (second methylation reaction), and DMA/iAs (total methylation), because they did not speciate all As metabolites according to their valency states. A above reports [13, 37] reported a decreased value of DMA/MMA ratio, i.e. the inhibition of second methylation reaction was reported with increasing As exposure in Mexico, but not in the Finnish population. Since all As species in urine samples were stable up to 4 months of their collection (Table 2), the ratios MMA + DMA/iAs Met - iAs^{V}, DMA/MMA + DMA, and DMA/iAs Met - iAs^{V} were used for first methylation, second methylation, and total methylation capacities, respectively in the present study (Table 5 and 6). Bivariate scattergram

between methylation capacities and urinary iAs Met (µg/l) (Figures not shown) stated that the methylations were proportional to iAs exposure, which corroborated to the Finnish population, but not to the Mexican population. Also, the values of MMA + DMA/iAs Met - iAs^V and DMA/iAs Met - iAs^V ratio for children and males were lower than that for females in the present study (Table 5), which suggested that the children and males might be more susceptible to iAs toxicity than females, but it was not significant statistically ($p>0.05$). Further, it did not support our findings of the lowest percentage of arsenical dermatological manifestations among the children within the age of 10 years in West Bengal, India and Bangladesh, which implied that there would be another key factors for such findings.

Kurttio et al. [13] reported that older persons were better methylators of iAs than younger individuals, but no conclusion was drawn in the present study regarding age (Tables 8 and 9). A recent report of the study in Mexico indicated that exposed individuals with skin alterations had a lower DMA/MMA ratio than exposed persons without skin effects [37, 47]. Although values of first and third methylation capacities of people with skin alterations were greater than that of people without skin alterations they were not statistically significant ($p>0.05$) and hence our results did not support this (Tables 5).

Hopenhayn-Rich et al. [15] compared methylation patterns in Chilean subjects (n = 73) before and after changing from higher (600 µg As/l) to lower (45 µg As/l) As-containing drinking water. There was a small but significant decrease in urinary iAs (17.8 % to 14.1 %) and a decrease in the MMA to DMA ratio (0.23 to 0.18). In the present study, after changing As contaminated water to safe water (<3.0 µg As/l) the methylation capacities of ex-user of As contaminated water became almost parallel to urinary iAs Met (µg As/l) (Bivariate Scattergrams not shown).

Hopenhayn-Rich et al. [15-16] suggested the inhibition of methylation of As by smoking. But, in the present study, no data were collected on smoking from the studied populations. Although nutrition [1, 48-49] and ethnicity [15] of the studied populations influenced the methylation capacity, our studied population was of similar nutrition and ethnicity status.

Besides the differences found between population groups, there was a substantial inter-individual variation in the methylation of As (for reviews, see Ref. [19, 22]. These variations might indicate a polymorphism of the genes regulating the expression of As methyltransferases. So, genetic polymorphism in As–methylating enzymes and other co-factors were likely to contribute to some of the unexplained variation.

In the present study, the values of MMA + DMA/iAs Met - iAs^V, DMA/MMA + DMA, and DMA/iAs Met - iAs^V had an increasing trend with urinary iAs Met in both adults and child of current users (Table 7). These results suggested that all methylations steps of arsenicals were directly proportional to the exposure. Table 5 showed that first and total methylations were statistically significant among groups ($p<0.05$). In all individual groups, first methylation capacity was satistically significant ($p<0.05$), but total methylation was significant individually only in groups B and D (Table 5). Although second methylation capacity was significant ($p<0.05$) individually in groups B and D overall it was not significant ($p>0.05$) among groups (Table 5). Similarly, first and total methylations were significant ($p<0.05$) in all individual sex,

age, and patient groups, but overall they were not significant ($p>0.05$) whereas second methylation was not significant both individually and overall among various groups (Table 5). Again, Table 8 showed that first and total methylations were statistically significant among groups and people with and without arsenism ($p<0.05$), but not among age and gender ($p>0.05$) in current users. Only second methylation was statistically significant among ages ($p<0.05$) (Table 8). In case of ex-users, no statistical significant methylation capacities were observed among sex, age, and people with and without arsenism ($p>0.05$)(Tables 6 and 9), but second methylation was significant ($p<0.05$) individually in group, male, and non-patient; total methylation individually in male and non-patient groups (Table 6). Table 10 showed that both first and third methylation capacities were statistically significant ($p<0.0001$) between current and ex-users of arsenic contaminated water. Table 11 showed that no methylation capacities were significant between child, adult-males and adult-females from current users of arsenic contaminated water ($p>0.05$), while first and total methylations were significant ($p<0.05$) individually in child, adult-males and adult-females (Table 5).

In the present study the values of ratios for first and second methylation capacities were presented considering both oxidation states of arsenic for one sets and /considering simply As, MMA, and DMA for other sets (Tables 5-11). These results (Tables 5-11) suggested that only MMA + DMA/iAs (first methylation capacity) was not satistically significant ($p>0.05$) among people with and without arsenism of the current users of arsenic contaminated water, whereas MMA + DMA/iAs Met - iAs^{V} (first methylation capacity) was satistically significant ($p<0.05$) among people with and without arsenism (Table 8). All other sets of results were same in both cases, which suggested that consideration of both oxidation states of arsenic did not alter the outcome of the present study.

Table 8 showed that all reduction capacities were statistically significant among groups of current users ($p<0.05$) and first reduction capacity was statistically significant ($p<0.05$) among people with and without arsenism of current users. It was difficult in this study to consider all responsible factors for the reduction steps in the biotransformation of As such as GSH content, arsenate reductase, and monomethylarsonic acid reductase in the present study to use these ratios for the interpretation of methylation capacity due to lack of information/data regarding these matters. Since Zakharyan et al. [50] informed that MMA^{V} reductase, which catalyzed the synthesis of MMA^{III}, was the rate-limiting enzyme, these reduction steps might be the determining factor for the methylation efficiency in the As-affected people. Alternatively, it might be the cause for inter-individual variation in As metabolism in humans.

Table 10. GLM table (current vs. ex-users of arsenic contaminated water) using the ratios MMA + DMA/iAs Met, MMA + DMA/iAs Met - iAs^V, DMA/MMA + DMA, DMA/iAs Met - iAs^V, iAs Met - iAs^V/iAs Met, MMA^{III} + DMA/MMA + DMA, and DMA^{III}/DMA as the dependent variables.

Predictor variables	Df[a]	Type III sum of squares	Mean square	F-value
MMA + DMA/iAs Met (n = 356)	1	3.189	3.189	14.14**
MMA + DMA/iAs Met - iAs^V (n = 326)	1	61.48	61.48	19.53**
DMA/MMA + DMA (n = 355)	1	0.0013	0.0013	0.14
DMA/iAs Met - iAs^V (n = 329)	1	42.98	42.98	18.48**
iAs Met - iAs^V/iAs Met (n = 329)	1	0.108	0.108	9.24*
MMA^{III} + DMA/MMA + DMA (n = 182)	1	0.0058	0.0058	0.44
DMA^{III}/DMA (n = 270)	1	0.4081	0.4081	7.32*

[a]Degrees of freedom; *Significant (p<0.05); **Significant (p<0.001).

Table 11. GLM table (child, adult-males, and adult-females among current users of arsenic contaminated water) using the ratios MMA + DMA/iAs Met, MMA + DMA/iAs Met - iAs^V, DMA/MMA + DMA, DMA/iAs Met - iAs^V, iAs Met - iAs^V/iAs Met, MMA^{III} + DMA/MMA + DMA, and DMA^{III}/DMA as the dependent variables.

Predictor variables	Df[a]	Type III sum of squares	Mean square	F-value
MMA + DMA/iAs Met (n = 326)	2	0.629	0.314	1.03
MMA + DMA/iAs Met - iAs^V (n = 326)	2	9.325	4.662	1.18
DMA/MMA + DMA (n = 355)	2	0.016	0.008	0.79
DMA/iAs Met - iAs^V (n = 329)	2	7.070	3.535	1.21
iAs Met - iAs^V/iAs Met (n = 329)	2	0.068	0.034	2.02
MMA^{III} + DMA/MMA + DMA (n = 182)	2	0.111	0.055	0.46
DMA^{III}/DMA (n = 270)	2	0.022	0.011	0.21

*[a]Degrees of freedom; *Significant (p<0.05); **Significant (p<0.001).*

CONCLUSION

Trivalent arsenicals were 97.6-99.8% stable when they were spiked in control urines, kept in –28^o C and analyzed after 66 days in the present study without any preservatives i.e. it varied 2-3% than our recommended best method for urine samples collection in epidemiological studies of arsenic The limited data on stabilities of arsenic metabolites in the collected urine samples suggested that they (arsenicals) were stable for up to 4 months when urine samples were stored at –28^o C without any preservatives in the present study which supported the findings of Gong et al.

[28] for MMA^{III}, but not for DMA^{III}. The stability experiments of spiked arsenicals in urine also suggested that if possible to carry nitrogen cylinder to the field and samples were kept in nitrogen medium, all arsenicals in urine might be stable for a long time. For longer period of storage, the stability of As species depended on urine matrices and preservatives had no effect. All As metabolites in human urine were dose dependent. In the present study, females appeared to be better methylators than males and children, but it was not statistically significant ($p>0.05$). Only second methylation capacity between different age groups was significant ($p<0.05$). The first and total methylation capacities were statistically significant ($p<0.05$) between groups, people with and without arsenism, and current and ex-users of As contaminated water. Based on the results of the present study, the hypothesis of methylation threshold was not established in these exposure ranges. Although methylation capacity is not statistically conclusive in the present study, this is the first study, which documents the results on reduction capacity of the As-affected population in the endemic areas. Another outcome of this study is that researchers must consider the intake route of As via food-chain during the preparation of toxicity model for the As-affected population. Further researches on dose-response, enzymology of reduction-methylation reactions of As biotransformation, and genetic polymorphism would be conducted to correlate all hypotheses regarding the efficiency of As metabolism in human.

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