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EXPOSURE TO INORGANIC ARSENIC IN PREGNANCY AND METABOLISM-NUTRITION INTERACTION

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ABSTRACT

Inorganic arsenic is metabolized by most mammals, including humans, via series of reduction and methylation reactions with S-adenosylmethionine as main methyl donor. Thus, it seems likely that it is influenced by the availability of methyl groups. The main arsenic metabolites excreted in human urine are monomethylarsonic acid (MMA), dimethylarsinic acid (DMA), besides some un-methylated inorganic arsenic (arsenate [As(V)] and arsenite [As(III)]). The aim of the present thesis was to investigate the arsenic exposure via drinking water in Matlab, Bangladesh, an area with high-elevated concentrations of arsenic in tubewells, and arsenic metabolism in relation to nutritional status in people chronically exposed to inorganic arsenic

For assessment of arsenic exposure, total arsenic concentrations in urine and drinking water were determined by hydride generation atomic absorption spectrophotometry (HG-AAS). In order to assess methylation capacity, arsenic metabolites in urine were speciated by high performance liquid chromatography coupled with hydride generation and inductively coupled plasma mass spectrometry (HPLC-HG-ICPMS).

There was a considerable variation in urinary concentrations of arsenic (total range 1-1,470 µg/L, adjusted to specific gravity 1.012 g/mL) measured in 3,426 pregnant women in about gestational week 8, with an overall median concentration of 80 µg/L. Similar concentrations were found in gestational week 30, indicating no trend of decreasing exposure, despite the initiated mitigation activities in the area. Arsenic exposure was negatively associated with socioeconomic groups and achieved educational level.

We studied the effect of macro-nutritional status, assessed by body mass index (BMI) and urinary creatinine excretion, among 442 pregnant women in gestational week 9, and the effects of micro-nutritional status, assessed by serum folate, vitamin B12, zinc, and ferritin as well as urinary selenium, among 753 women in gestational week 14. The average proportions of iAs, MMA, and DMA in urine in gestational week 9 were 15%, 11% and 74%, respectively, indicating an efficient arsenic methylation in spite of the malnutrition (about one third of the women had BMI below 18.5 kg/m^2). Also, we found that the metabolism of inorganic arsenic was only marginally influenced by micro-nutritional status, except for selenium and, probably, zinc. Urinary %MMA increased with increasing serum zinc and %DMA increased with increasing urinary selenium, indicating a role of both essential elements in arsenic methylation. However, the arsenic exposure level assessed by the urinary arsenic had the greatest impact on arsenic methylation among the studied factors. The further research will focus on changes in arsenic methylation during pregnancy and food and micronutrient supplementation as well as the impact on arsenic-related effects on birth weight.

Keywords: *Arsenic, metabolism, methylation, nutrition, women, pregnancy, drinking water*

LIST OF PUBLICATIONS

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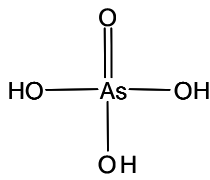
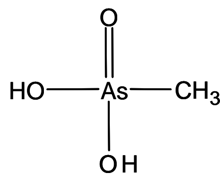
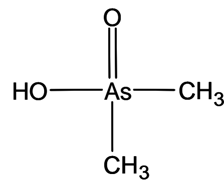
Structures of arsenic species relevant to this thesis



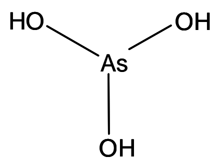
Arsenic acid
As(V)



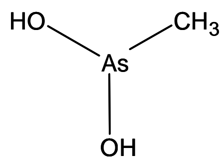
Monomethylarsonic acid
MMA



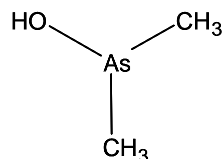
Dimethylarsinic acid
DMA



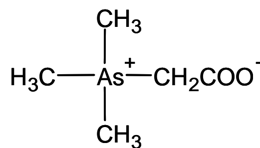
Arsenous acid
As(III)



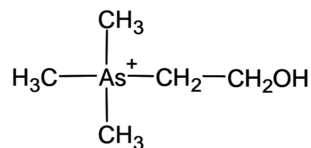
Monomethylarsonous acid
MMA(III)



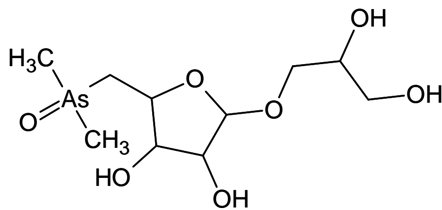
Dimethylarsinous acid
DMA(III)



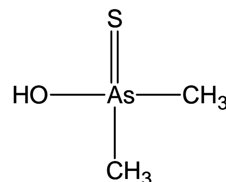
Arsenobetaine



Arsenocholine



Arsenosugars



Thio-dimethylarsinic acid
Thio-DMA

LIST OF ABBREVIATIONS

μg/L	Micrograms per litre
AAS	Atomic absorption spectrophotometry
AFS	Atomic fluorescence spectrometry
As(III)	Arsenite, arsenous acid
As(V)	Arsenate, arsenic acid
CYT19	a 369 amino acid residues protein, molecular mass of 37,969Da
DMA	Dimethylarsinic acid
DMA(III)	Dimethylarsinous acid
GSH	Glutathione
GSSG	GSH disulfide
GW	Gestational week
HG	Hydride generation
hGSTO 1-1	Human Glutathione-S-Transferase Omega Class
HPLC	High performance liquid chromatography
iAs	Inorganic arsenic, sum of arsenate As(V) and arsenite As(III)
ICPMS	Inductively Coupled Plasma Mass Spectrometry
MMA	Monomethylarsonic acid
MMA(III)	Monomethylarsonous acid
ppb	Parts per billion
SAH	*S*-adenosylhomocysteine
SAM	*S*-adenosyl-L-methionine
SG	Specific gravity
SH	Sulfhydryl
SRMs	Standard reference materials
U-As	Urinary arsenic, sum of iAs, MA and DMA

1 INTRODUCTION

1.1 Arsenic in environment

1.1.1 Arsenic sources

Arsenic (As) is a ubiquitous trace element and widely distributed throughout the earth's crust. Dissolution of arsenic from the soil can easily contaminate groundwater with up to mg/L levels of inorganic arsenic (iAs). Therefore, one of the most serious worldwide environmental problems nowadays is drinking water polluted by arsenic (WHO, 2001; IARC, 2004). In groundwater, arsenic is present mainly as arsenate [As(V)] or arsenite [As(III)] depending on the pH and the presence of reducing or oxidizing substances.

Inorganic arsenic compounds have been widely used in wood preservatives (chromated copper arsenate), herbicides, insecticides, pigments, glass and medicines. Combustion of coal is considered one of the main sources of air pollution by arsenic. Although the concentrations of arsenic in sea water usually is between under detection limit to 4 µg/L according to previous studies (WHO/IPCS 2001), arsenic is biomagnified in the marine food chain. Most seafood contain elevated concentrations of arsenic, mainly in the form of organic arsenic compounds, of which arsenobetaine is the major species in marine animals, while arsenosugars (a collective name of arsenic bound to carbohydrate compounds) are common in seaweeds and bivalves. Arsenocholine is also found in certain kind of fish and shrimps. **Figure 1** describes schematically the main human exposure pathways for arsenic.

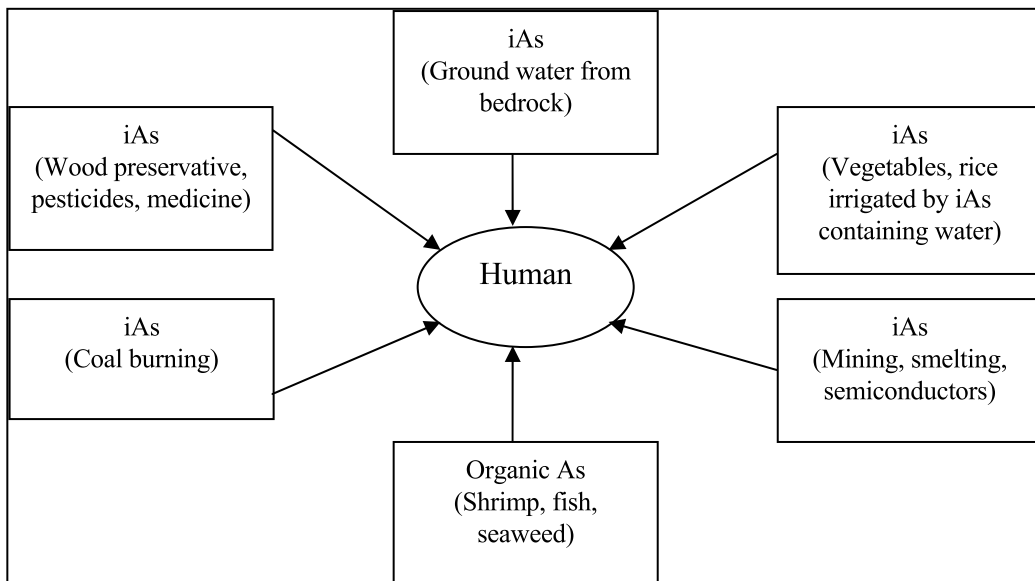


Figure 1.

Human exposure pathways for arsenic.

1.1.2 Arsenic in drinking water

The extent of the arsenic problem worldwide is still unknown. The regions with high concentrations of arsenic in drinking-water studied so far include Bangladesh, China (Taiwan and Inner Mongolia), India (West Bengal), Argentina, Australia, Chile, Mexico, the USA (Nevada, California and Arizona), Hungary, Romania, Ghana, Nepal and Vietnam, where drinking water is naturally contaminated by arsenic-rich geological formations (BGS 2001; WHO 2001; WHO 2003). In some areas of Japan, Mexico, Thailand and some African countries, arsenic in many polluted water sources comes from mining, smelting and other industrial activities. Many other countries, including Sweden, have initiated screening of public and private ground water sources for arsenic concentration. The estimated number of people worldwide exposed to arsenic via drinking water is approximately 80 millions. Among these, up to 57 millions in Bangladesh are drinking water with an arsenic concentration exceeding the WHO guideline value of 10 μg/L, and up to 35 millions are drinking water with concentrations in excess of the Bangladeshi standard of 50 μg/L.

1.2 Arsenic metabolism and health effects

1.2.1 Metabolism of inorganic arsenic

1.2.1.1 Absorption

Both As(III) and As(V) in dissolved forms are well absorbed in the gastrointestinal tract of humans and experimental animals; up to about 80 to 90% of a single dose (Pomroy et al. 1980; Tam et al. 1979). Arsenic is also absorbed from the respiratory tract; however sufficient information for quantitative estimation of arsenic absorption after inhalation is lacking due to potential interferences from oral exposure (WHO/IPCS 2001). The absorption through human skin is low; about 2% of low doses of AsV in water and about 1% of that in soil over a 24-h period (Wester et al. 1993).

1.2.1.2 Metabolism

It is clearly demonstrated that inorganic arsenic is metabolized by most mammals, including humans, via a series of reduction and methylation reactions, with S-adenosylmethionine (SAM) as main methyl donor (Cullen and Reimer 1989; Marafante and Vahter 1984; Radabaugh and Aposhian 2000; Vahter 2000; Zakharyan and Aposhian 1999). Only a few animal species, i.e. chimpanzees, marmoset monkeys, and guinea pigs seem unable to methylate arsenic (Vahter 1999). The classical metabolic pathway of inorganic arsenic, shown in **Figure 2,** consists of alternating reduction from pentavalent arsenic to trivalent and oxidative methylation of the trivalent forms. The six arsenic species involved in the pathway are As(V), As(III), monomethylarsonic acid (MMA), monomethylarsonous acid [MMA(III)] dimethylarsinic acid (DMA) and dimethylarsinous acid [DMA(III)].

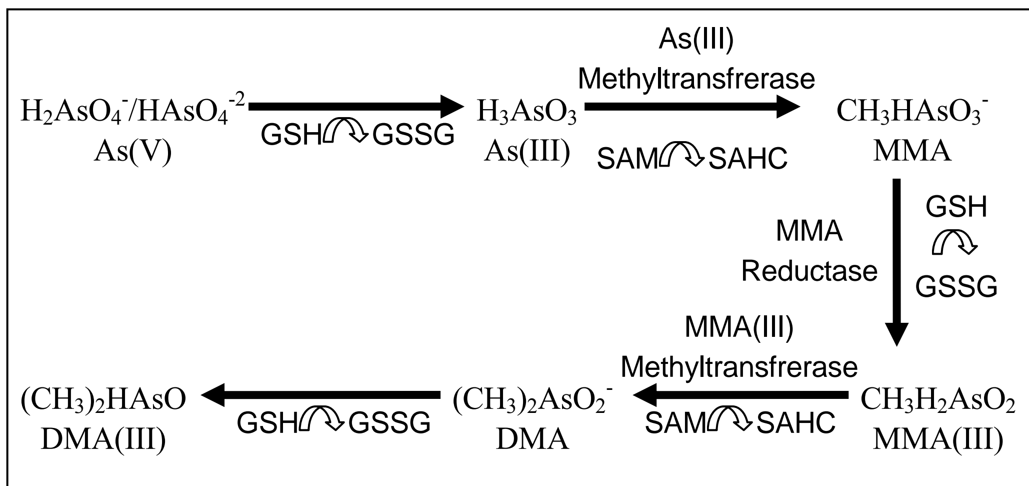


Figure 2.

The classical pathways for the metabolism of iAs in mammalian systems (Cullen and Reimer 1989; Gebel 2002; Vahter and Marafante 1983) Arsenate [As(V)] is reduced to arsenite [As(III)] by receiving two electrons donated via the conversion from reductants such as glutathione (GSH) to glutathione disulfide (GSSG). As(III) then undergoes an oxidative methylation, with S-adenosylmethionine (SAM) as the methyl donor and As(III) methyltransfrerase (e.g. CYT 19), forming MMA and S-adenosylhomocysteine (SAH) as the by-product of SAM. MMA then is reduced to MMA(III) by MMA reductase (e.g. hGSTO 1-1) before a subsequent oxidative methylation step to DMA catalyzed by MMA(III) methyltransfrerase, e.g. CYT 19. DMA might be further reduced to DMA(III).

The chemical form and valence state of arsenic can affect the cellular uptake and the affinity to proteins/tissues, subsequently influencing the toxicological response. Under physiological conditions, the As metabolites are charged to a different extent in blood and tissues (Gebel 2002). At pH 7.4, As(III), possibly also MMA(III) and DMA(III), are quantitatively uncharged and exist as protonized weak acids. In contrast, the three pentavalent metabolites [As(V), MMA and DMA] are almost quantitatively charged in blood and tissues. These differences are the main reason why As(III), but not As(V), is easily taken up by the hepatocytes (Lerman and Clarkson 1983). Likewise, the trivalent arsenic species are more reactive and bind to thiol groups of peptides, proteins etc. (Styblo and Thomas 1997). The pentavalent arsenicals are more easily and more rapidly excreted from cells, tissues and the body, and, thus, have a shorter biological half-life in comparison to trivalent As species. However, arsenate may be taken up by certain other cells, e.g. in the proximal renal tubular and the skeleton, possibly via the phosphate transporters (Lerman and Clarkson 1983; Lindgren et al. 1982).

The metabolism of arsenic is not completely understood. Experimental studies show that absorbed As(V), is rapidly reduced to As(III) already in the blood (Vahter and Marafante 1983; Vahter et al. 1984). In rabbits and marmoset monkeys, as much as 50-70% of a single dose of As(V) was rapidly reduced to As(III). There are no reports on species lacking the ability to reduce As(V) (Wildfang et al, 2001).

In the classical metabolic pathway of inorganic arsenic (**Figure 2**), the pentavalent arsenic species are formed before the trivalent ones, e.g. MMA before MMA(III) and DMA before DMA(III). But, in a newly proposed pathway by Hayakawa both MMA

and DMA as end products of arsenic metabolism (Hayakawa et al. 2005), which are the major arsenic metabolites found in urine. Those principles are supported by a recent study on rats (Naranmandura et al. 2006). However, the structures of the new intermediates involved, i.e. protein- and glutathione-bound arsenic, have not been confirmed.

Only two enzymes in the arsenic methylation pathway, MMA reductase and arsenic methyltransferase have been extensively studied. Human MMA reductase was found to be identical to hGSTO 1-1 (Human Glutathione-S-Transferase Omega Class), which belongs to the glutathione-S-transferase family (Zakharyan et al. 2001). In the reduction reactions, glutathione, and possibly other thiols, are required. In *in vitro* studies, DMA and As(V) were also reduced by MMA reductase (Zakharyan et al., 2001). The human CYT 19 was recognized as arsenic methyltransferase (Lin et al. 2002; Styblo et al. 1995), which has been produced only by DNA recombinant technology but not from human tissue. However, there are increasing evidence for alternate enzymes involved in the arsenic methylation pathway (Chowdhury et al. 2006).

Although most tissues are capable of methylating arsenic, as shown in *in vitro* studies on mouse cytosol, the liver appears an important site of arsenic methylation (Healy et al. 1998; NRC 1999). The highest activity of As(III) methyltransferase was observed in cytosol of the testis, followed by cytosol from the kidney, liver, and lung. A much higher rate of arsenic methylation was observed in primary human hepatocytes compared with human keratinocytes and bronchial cells, and no methylation activity was detected in human urinary-bladder cells (Styblo et al. 2000).

The metabolism of arsenic implies both detoxification and activation. The end products the pentavalent forms of MMA and DMA are less react with tissue constituents more readily excreted in urine compared to iAs. More efficient methylation, especially to DMA, means faster overall excretion of arsenic (Vahter 1999). Thus, the methylation has long been considered as a detoxification process. However, the recent studies confirmed that the reduced trivalent forms, in particular MMA(III) and DMA(III) involved in the methylation pathway are more reactive, binding preferentially to SH-groups in proteins, and more toxic than the pentavalent's (Styblo et al. 2000; Styblo and Thomas 1997). Recent studies indicate that MMA(III) might be the most toxic intracellular form of arsenic in terms of oxidative stress, enzyme inhibition and damage (NRC 2001).

It is generally accepted that the main arsenic metabolites excreted in human urine are iAs [As(V) + As(III)], MMA and DMA (Vahter 1994; Vahter 2002). However, a series of reports have shown the presence of MMA(III) and DMA(III) in human urine following exposure to inorganic arsenic (Aposhian et al. 2000; Le et al. 2000; Mandal et al. 2001). Recently, we found a thio-dimethylarsinic acid (thio-DMA) in urine of women exposed to inorganic arsenic via drinking water (Raml et al. 2006). This arsenical has chemical properties similar to those of DMA(III), and it has been suggested that it has been mistaken for DMA(III) in the previous studies reporting on the presence of DMA(III) in urine (Hansen et al. 2004; Raml et al. 2006).

In general, human urine contains 10-30% iAs, 10-20% MMA and 60-80% DMA, but there is a wide inter-individual variation in the metabolism of arsenic (Vahter 2002). Because of the variation in toxicity among the arsenic metabolites, it is essential to identify reasons for the marked inter-individual variations in arsenic metabolism. Obviously, genetic polymorphisms in the enzymes involved in reduction and methylation of arsenic may play a critical role in the variability in arsenic metabolism, but there may be several other factors. The proportions of iAs, MMA and DMA in urine together with methylation indexes (primary methylation index (PMI), defined as the ratio between MMA and iAs, and secondary methylation index (SMI), as the ratio between DMA and MMA) are used for assessment of arsenic metabolism.

Because arsenic is methylated via the general methylation pathway, it is likely that the availability of methyl groups influences arsenic methylation. Experimental studies have shown that a low intake of protein, choline or methionine decreases arsenic methylation to DMA and increases tissue retention (Vahter and Marafante 1987). The various enzymes and co-factors involved in one carbon metabolism (**Figure 3**), e.g. folate, and vitamin B12 may influence arsenic metabolism (Spiegelstein et al. 2005; Spiegelstein et al. 2003). Given that deficiency in folate and vitamin B12 may decrease the level of S-adenosylmethionine (Newman 1999), it is likely to decrease methylation of arsenic. Indeed, folate was found to protect mouse embryo fibroblasts against acute cytotoxicity of arsenic (Ruan et al. 2000). In addition, there is also evidence for the involvement of essential trace elements like zinc, which regulates the expression of many enzymes (De Kimpe et al. 1999; Tseng et al. 2000), and selenium (Biswas et al. 1999; Csanaky and Gregus 2003).

The evidence for a nutritional status influencing arsenic methylation in humans is limited. Two recent studies indicate that folate or intake of proteins, iron, zinc, niacin alter the arsenic methylation, although these two studies have their own limitations (Gamble et al. 2005; Steinmaus et al. 2005). The other recent studies showed that the positive correlations between As methylation and urinary selenium (Hsueh et al. 2003; Jay Christian et al. 2006). Thus, there is lack of understanding of the role of nutritional status for arsenic metabolism.

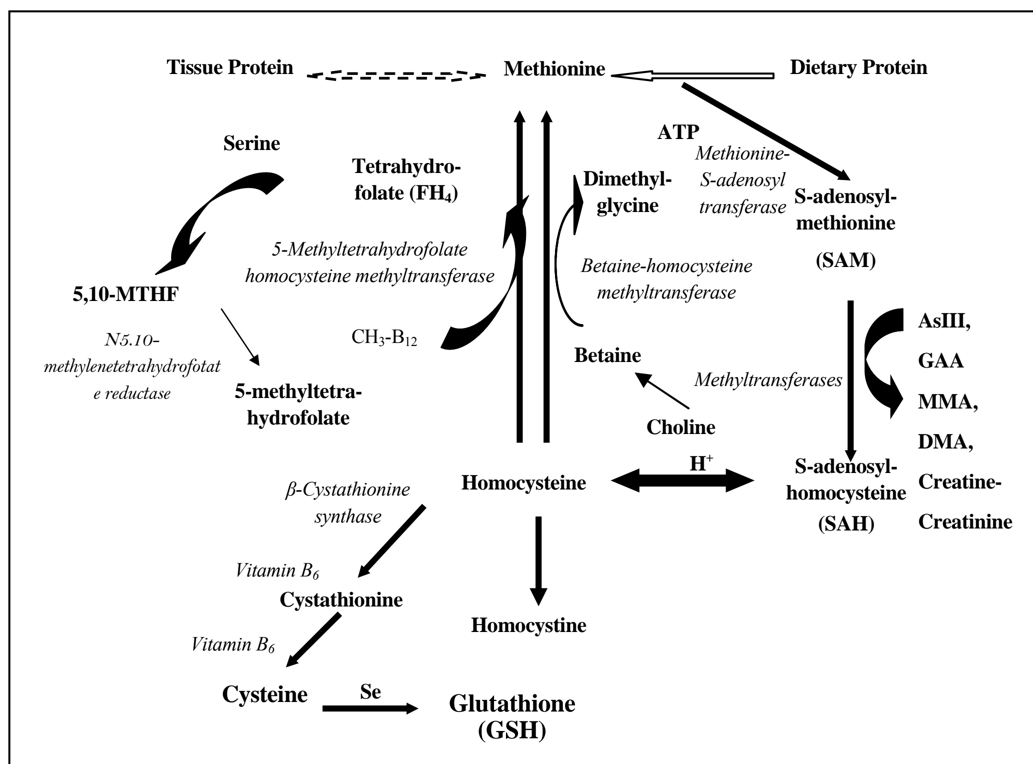


Figure 3.

Overview of one-carbon metabolism and the methylation of arsenic. Methionine comes from dietary protein, as well as tissue protein. It is activated by methionine adenosyltransferase to form S-adenosylmethionine (SAM). SAM is a universal methyl donor providing methyl groups for numerous methylation reactions, e.g. methylation of guanidinoacetate (GAA) to creatine (which is converted to creatinine), iAs to MMA, MMA to DMA as well as DNA methylation. Methyltransferases are required for the methylations. S-adenosylhomocysteine (SAH), the by-product of SAM in the methylation reactions, is hydrolyzed to generate homocysteine, which either is methylated to regenerate methionine or forming cysteine and glutathione (GSH) under trans-sulfuration pathway. In one methionine regeneration pathway, the methyl group is offered by 5-methylterahydrofolate catalyzed by the vitamin B12-containing enzyme; in the other one, the methyl group is offered by betaine catalyzed by betaine-homocysteine methyltransferase. In the trans-sulfuration pathway, selenium-containing enzyme is involved.

1.2.1.3 Distribution

Analysis of human tissues reveals that arsenic is widely distributed in the body after either long-term relatively low-level exposure or poisoning as in experimental animals, however the arsenic concentrations are rather low in blood and brain relative to other tissues (WHO/IPCS 2001). Keratin-rich tissues, such as skin, hair and nails, show the longest retention and the highest concentrations of arsenic. Arsenite reacts readily with sulfhydryl (SH) groups of a variety of essential enzymes and proteins. It is the affinity of arsenite for SH groups that accounts for its accumulation in keratin-rich tissues (WHO/IPCS 2001). As mentioned above, arsenate may be taken up by phosphate transporters in e.g. kidney and skeleton (Lindgren et al. 1982). Inorganic arsenic was

the predominant form in tissues, followed by DMA (Yamauchi and Yamamura 1983). MMA levels were considerable low and detected only in liver and kidney. Interestingly, lungs (Hughes et al. 2000; Vahter et al. 1984) and malignant tissue had significantly higher levels of arsenic than normal tissue, and plasma arsenic levels were also significantly higher in cancer patients than in controls (Collecchi et al. 1986). Both human and animal studies showed that arsenic accumulates in tissues with age (Raie 1996; Vahter et al. 1982; Yamauchi and Yamamura 1983).

1.2.1.4 Excretion

Humans exposed to inorganic arsenic eliminate various arsenic metabolites mainly via urine, to a minor extent via bile, sweat, epidermis and hair. Renal excretion plays the leading role among all excretion paths (Y. Zheng et al 2002). The half time of a single ingested dose of inorganic arsenic in the human body is about 3-4 days, and urinary arsenic has been commonly used as a biomarker of recent exposure to inorganic arsenic (WHO/IPCS 2001). It should be noted that also several other forms of arsenic, e.g. arsenobetaine, arsenocholine and arsenosugars in food are rapidly excreted in urine (Vahter 1994). Since the metabolism of iAs is remarkably stable for each individual over time (Concha et al. 2002; Steinmaus et al. 2005), spot urine sample is often collected for analysis of arsenic exposure and metabolism.

1.2.2 Health effects of inorganic arsenic

Inorganic arsenic is a documented potent human carcinogen (IARC 2004; WHO 2001). Chronic exposure to inorganic arsenic via drinking water causes cancer of the skin, urinary bladder, lungs, kidneys, and possibly liver. Arsenic-related non-cancer effects include skin effects, such as pigmentation changes and hyperkeratosis, cardiovascular and respiratory effects, diabetes, hypertension, and liver- and neurotoxicity. A drinking water guideline of 10μg/L is recommended by the WHO. Recent risk assessment indicated that even this concentration of arsenic is associated with a significant cancer risk (NRC 2001). At a concentration of 10 μg/L arsenic in drinking water, the estimated excess lifetime bladder cancer risk is 23 per 10,000 people for men and 12 for women, and the excess lifetime lung cancer risk is 14 per 10,000 people for men and 18 for women. Arsenic is known to induce deletion mutations and chromosomal alterations such as aberrations, aneuploidy, and sister-chromatid exchanges, but not point mutations (NRC 1999; NRC 2001). Proposed mechanisms of action include induction of oxidative damage to DNA, altered DNA methylation and gene expression, changes in intracellular levels of mdm2 protein and p53 protein, induction of protein-DNA cross-links, induction of apoptosis and etc. (NRC 1999; NRC 2001).

The evidence for reproductive and developmental effects of arsenic in humans is not conclusive (NRC 2001). Arsenic is easily transferred to the fetus (Concha et al. 1998), both the inorganic arsenic and the methylated metabolites pass the placenta. Malformations have been observed in experimental animals exposed to arsenic (Golub et al. 1998; Holson et al. 2000). Several studies indicate association between arsenic in drinking water (Ahmad et al. 2001; Aschengrau et al. 1989; Hopenhayn-Rich et al. 2000; Yang et al. 2003) or industrial emissions (Nordstrom et al. 1978) and fetal loss, impaired fetal growth, neonatal deaths and low birth weight. Our initial retrospective

study showed that drinking tubewell water with more than 50 µg/L of arsenic during pregnancy significantly increased the risk of fetal loss and infant death (relative risk 1.14 and 1.17, respectively) (Rahman et al. Submitted).

As addressed above, the metabolism of arsenic implies both detoxification and activation. The reduced trivalent forms, in particular MMA(III), are more toxic than the pentavalent forms (Bredfeldt et al. 2006; Lin et al. 1999; Petrick et al. 2000; Petrick et al. 2001; Styblo et al. 2000; Styblo et al. 2002). A high concentration of MMA in the urine indicates a low capacity of further methylation to DMA and, probably, a high concentration of the highly toxic MMA(III) in the cells. There is increasing evidence of positive associations between urinary MMA and the prevalence of arsenic-related skin cancer (Chen et al. 2003; Hsueh et al. 1997; Yu et al. 2000), bladder cancer (Chen et al. 2003; Steinmaus et al. 2006), other skin effects (Del Razo et al. 1997), structural chromosomal aberrations populations (Maki-Paakkanen et al. 1998), cardio-vascular effects (Tseng et al. 2003), and retention of ingested arsenic (Vahter 2002) in arsenic-exposed individuals. However, the association between MMA and arsenic related reproductive effects has not been studied. It seems likely that the variation in susceptibility to inorganic arsenic could partly be explained by differences in metabolism.

2 AIM OF THE THESIS

The overall aim of the present thesis is to assess to what extent malnutrition affects the metabolism of arsenic in a large number of women in Matlab, Bangladesh, exposed to a wide range of inorganic arsenic via drinking water.

Specifically, we aimed at elucidating:

- Arsenic exposure in Matlab, Bangladesh
- Arsenic metabolism in relation to exposure
- Arsenic metabolism in relation to macro-nutritional status
- Arsenic metabolism in relation to micro-nutritional status

3 METHODS

For more specific details concerning the methods used, reference is made to the individual papers.

3.1 Study area and population

The studies were carried out in Matlab, 53 km southeast of Dhaka, Bangladesh where groundwater is highly affected by the historic natural sedimentation of arsenic laden soil (**Figure 4**). Our ongoing research project on the effects of arsenic exposure via drinking water on reproduction and child development is nested into a food and micronutrient supplementation trial (Maternal and Infant Nutrition Interventions of Matlab, MINIMat). In Matlab, the ICDDR, B, Dhaka (Centre for Health and Population Research) is running a health and demographic surveillance system (HDSS) covering about 220,000 inhabitants in 142 villages. The HDSS records all vital events, such as births, deaths, marriages, pregnancies and different pregnancy outcomes, and in- and out-migrations. The databases are updated monthly based on information collected during home-visits by community health research workers.

In a parallel project [Arsenic in tube-wells in Matlab, (AsMat)] all households in the HDSS area were visited between January 2002 and August 2003 for screening of arsenic-related skin lesions and collection of information on current and past use of tubewell water by each person in the household (Rahman et al. 2006). Analysis of arsenic (by direct hydride generation-atomic absorption spectroscopy, HG-AAS) of water samples collected from all functional tubewells showed that more than 70% of the about 13,000 tube-wells in Matlab exceeded WHO guideline on arsenic in drinking water, 10 µg/L (Rahman et al. 2006).

In half of the HDSS surveillance area (**Figure 4**, blocks A, B, C, D with about 110,000 inhabitants), the ICDDR, B used the system to identify pregnant women for the MINIMat trial. Between November 2001 and October 2003 the trial identified pregnant women by urine test performed if a woman reported amenorrhea at the time of the monthly HDSS routine home visit performed by community health research workers. In case of positive pregnancy test, the woman was asked to donate a urine samples for arsenic analysis. Eligible women who gave their consent for the MINIMat study were followed throughout pregnancy, including repeated collection of urine samples.

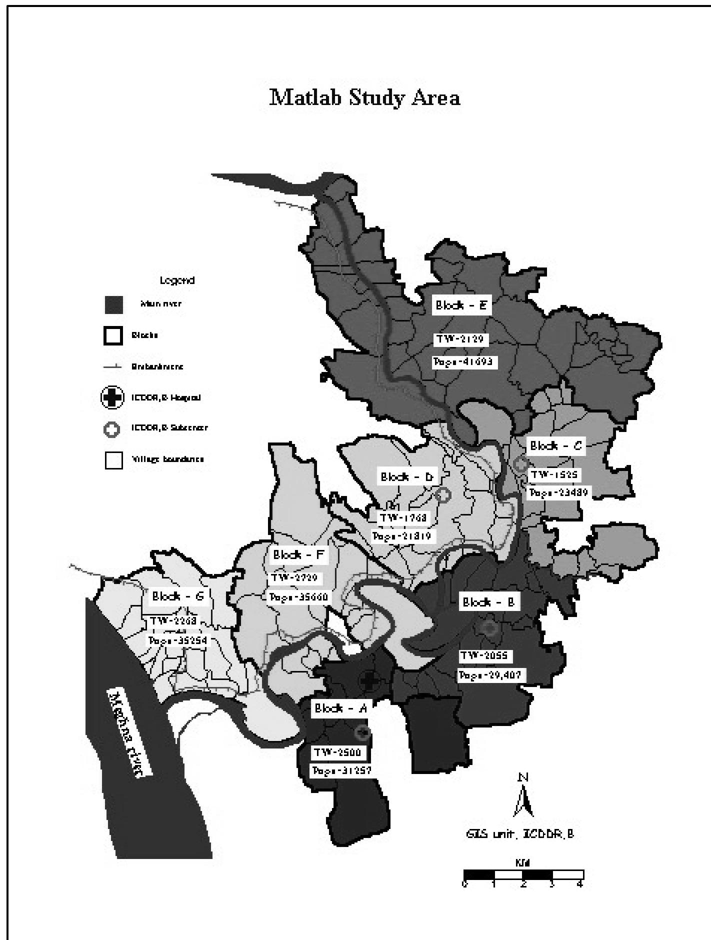


Figure 4.

Map of Matlab, Bangladesh showing the study areas (Blocks A, B, C and D).

The 3,418 women enrolled between January 2002–March 2003 were included in our Study I. To assess the exposure to arsenic, we measured the total concentrations of arsenic metabolites (by HG-AAS) in early and late pregnancy [about gestational weeks 8 (GW8) and 30 (GW30)]. We had early pregnancy urine samples from all of the 3,418 women enrolled and late pregnancy urine samples from 1, 944 women. For 2,330 of the women, we also had data on arsenic concentration in the drinking water samples used during pregnancy from the parallel AsMat project. The numbers of urine and water samples analyzed are shown in **Table 1**.

A random selection of 1,000 out of the 2,119 women enrolled between 1 January and 31 December 2002 was made for measurements of micronutrients in blood samples collected in gestational week 14. In study II, we used that cohort of 1,000 randomly selected women for further analyses of arsenic metabolites in urine. Out of the 1,000 women, 753 women donated both urine and blood samples. A subset consisting of the first 500 randomly selected women were used for the evaluation of effect of macro nutritional status on arsenic metabolism using urine samples collected in GW9, of which 422 women donated urine samples were included. The numbers of urine and blood samples analyzed are shown in **Table 1**.

Table 1.
Summary of the study design and sample collection.

	Study I			Study II		
	Subjects	Urine	Water	Subjects	Urine	Blood
GW8/9	3418	√	√	422	√	
GW14				753	√	√
GW30	1944	√				

GW: gestational week.

3.2 Determinations of urinary As and water As by HG-AAS

Throughout this study we have used concentrations of arsenic metabolites in spot urine samples for evaluation of arsenic exposure and metabolism. Spot-urine samples were collected at the health clinics except those of the first urine sampling (GW 9), which were collected in the women's homes, following positive results of the urine-based pregnancy test. Spot-urine samples were collected in disposable plastic urine-collection cups and then immediately transferred to acid-washed 24 mL plastic vials. The urine samples were stored in freezers (-80 °C) at the Matlab hospital until being transported to the Karolinska Institute in Stockholm. During transportation, the samples were kept in insulated containers with cooling blocks.

Since arsenic and its metabolites can form volatile hydrides (the arsines AsH_3, CH_3AsH_2, $(CH3)_2AsH$) upon reduction by, for instance, sodium borohydride ($NaBH_4$), the concentrations of U-As and W-As were measured by HG-AAS (Vahter et al. 1995). This method is specific for the sum of iAs and its metabolites. Other arsenic species in urine such as arsenobetaine and arsenocholine, will not interfere with the results, even though the concentrations of arsenobetiane in urine may exceed 1,000 µg/L (Norin and Vahter 1981). However, exposure to DMA and arsenosugars via food may bias the results, because arsenosugars are also metabolized to DMA as shown in our pilot study on 4 female volunteers ingesting seaweed (unpublished data).

The detection limit of the AAS method was 1.3±0.27 µg/L. For quality control purpose, certified reference materials (NIST 2670 urine HL with certified 480±100 µg As/L and NIST 1643d water with certified 56.02±0.73 µg As/L) were included in each analytical run. As certified reference materials exist only for total arsenic, we also participated in inter-laboratory comparisons of arsenic metabolites in urine to verify the analytical accuracy (within the EU Ashram project). There was a good agreement between our results and the results of University of Graz, Austria, using HPLC-HG-ICPMS (correlation 0.997 within the concentration range 10-150 µg/L, n=7).

3.3 Speciation of arsenic metabolites in urine by HPLC-HG-ICPMS

The analytical method for arsenic speciation (Lindberg et al. 2006) included chromatographic separation [in order of As(III), DMA, MMA and As(V)] on line with hydride generation [volatile arsines AsH3, $(CH_3)_2AsH$, CH_3AsH_2 formed] and detection of arsenic by inductively coupled plasma mass spectrometry to reach a very high sensitivity. With this method the following determination limits were obtained: 0.1 µg/L for As(III) and MMA, and 0.2 µg/L for DMA and As(V) (**Figure 5**). Total urinary arsenic (U-As) was defined as the sum of arsenic metabolites (iAs+MMA+DMA), the determination limit for U-As by HPLC-HG-ICPMS was about 0.6 µg/L.

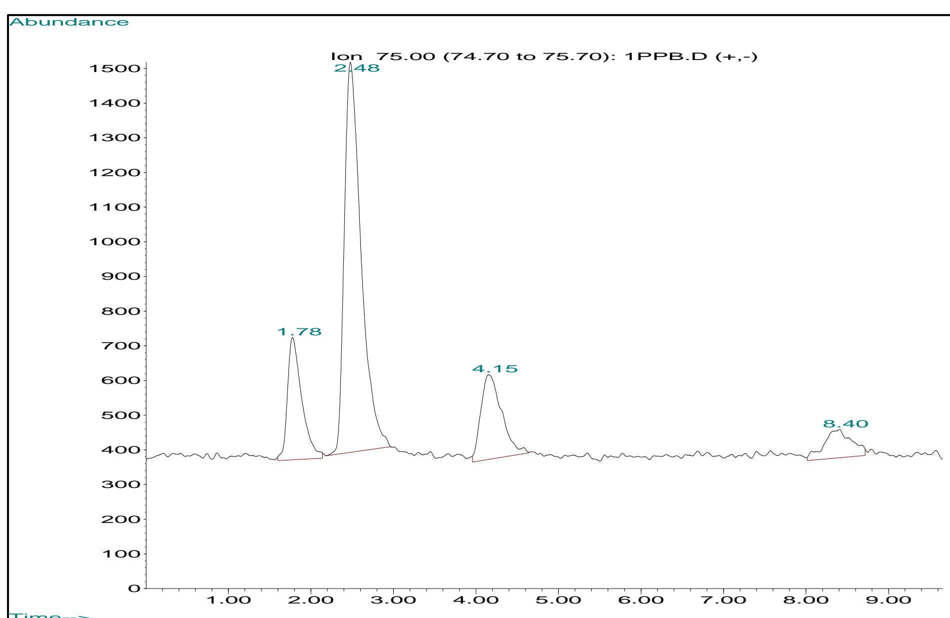


Figure 5.

Determination limits of arsenic speciation. The peaks from left to right are As(III), DMA, MMA and As(V). The retention time of each peak are shown on the top. The chromatogram shows the lowest standard solution containing 1µg/L DMA and 0.2 µg/L MMA, As(III) and As(V). The determination limits for each metabolites are 0.1 µg/L for As(III) and MMA, and 0.2 µg/L for DMA and As(V), the determination limit for urinary arsenic is about 0.6 µg/L.

There are no commercially available standard reference materials (SRMs) for all the different arsenic metabolites in urine. For quality control (QC) purposes, we therefore relied mainly on comparisons of results obtained by different analytical methods. We analyzed arsenic metabolites in a number of urine samples also by atomic fluorescence spectrometry (AFS) and we compared the results on the sum of arsenic metabolites obtained by HG-AAS, AFS and HPLC-HG-ICPMS. The results of ICPMS and AFS showed good agreement for each metabolite; iAs (As(III)+As(V)), MMA and DMA (R^2=0.91, R^2=0.91 and R^2=0.97, respectively, N=221). Also, good agreement for the sum of arsenic metabolites between the three different methods was found (ICPMS vs.

AFS: R^2=0.97 and ICPMS vs. AAS: R^2=0.96, N=221). This shows that the error caused by the different signal responses for the different metabolites in the AAS method was negligible. Also, the Certified Reference Material (CRM NO. 18, The National Institute for Environmental Studies, Ibaraki, Japan) with a certified value for DMA of 36 ± 9 µg/L was analyzed together with the collected urine samples. The found average concentration of DMA was 41 µg/L (S.D 3.4, n = 18), which is within the indicated range. An internal quality control urine was prepared by spiking a human urine sample (background concentration 6 µg/L of total arsenic metabolites) with 50 µg/L DMA and 10 µ/L of each of As(V), As(III) and MMA. This QC sample was included in each analytical run.

3.4 Urinary adjustment

Spot urine samples vary in dilution due to variation in intake of fluids, physical activity, temperature, etc. Therefore, the analyzed arsenic concentrations had to be adjusted in order to eliminate the influence of urine dilution. This is commonly done by adjusting the concentrations to urinary creatinine or specific gravity (SG). However, cretainine excretion is more influenced by individual features, such as muscle mass, BMI, meat intake than is specific gravity (Suwazono et al. 2005) The mean of adjusted urinary creatinine was 0.57 g/L (N=442, GW9), which is rather low and in line with the low BMI and reported low intake of meat. Thus, adjusting the arsenic concentrations in the urine samples of the present study by creatinine values gave almost double the U-As values obtained by adjusting to SG (mean, 288 µg/g creatinine and 159 µg/L, respectively). In women with adequate nutrition, the results from these two adjustments are usually rather close. Because of the prevalent malnutrition among the women in the present study, we chose to apply the SG adjustment method. The average SG was 1.012 g/mL.

3.5 Determination of micro-nutrients

Venous blood samples were collected in GW 14 at the Matlab health clinics. The samples were transported to the hospital laboratory for separation of plasma, which was transferred to 2 mL cryotubes and stored at -80ºC until analyses of micronutrients at the University of California, Davis (UCD), US. Serum ferritin (sFt) was measured using radioimmunoassay, serum zinc by atomic absorption spectrometry (Clegg et al. 1981), folate and vitamin B12 was determined by SimulTRAC-SNB radioassay kit (MP Biomedicals; Orangeburg, NY). Urinary Se (U-Se) was measured by ICPMS at Karolinska Institutet, Sweden.

3.6 Statistical methods

For statistical computations, we used STATISTICA 7.1 (StatSoft Inc., Tulsa, OK, USA). Descriptive analysis of data included evaluations of central tendency (mean/median) and variation (standard deviation, frequency distribution and percentiles). Neither the arsenic concentration in water, nor the concentration of arsenic metabolites in urine was normally distributed and analyses using parametric test, such as linear regression were done on transformed variables to meet the assumptions of normality and equality of variances and to achieve approximately normal distribution.

Mann-Whitney U test was used for comparisons of medians values between groups. Spearman's rank correlation was applied for bivariate comparisons and for identification of potentially confounding factors i.e. factors associated both to arsenic metabolites and the independent variables of interest. A *p* value below 0.05 was considered as statistically significant.

3.7 Ethics

The two underlying studies (MINIMat and AsMat) were reviewed by the ethical research committee at ICDDR,B. Participants in the studies were informed about the purpose of the studies and the procedures they involved including collection of blood and urine samples. They were also informed that participation was voluntary and that they could withdraw at any time of studies without any further consequences. Verbal and written consent was obtained before enrollment in any of the studies. Our project focusing on the reproductive effects of arsenic was reviewed both at the ethical research committee at ICDDR,B as well as the Regional Ethical Committee at the Karolinska Institute.

Con-currant with the initiation of the AsMat study an arsenic mitigation program was implemented in Matlab whereby all tubewells in the area were tested for arsenic content and color marked whether the results was above or below the local water standard for arsenic. The program, run by the government in collaboration with a local non-governmental organization (BRAC) also assisted the participants to identify alternative safe water sources (Jakariya Md RM 2005).

4 RESULTS AND DISCUSSION

For details see the individual papers. Some data and discussions not presented in the individual papers are included in this section.

4.1 Arsenic exposure

4.1.1 Arsenic in water and urine

4.1.1.1 Estimate of arsenic exposure

Accurate estimation of the exposure is critical for risk assessment, for follow-up of mitigation activities and for assessment of dose-response relationships in epidemiological studies of adverse health effects. The most common measures of arsenic exposure are: i) arsenic concentration in drinking water, sometimes in combination with estimates of intake of water; and ii) arsenic concentration in urine. There is a great inter-individual variation in the intake of water (NRC 2001) and it is not always feasible in epidemiological studies to measure the exact amount of water intake of each individual, resulting in varying arsenic doses, in spite of the same water concentration. Often, people use different sources of water e.g. at home, at the work place and at restaurants. One study indicated that the concentration of As in drinking water gave a better prediction of the concentration of As in urine than did the estimated intake of As from drinking water (Calderon et al. 1999).

Screening of arsenic concentrations in water from all the tubewells in Matlab (N=13,286) in the parallel AsMat project, conducted between January 2002 and August 2003, showed more than 60% of the functioning tubewells had arsenic concentrations exceeding the local drinking water standard of 50 µg/L (Jakariya et al. 2006; Rahman et al. 2006; Wahed et al. 2006). The tubewells containing more than 50 µg/L arsenic were painted red and the users were encouraged to take water from a nearby green-painted tubewell, with water containing less than 50 µg/L. Prior to water sampling for As analysis, all individuals above 4 years of age were interviewed about drinking water sources used currently and in the past.

In the present study (**Paper I**), the average urine to water ratio of arsenic was about 1, i.e. about the same concentrations in urine as in drinking water. However, it has been reported that the Andean women with a sole source of fluid intake and for preparation of food had urinary arsenic concentration about 2-fold higher than the water arsenic concentration. Although the mean urine and water arsenic concentrations in the present study were about the same (161 vs. 155 µg/L, medians 91 vs. 78 µg/L), only around 2% women had urinary As concentrations below 10 µg/L, whereas 34% of women reported drinking water containing less than 10 µg/L arsenic (**Figure 6**) . This indicates additional exposure to arsenic via food. Although there was a significant association between arsenic in urine (U-As) in GW8 and arsenic in the drinking water used during pregnancy (W-As; R^2= 0.39; N=2,330; p<0.001), there was a considerable variation among individuals. In fact, a large part (about 60%) could not be explained by this association (**Paper I**). Probably, this can partly be explained by additional exposure via

food and other water sources, the ongoing mitigation activities, which involve recommendations to use other tubewells than the red-painted ones with elevated arsenic levels, and visiting homes with other water sources, e.g. the homes of the parents. The advantage of urinary arsenic as measure of exposure is that it reflects the total exposure to inorganic arsenic via all sources. It should be noted that urinary arsenic reflects current exposure, because of the fairly short half-time of arsenic in the body; about 3-4 days. However, in the case of continuous exposure via drinking water, urinary arsenic gives a good estimate of the exposure. We therefore conclude that the arsenic concentration in urine most likely provides a more accurate estimate of the true current exposure than the arsenic concentration in drinking water in our study. Obviously, the repeated measurements of urinary arsenic over time provide a more precise measure of a more long-term average exposure.

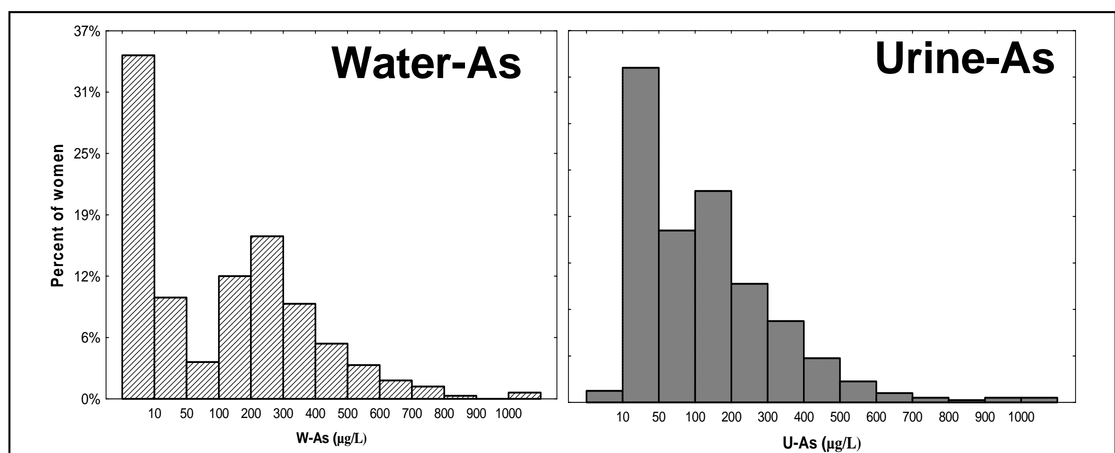


Figure 6.

Distributions of water arsenic (W-As) and urinary arsenic (U-As). The left picture presents As concentration in drinking water and the right presents arsenic concentration in urine. The average U-As and W-As are both about 160 µg/L

4.1.1.2 Correlation of urinary arsenic in early and later pregnancy

In general, the concentrations of arsenic in urine in late gestation, about GW 30, were similar to those in early pregnancy (median 83 vs. 81 µg/L, mean 164 vs. 150 µg/L; N=1,944), but with a considerable variation. As a consequence of the ongoing mitigation activities (changing drinking water sources), women who had had their tubewell tested during pregnancy showed lower urinary arsenic concentrations in late pregnancy GW 30 (median 89 µg/L) than in early pregnancy GW 8 (median 137 µg/L; N=442), however they were still highly exposed. In contrast, a number of women have a considerably higher arsenic concentration in GW 30 than in GW 8, which is unexpected. It might be due to the problems involved in fetching family drinking water from far-away green-pained tubewells for pregnant women or mobility. Women often deliver their children at their parents' home, especially the first delivery. In fact, the nulliparous women showed a higher average U-As in GW 30 than in GW 8 (179 vs. 155 µg/L, $p<0.05$, N=511).

4.1.2 Arsenic metabolites in urine

4.1.2.1 Valence forms of arsenic metabolites in urine

For evaluation of arsenic metabolism, we have measured iAs, MMA and DMA in urine. In a number of previous publications, MMA(III) and DMA(III) have been reported to be present in human urine (Aposhian et al. 2000; Le et al. 2000; Mandal et al. 2001) and in a recent study DMA(III) was claimed to be the major metabolite in people exposed to inorganic arsenic via drinking water in Mexico (Valenzuela et al., 2005). However, the identification of MMA(III) and DMA(III) was only based on comparison of chromatographic retention times between the peaks in standard solutions with the peaks in urine samples. These are indeed unexpected results, because the high reactivity of MMA(III) and DMA(III) would rather render them to bind in tissues than to be excreted in urine (Vahter 2002). So far, we have speciated arsenic metabolites in more than 5,000 urine samples in our laboratory and no extra peaks that would indicate MMA(III) or DMA(III) were found.

In urine, the percentage of iAs [sum of As(III) and As(V)] often amount to 10-30% of the total arsenic excreted. However, there are exceptions. In children exposed to drinking water with 300-400 μg/L arsenic in Argentina, about 49% of the urinary arsenic was in the form of iAs (Concha et al. 1998; Vahter 2002). The proportions of As(III) and As(V) in the excreted iAs may vary substantially. In a pilot study (N=75) we found that the proportions of As(III) and As(V) depended on the pH of the urine (**Figure 7**), with more arsenic being present as As(III) at lower pH (less than 7.4) and more As(V) at higher pH. Urinary pH varies depending on the type of food, e.g. meat products give a lower pH, while most vegetables and fruits give a higher pH, as well as the regulation of pH in the renal tubuli. Obviously, the As(III)/As(V) ratio is also depending on the presence of reducing or oxidizing substances in the urine and the air contact of the urine sample. Leaving the urine sample a few hours in the open air would convert much As(III) to As(V). We also observed that a standard solution of As(III) can be partly oxidized to As(V) after 24 hours of analytical run. Therefore, special precaution is needed for identification of the exact chemical form of iAs in urine. Still, the toxicological significance of the different forms of iAs in urine is not clear.

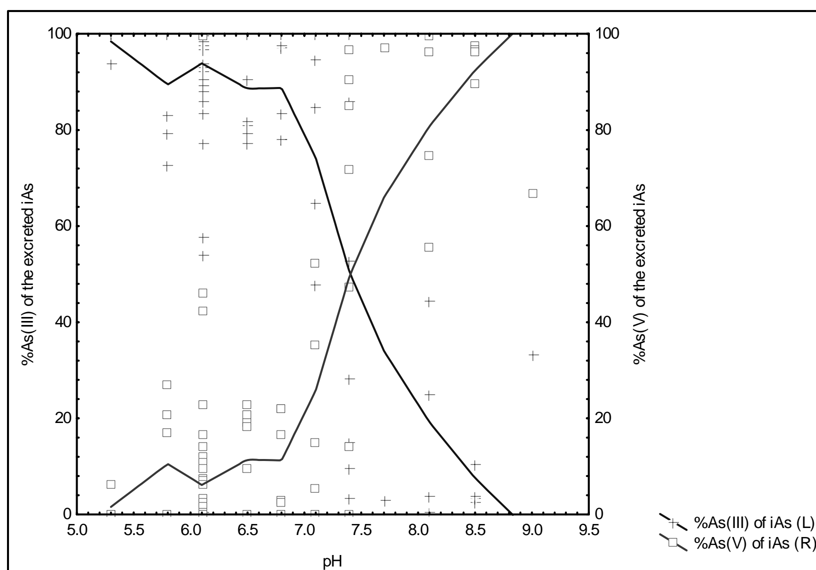


Figure 7

The proportions of As(III) (cross) and As(V) (square) in the excreted inorganic arsenic in urine in relation to pH (range 5.3 -9.2) of urine. Lowess, stiffness=0.38.

4.1.2.2 The newly discovered metabolite

When we started speciating As metabolites by using our newly established HPLC-HG-ICPMS system in April, 2005, we found that 19 of 442 urine samples (**Paper I**) had an extra peak at retention time of 15 min (**Figure 8**). In cooperation with University of Graz, Austria, this substance was later identified as Thio-DMA.

Additional urine samples from 75 arsenic-exposed women from Matlab were analyzed by HPLC-ICPMS under conditions designed to distinguish between DMA(III) and thio-DMA. The total arsenic concentrations in the urine ranged from 25 to about 900 µg As/L, and thio-DMA was present in 47% of the samples at concentrations ranging from 0.4 to 24 µg As/L (Raml et al. 2006). Probably, the concentrations had been much higher at the time of urine sampling, as it was found that the thio-DMA is easily oxidized to DMA and most of the samples had been thawed for the measurements of total arsenic in urine. In none of the urine samples could we detect DMA(III). Cytotoxicity testing with HepG2 cells derived from human hepatocarcinoma indicated that thio-DMA was considerably more cytotoxic than DMA. This is the first report on the presence of thio-DMA in urine of people exposed to inorganic arsenic. It has previously been found in human urine after ingestion of arsenosugars (Raml et al. 2005). These findings suggest that DMA(III) might at times have been misidentified, because its chromatographic properties are similar to those of thio-DMA (Hansen et al. 2004; Raml et al. 2006).

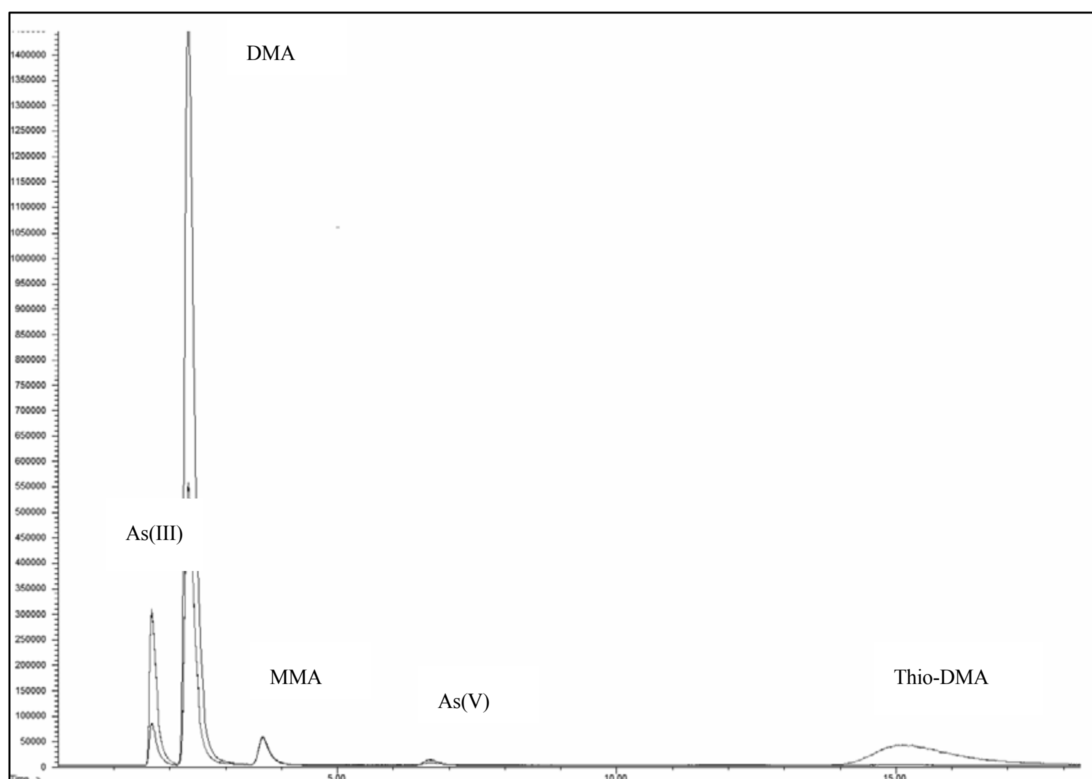


Figure 8

The discovery of the new As metabolite Thio-DMA in human urine. In the chromatogram of As speciation, the peaks from left to right are As(III), DMA, MMA, As(V) and at a retention time of 15 min is the new As metabolite, Thio-DMA.

4.2 Variability in arsenic metabolism

Factors suggested to be associated with arsenic metabolism include age, gender, exposure level of inorganic arsenic, pregnancy, nutritional status, ethnicity, diseases, cigarette smoking and alcohol intake (Vahter 2002). However, the relative importance of the various factors is not clear. Our study population is particularly suitable for studying the effects of exposure level, nutrition and pregnancy as it is fairly homogeneous, i.e. rural pregnant Bangladeshi women with a small age span (14 -49 years), of whom almost no one is smoking (<1%) or using alcohol.

4.2.1 Population variation in arsenic metabolism

The average distribution of arsenic metabolites in urine is fairly consistent among various populations exposed to inorganic arsenic: 10-30% iAs, 10-20% MMA and 60-80% DMA (Vahter, 2002). However, there are a few exceptions (**Figure 9**). Indigenous people in the Andean part of Argentina and Chile were found to excrete just a few percent of MMA in urine (Smith et al. 1993; Vahter et al. 1995). In contrast, people in Taiwan were reported to have 20-30% MMA in urine (Chiou et al. 1997). Our study in Matlab (**Paper II**) shows a rather efficient methylation among the studied women with an average of 11% MMA and 74% DMA, which is similar to those reported from a

recent study in Araihazar region, Bangladesh (Gamble et al. 2005). Thus, the methylation efficiency of inorganic arsenic in the Bangladeshi women is better than in many other populations studied.

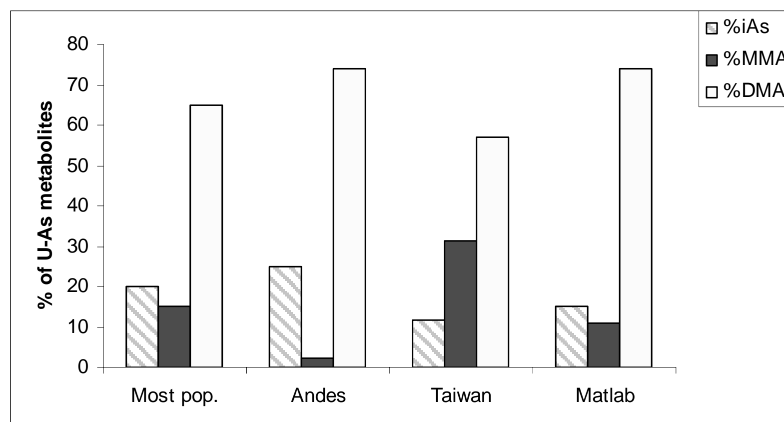


Figure 9.

Proportions of arsenic metabolites, iAs, MMA and DMA, in urine in different populations: most populations studied (Hopenhayn-Rich et al. 1993; Vahter 2000), indigenous people in Argentinean Andes (Vahter et al. 1995), Taiwanese in south-west Taiwan (Chiou et al. 1997), and women in Matlab. (Paper II).

4.2.2 Intra-individual variation

Only two studies on the intra-individual variation in arsenic metabolism have been published so far. Our previous study on 15 women chronically exposed to about 300 µg/L iAs via drinking water found that the distribution of the various arsenic metabolites in urine was remarkably stable over a 5-day period, indicting that the distribution of arsenic metabolites in a spot urine sample is representative of that individual's methylation of inorganic arsenic (Concha et al. 2002). A recent study on 81 bladder cancer patients exposed about 100 µg/L iAs in drinking water showed that individual methylation pattern remained fairy stable over a one-year period (Steinmaus et al. 2005). Thus, it is suggested that the predominant factors controlling metabolism of arsenic are genetically influenced and/or related to environmental factors, such as long-term dietary patterns, which also remain stable over time.

4.2.3 Inter-individual variation

There was a wide inter-individual variability in arsenic metabolism in the studied women (**Paper II**). The 10th and 90th percentiles of % iAs were 7 and 24%, 5 and 17% for MMA and 62 and 85% for DMA, respectively. Although genetic polymorphism in the enzymes responsible for arsenic methylation may explain a part of the observed variation in the excretion of arsenic metabolites (Meza et al. 2005; Schläwicke Engström et al. 2007), other factors such as exposure level and nutrition may be important.

4.2.4 Effect of arsenic exposure on arsenic metabolism

Of the studied factors in our study (**Paper II**), the arsenic exposure level had the greatest impact on arsenic methylation. The proportion of MMA increased and that of DMA decreased with increasing exposure level (**Figure 10**), indicating that the second methylation step (from MMA to DMA) is more sensitive to inhibition by inorganic arsenic than is the first methylation step. This is in agreement with *in vitro* studies showing that the second arsenic methylation step is particularly sensitive to interference from excess inorganic arsenic (De Kimpe et al. 1999; Styblo et al. 2000). It seems that the inhibition of arsenic starts at rather low exposure among our studied women in **Figure 10**, well below 100 µg/L. The high percentage of DMA at the lowest urinary arsenic concentrations, i.e. below about 30 µg/L, is probably due to exposure to DMA via food (see section 4.1.1.1).

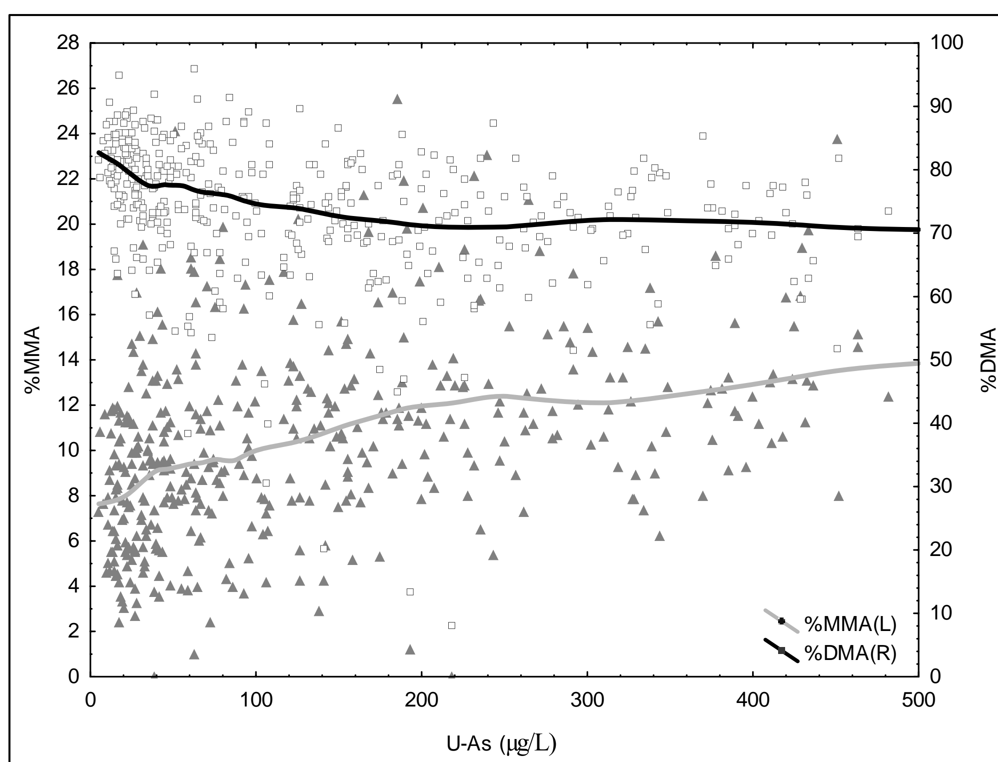


Figure 10

Effect of arsenic exposure on the proportions of MMA (triangle) and DMA (square) in urine. Scatter plot, axis Y left is percentage of MMA with triangle spots; axis Y right is percentage of DMA with square spots; and axis X presents arsenic concentration in urine (µg/L).

4.2.5 Effect of nutritional status on arsenic metabolism

The results of the present study show that the studied women have a remarkable efficient methylation of inorganic arsenic, in spite of the high prevalence of malnutrition (**Paper II**). The average percentage of DMA in urine (74%) was higher than that observed in many populations in developed countries, with much better nutritional status (Vahter 2002). We found that the metabolism of inorganic arsenic is only marginally influenced by various nutritional markers studied.

Although the nutritional status in general had only a marginal effect on arsenic metabolism in the present study, we found a synergistic interaction between high arsenic exposure and low serum folate and vitamin B12 concentrations for the arsenic methylation (Paper II). Only at high exposure levels (>200 µg/L) were low levels of serum folate and vitamin B12 associated with slightly impaired arsenic methylation. For example, low serum folate levels below 3.8 µg/L, were associated with about 2% lower DMA in urine.

We found a clear association between urinary selenium and the percentage of urinary DMA, which is consistent with recent studies (Hsueh, Ko et al. 2003; Jay Christian, Hopenhayn et al. 2006). Experimental studies showed that mice fed a diet with relatively high concentration of selenium had a faster elimination of single oral doses of arsenite, arsenate, or DMA compared with mice fed a selenium-deficient diet, indicating that selenium stimulates the excretion of arsenic (Kenyon et al. 1997). However, we found no association between U-Se and U-As.

Although the nutrition had a small impact on arsenic methylation, nutritional status may still influence the risk for health effects following arsenic exposure. In a recent review it was concluded that malnourished populations probably are more susceptible to the disease effects than others (Smith and Smith 2004). A recent case control study in Bangladesh (138 cases and 144 controls) showed that individuals with BMI below 18.5 kg/m^2, the cut off point for malnutrition, had a higher prevalence of arsenicosis, the risk ratio was 1.92 (95% CI= 1.33-2.78), supporting that poor nutritional status may increase the susceptibility for arsenic toxicity (Milton et al. 2004). Also, in West Bengal, India, subjects with poor nutritional status (weight below 80% of the standard body weight for their age and sex) were found to have a 1.6 fold increased prevalence of keratoses (Guha Mazumder et al. 1998). A study on Taiwanese individuals showed that undernourishment was associated with an increased prevalence of arsenic-induced skin cancer (Hsueh et al. 1995). Experimental studies showed that protein deficiency enhanced the developmental toxicity of inorganic arsenic in mice, (Lammon and Hood 2004).

5 CONCLUSIONS

Based on our studies on arsenic exposure and metabolism among pregnant women, exposed to inorganic arsenic via drinking water in rural Bangladesh, we can conclude the following:

- Most women were exposed to inorganic arsenic during the entire pregnancy with a wide range of arsenic concentration in urine (1 – 1,470 µg/L). It is essential to follow up the consequences of such exposure.
- The studied women were not only exposed to inorganic arsenic via drinking water but also via food, probably due to irrigation of rice and vegetables by arsenic-rich water.
- Arsenic in urine was a better marker of exposure than arsenic in drinking water.
- There was a considerable inter-individual variation in arsenic methylation. The percentage in urine of the monomethylated metabolite, assumed to be associated with increased risk of health effects, varied between 0 and 26%.
- Arsenic methylation to DMA decreased with increasing exposure, while the percentage of MMA increased, indicating inhibition of the methylatransferases involved in the second methylation step by excess arsenic.
- Despite the high prevalence of malnutrition, the studied women had a remarkably efficient methylation of arsenic; on average 74% DMA in urine.
- Nutritional status had a marginal effect on arsenic metabolism except for a positive effect of selenium, and probably zinc, on arsenic methylation.
- Only at high arsenic exposure levels was serum folate and vitamin B12 concentrations positively associated with arsenic methylation; and even then was the effect small.
- Arsenic methylation increased with increasing urinary selenium and increasing serum zinc. Considering the low selenium and zinc levels (average serum Se of 60 µg/L, serum Zn of 0.61 µg/L), the results indicate that selenium and zinc are essential for the methylation of arsenic.

6 FURTHER RESEARCH QUESTIONS

The following research questions are going to be elucidated:

- The influence of arsenic metabolism on arsenic-related low birth weight; study of about 500 women in gestational week 8.
- Arsenic metabolism in relation to pregnancy in about 350 women in gestational week 8, 14 and 30.
- Arsenic metabolism in relation to food and nutrition supplementations; comparison of arsenic metabolism before and after food and multi-micronutrient supplementations in about 500 women.

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