NOVEL BIOMARKERS FOR THE RISK ASSESSMENT OF EXPOSURE TO
ARSENIC IN DRINKING WATER

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ABSTRACT

JENNA MARIE CURRIER: Novel Biomarkers for the Risk Assessment of Exposure to
Arsenic in Drinking Water
(Under the direction of Miroslav Stýblo)

The toxic methylated trivalent metabolites of inorganic arsenic (iAs), methylarsonite (MAs^{III}) and dimethylarsinite (DMAs^{III}), play an important role in the etiology of As-induced diseases, including diabetes mellitus. However, the rapid oxidation of DMAs^{III} and, to a lesser extent, MAs^{III} leads to difficulties in the analysis of these metabolites in biological samples and assessment of the risk associated with As exposure. The goal of this project was to determine if a recently optimized analytical technique, hydride generation-cryotrapping-atomic absorption spectroscopy (HG-CT-AAS), can be used to quantitate trivalent As species in complex biological matrices, and determine whether concentrations of these species in biological systems can predict susceptibility to the diabetogenic effects of As exposure.

First, we established that HG-CT-AAS is suitable for the quantification of MAs^{III} and DMAs^{III} and that these species are relatively stable in cells and tissues. We then used HG-CT-AAS to compare As speciation in tissues of wild-type (WT) mice that methylate As and mice null for As (3+ oxidation state) methyltransferase (*As3mt*-KO), the key enzyme in the pathway for As methylation, focusing on tissues regulating glucose homeostasis, including liver, pancreas, skeletal muscle, and adipose tissue. In WT mice, MAs^{III} and DMAs^{III} were extensively retained in these tissues, while iAs^{III} and iAs^{V} were

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predominantly retained in tissues of A*s3mt*-KO mice. Lastly, we used HG-CT-inductively coupled plasma-mass spectrometry, which provides lower detection limits, to examine concentrations of tri- and pentavalent As species retained in bladder exfoliated cells (BECs) recovered from individuals exposed to iAs in drinking water, and determined associations between the concentrations of As species in BECs and urine and the individual risk of developing diabetes. As species retained in BECs were positively correlated with other markers of exposure. More importantly, iAs^{III} and MAs^{III} retained in BECs were positively associated with risk of diabetes. Taken together, this work demonstrates the robustness of the optimized HG-CT-based techniques for the oxidation state specific analysis of As species in a variety of biological samples, and provides the first evidence that measurements of trivalent arsenicals in these samples can provide sensitive markers for assessment of health risks associated with iAs exposure.

To those who trudge the road of happy destiny.

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LIST OF ABBREVIATIONS

2HBG   2-hour blood glucose

2HPG   2-hour plasma glucose

AAS   Atomic absorption spectrometry

AFS   Atomic fluorescence spectrometry

ANOVA   Analysis of variance

APL   Acute promyelocytic leukemia

AQP   Aquaglyceroporin

As   Arsenic

As^{III} Trivalent arsenic

As^{V} Pentavalent arsenic

AS3MT   Arsenic (3+ oxidation state) methyltransferase

AsB   Arsenobetaine

AsC   Arsenocholine

AT   Autotransformer

BECs   Bladder exfoliated cells

BSO   Buthionine sulfoximine

CCA   Chromated copper arsenate

CI   Confidence interval

CT   Cryotrapping

DIW   Deionized water

DMAs   Dimethylated arsenic

$DMAs^{III}$ Dimethylarsinite (dimethylarsinous acid)

DMAs^{III}I	Iododimethylarsine
DMAs^{V}	Dimethylarsinate (dimethyarsinic acid)
DMTA	Dimethylthioarsinic acid
DWF	Dewar flask
FBG	Fasting blood glucose
FDA	Food and Drug Administration
FIAS	Flow injection for atomic spectroscopy
GF	Glass flask
GLS	Gas-liquid phase separator
GLUT	Glucose transporter
GSH	Glutathione
GSTO	Glutathione S-transferase omega
GSIS	Glucose-stimulated insulin secretion
HbA1c	Glycosylated hemoglobin
HG	Hydride generation
HPLC	High performance liquid chromatography
iAs	Inorganic arsenic
iAs^{III}	Arsenite
iAs^{V}	Arsenate
ICP-MS	Inductively coupled plasma mass spectrometry
INJ	Injection valve
KO	Knockout
LOD	Limits of detection

MAs	Methylated arsenic
MAs^{III}	Methylarsonite (methylarsonous acid)
MAs^{III}O	Oxomethlarsine
MAs^{V}	Methylarsonate (methylarsonic acid)
MC	Mixing coil
MCL	Maximum contaminant level
MRP	Multidrug resistance protein
m/z	Mass-to-charge ratio
NHANES	National Health and Nutrition Examination Survey
NTP	National Toxicology Program
OAT	Organic anion transporting polypeptide
ODS	Octadecylsilane
OGTT	Oral glucose tolerance test
OR	Odds ratio
PBS	Phosphate-buffered saline
PEEK	Polyether ether ketone
PP	Peristaltic pump
R	Remote
RBC	Red blood cell
RC	Reaction coil
ROS	Reactive oxygen species
RT	Rotamer tube
SAM	S-adenosylmethionine

SD	Standard deviation
SNP	Single nucleotide polymorphism
ST	Separating transformer
SV	Solenoid valve
SW	Relay switch
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TBAH	Tetrabutylammonium hydroxide
TI	Thermal insulation
TMAs^{V}O	Trimethylarsine oxide
TRIS	Tris(hydroxymethyl)aminomethane
US EPA	United States Environmental Protection Agency
V	Voltmeter
WHO	World Health Organization
WT	Wild-type

CHAPTER I

Introduction

1.1 Biomarkers of Arsenic exposure and disease susceptibility

Environmental and occupational exposures to inorganic arsenic (iAs) have been linked to several adverse health effects, including cancer of the skin, lungs, liver, and urinary bladder (1). Other effects of iAs exposure include vascular disease, hypertension, skin lesions, and diabetes (1-4). The presence of arsenic (As) in the earth's crust and certain industrial activities contribute to its contamination of aquifers used for drinking water across the globe. Recently, exposure to high levels of iAs through the ingestion of contaminated drinking water has been implicated in an As-induced form of diabetes characterized by impaired glucose tolerance in the absence of fasting hyperinsulinemia and insulin resistance (5).

Exposure to iAs and susceptibility to disease have commonly been assessed through analysis of total As and biological speciated metabolites of As in urine and other easily attainable and minimally invasive samples, including blood, hair, and nails. The United States Environmental Protection Agency (US EPA) and World Health Organization (WHO) recently lowered the safe levels for As in drinking water from 50 to 10 ppb in response to evidence of increased risk of cancer associated with chronic ingestion of As, even at low levels (6, 7). Current evidence suggest that the toxic methylated trivalent metabolites of iAs, methylarsonite (MAs[III]) and dimethylarsinite (DMAs[III]) produced in the course of As metabolism, play a key role in the etiology of As-induced diseases (8-11). While MAs[III] and DMAs[III] have been detected in urine of

subjects exposed to iAs (12, 13), the rapid oxidation of DMAs^{III} and, to a lesser extent, MAs^{III} to their pentavalent counterparts, methylarsonic acid (MAs^{V}) and dimethylarsinic acid (DMAs^{V}), complicates their analysis in samples of urine collected in field studies (13, 14). Furthermore, comparatively little work has been conducted toward the direct analysis of trivalent arsenicals in more complex biological matrices.

This project was designed to develop methods for assessing trivalent arsenicals as biomarkers of iAs exposure and susceptibility to As-induced diabetes. Establishing methods for the determination of As metabolites in target human tissues and linking As concentrations in tissues to adverse health effects will help inform the risk assessment of As-induced diabetes and other chronic diseases in exposed populations worldwide.

1.2 Human As exposure

As is ubiquitous in the environment and has been detected in nearly every medium imaginable; it exists in water sources, soil, plants, and animal and human tissues. As is the 20th most abundant element present in earth's crust at an average concentration of approximately 0.5 mg/kg and is highly concentrated in sulfide ores (15). Arsenopyrite, a mineral containing As, sulfur, and iron, is the main form of As in the environment. Due to the presence of iAs in geological formations, As accumulates in aquifers naturally or due to anthropogenic activities depending upon several factors, including temperature, pressure, pH, redox state, and mineral content (16). Volcanic activity largely contributes to the natural emission of As into the atmosphere (17).

A variety of anthropogenic activities and substances contribute to As accumulation in the environment. Notably, As is currently at the top of the Superfund Substance Priority List published by the Agency for Toxic Substances and Disease

Registry (18). During mining, sulfide minerals, which can include arsenopyrite, are exposed to air and often acidic water, resulting in favorable conditions for As leaching and potential As contamination of mine drainage (19). Additionally, the combustion of coal containing As generates fly ash, the disposal of which can contaminate nearby soil and water sources (20). Moreover, the use of geothermal waters, often containing high levels of iAs, during geothermal power production can inadvertently increase the concentration of As in nearby waterways (21). Manmade sources of As, including As-based pesticides, herbicides, animal-feed additives, and wood treatments contribute to the persistence of As in the environment. Lead arsenate, a widely used pesticide until the 1960's, may persist in soils where it was employed. Monosodium methyl arsonate (CH_4AsNaO_3), an organic arsenical, is commonly used to for weed management on golf courses and cotton crops. Although, the chicken-feed additive, 4-hydroxy-3-nitrobenzenearsonic acid (Roxarsone), is an organic arsenical, iAs accumulation has been detected in the tissues and litter from treated animals. Additionally, chromated copper arsenate (CCA) has been used as a wood preservative since the 1930's; however, the US EPA limited the residential use of CCA-treated wood in 2004 because of potential leaching and exposure concerns (22). While the most common route of iAs exposure is through the ingestion of contaminated drinking water, other exposures include eating As-rich foods, certain occupational settings, and medical treatments with As containing drugs.

1.2.1 Ingestion of As in drinking water

Worldwide, tens of millions of people are chronically exposed to hazardous levels of As. Sources of drinking water with high levels of iAs occur across the globe (**Figure 1.1**) (16).
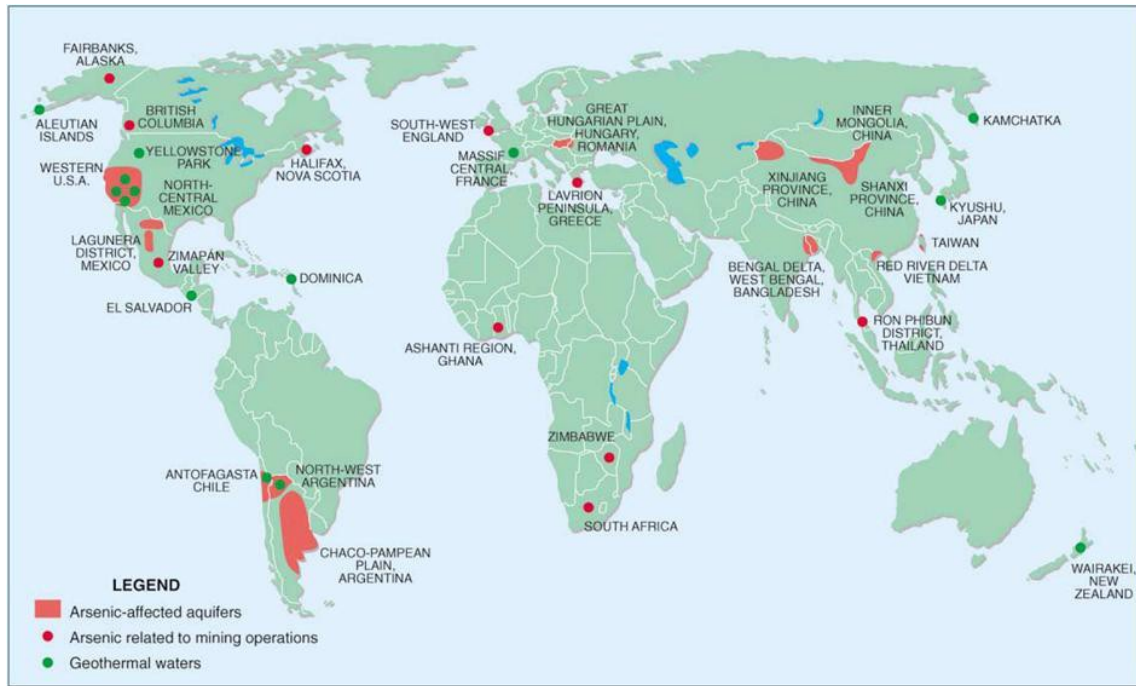
Figure 1.1: An overview of the prevalence of groundwater As contamination according to the 2002 British Geological Survey (16).

In affected areas, including regions of Mexico, China, India, and Bangladesh, contaminated water can reach upwards of 1000 ppb (16). In the United States, levels of As can exceed 50 ppb (**Figure 1.2**), but municipal sources are generally below the EPA's Maximum Contaminant Level (MCL) of 10 ppb (19). However, domestic well water in the US is only partially regulated and may not meet federal standards for safe drinking water. Speciation analysis of As in water reveals that arsenite (iAs^{III}) and arsenate (iAs^{V}) are the major species present, although MAs and DMAs have been reported in some

samples (23). Remediation techniques involving reverse osmosis, anion exchange, or adsorption are efficient, but often unattainable or difficult to maintain in regions that are plagued with water containing high levels of iAs (24).
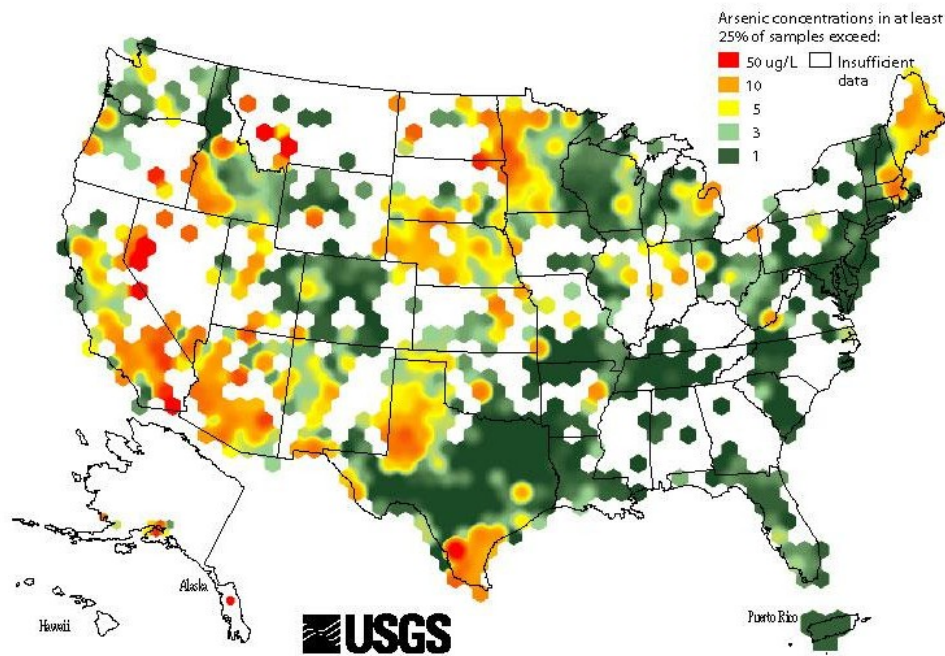
Figure 1.2: As contamination of municipal groundwater supplies in the United States (25).

1.2.2 As exposure through food

There are numerous reports of As in food items, including rice, seafood, seaweed and fruit juices. The use of water containing high iAs levels in agriculture can increase As in plants and animals used for consumption. Additionally, plants can bioaccumulate iAs through uptake from contaminated soils. Cooking with water high in iAs can also increase exposure. Seafood often contains organic arsenicals, including arsenobetaine (AsB), arsenocholine (AsC), and arsenosugars that can be detected in urine and increase

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urinary excretion of dimethylated As (DMAs) (26). Additionally, unsafe levels of iAs ranging from 67–96 mg/kg have been found in samples of hijiki seaweed native to Japan, Korea, and China (27). Until recently, chickens for consumption were routinely fed Roxarsone, an antiparasitic agent used to prevent disease and stimulate growth. While Roxarsone, an organoarsenical, was not thought to breakdown into toxic iAs species, the Food and Drug Administration (FDA) recently published evidence of iAs accumulation in livers and muscle tissue from chickens even after the mandatory 5-day wash-out period (28). These findings prompted Pfizer, the drug's manufacturer, to voluntarily halt sales in June 2011.

1.2.3 Occupational and other exposures

As exposure from coal fly ash, cigarette smoking, and certain occupational settings primarily result in the inhalation of As-containing particles. Non-ferrous metal smelting, including copper and lead, creates As laden dust and particles that can be inhaled and even emitted as air pollution. A rising industry with potential for As exposure is semiconductor chip manufacturing, where gallium arsenide is used to make electronic wafers (29). Furthermore, As has been measured in cigarette tobacco (30) leading to increased levels of As in hair (31) and urine (32) collected from smokers. Other industries with risk for As exposure include battery assembly, coal-fired power plants and the manufacture CCA-treated wood and glass. Additionally, activities that include handling of CCA-treated wood or As-based pesticides may lead to As exposure through dermal absorption or ingestion.

1.2.4 Current medical uses for As

As containing compounds have been used for centuries to treat a variety of ailments, including several cancers and infectious diseases. Notably, iAs and Salvarsan, an As-containing drug, were routinely used to treat syphilis until after the mass production of effective antibiotics during WWII (33). As trioxide (Trisenox) and other arsenicals are currently being used or investigated for the treatment of certain cancers, including leukemias, lymphoid malignancies, and solid tumors (34). Trisenox used for the treatment of acute promyelocytic leukemia (APL) is the only As-based drug approved by the FDA (35).

1.3 As Metabolism

As is ubiquitous in the environment, and thus, human exposure is inevitable. Its toxicity and individual susceptibility to disease are largely dependent on the chemical form and oxidation state of As. Additionally, bioavailability, the ability to be absorbed by the body and enter circulation, influences As toxicity. From soils and As-containing foods, including rice, gastrointestinal uptake after oral exposure is largely dependent on speciation and matrix, with soluble As compounds being more bioavailable (36, 37); unabsorbed As is largely eliminated in the feces, while absorbed As is primarily eliminated in urine. This section will focus on the fate of As after oral exposure in humans and other mammals and factors affecting uptake, metabolism, retention, and excretion.

1.3.1 As uptake and transport

The mechanism of transport of As across membranes is dependent on its form and oxidation state along with the varied expression of transporters between organs. Several

protein families are thought to mediate the transport of As across cell membranes, including organic anion transporting polypeptides (OATs), aquaglyceroporins (AQPs), glucose transporters (GLUTs), phosphate transporters and multidrug resistance proteins (MRPs) from the ATP-binding cassette transporter peptide family. A recent study in the human intestinal epithelial cell line, Caco-2 indicates that iAs^{III} is largely transported by OATPB, AQP10 and GLUT5; conversely, iAs^{V}, which competes with phosphate for uptake, is dependent on the phosphate transporter, NaPiIIb (38). GLUT1, which is expressed in the neonatal heart and epithelial cells of blood brain barrier, transports As^{III} species using a different mechanism than for glucose transport (39). In primary human hepatocytes, the expression of MRP2 may mediate the efflux of DMAs, while GLUT2 expression is associated with increased cellular accumulation of iAs and MAs (40). Moreover, AQP9, a membrane water channel expressed in the liver, preferentially transports trivalent arsenicals, mediates the accumulation of iAs^{III} and MAs^{III} in hepatocytes, and likely contributes to the efflux of pentavalent arsenicals into the blood stream (41). APQ9 null mice were shown to have reduced clearance and increased susceptibility to iAs toxicity (42). Furthermore, MRPs largely mediate the efflux of GSH conjugates, which are critical for detoxification of xenobiotics, and are thought to aid in the clearance of As species in the bile (43). GSH conjugates of iAs^{III} and MAs^{III} have been measured in rat bile after exposure to iAs species (44, 45).

1.3.2 Biotransformation

Methylated As species were discovered in human urine in 1973 (46) and further experiments confirmed that inhaled or ingested iAs was converted to methyl- and dimethylated As species (47, 48). Humans and many common laboratory animals

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methylate As with the exception of guinea pigs (49), chimpanzees (50) and marmoset and tamarin monkeys (51). As (+3 oxidation state) methyltransferase (AS3MT) catalyzes the S-adenosylmethionine (SAM) dependent methylation of trivalent As species (52). This 42 kDa protein was first purified from the liver of male Fischer 344 rats, and AS3MT mRNA has been found in several other tissues, including, kidney, urinary bladder, heart, lung, testes, and adrenal gland (53). Notably, the SV-40 transformed urothelial cell line, UROtsa, does not methylate As; however, cultured HepG2 cells and UROtsa cells expressing rat As3mt produce tri- and pentavalent arsenicals when exposed to iAs (54). Disruption of the *As3mt* gene in mice leads to a greater accumulation of whole-body As, almost exclusively as iAs, and an increased susceptibility to iAs toxicity (55, 56). These data indicate that *As3mt* genotype affects As methylation phenotype, a potential factor in the development of iAs-induced diseases.

Current evidence supports two distinct mechanisms for the AS3MT-mediated biomethylation of As (**Figure 1.3**). The first pathway, characterized by the reduction of As^V species followed by an oxidative methylation of the As^{III} species, was proposed by Challenger in 1945 and later revised by Cullen in 1984 (57, 58). It is hypothesized that pentavalent arsenicals are first reduced to As^{III} species by a reducing equivalent, including glutathione (GSH) or the enzyme itself before the addition of a methyl group from SAM by AS3MT (59). After methylation, the pentavalent arsenicals, MAs^V and $DMAs^V$ are available for reduction to their trivalent counterparts, MAs^{III} and $DMAs^{III}$ for storage, transportation or excretion (60). While DMAs is the predominant end product of iAs metabolism in humans and laboratory animals, the major end product in some organisms is trimethylarsine oxide ($TMAs^VO$) (61).

An additional proposed pathway for the biotransformation of iAs includes a reductive methylation where As^{III} species are bound to GSH or AS3MT during methylation, resulting in the formation of methylated pentavalent arsenicals as end products due to oxidation as opposed to intermediates (62). While some evidence has suggested As-GSH complexes at substrates for AS3MT (63), *in vitro* evidence indicates that GSH is not necessary for enzymatic function, and GSH alone in the absence of other endogenous dithiol reductants, including thioredoxin, only exhibits limited catalytic activity (59). The structure of CmArsM, a human AS3MT orthologue, has recently been published, and homology modeling of the human enzyme suggests that AS3MT lacks a reductase domain (64). Moreover, catalytic studies of CmArsM reveal that iAs- and MAs-GSH conjugates bind faster than iAs or MAs (65).
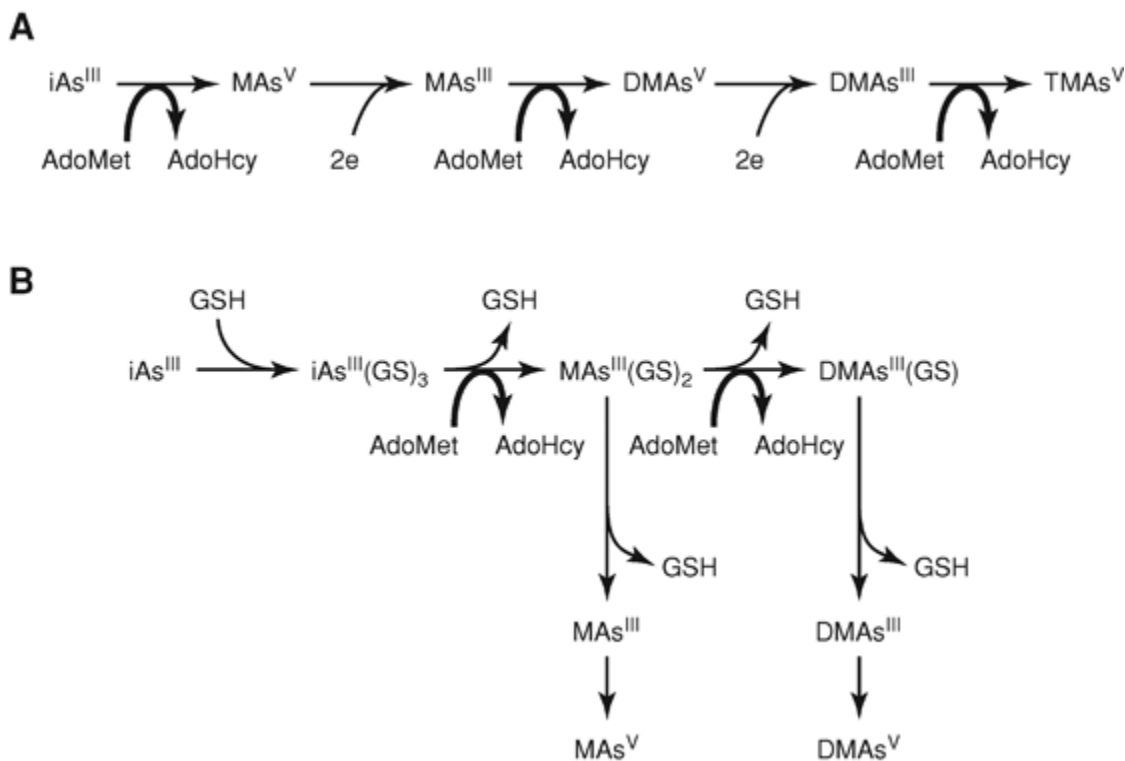
Figure 1.3: Schematic representations of the oxidative (A) and reductive (B) pathways for As biomethylation (66).

1.3.2.1 Genetic factors affecting As biotransformation

Genetic differences altering the As methylation phenotype in humans have been described for several enzymes, including AS3MT. Single nucleotide polymorphisms (SNPs) of the human *AS3MT* gene have been reported in both intronic and exonic locations, potentially altering As biomethylation and contributing to inter individual differences in As metabolite excretion. In primary human hepatocytes, a single allele missense mutation at position 14458 in *AS3MT* resulting in the Met287Thr (T$\rightarrow$C) amino acid substitution produced an altered methylation phenotype (67). While altered methylation phenotypes linked to several *AS3MT* SNPs of have been inconsistent across populations, results associated with the variants at positions 12390, in intronic site, and 14458 have been consistent regardless of the populations examined (68). For example, increased urinary %MAs suggesting reduced overall methylation capacity has been linked to the Met287Thr AS3MT variant in regions of Mexico (69, 70), Europe (71), and Vietnam (72). Other genetic associations to the methylation of iAs in human populations include SNPs in glutathione S-transferase omega 1 (GSTO) (73), critical for detoxification of xenobiotics and enzymes associated with SAM biosynthesis, methylenetetrahydrofolate reductase (71) and cystathionine ß synthase (74).

1.3.2.2 Dietary factors affecting As biotransformation

Dietary factors that affect one-carbon metabolism are likely to influence As methylation profiles. SAM, the methyl donor required in the biomethylation of iAs, is generated by a set of cyclic reactions requiring several dietary constituents, including folate, amino acids, and vitamins B12 and B6. In a U.S. population, lower protein, iron, zinc, and niacin intakes were associated with increased %MAs and decreased %DMAs

composition in urine (75). In Bangladesh, increased intake of protein, methionine and cysteine were associated with a 10-15% increase in total As excretion (76). Moreover, in a double-blind study in folate-deficient individuals, folate supplementation led to increased %DMA and decreased %MAs and %iAs in urine compared to individuals given placebo (77).

1.3.3 As retention, and excretion

In humans and laboratory animals, ingested As is primarily excreted through the kidneys into urine as DMAs; however, urinary As levels reflects only recent As exposures and may not present an accurate picture of As species retained in target tissues. In a controlled human study, approximately 60% of ingested iAs was excreted in the urine, with DMAs representing 42-73% of total As (78). Individuals with higher urinary MAs/DMAs concentrations exhibit an increased risk for several adverse effects associated with As exposure, including skin lesions (79), cancer (80, 81), and cardiovascular disease (82), and. In human tissues analyzed at autopsy from individuals exposed to low levels of As, primarily through their diets, iAs and DMAs predominated with the highest total As present in the aorta (83). Studies of gestational exposure indicate that in humans, As crosses the placenta and is measured in maternal and cord blood primarily as DMAs (84). Because of the logistical complications involved in collecting human tissues, little research has focused on the speciation of As in target tissues of individuals exposed to iAs. However, a recent analysis of As speciation in bladder exfoliated cells (BECs) and urine from residents living in an As endemic area of Zimapan, Mexico indicates that As retention in this target tissue may not correlate with urinary excretion (85).

The retention of As species in laboratory animal tissues varies based on length of exposure and dosing protocol. Several recent *in vivo* studies in mice have examined tissue distribution after exposure to iAs^{III} or iAs^{V} through drinking water; however, oxidation state specific analysis was not performed. In mice subchronically exposed to 25 or 50 ppm As as iAs^{III}, DMAs predominated in tissues critical for glucose homeostasis, including adipose, skeletal muscle, pancreas, and liver (86, 87). Similarly, 12-week exposure to 50 ppm As as iAs^{V} revealed the predominant accumulation of DMAs in lung, but MAs in kidney, with the highest total As levels in kidney, lung and bladder (88). Gestational studies of exposures to iAs^{III} in pregnant mice indicate that As readily crosses the placenta and accumulates as DMAs in fetal lung, liver, and blood (89).

Recently developed, *As3mt*-knockout (KO) mice lacking the ability to methylate iAs (55) have been shown to retain significantly higher total levels of As (55, 90, 91). These mice are more sensitive to iAs-induced toxicity compared to wild-type (WT) mice (56, 92). As speciation in tissues from *As3mt*-KO mice exposed to iAs primarily retain iAs; however, methylated metabolites have been detected in liver and plasma, suggesting As methylation mediated by additional methyltransferases or intestinal microbiota (55, 93). Because *As3mt* genotype produces significantly different As methylation phenotypes, the *As3mt*-KO mouse model provides a unique model to study the toxic effects of methylated metabolites or individual arsenicals.

1.4 As and Diabetes

Worldwide, over 280 million people are estimated to be living with diabetes (94), and in the United States, the prevalence of diabetes in adults is 11.3% (95). Current classifications of diabetes includes insulin-dependent (type 1), non insulin-dependent

(type 2), and gestational (type 3), depending on etiology and disease presentation. Type 1 diabetes (T1D) is characterized by a deficiency in insulin secretion (hypoinsulinemia) that leads to high levels of circulating glucose (hyperglycemia) due to a loss of pancreatic ß-cells within the islets of Langerhans, which are responsible for insulin production and secretion. This process is mediated by an auto-immune response that usually occurs in early childhood. Ketoacidosis, a characteristic of T1D, is a condition triggered by the β-oxidation of fatty acids which replace glucose as the major source of energy, resulting in the accumulation of ketone bodies and harmful reduction of blood pH. In contrast, type 2 diabetes (T2D) is characterized by insulin resistance in the liver and peripheral tissues, including skeletal muscle and adipose tissue that results in hyperglycemia. Thus, more insulin is needed to achieve insulin-stimulated glucose uptake in these tissues, resulting in hyperinsulinemia, and eventually pancreatic ß-cell function is diminished due to apoptosis (96). Type 3 diabetes occurs in women during pregnancy and generally resolves after childbirth. Current WHO diagnostic criteria for diabetes include individuals presenting with fasting plasma glucose levels $\geq$ 126 mg/dL or a two-hour plasma glucose level (2HPG) $\geq$ 200 mg/dL after an oral glucose tolerance test (OGTT) (97). Moreover, glycosylated hemoglobin (HbA1c) can be measured to estimated average plasma glucose levels over a 2 to 3 month period with levels $\geq$ 6.5% sufficient for a diagnosis of diabetes (98).

While obesity and metabolic syndrome are implicated in 70% of T2D cases, environmental chemicals, including iAs, may contribute to its development (4). A recent National Toxicology Program (NTP) workshop on the effects of environmental chemicals on the development of diabetes and obesity discussed several suspected diabetogenic and

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obesogenic environmental factors or chemicals, including maternal smoking, nicotine, As, bisphenol A, pesticides, organochlorine-based persistent organic pollutants, and peroxisome proliferator-activated receptor activators (e.g., organotins and phthalates). The As breakout panel concluded that there was "limited to sufficient" evidence to link iAs exposures to an increased prevalence of diabetes in populations exposed to levels of iAs in drinking water greater than 150 ppb (5). This section will discuss the current *in vivo* and *in vitro* data assessing the associations between As exposure and diabetes.

1.4.1 Epidemiological evidence for associations between As and diabetes

Early epidemiological evidence of an association between As and diabetes were published in the 1990's from areas of Taiwan (99) and Bangladesh (100) with very high As levels in drinking water. The strongest associations are found in areas of Taiwan (99, 101-103) and Bangladesh (100, 104) with drinking water As levels above 500 ppb. Many concerns regarding diabetes diagnosis, exposure assessment, the "healthy worker" effect and study power have been brought up for earlier epidemiological studies examining the associations between As and diabetes (5, 105). For example, some studies have used death certificates, glucose measurements in urine, or glycosylated hemoglobin for diagnosis, which may not accurately reflect diabetic status. A recent study of individuals in Bangladesh primarily exposed to < 300 ppb As in drinking water showed no associations between time weighted As exposure or urinary As and self-reported diabetes or glycosuria (106). However, FPG or 2HPG levels after an OGTT are preferred measurements for diagnosing diabetes; FPG and 2HPG levels greater than 126 and 200 mg/dL, respectively are indicative of diabetes (97).

In U.S. residents participating in the 2003-2004 National Health and Nutrition Examination Survey (NHANES), a population exposed to low levels of iAs, data on the association between As exposure and T2D yield conflicting reports depending on exposure assessment used for the statistical model. Navas-Acien and associates found significantly increased odds of T2D correlated to total urinary As measurements; however, the total urinary As measurement included in the model contains AsB, a metabolite of seafood intake not considered to be toxic (107). While the authors excluded participants who reported seafood consumption within the past 24 hours and included urinary AsB in their statistical model, some criticisms have been made concerning the contribution of AsB metabolism to urinary DMAs levels. A reanalysis of the 2003-2004 NHANES data by Steinmaus and associates included a model where AsB and AsC were subtracted from the total urinary As measurement, but seafood intake was not included as an independent variable in the statistical model, resulting in no increase in the odds of T2D (108). This method for estimated urinary As resulting from exposure to iAs does not account for other organoarsenicals from seafood consumption. Notably, consumption of arsenosugars increases urinary DMAs excretion (109). A more recent analysis of expanded NHANES data (2003-2006) further supporting the association between iAs exposure and diabetes using only participants with undetectable AsB levels eliminates the concerns regarding contribution of DMAs from seafood consumption (110).

Despite this controversy, recent data provide consistent associations between As and diabetes (70, 111, 112) or gestational diabetes (113) in populations exposed to less than 150 ppb As diagnosed by preferred methods, including FPG, OGTT, or HbA1c levels. In As-endemic regions of Mexico, Del Razo and associates found associations

between urinary DMAs^{III} concentration and diabetes (70). Moreover, the AS3MT M287T genotype in this population was found to be associated with increased levels of FPG, 2HPG, HbA1c, and urinary DMAs^{III} (114). Additional improvements in study design, including increasing population size, performing prospective studies, conducting more accurate exposure assessment, and using consistent diagnostic criteria for diabetes are likely to strengthen the body of evidence for an As-induced diabetes.

Exposure assessment varies widely among studies examining the relationship between As and diabetes. Measurements of As concentration in drinking water does not reflect individual exposures through drinking water ingestion because water sources and consumption pattern may vary; however, some studies have combined historical water As data with self-reported use of drinking water. Other options include measuring biomarkers of the exposure, e.g., As in biological samples, including blood, hair, nails or urine, which may not be accurate indices of exposure. Hair and nail matrices are sulfhydryl rich leading to As accumulation and are not recommended for As exposure analysis (115). Moreover, the half-life of As in blood and urine are approximately 1 hour and a few days, respectively, reflecting only recent As exposures. In urine, As concentration can be affected by hydration status and normalization techniques have limitations. Urinary creatinine has been associated with total urinary As (116) and increased urinary DMAs excretion (117). Furthermore, the condition of diabetes was shown to result in an over-estimation of urinary As levels adjusted using creatinine measurements (118). It has been suggested that specific gravity could be used to adjust urinary As levels for hydration status; however, certain disease states, including diabetes can lead to abnormal urinary glucose and albumin excretion, which would overestimate

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urine density (119, 120). Internal retention of As in human tissues has not been widely researched, on the other hand, exposure assessments in bladder exfoliated cells (BECs) (85) and saliva (121, 122) have been performed. In BECs from 21 individuals living in an As-endemic region of Mexico, the retention of speciated As did not correlate with urinary excretion (85); however, As species in saliva samples from individuals exposed to As in China did correlate with drinking water As concentrations (121).

1.4.2 Effects of As on glucose metabolism and homeostasis: laboratory evidence

Several laboratory-based *in vitro* and *in vivo* investigations link As exposure to altered glucose homeostasis and negative effects on mechanisms associated with glucose metabolism. In mice, impaired glucose tolerance has been observed after exposure to iAs^{III} in drinking water (86, 87, 123) and iAs^{V} (124). Notably, chronic iAs^{III} exposure combined with high fat diet produced a unique diabetic phenotype in mice which was characterized by impaired glucose tolerance in the absence of pronounced obesity, fasting hyperinsulinemia, or insulin resistance (87). While additional studies have shown As-induced impaired glucose homeostasis in rats (125-128), DMAs, an iAs metabolite, forms hemoglobin adducts and accumulates in the red blood cells (RBCs) of this species unlike humans and other laboratory animal models (129). Therefore, rats are not an appropriate model for As toxicity studies relating to human health.

In vitro studies have revealed several pathways for the pathogenesis of As-induced diabetes, including pancreatic β-cell function and glucose uptake in peripheral tissues. A reduction in glucose-stimulated insulin secretion (GSIS) has been observed in a variety of β-cell cultures exposed to As (130). These studies noted several mechanisms for the diminished GSIS, including reduced insulin mRNA levels (131), alterations in

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free Ca^{2+} oscillations (132), and a suppression of reactive oxygen species (ROS) due to an Nrf2-mediated antioxidant response (133, 134). It is suggested that ROS produced in the course of glucose metabolism may act as homeostatic regulators of insulin secretion, and thus, an Nrf2-mediated antioxidant response would hinder ROS signaling (135). In murine pancreatic islets, exposure to subtoxic concentrations of As^{III} species inhibited GSIS; however, basal insulin secretion or insulin content and expression were not affected, suggesting that trivalent arsenicals inhibit insulin transport vesicle packaging or translocation to the plasma membrane (136).

Mechanisms for peripheral tissue alterations in glucose homeostasis include inhibition of cellular differentiation and inhibited insulin-stimulated glucose uptake. iAs^{III} has been shown to inhibit differentiation of adipocytes (137, 138) and myoblasts (139). Moreover, trivalent arsenicals inhibit insulin-stimulated glucose uptake by adipocytes through reducing GLUT 4 translocation to the plasma membrane (140, 141). Subtoxic concentrations of iAs^{III} and MAs^{III} were shown to inhibit the phosphorylation of protein kinase B (Akt) by 3-phosphoinositide-dependent kinase-1,2 (PI3K) in cultured murine adipocytes (141).

1.5 As Speciation Analysis

As analysis became widespread with the development of the Marsh test in 1836, which sought to detect As in suspected poisoning cases (142). Quantitative determination of As has been conducted using a variety of techniques, including thin-layer chromatography with autoradiography using radiolabeled As and hydride generation (HG) or liquid chromatography coupled to various spectrometric detention techniques. After the discovery of methylated As species and the toxic MAs^{III} and $DMAs^{III}$

metabolites, speciation analysis became critical to assess exposure and potential disease mechanisms. However, due the instability of trivalent arsenicals, oxidation state specific analysis has not been widely explored until the last two decades. Currently, two main techniques, HG with cryotrapping (CT) and high performance liquid chromatography (HPLC), are used to separate As species for oxidation state analysis. These separation methods are coupled to a variety of detection devices, including the atomic absorption spectrometer (AAS), atomic fluorescence spectrometer (AFS), or inductively coupled plasma-mass spectrometer (ICP-MS). With these methods, the retention and excretion of As species can be assessed in a variety of biological samples to better characterize the metabolism and adverse effects of chronic As exposure. This section will focus on separation and detection techniques used for oxidation state specific analysis of As species in a variety of biologically relevant samples.

1.5.2 Separation of As species

The formation of arsine gas using HG has a distinct history in the analysis of As. In the Marsh test, zinc and sulfuric acid are mixed with the sample to generate arsine that was then oxidized with a flame to form pure As, generating a black stain that could be compared to known standards for quantification purposes. Modern HG techniques use a strong reducing agent, e.g., sodium borohydride, to generate arsine and methyl substituted arsines from seven As species, included tri- and pentavalent iAs, MAs and DMAs, and TMAs^{V}O using sample volumes up to 1 mL. The generation of arsine gases from different oxidation states of As can be manipulated by measuring two aliquots of each sample and altering the reagents used for HG. Early optimization of the HG technique for detecting As^{III} species and As^{III+V} involved performing the HG reaction at

pH 6 and 2, respectively (143, 144). The acidic environment allows for the generation of arsines and methyl substituted arsines from both trivalent and pentavalent species in one analysis, while the reaction mixture buffered at pH 6 selectively generates arsines from trivalent As species only. Further development paired the direct analysis at pH 6 to detect As^{III} species with the pretreatment of an additional samples aliquot with L-cysteine to detect As^{III+V} species. The L-cysteine forms a conjugation with As^V species reducing them to trivalency and likely forming As-sulfhydryl conjugates, resulting in arsine and methyl-substituted arsine generation from both As^{III} and As^V species. Pentavalent arsenicals can then be quantified by taking the difference of the results from the L-cysteine pretreated and directly analyzed aliquots. The online prereduction of As^V species using thioglycolic acid has been explored, but is not widely used (145).

Because arsine and methyl-substituted arsines are generated simultaneously from the sample, HG used as the primary separation technique is combined with a CT step for separation based on boiling points of individual arsines. Current techniques use a glass capillary U-tube wrapped with Ni-Cr wire and filled with Chromosorb, a diatomaceous earth filling often used for gas chromatography, which is submerged in liquid N_2. After sufficient reaction time for the generation of hydrides, the U-tube is removed from the liquid N_2 either manually or automatically using a doubled walled flask connected to a remotely activated solenoid valve. A power source then heats the wire wrapped U-tube allowing for separation of arsine and the methyl-substituted arsines based on boiling point. The boiling points of arsine and methyl-, dimethyl- and trimethylarsine are -62.5, 1.2, 35.6, and 53.8°C, respectively and can be fully resolved in less than one minute

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(146). A schematic of the HG-CT system coupled to an atomic absorption spectrometer (AAS) is presented in **Figure 1.4**.

An additional method widely used for the separation of As species is HPLC, most commonly ion exchange and reversed-phase. The high pressures generated during HPLC provide for improved resolution and greatly reduced separation times over conventional liquid chromatography; however, sample volumes are generally limited to a few hundred microliters. Separation of sample components is based on interactions with the stationary phase of the column and mobile phase used for elution and can be optimized for the oxidation state separation of As species.

Reversed-phase HPLC is characterized by a hydrophobic column packing combined with a hydrophilic mobile phase. The most common stationary phase for As speciation is octadecylsilane (ODS), which consists of C18 chains bonded to silicone. To enhance separation of ionic species, an ion-pairing reagent can be used to interact with the column matrix and alter analyte retention times. Reversed-phase ion pairing HPLC was first used for the oxidation state speciation of the more toxic MAs[III] and DMAs[III] species in 2000 (12). Here, a mobile phase of tetrabutylammonium hydroxide (TBAH), malonic acid, and methanol was used with an ODS-3 C18 column to separate six As species. Rabieh and associates further optimized the concentrations of mobile phase components for improved separation of six As species in less than seven minutes (147).
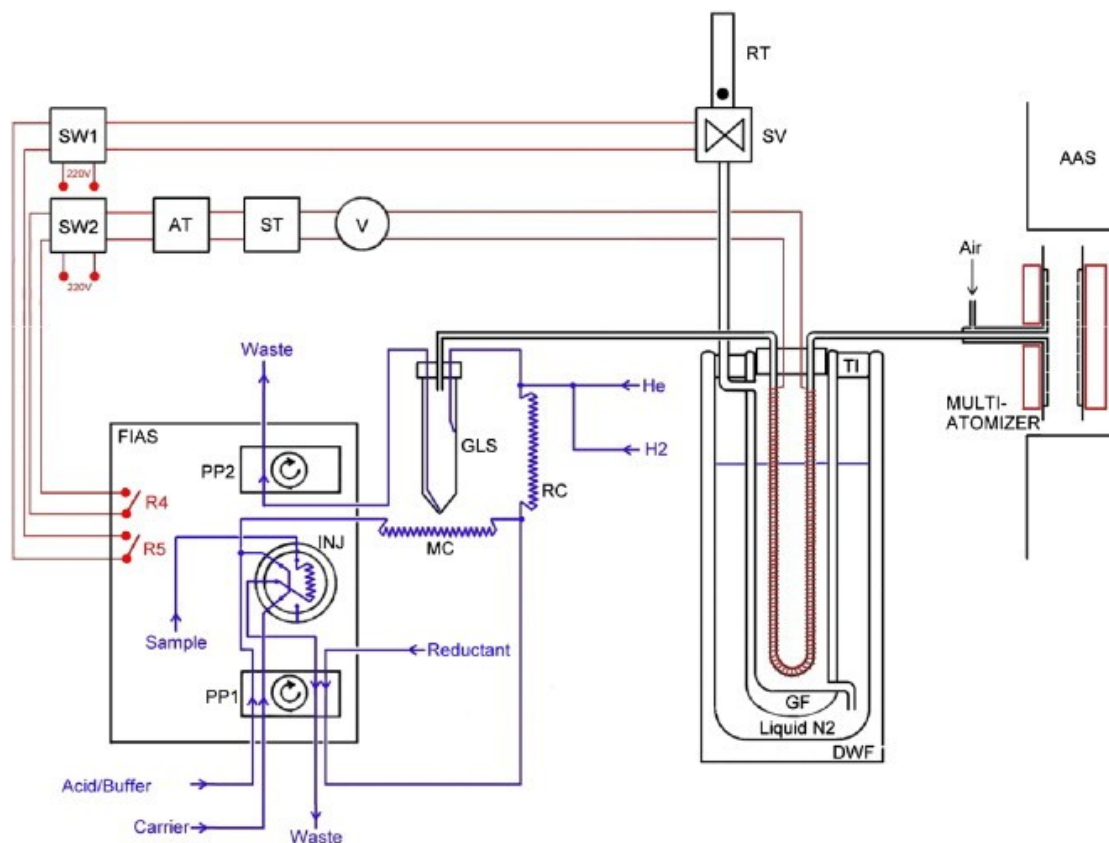
Figure 1.4: A schematic representation of the semi-automated HG-CT-AAS method. The following abbreviations are used: Flow injection for atomic spectroscopy (FIAS) peristaltic pump (PP1 and PP2); injection valve (INJ, 500 µl loop); mixing coil (MC); reaction coil (RC); gas-liquid phase separator (GLS); Dewar flask (DWF); glass flask (GF); thermal insulation (TI); FIAS remotes (R4 and R5); relay switches (SW1 and SW2); autotransformer (AT); separating transformer (ST); voltmeter (V); solenoid valve (SV); and rotameter tube for gas outlet monitoring (RT) (146).

Another common HPLC technique used to separate tri- and pentavalent As species is ion-exchange chromatography, commonly referred to as either cation or anion exchange chromatography depending on the composition of the stationary phase. In this method, a charged stationary phase attracts and retains ionic analytes of the opposite charge. Hence, cation exchange involves negatively charged functional groups attached to the stationary phase, which increases the retention time of positively charged analytes. Conversely, in anion-exchange HPLC, positively charged functional groups are attached

to the stationary phase, which interacts and increases the retention time of negatively charged analytes. A combination of anion- and cation-exchange HPLC has been used to separate 6 tri- and pentavalent As species and AsB in urine; however, two separate columns with nearly 10 minutes of separation each are needed (148).

1.5.3 Analytical detectors

Systems for the separation of As species are generally coupled to AAS, AFS, or ICP-MS for the detection of As species. These methods measure elemental As, but several factors affect their use for As speciation analysis. AAS is often coupled with HG-CT and the gaseous hydrides are atomized at high temperatures. As detection is facilitated by an electrodeless discharge lamp which emits light at a wavelength that elemental As absorbs, 193.7 nm, through the atomizer where the unabsorbed light is detected. AAS offers low detection limits and is relatively inexpensive to purchase and operate. Current HG-CT-AAS methodologies have produced detection limits from 16 to 40 ppt in biological matrices (149). AFS, has been widely used to speciate As both with HG-CT and HPLC-HG separations. It offers greater sensitivity than AAS (150) and is less expensive to operate than ICP-MS (151), but may be complicated by quenching and spectral interferences (152). In ICP-MS, nebulized samples are ionized in an argon-generated plasma flame prior to detection of the charged particles based on the mass-to-charge ratio (m/z), approximately 75 for As. Despite the expenses involved with ICP-MS analyses, this method offers several advantages, including very low detection limits, a wide dynamic range, and multielemental capabilities. In addition to the increased cost, polyatomic inferences, specifically by atoms of $^{40}Ar^{35}Cl$, must also be considered.

1.5.4 Biological matrices

The quantification of As metabolites in laboratory- and population-based samples can provide critical information on the toxicity of individual As species and explore mechanisms of action for the development of iAs-induced diseases. Recent developments for the speciation of As in biological samples have focused on HG-CT-AAS and HPLC-ICP-MS. However, HG steps have been used after HPLC separation in conjunction with AFS and ICP-MS to minimize interferences. Here, biologically relevant samples available for As speciation will be discussed.

Laboratory-based samples used for As speciation are an excellent tool to assess methylation and even tissue retention under experimental conditions. Simple *in vitro* assays using cytosolic liver fractions (153) or recombinant AS3MT (154) have been used to measure enzymatic activity and the effects of relevant SNPs. The HG-CT-AAS technique has been recently optimized for the oxidation state specific analysis of As species. Here, As^{III} and As^{V} species have been quantified in reaction mixtures containing recombinant AS3MT and in HepG2 cells and primary rat hepatocytes after *in vitro* exposure to iAs^{III} (143, 149). While the speciation of As in animal tissues after exposure to a variety of arsenicals has been extensively studied, no data on the oxidation state of these As metabolites has been available because acid digestion or extractions were performed prior to analysis converting As^{III} to As^{V} species.

Urine is often used for the speciation of As in population studies because it is minimally invasive to collect and provides robust information on exposure to iAs. Other biological samples routinely collected in population studies for the speciation of As species include blood, hair, nails, saliva. However, these specimens might not reflect

internal As exposures in target tissues critical for disease development. Recent research has focused on target tissues and the analysis of As metabolites in BECs has been performed (85). Despite the developments in As speciation analysis, little work has focused on the quantification of the more toxic trivalent As species, MAs^{III} and DMAs^{III} in target human tissues.

1.6 Conclusions

In conclusion, increasing evidence indicates an increased prevalence of diabetes in populations exposed to high levels of iAs in drinking water. The trivalent methylated metabolites of iAs, MAs^{III} and DMAs^{III}, likely contribute to its adverse effects. However, internal exposures to these toxic metabolites in humans are not well characterized. The following chapters will present an optimized method for analyzing these toxic iAs metabolites in biological samples and its application to laboratory- and population-based studies examining the associations between the more toxic, trivalent As metabolites and development of iAs-induced diabetes. These results will ultimately aid in the risk assessment of exposure to iAs through contaminated drinking water.

CHAPTER II

Direct Analysis of Methylated Trivalent Arsenicals in Mouse Liver by Hydride

Generation-Cryotrapping-Atomic Absorption Spectrometry[1]

2.1 Overview

Growing evidence suggest that the methylated trivalent metabolites of inorganic arsenic (iAs), methylarsonite (MAs^{III}) and dimethylarsinite ($DMAs^{III}$), contribute to adverse effects of iAs exposure. However, the lack of suitable methods has hindered the quantitative analysis of MAs^{III} and $DMAs^{III}$ in complex biological matrices. Here, we show that hydride generation-cryotrapping-atomic absorption spectrometry can quantify both MAs^{III} and $DMAs^{III}$ in livers of mice exposed to iAs. No sample extraction is required, thus limiting MAs^{III} or $DMAs^{III}$ oxidation prior to analysis. The limits of detection are below 6 ng As/g of tissue, making this method suitable even for studies examining low exposures to iAs.

2.2 Introduction

Inorganic arsenic (iAs), a carcinogenic metalloid found in the earth's crust, can accumulate in aquifers naturally or due to industrial activities (16). Chronic iAs exposure through contaminated drinking water has been linked to risks of various diseases, including cancer, hypertension, and diabetes (1, 3, 155). The individual susceptibility to

these diseases varies considerably, even at similar exposure levels, complicating the risk assessment. Current evidence suggests that the trivalent methylated metabolites of iAs, methylarsonite (MAs^{III}) and dimethylarsinite ($DMAs^{III}$), are more toxic than pentavalent methylarsonate (MAs^V) and dimethylarsinate ($DMAs^V$), or iAs species, arsenite (iAs^{III}) and arsenate (iAs^V) (8). Both MAs^{III} and $DMAs^{III}$ are products of iAs methylation by arsenic (+3 oxidation state) methyltransferase (149) and by cultured human hepatocytes (144). Both MAs^{III} and $DMAs^{III}$ are present in urine of individuals exposed to iAs in drinking water (156). However, results of recent studies indicate that the concentrations and proportion of iAs metabolites in urine do not necessarily reflect the concentrations and speciation of As in tissues targeted by iAs exposure (85, 157, 158). Other studies demonstrate an organ specific accumulation of iAs and methylated arsenicals after exposure to iAs (88). Thus, the quantitative analysis of iAs metabolites in target tissues is crucial for elucidating the mechanisms of the adverse effects of iAs exposure and for understanding the inter-individual variations in manifestation and severity of the diseases associated with this exposure.

Several methods have been developed for the speciation analysis of As in aqueous samples, including human urine (14, 159, 160). However, only hydride generation-cryotrapping-atomic absorption spectroscopy (HG-CT-AAS) is uniquely suited for the oxidation state specific analysis of As in complex biological matrices (143, 146, 149). Unlike HPLC techniques, HG-CT-AAS does not require digestion or extraction of biological samples and, therefore, limits the artifacts associated with the oxidation or with on-column binding of the reactive, but unstable methylated trivalent metabolites (161). We have previously used HG-CT-AAS for quantitative analysis of iAs metabolites,

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including MAs^{III} and $DMAs^{III}$ in cultured mammalian cells capable of methylating iAs (149). Here, speciation analysis of As is carried out in two sample aliquots. In the first aliquot, hydrides (arsine and methyl-substituted arsines) from the trivalent As species (iAs^{III}, MAs^{III} and $DMAs^{III}$) are selectively generated at pH 6 and measured directly without sample pretreatment. The second aliquot is treated with 2% L-cysteine to reduce the pentavalent As species (iAs^{V}, MAs^{V} and $DMAs^{V}$) to trivalency; thus, arsines generated from this sample aliquot represent both tri- and pentavalent As species (iAs^{III+V}, MAs^{III+V} and $DMAs^{III+V}$) present in the sample. The concentrations of the pentavalent As species are then determined by subtracting the results of analysis in the 1st sample aliquot from results of the analysis in the 2nd aliquot. The goal of the present study was to examine whether this HG-CT-AAS technique is also suitable for quantitative, oxidation state specific analysis of As species in tissues.

2.3 Materials and Methods

2.3.1 Arsenicals

Sodium arsenite (iAs^{III}) and sodium arsenate (iAs^{V}), (>99% pure) were purchased from Sigma-Aldrich (St. Louis, MO). A 1000 μg/L As AAS standard solution was purchased from Merck (Darmstadt, Germany). Because sodium arsenite is commonly contaminated with arsenate, arsenic trioxide (As_2O_3; >99.9% pure from Sigma-Aldrich) was used as the trivalent inorganic As specie for validation of the oxidation state specific analyses. Methylarsonic acid (MAs^{V}), disodium salt ($CH_3As^{V}O(ONa)_2$) and dimethylarsinic acid ($DMAs^{V}$) both better than 98% pure were purchased from Chem Service (West Chester, PA). The methylated trivalent arsenicals, oxomethylarsine ($MAs^{III}O$) and iododimethylarsine ($DMAs^{III}I$), were custom synthesized by Dr. William

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Cullen (University of British Columbia, Vancouver, Canada). In aqueous solutions, $MAs^{III}O$ and $DMAs^{III}I$ hydrolyze to form the methylarsonous (MAs^{III}) and dimethylarsinous ($DMAs^{III}$) anions, respectively.

2.3.2 Speciation Analysis of As

We used a semi-automated HG-CT-AAS system similar to that described in our previous reports (146, 149). AAnalyst 800 spectrometer (Perkin-Elmer, Norwalk, CT, USA) equipped with a multiple microflame quartz tube atomizer (multiatomizer) and coupled to a cryotrap was controlled by FIAS 400 flow injection accessory (Perkin-Elmer). As compared to the previously described HG-CT-AAS setup (146), the injection port of the FIAS 400 was bypassed to avoid plugging with homogenate slurries. Using this system, iAs and the methylated arsenicals in samples are reduced to the corresponding arsines (arsine, methylarsine, and dimethylarsine) in a reaction mixture containing sodium borohydride ($NaBH_4$; Sigma-Aldrich, St. Louis, MO) and a 0.75M Tris(hydroxymethyl)aminomethane (TRIS)-HCl buffer (pH 6; Sigma-Aldrich). The arsines are cryotrapped and then separated by their boiling points prior to detection by the AA spectrometer. Here, two aliquots of each sample are analyzed to determine As^{III} species and As^{III+V} species. Trivalent arsenicals (iAs^{III}, MAs^{III} and $DMAs^{III}$) are analyzed directly without sample extraction or pretreatment. For analysis of total, tri- and pentavalent arsenicals (iAs^{III+V}, MAs^{III+V} and $DMAs^{III+V}$), the 2nd sample aliquot is pretreated with 2% L-cysteine (biochemistry grade, EMD Chemicals, Inc., Gibbstown, NJ) for 1 hour at room temperature. L-cysteine reduces the pentavalent arsenicals to trivalency, allowing arsine generation from both As^{III} and As^{V} species. The

concentrations of iAs^V, MAs^V, and $DMAs^V$ are then determined by subtracting the results of analysis in the 1st sample aliquot from results of the analysis in the 2nd aliquot.

Calibration curves for quantification of As species were generated using aqueous solutions of the pentavalent standards, iAs^V, MAs^V, and $DMAs^V$ treated with L-cysteine. To account for contamination, the As content in each of the standards was determined by graphite furnace-AAS (146). We have shown that the slopes of these curves are identical with slopes of curves generated from solutions of the trivalent arsenicals in the absence of cysteine (149). There are no standard reference materials available with certified levels of MAs^{III} and $DMAs^{III}$. We have routinely used custom synthesized MAs^{III} and $DMAs^{III}$ standards ($MAs^{III}O$ and $DMAs^{III}I$) for quality control, including calibration (when needed) or the standard addition techniques with relevant biological matrices.

2.3.3 Animals

Twelve week-old C57BL/6 mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA) and housed in polycarbonate cages (2/cage) with corn cob bedding at the University of North Carolina Animal Facility (12 h light/dark cycle, 22 ± 1 °C and humidity of $50 \pm 10\%$), accredited by the American Association for Accreditation of Laboratory Animal Care. Mice were allowed free access to food (Lab Diet 5058, Nutrition International, Brentwood, MO) and pure deionized water (DIW) or DIW containing iAs^{III} (50 mg As/L).

2.3.4 Tissues

Freshly dissected mouse livers were collected from unexposed (control) mice and from mice exposed to iAs^{III} in drinking water for 9 days. Liver homogenates were prepared in ice cold DIW (10% w/v). Liver homogenates from control mice were used

for calibration and to prepare homogenates spiked with As standards. Liver homogenates from mice exposed to iAs^{III} were used for the method validation. Here, the As^{III} species were analyzed directly in freshly prepared homogenates; As^{III+V} species were analyzed in homogenates pretreated with 2% L-cysteine. To determine % recovery of As during these analyses, aliquots of the homogenates were digested using a MARS Microwave Reaction System equipped with MarsXpress Temperature Control (CAM, Matthews, NC). Briefly, 100 μL of 10% homogenate was digested in 3 mL of 2 M ultrapure phosphoric acid (EMD Chemicals, Inc.) for 10 hours at $90 \pm 4°C$. After digestion, 1 mL of each digestate was neutralized with 0.25 mL of 10 N NaOH to a final pH between 6 and 7. Since this digestion oxidizes all trivalent arsenicals to their pentavalent counterparts, the neutralized digestates were treated with 2% L-cysteine by the addition of 20% L-cysteine and incubated at room temperature for 1 hour prior to analysis.

2.3.5 Statistical Analyses

All statistical analyses were performed using GraphPad Instat software package (GraphPad Software Inc., San Diego, CA). Linear regression and correlation analyses were employed to determine characteristic of calibration curves of As^V species in liver homogenates and DIW. ANOVA followed by Bonferroni's multiple comparison posttest was employed to determine differences in As recovery between DIW and homogenate solutions spiked with As standards. Statistical significance was considered at the level of $p < 0.05$.

2.4 Results and Discussion

In the first step, we compared the efficiency of generation of As species in aqueous solutions and in mouse livers. Here, we used liver from an untreated mouse fed a

regular diet and drinking deionized water (DIW). Ten percent liver homogenates (w/v) were prepared in DIW on ice and spiked with iAs^V, MAs^V, and $DMAs^V$ standards to generate calibration curves. In parallel, calibration curves were generated for solutions of these standards in DIW. Both, the aqueous standard solutions and the spiked homogenates were treated with 2% L-cysteine and analyzed by HG-CT-AAS (143, 146, 149) using AAnalyst800 atomic absorption spectrometer equipped with FIAS400 flow injection accessory (PerkinElmer Norwalk, CT, USA). We found that the slopes of the calibration curves generated for the aqueous standards and spiked homogenates are very similar (**Table 2.1**), suggesting that the complex matrix of the liver homogenate does not interfere with the analysis.

Table 2.1: Characteristics of the calibration curves for As^V standards spiked into DIW or 10% liver homogenate (a)

Matrices	As Standard	Linear Regression (b)	Correlation Coefficient
DIW	iAs^V	0.821x - 0.019	0.999
	MAs^V	0.825x - 0.024	0.999
	$DMAs^V$	0.835x - 0.024	0.999
Homogenate	iAs^V	0.873x + 0.00004	0.999
	MAs^V	0.880x - 0.005	0.998
	$DMAs^V$	0.941x - 0.027	0.997

(a) Arsines and methyl substituted arsines were generated after 1-hour pretreatment with 2% L-cysteine.

(b) The linear regression for each As^V standard was determined over the range of 0.125 to 4 ng As/mL.

In the second step, we compared the recovery of As standards from a liver homogenate and aqueous solutions. Here, a mixture of pentavalent iAs^V, MAs^V, and $DMAs^V$ standards (10 ng As each) and individual trivalent iAs^{III}, MAs^{III}, and $DMAs^{III}$ standards (10 ng As each) were prepared in DIW or in a 10% liver homogenate from an

untreated mouse and analyzed with or without L-cysteine pretreatment. Figure 2.1 shows

that, regardless of the matrix, the trivalent arsenicals can be quantitatively analyzed in the

absence or presence of L-cysteine with As recoveries of ~92 - 105%. Lower As
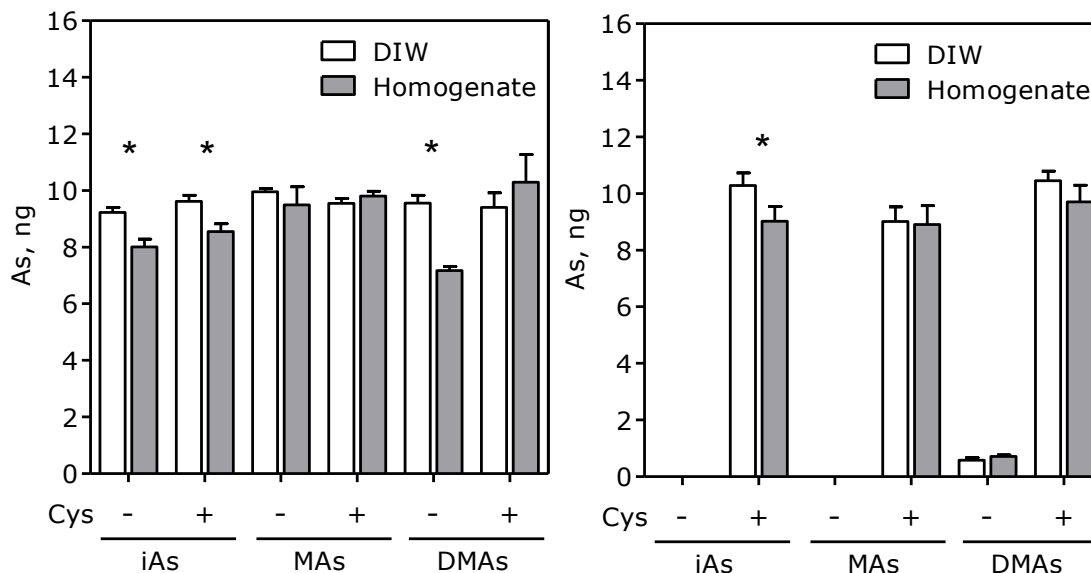
Figure 2.1: HG-CT-AAS analysis of DIW and 10% liver homogenate spiked with As^{III} (A) and As^V (B) standards. DIW and aliquots of the homogenate were spiked with a mixture of As^V standards (10 ng each) or with individual As^{III} standards (10 ng each) and analyzed before and after pretreatment with 2% L-cysteine (Cys) (mean with SD, n = 3). *Statistically significant differences ($p < 0.05$) between the amounts of an As species detected in DIW and in the homogenate as determined by ANOVA with a Bonferroni multiple comparison post-test.

recoveries were found only for homogenates spiked with iAs^{III} (~80%) or $DMAs^{III}$

(~72%) that were not treated with L-cysteine. In contrast, the pentavalent arsenicals were

detected only after the pre-reduction with L-cysteine. Consistent with our previous

findings (146), only $DMAs^V$ generated a small amount of dimethylarsine (~6-7% of total

As) in the absence of L-cysteine. The recoveries of As for DIW and homogenates spiked

with pentavalent standards ranged from ~90 to 102%. Detection limits calculated for a

blank liver homogenate with and without L-cysteine pretreatment ranged from 9 to 14 pg

As, which translate to $\leq$ 6 ng As/g of the tissue (**Table 2.2**).

Table 2.2: The instrumental and tissue detection limits for analysis of As^{III} and As^{III+V} species in mouse liver homogenate.

Detection Limit	iAs^{III}	MAs^{III}	DMAs^{III}	iAs^{III+V}	MAs^{III+V}	DMAs^{III+V}
Instrumental (pg As) (a)	14	13	9	10	10	12
Tissue (ng As/g tissue) (b)	6	5	4	4	4	5

(a) The instrumental detection limits were calculated from the AAS spectra generated for blanks (control liver homogenates, n=8) as 3(SD/slope) for the absorbance areas with the retention times corresponding to arsine, methylarsine, and dimethylarsine signals.

(b) Tissue detection limits were calculated from the instrumental detection limits and reflect the concentration and dilution of the liver homogenates used for the analysis.

Finally, we used HG-CT-AAS to determine concentrations of As species in the liver of a mouse exposed to iAs^{III} in drinking water (50 mg As/L) for 9 days. Here, the freshly dissected liver was divided into 4 sections. Each section was homogenized in ice-cold DIW. Aliquots of the 10% homogenates were immediately analyzed for As^{III} species (without L-cysteine pretreatment) and for As^{III+V} species (after pretreatment with L-cysteine). Additional aliquots from each liver section were digested in phosphoric acid for 10 hours at 90°C using the MARS5 Microwave system (90). This digestion eliminates the biological matrix and oxidizes all trivalent As species to pentavalency (162). Thus, only the total iAs (iAs^{III+V}), MAs (MAs^{III+V}) and DMAs (DMAs^{III+V}) can be measured in the digested samples. **Table 2.3** compares results of the direct speciation analysis and analysis of the digested homogenate from one of the liver sections. Based on the direct analysis, MAs^{III} and DMAs^{III} represented respectively 12% and 45% of the total speciated As, while their pentavalent counterparts, MAs^{V} and DMAs^{V}, accounted for 8% and 18%. Notably, the sums of tri- and pentavalent MAs and DMAs determined by the

direct analysis were in a good agreement with the concentrations of MAs^{III+V} and $DMAs^{III+V}$ determined in the digested homogenates. However, the amount of iAs recovered during the direct analysis represented only ~79% of iAs recovered after the digestion. **Figure 2.2** shows the recoveries of iAs, MAs and DMAs in directly analyzed homogenates from all four sections of the liver as compared to the analyses in digested homogenates. Here, the direct analysis recovered approximately 99% of total As, with individual recoveries of 83±5% for iAs^{III+V}, 100±6% for MAs^{III+V}, and 107±1% for $DMAs^{III+V}$. Together with data in **Table 2.3**, these results suggest high As recoveries and a good reproducibility of the speciation analysis in liver homogenates.

In conclusion, this work shows that HG-CT-AAS is suitable for the quantitative, oxidation state specific analysis of As species, including the unstable MAs^{III} and $DMAs^{III}$, in mammalian tissues. Our data show that approximately 66% of As in the liver of a mouse exposed to iAs is represented by trivalent species; ~12% by MAs^{III} and ~45% by $DMAs^{III}$. These results further strengthen the hypothesis that methylated trivalent arsenicals contribute to the adverse effects in tissues targeted by iAs exposure. Additional optimization may be needed to improve recoveries of iAs which is likely bound to high-affinity binding sites in tissue homogenates and to prevent the oxidation of $DMAs^{III}$ which contributes to lower recoveries of this unstable As species.

Table 2.3: The concentration of As species in one section of the liver (ng As/g of tissue) from a mouse exposed to iAs^{III} in drinking water (50 ppm As) for 9 days. Results of the direct analysis of fresh liver homogenate and the homogenate digested in phosphoric acid.

As species	Analysis of digested liver homogenate (a)	Direct analysis of fresh liver homogenate (b)	
	ng As/g of tissue	ng As/g of tissue	% Recovery (c)
iAs^{III}		151 ± 15	
iAs^{V}		158 ± 16	
iAs^{III+V}	392 ± 12	309 ± 7	79 ± 3
MAs^{III}		220 ± 5	
MAs^{V}		143 ± 6	
MAs^{III+V}	390 ± 1	363 ± 4	93 ± 1
$DMAs^{III}$		828 ± 11	
$DMAs^{V}$		324 ± 16	
$DMAs^{III+V}$	1069 ± 10	1152 ± 11	108 ± 2
Total As^{III+V}	**1851 ± 16**	**1824 ± 14**	**99 ± 1**

A total of 9 aliquots of the homogenate prepared from 1 section of the liver were analyzed by HG-CT-AAS:

(a) 3 aliquots were microwave digested in phosphoric acid at $90^{o}C$ for 10 hours and analyzed for As^{III+V} species. (Mean $\pm$ SD are shown for n = 3)

(b) 6 aliquots were analyzed directly: 3 aliquots were analyzed for As^{III} species (without pretreatment) and 3 aliquots were analyzed for As^{III+V} species after pretreatment with 2% L-cysteine. The concentrations of As^{V} species were calculated as the difference between the two measurements. (Mean $\pm$ SD are shown for n = 3 replicate measurements of homogenate from 1 liver section)

(c) % recovery = (ng As^{III+V} determined by the direct analysis / ng As^{III+V} determined in the digested homogenate) x 100
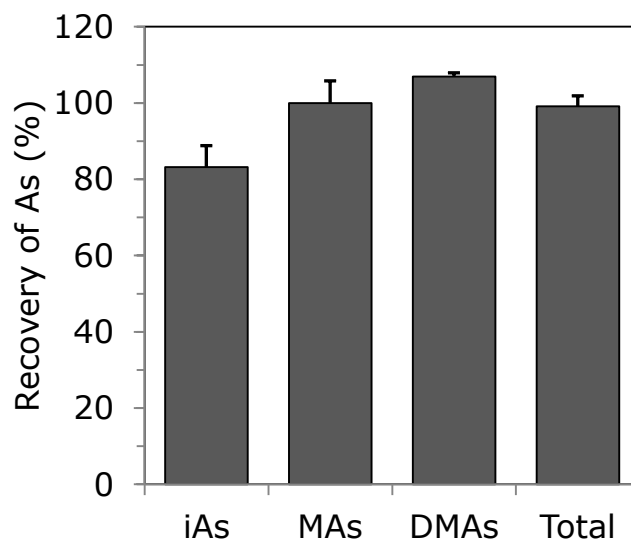
Figure 2.2: Recovery of total speciated As during the direct analyses of fresh (undigested) homogenates prepared from four sections of the liver of a mouse exposed to iAs^{III} (50 ppm As) for 9 days (mean ± SD, n = 4). The homogenates were analyzed by HG-CT-AAS as described for **Figure 2.1**. The % recovery was calculated as (As^{III+V} analyzed directly/ As^{III+V} after digestion) x 100.

CHAPTER III

Direct Analysis and Stability of Methylated Trivalent Arsenic

Metabolites in Cells and Tissues[2]

3.1 Overview

Chronic ingestion of water containing inorganic arsenic (iAs) has been linked to a variety of adverse health effects, including cancer, hypertension and diabetes. Current evidence suggests that the toxic methylated trivalent metabolites of iAs, methylarsonous acid (MAs^{III}) and dimethylarsinous acid ($DMAs^{III}$) play a key role in the etiology of these diseases. Both MAs^{III} and $DMAs^{III}$ have been detected in urine of subjects exposed to iAs. However, the rapid oxidation of $DMAs^{III}$ and, to a lesser extent, MAs^{III} in oxygen-rich environments leads to difficulties in the analysis of these metabolites in samples of urine collected in population studies. Results of our previous work indicate that MAs^{III} and $DMAs^{III}$ are relatively stable in a reducing cellular environment and can be quantified in cells and tissues. In the present study, we used the oxidation state-specific hydride generation-cryotrapping-atomic absorption spectroscopy (HG-CT-AAS) to examine the presence and stability of these trivalent metabolites in the liver of mice and in UROtsa/F35 cells exposed to iAs. Tri- and pentavalent metabolites of iAs were analyzed directly (without chemical extraction or digestion). Liver homogenates prepared in cold deionized water and cell culture medium and lysates were stored at either 0°C or -80°C for up to 22 days. Both MAs^{III} and $DMAs^{III}$ were stable in homogenates stored at

-80°C. In contrast, DMAs^{III} in homogenates stored at 0°C began to oxidize to its pentavalent counterpart after 1 day; MAs^{III} remained stable for at least 3 weeks under these conditions. MAs^{III} and DMAs^{III} generated in UROtsa/F35 cultures were stable for 3 weeks when culture media and cell lysates were stored at 80°C. These results suggest that samples of cells and tissues represent suitable material for the quantitative, oxidation state-specific analysis of As in laboratory and population studies examining the metabolism or toxic effects of this metalloid.

3.2 Introduction

Inorganic arsenic (iAs) is a natural, carcinogenic metalloid found in water sources worldwide, most commonly as arsenite (iAs^{III}) and arsenate (iAs^V) (16). The ingestion of drinking water containing high levels of iAs is associated with an array of adverse health effects, including cancer of the skin, lungs, liver and urinary bladder (1). Non-neoplastic effects of iAs exposure include vascular disease, hypertension, skin lesions and diabetes (3, 155, 163). Chronic toxicity due to drinking water with high levels of iAs can lead to a collection of these symptoms, known as arsenicosis (164). The United States Environmental Protection Agency and World Health Organization lowered the safe level for As in drinking water from 50 to 10 ppb in response to evidence of iAs toxicity even at low exposure levels (6, 7). For the tens of millions of people chronically exposed to iAs, outcomes vary widely and depend not only on the level of exposure but also on the inter-individual differences in As metabolism. Exposure levels and the pattern of iAs metabolism are commonly assessed through analysis of iAs metabolites in urine; however, concentrations of these metabolites in human tissues are not well researched.

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The metabolism of iAs in humans is mediated by arsenic (+3 oxidation state) methyltransferase (AS3MT) (53). The AS3MT-catalyzed and S-adenosylmethionine-dependent methylation of iAs yields both trivalent and pentavalent methylated arsenicals, including methylarsonic acid (MAs^V), methylarsonous acid (MAs^{III}), dimethylarsinic acid (DMAs^V) and dimethylarsinous acid (DMAs^{III}) (59, 60). Several intronic and exonic polymorphisms have been described for the human AS3MT gene, including Met287The (T - C), potentially altering the rates and yields of iAs methylation and contributing to the inter-individual differences in susceptibility to iAs toxicity (67, 72).

While the methylation of iAs is critical for its detoxification, recent evidence suggests that methylated trivalent As metabolites (MAs^{III} and DMAs^{III}) generated in the course of iAs metabolism in human cells and tissues are more cytotoxic and genotoxic than their pentavalent counterparts or iAs species (8). MAs^{III} and DMAs^{III} are also potent enzyme inhibitors, alter cell signaling pathways, and induce oxidative damage (9-11). Moreover, these As metabolites have been shown to inhibit insulin signaling and insulin-stimulated glucose uptake in cultured murine adipocytes, providing a potential mechanism for the diabetogenic effects of iAs exposure (86, 140).

The analysis of iAs metabolites in human population studies has been typically limited to urine. The half life of iAs in the human body measures in days. Thus, although urine is an important source of information about exposure to iAs, the urinary metabolites reflect only recent exposures (165). Furthermore, analysis of As species in urine of chronically exposed individuals produces a wide range of responses, making it difficult to elucidate the mechanisms responsible for iAs toxicity and the direct effects on target human tissues, such as, skin, urinary bladder, lungs, and liver. Interestingly, *in vivo*

studies indicate that urinary excretion of iAs and its metabolites does not correspond to tissue distribution of these As species (157, 158). For example, in mice exposed to iAs^V in drinking water the predominant accumulation of MAs in the kidney and DMAs in the lungs has been reported (157, 158). Furthermore, a study comparing human urinary metabolites of iAs to As species retained in bladder exfoliated cells found no correlation, suggesting that monitoring urine may not give a clear idea of As species stored in specific tissues (85). Thus, identification and quantification of iAs metabolites in human tissues, including the target organs, or in cells originating from these tissues could provide important information about the risk associated with iAs exposure and about the mechanism underlying the adverse effects of this exposure.

The oxidation state-specific speciation analysis of As in urine is complicated mainly by the low stability of methylated trivalent arsenicals, particularly DMAs^{III}. DMAs^{III} can be completely converted to DMAs^V in several hours even in frozen urine; MAs^{III} can oxidize in several months (14). However, the rate of oxidation varies and depends, in part, on individual urine composition, further confusing the interpretation of the analytical data. To date, no attempt has been made to characterize the stability of the methylated trivalent As species in tissues and cells. Mammalian cells contain millimolar concentrations of a potent low-molecular weight reductant, glutathione (GSH), which is known to bind trivalent arsenicals, producing As^{III}–thiol complexes (166). Thus, the binding to GSH or to protein thiols is likely to protect the methylated trivalent arsenicals from oxidation and extend their lifetime inside the cells. The main objective of this study was to optimize the hydride generation-cryotrapping-atomic absorption spectroscopy (HG-CT-AAS) system for analysis of tissues and cells and to characterize the stability of

trivalent arsenicals, particularly DMAs^{III} in these complex biological matrices. Here we report, for the first time, that DMAs^{III} is relatively stable in tissue homogenates and cell lysates under conditions that are typically used for storage and transport of biological samples collected in laboratory and population studies. We, therefore, suggest that samples of the tissues targeted by iAs exposure or cells originated from these tissues are used to obtain more complete information about the metabolism of iAs or about the mechanisms of iAs toxicity in animals and humans.

3.3 Methods

3.3.1 Arsenicals

The following arsenicals were used for calibration and in cell culture experiments: sodium arsenite (NaAs^{III}O$_2$) and sodium arsenate (Na$_2$HAs^{V}O$_4$, 99% pure) were purchased from Sigma-Aldrich (St. Louis, MO). Methylarsonic acid, disodium salt (CH$_3$As^{V}O(ONa)$_2$), and dimethylarsinic acid ((CH$_3$)$_2$As^{V}O(OH)) both better than 98% pure were purchased from Chem Service (West Chester, PA). Oxomethylarsine (CH$_3$AsO)$_4$ which forms MAs^{III} in aqueous solution was provided by Dr William Cullen (University of British Columbia, Vancouver, Canada). The As content in each of the standards was determined by graphite furnace-AAS (146).

3.3.2 Mice and treatments

Twelve week-old C57BL/6 mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA) and housed in polycarbonate cages (2 per cage) with corn cob bedding at the University of North Carolina Animal Facility (12 h light/dark cycle, 22 ± 1°C and humidity 50 ± 10%), accredited by the American Association for Accreditation

of Laboratory Animal Care. Mice were allowed free access to food (Lab Diet 5058, Nutrition International, Brentwood, MO) and pure deionized water (DIW) or DIW containing iAs^{III} (50 mg As per L, i.e. 50 ppm). Mice were euthanized by cervical dislocation. Freshly dissected mice livers were processed as previously described (167). Liver homogenates were prepared in ice cold DIW (10% w/v). All procedures involving mice were approved by the UNC Institutional Animal Care and Use Committee.

3.3.3 Cultured cells and treatments

The SV-40 transformed human urothelial cell line, UROtsa, was obtained from Dr Unimye (Department of Urology, West Virginia University). The UROtsa/F35 clonal cell line expressing rat As3mt and capable of As methylation was previously generated from the parental UROtsa cell line using a retroviral pLEGFP-N1 gene delivery system (54). In the present study, UROtsa/F35 cells were cultured in MEM (Mediatech, Manassas, VA) supplemented with 10% heat inactivated FBS (Gemini Bio-Products, Sacramento, CA), 50 U penicillin (Sigma) per mL and 50 mg streptomycin (Sigma) per mL. Cells were cultured in 6-well plates (Corning, Inc., Corning, NY) at 37°C in a humidified incubator with a 95% air/5% CO_2 atmosphere.

To generate $DMAs^{III}$, near confluent cells in 6-well plates were incubated with MAs^{III} in 2 mL of MEM/well for varying lengths of time. After incubation, media were aspirated into capped tubes, cells were lysed on ice in pre-chilled DIW for 20 minutes and harvested by scraping with a pipette tip to reduce transfer losses. In some experiments, cells were lysed in 0.5% Triton X-100. Control, unexposed UROtsa/F35 cells were used to establish the background levels of As species in the cell culture.

3.3.4 Depletion of intracellular GSH

In some experiments, the cultured UROtsa/F35 cells were treated with butathione sulfoximine (BSO), the inhibitor of GSH synthesis. BSO was added to the culture medium at a final concentration of 250 mM BSO for 24 hours. The GSH concentrations were measured before and after BSO treatment in cell lysates prepared in 2% sulfosalicylic acid, using a previously described enzymatic recycling assay (168).

3.3.5 Speciation analysis of As by HG-CT-AAS

Arsenic species were analyzed by a semi-automated HG-CT-AAS using a AAnalyst 800 spectrometer (Perkin-Elmer, Norwalk, CT, USA) equipped with a multiple microflame quartz tube atomizer (multiatomizer) and coupled to a cryotrap. The HG and CT steps were controlled by a FIAS 400 flow injection accessory (Perkin-Elmer) (146, 149). Using this system, arsines from trivalent arsenicals (iAs^{III}, MAs^{III} and DMAs^{III}) are generated directly at pH 6; to generate arsines from both tri- and pentavalent arsenicals (iAs^{III+V}, MAs^{III+V} and DMAs^{III+V}), the As^V-standards and samples are pre-reduced with L-cysteine (EMD Chemicals Inc., Gibbstown, NJ) (146, 149). The concentrations of iAs^V, MAs^V, and DMAs^V are then determined as a difference between AAS signals obtained for cysteine-treated and untreated sample aliquots.

We have previously shown that the slopes of the calibration curves for the cysteine-treated As^V-standards prepared in DIW are identical with the slopes of curves generated for the trivalent arsenicals in the absence of cysteine (149). We have also shown that the slopes of the calibration curves for the cysteine-treated aqueous solutions of As standards do not significantly differ from the slopes of curves prepared for liver homogenates spiked with these standards and treated with cysteine (167). Therefore,

calibration curves for quantification of tri- and pentavalent As species in this study were generated using aqueous solutions of the pentavalent standards (iAs^V, MAs^V, and $DMAs^V$) treated with cysteine.

3.3.6 Acid digestion

To determine the recovery of As species during the direct analyses of mouse liver homogenates, aliquots of the homogenates were acid digested using a MARS Microwave Reaction System equipped with MarsXpress Temperature Control (CAM, Matthews, NC). Here, 100 mL of 10% homogenate was mixed with 3 mL of 2M ultrapure phosphoric acid (EMD Chemicals, Inc.) and microwaved for 10 hours at 90°C. One milliliter of the digestate was neutralized with 0.25 mL of 10 N NaOH to a final pH of ~6. Because this digestion oxidizes all trivalent arsenicals to their pentavalent counterparts, the neutralized digestates were treated with 2% cysteine for 1 hour prior to analysis (167).

3.3.7 Statistical analysis

All statistical analyses were performed using GraphPad Instat software package (GraphPad Software Inc., San Diego, CA). Linear regression and correlation analyses were employed to characterize the calibration curves. ANOVA followed by Bonferroni's multiple comparison posttest was used to determine significant differences in As concentration between fresh, immediately analyzed samples and each time point during the storage of samples at either 0°C or -80°C. This test was also used to determine differences in $DMAs^{III}$ present in cell lysates after lysis with either DIW or 0.5% Triton X-100. Statistical significance was considered at the level of $p < 0.01$.

3.4 Results and discussion

3.4.1 Speciation analysis of As in biological matrices

The growing number of reports on widespread exposures to iAs in drinking water has triggered an intensive search for biomarkers that could serve as indicators of these exposures or could predict the susceptibility of individuals to diseases associated with chronic toxicity of iAs. For many years, the urinary concentrations of As species that originate from iAs metabolism in human tissues have been used as the exposure indicators. In addition, the composition of iAs metabolites in human urine (e.g., DMAs/MAs ratio) is believed to provide information about the capacity of individuals to methylate and thus to detoxify iAs (169, 170). The attempts to determine concentrations of iAs metabolites, especially the toxic MAs^{III} and DMAs^{III} in tissues, have been rare and suffered problems associated with the lack of suitable analytical techniques and with low stability of these methylated trivalent arsenicals.

We have shown that in rat liver cytosol large portions of iAs and its methylated metabolites are bound to proteins (153). The binding of As species to proteins that has also been reported by others (62, 171) makes it necessary to perform digestions or extraction when chromatographic methods are used for speciation analysis of As in cells, tissues, or tissue fractions. However, both digestion and extraction can lead to oxidation or losses of the unstable and highly reactive methylated As^{III} species (148). Our previous work has provided strong evidence that, in spite of their limited specificity, HG-based techniques coupled with a sensitive detector may provide a unique tool for the detection and quantification of MAs^{III} and DMAs^{III} in the biological matrices as complex as cultured cells or tissue (144, 146, 149, 167). The present study continues this work by

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examining stability of the methylated trivalent arsenicals in liver homogenates and cell lysates using a HG-CT-AAS technique developed by our laboratories.

3.4.2 Optimization of HG-CT-AAS for analysis of slurries

The previously described HG-CT-AAS system was developed for analysis of solutions and relatively simple liquid biological matrices (146, 149). This system was optimized in the present study for analysis of complex biological slurries, including tissue homogenates and crude cell lysates (**Figure 3.1**). To avoid plugging of the FIAS 400 injection port with tissue and cell fragments, the homogenates and cell lysates were introduced directly to the manifold. The sample (typically 500 mL) was pipetted into a 1 mL pipette tip connected to the FIAS 400 peristaltic pump. From the pipette tip, the sample was pumped into the system followed by 500 mL of DIW. The sample was then mixed with equal flows (2 mL min^{-1}) of Tris-HCl buffer (pH 6) and NaBH$_4$ solutions prior to entering the reaction coil and the gas–liquid separator. For drying of the gaseous phase prior to the cryotrapping stage, a cartridge filled with NaOH pellets was included (172). The cryotrapping step and AAS detection were performed without modifications as previously described (146).
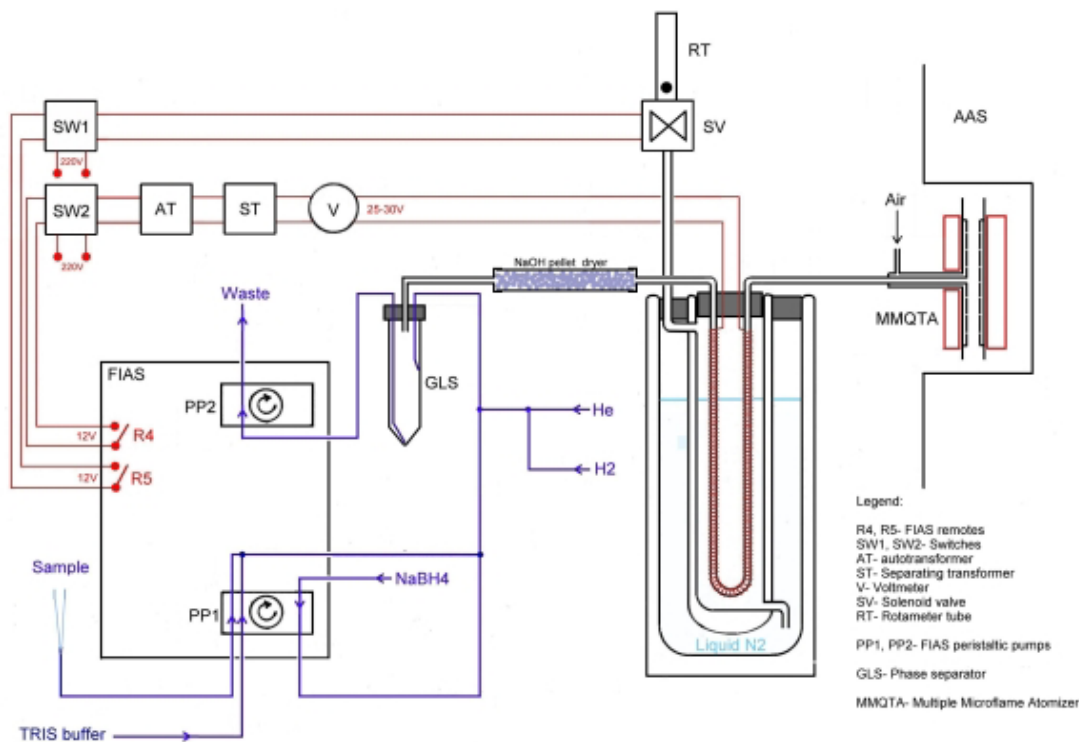
Figure 3.1: The optimized HG-CT-AAS system for slurry sampling.

Further optimization focused on the pre-reduction with cysteine and on the components of the HG mixture. Here, liver homogenate from a single mouse exposed to iAs^{III} (50 ppm As) in drinking water was aliquoted, pre-treated with cysteine (0.5, 2 or 4%) for up to 80 minutes and analyzed directly by HG-CT-AAS. Results of the direct analysis suggest that treatment with 0.5–2% cysteine for at least 20 minutes is optimal for generation of arsines from all As species present in the homogenate (**Figure 3.2**). It is unclear why the AAS signals were lower for homogenate aliquots treated with 4% cysteine. It is possible that portions of arsenicals in homogenates were reduced by high cysteine concentration to form volatile arsines, thus decreasing the amounts of arsines generated during the HG step.
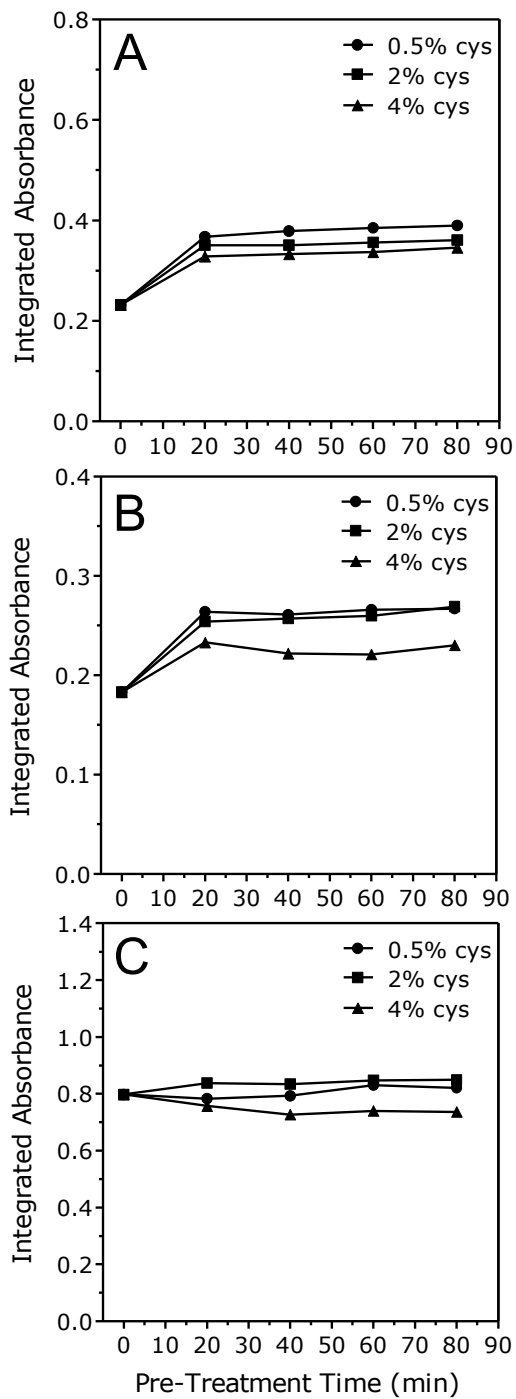
Figure 3.2: Peak areas recorded for iAs^{III+V} (A), MAs^{III+V} (B), and DMAs^{III+V} (C) during the direct HG-CT-AAS analysis of aliquots of a mouse liver homogenate pre-treated with 0.5, 2, or 4% cysteine for up to 80 minutes. Time 0 minutes shows results of the direct analysis of liver homogenates prior to the cysteine treatment. Each point represents a single measurement.

In the next step, AAS signals were tested in the reaction mixtures containing liver homogenates treated for 1 hour with 2% cysteine and varied concentrations of Tris-HCl (pH 6) or $NaBH_4$. For this test we pooled liver homogenates from several mice exposed to 50 ppm As in drinking water. No significant differences in AAS signals were measured in mixtures containing 1% $NaBH_4$ and 0.2 M to 1 M Tris, even though the pH measured after the HG reaction decreased from 8.3 to 6.9 (**Figure 3.3**). Similarly, changing $NaBH_4$ concentration (0.5% to 2.5%) in a reaction mixture containing 0.75 M Tris had little effect on the HG efficiency (Fig. 3.4).
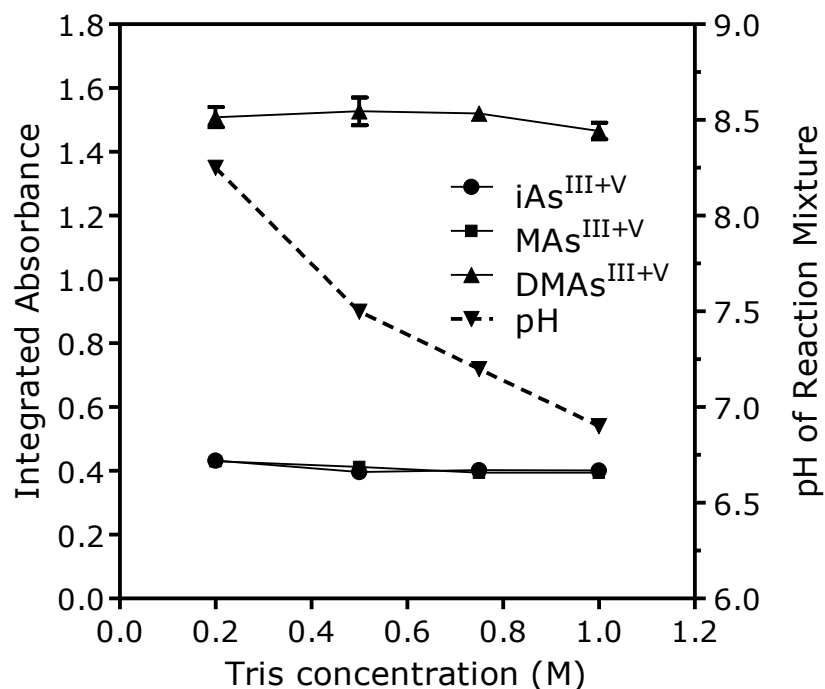
Figure 3.3: HG-CT-AAS analysis of pooled mouse liver homogenates pre-treated with 2% cysteine for 1 hour: effect of Tris buffer concentration in the HG reaction mixture on AAS signals for iAs^{III+V}, MAs^{III+V}, and $DMAs^{III+V}$. Each point represents mean ± SD for n = 3.
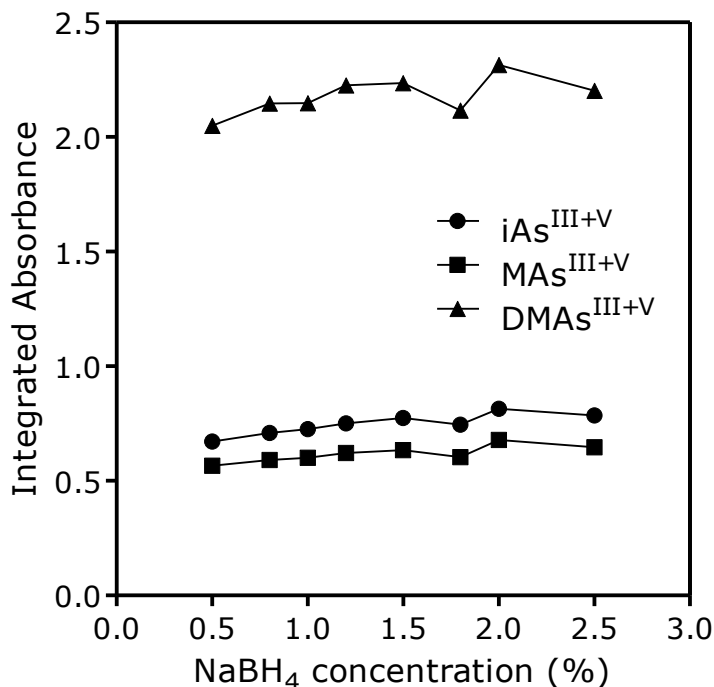
Figure 3.4: HG-CT-AAS analysis of pooled mouse liver homogenates pre-treated with 2% cysteine for 1 hour: effect of NaBH$_4$ concentration in the HG reaction mixture on AAS signals for iAs^{III+V}, MAs^{III+V}, and DMAs^{III+V}. Each point represents mean ± SD for n = 3.

Thus, each of the experimental parameters we examined exhibits a relatively broad optimum, which is important for achieving robust and reliable analytical conditions. Based on these results, we concluded that the 1 hour treatment with 2% cysteine and the HG mixture containing 0.75 M Tris and 1% NaBH$_4$ is optimal for analysis of liver homogenates. Notably, the same reaction conditions were previously used in our laboratories for analyses of liquid samples (146, 149). To examine As recoveries under these conditions, additional aliquots of the homogenates were analyzed after digestion in phosphoric acid. The analysis of acid-digested homogenates showed that the direct analysis of the homogenates treated with 2% cysteine recovered ~100% of

MAs^{III+V} and DMAs^{III+V}. Consistent with our previous report, (167) the average recovery of iAs^{III+V} was lower, reaching only about 85%.

The 1 hour treatment with 2% cysteine and the HG mixture containing 0.75 M Tris and 1% NaBH$_4$ was used in all following experiments examining the stability of trivalent arsenicals in mouse liver homogenates and cell lysates.

3.4.3 Stability of trivalent arsenicals in mouse liver homogenates

The stability of As^{III} species was examined in a 10% homogenate prepared from a liver of a single mouse exposed to iAs^{III} in drinking water (50 ppm As) for 9 days. The homogenate was prepared in ice-cold DIW and immediately aliquoted. Three freshly prepared aliquots were analyzed directly for As^{III} species; another 3 aliquots were pretreated with 2% cysteine and analyzed for As^{III+V} species. The As^{III+V} species were also analyzed in acid digested aliquots of the homogenate. To determine the stability of trivalent arsenicals, remaining aliquots were stored at 0°C (on ice) or -80°C and analyzed for As^{III} and As^{III+V} species after 1, 6, 13, and 22 days (**Figure 3.5**).

In the fresh homogenate, trivalent arsenicals accounted for 65% of the sum of As species: iAs^{III} (8%), MAs^{III} (12%), and DMAs^{III} (45%). However, the recovery of iAs^{III+V} was only about 82% (**Figure 3.5A,B**), suggesting that a part of iAs, possibly protein-bound iAs^{III}, is not available for the HG reaction under these conditions. The recoveries of iAs remained low for up to 13 days, but increased at day 22 for aliquots of the homogenate stored either at 0°C (105%) or at -80°C (113%) due to increased levels of iAs^V. These data suggest that a prolonged storage may result in the release of iAs^{III} from high-affinity binding sites, followed by oxidation of iAs^{III} to iAs^V. Notably, both MAs^{III} and DMAs^{III} were relatively stable in aliquots of the homogenate stored at -80°C for up

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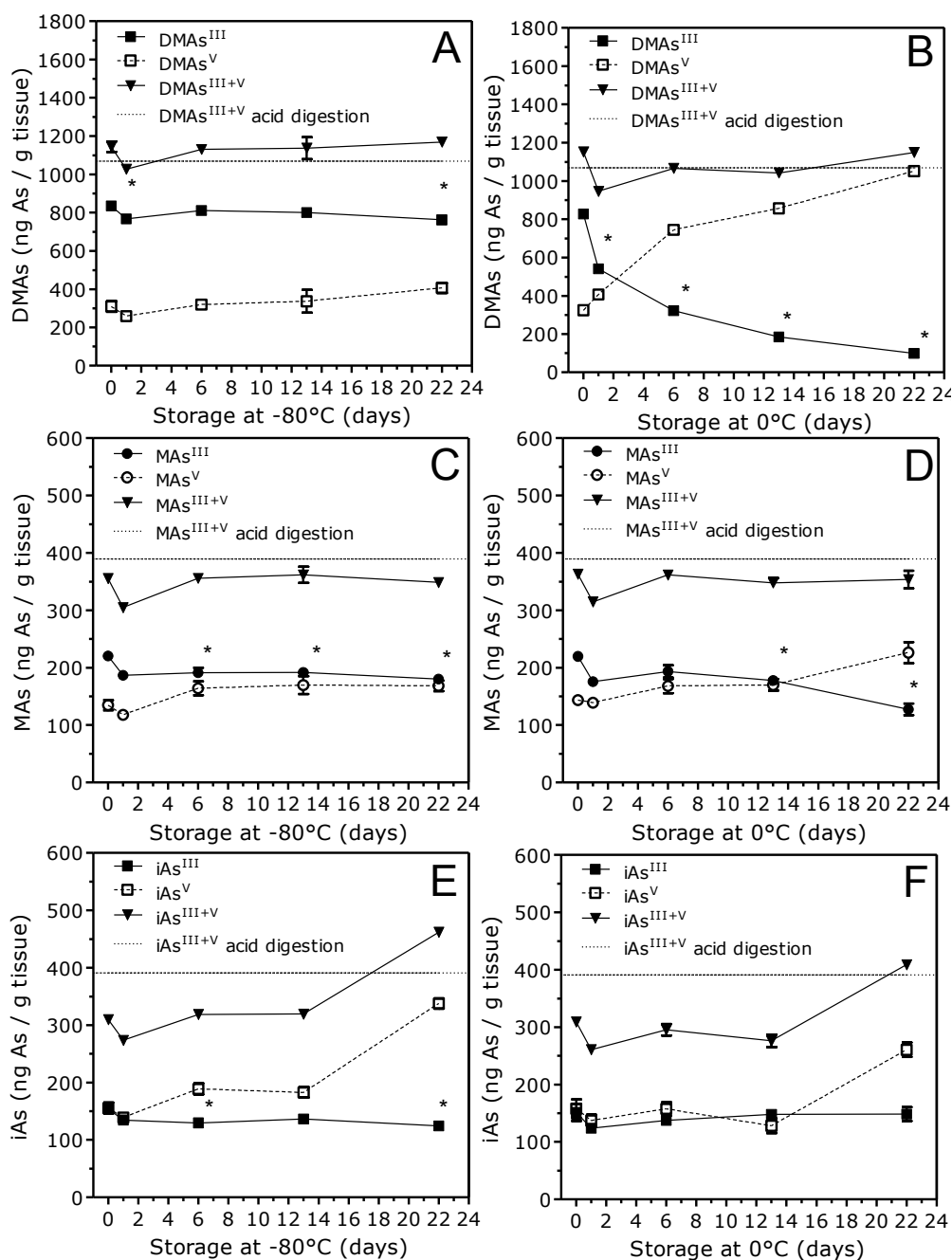
Figure 3.5: Stability of trivalent arsenicals in a liver homogenate from a mouse exposed to iAs[III] in drinking water (50 ppm As) for 9 days: liver homogenate was prepared in DIW. The direct HG-CT-AAS analysis was used to determine the concentrations of iAs (A, B), MAs (C, D) and DMAs (E, F) species in aliquots of the fresh homogenate and in aliquots stored at –80°C (A, C, E) or 0°C (B, D, F) for up to 22 days (mean ± SD, n = 3). To control for As recoveries during the direct analyses, iAs[III+V], MAs[III+V], and DMAs[III+V] were determined in aliquots of the fresh homogenate digested in phosphoric acid (mean, n = 3). *The concentration is significantly different from that found in the fresh homogenate (p < 0.01).

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to 22 days. Only about 18% of MAs^{III} and 9% of DMAs^{III} oxidized under these conditions (**Figure 3.5C,E**). In contrast, 42% of MAs^{III} and 88% of DMAs^{III} oxidized in aliquots of the homogenate stored at 0°C (**Figure 3.5 D,F**).

3.4.4 Generation and stability of DMAs^{III} in UROtsa/F35 cells

We have previously shown that cultured UROtsa/F35 cells treated with MAs^{III} produce DMAs^{III} and DMAs^{V} (54). In the present study, DMAs^{III} was generated in UROtsa/F35 culture exposed to 0.1 mM MAs^{III} (15 ng As per well) for 18 hours. DMAs^{III} levels were monitored in the culture medium and in cell lysates that were prepared in ice-cold DIW. **Figure 3.6** shows that DMAs^{III} was the major product of MAs^{III} methylation and that a substantial portion of DMAs^{III} was retained by cells. DMAs^{III} represented almost 50% and 90% of all As species associated with the culture medium and cells, respectively. The stability of DMAs^{III} was examined in both the medium and cell lysates prepared in DIW and stored at either 0°C or -80°C for up to 23 days. DMAs^{III} in cell lysates was stable regardless of the storage temperature; DMAs^{III} in culture medium was stable only at -80°C (**Figure 3.7**). In contrast, almost 50% of DMAs^{III} oxidized in the medium stored at 0°C.
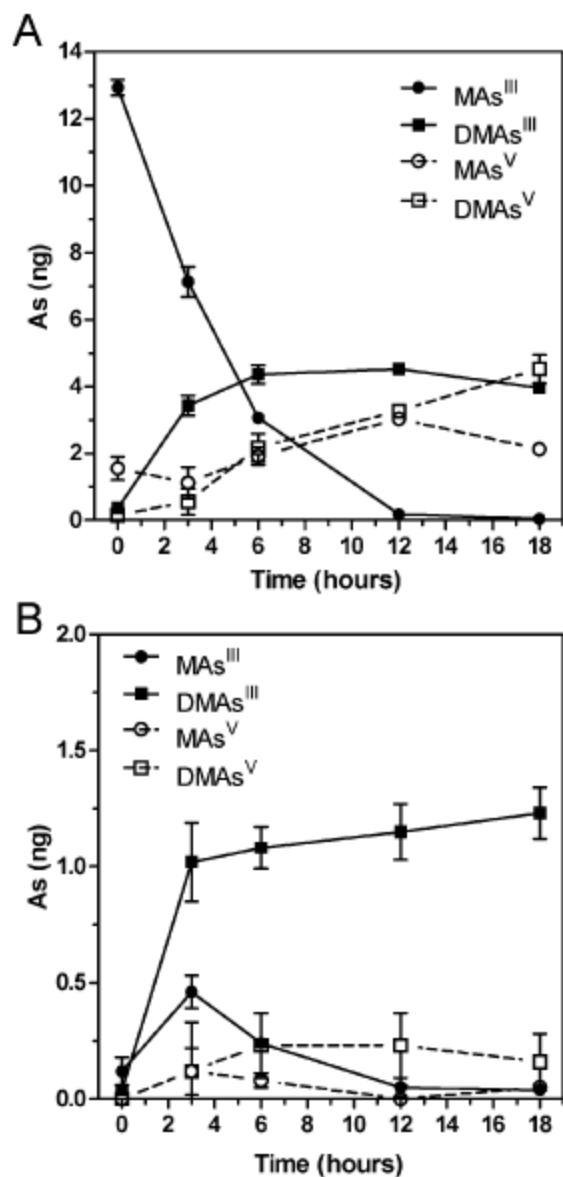
Figure 3.6: Generation of DMAs^{III} in UROtsa/F35 culture exposed to 0.1 mM MAs^{III} (15 ng As per well) for up to 18 hours. Tri- and pentavalent As species were measured by HG-CT-AAS in culture medium (A) and cell lysates (B) before and after pretreatment with 2% cysteine (mean ± SD, n = 3).

We also tested the stability of DMAs^{III} in cell lysates prepared in an ice-cold solution of 0.5% Triton X-100, a non-ionic laboratory detergent that is commonly used in biomedical studies for sample processing. We found that fresh cell lysates prepared in

Triton X-100 contained significantly less DMAs^{III} than the lysates prepared in DIW. The oxidation state specific analysis showed that 54% of DMAs^{III} in these lysates oxidized to DMAs^{V} (**Figure 3.8**).
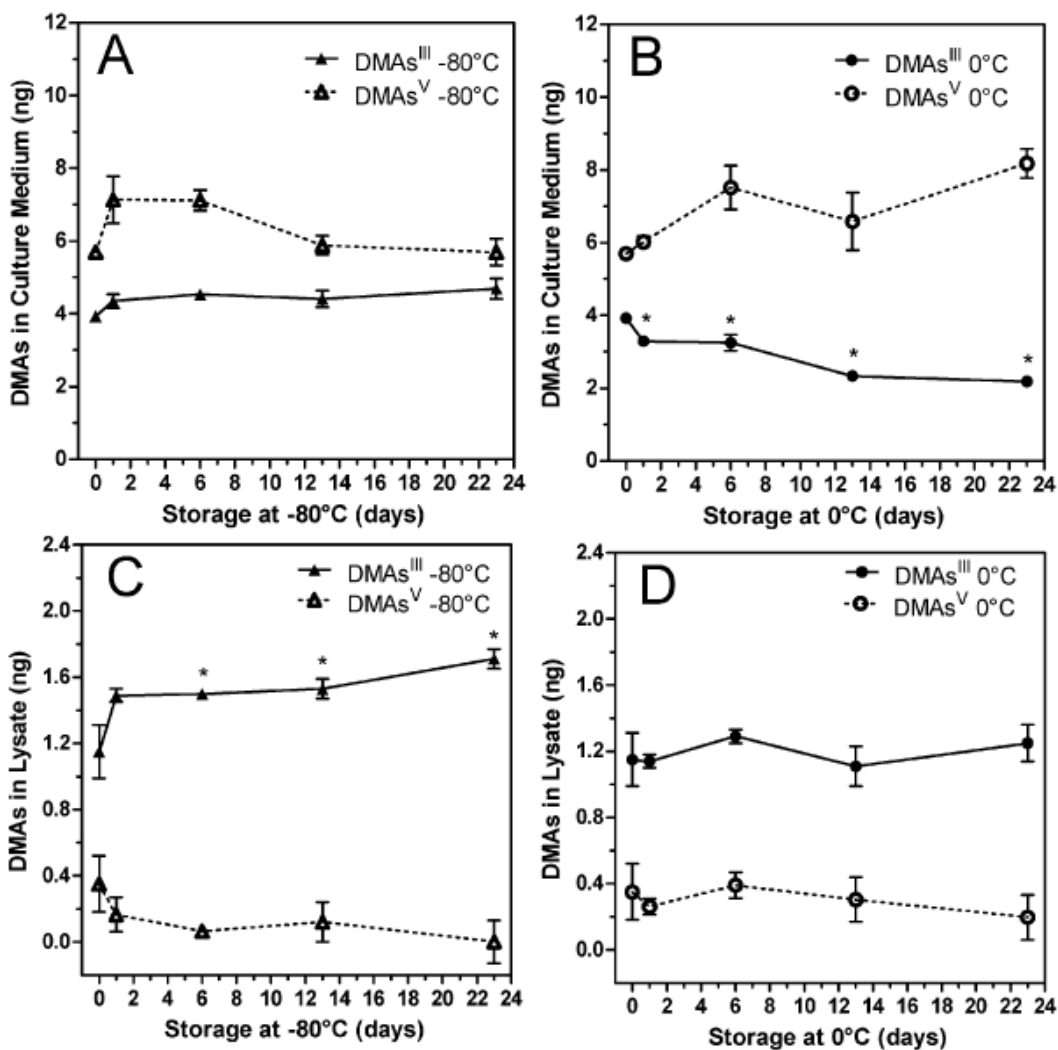
Figure 3.7: Stability of DMAs^{III} in medium and cell lysates from UROtsa/F35 culture exposed to 0.1 μM MAs^{III} (15 ng As per well) for 18 hours: DMAs^{III} and DMAs^{V} were analyzed in culture medium (A, B) and cell lysates (C, D) immediately after the exposure and after storage at -80°C or 0°C for up to 23 days (mean ± SD, n = 3). *The concentration is significantly different from that found in fresh medium or cell lysate (p < 0.01).

To assess the role of GSH in the stability of DMAs^{III}, UROtsa/F35 cells were incubated with BSO for 24 hours and then exposed for 18 hours to MAs^{III}. The treatment with BSO decreased the intracellular GSH concentration to 15% of the original level, but had no effect on the stability of DMAs^{III} in cell lysates prepared in DIW and stored at either -80°C or 0°C (data not shown). Thus, it is possible that the remaining GSH provided sufficient protection for DMAs^{III} in cell lysates or that DMAs^{III} was primarily bound to protein thiols and could not be affected by BSO treatment.
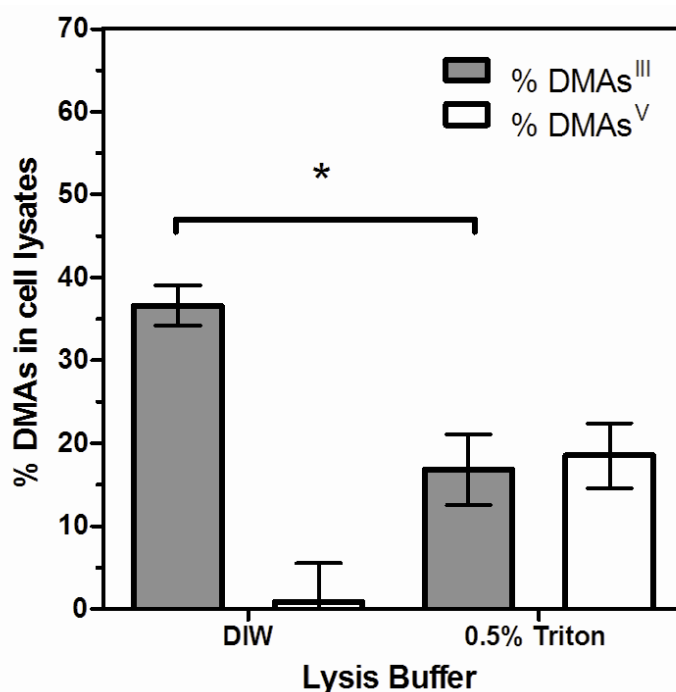
Figure 3.8: Effect of Triton X100 on DMAs^{III} stability in cell lysates: UROtsa/F35 cells were exposed to 0.1 μM iAs^{III} (15 ng As/well) for 24 hours. Cells were then lysed in either DIW or 0.5% Triton X-100. DMAs^{III} and DMAs^{V} were analyzed in fresh cell lysates by HG-CT-AAS. Values represent the percentage of total As in lysate (mean ± SD, n=3). * The percentage of DMAs^{III} in lysates prepared in Triton X100 is significantly different from that in lysates prepared in DIW ($p < 0.01$).

Finally, we examined the stability of DMAs^{III} under conditions that are consistent with a shipment of samples from field studies to analytical laboratories. Here,

UROtsa/F35 cells were exposed to 0.5 mM MAs^{III} (75 ng As per well) for 18 hours; aliquots of cell lysates prepared in DIW and aliquots of culture medium were placed in two polystyrene shipping containers filled with dry ice and two additional containers filled with ice packs that were pre-frozen at -80°C. One of the containers with dry ice and one with ice packs were stored at UNC Chapel Hill and DMAs^{III} was analyzed in aliquots of the stored samples for up to 7 days. The other containers were shipped by an express postal service to Prague, Czech Republic for analysis in the Institute of Analytical Chemistry. The HG-CT-AAS analysis at UNC found that DMAs^{III} is stable in both cell lysates and media stored in dry ice for 2 days; however, significant loses of DMAs^{III} occurred at day 5 and 7 (**Figure 3.9**). DMAs^{III} oxidized faster in samples stored with ice packs. The shipment to Prague was delayed in customs for 4 days, resulting in a significant oxidation of DMAs^{III} in culture medium and cell lysates shipped in either dry ice or ice packs (**Figure 3.10**).
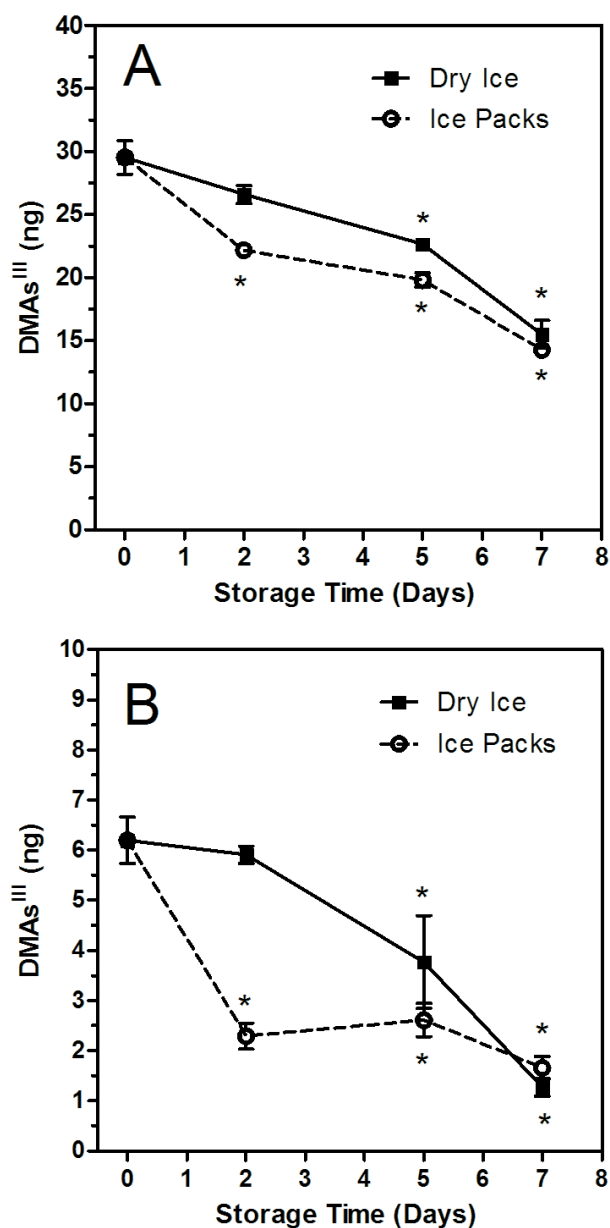
Figure 3.9: The oxidation of DMAs[III] in UROtsa/F35 culture medium (A) and cell lysates (B) placed in shipping containers packed with dry ice or with pre-frozen ice packs (mean ± SD, n = 3). Cells were exposed to 0.5 μM MAs[III] (75 ng As/well) for 18 hours, lysed in cold DIW and then aliquoted for storage and analysis by HG-CT-AAS. * The concentration of DMAs[III] is significantly different from that in fresh cell lysates or culture medium ($p < 0.01$).
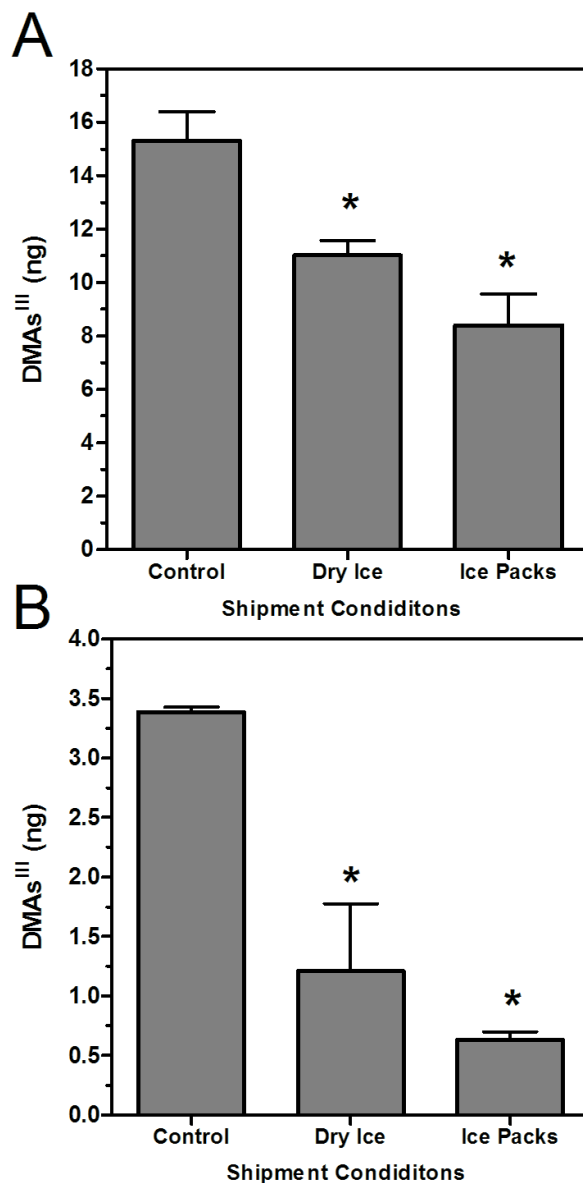
Figure 3.10: The oxidation of DMAs^{III} in UROtsa/F35 culture medium (A) and cell lysates (B) shipped to Prague, Czech Republic: DMAs^{III} concentrations cell lysates and medium were measured immediately after exposure to 0.5 μM MAs^{III} (75 ng As/well) for 18 hours and after shipment to Prague (mean ± SD, n = 4). The samples were shipped in containers filled with either dry ice or pre-frozen ice packs. The samples were analyzed in Prague 8 days after shipment due to a 4 day customs delay. * The concentration of DMAs^{III} is significantly different from the concentration determined in fresh cell lysates or culture medium (p < 0.01).

3.5 Conclusions

Previous work has shown that methylated trivalent metabolites of iAs, MAs^{III} and DMAs^{III} are unstable in human urine (14). We have recently reported that up to 50% of DMAs^{III} can oxidize during 24 hours even in urine stored in dry ice, (70) making it difficult to detect and quantify this metabolite in urine samples collected in population studies. The work presented here confirms that the trivalent metabolites of iAs, iAs^{III}, MAs^{III}, and DMAs^{III} can be detected and quantified in fresh cell lysates and tissue homogenates by direct HG-CT-AAS analysis. The optimum conditions for analysis of the pentavalent arsenicals include the pre-treatment with 2% cysteine for 60 minutes and generation of arsines in the reaction mixture containing 1% NaBH$_4$ and 0.75M Tris buffer (pH 6). Notably, the methylated trivalent metabolites of iAs, including DMAs^{III}, are stable for at least 3 weeks in cell lysates and tissue homogenates prepared in cold DIW and stored at -80°C. When packed in dry ice, these types of samples can be shipped without major losses of DMAs^{III}, as long as the shipping time does not exceed 2 days and dry ice is not depleted during the shipment.

In summary, results of the present study suggest that, unlike urine, samples of tissues or cells collected in human population studies provide suitable material for the quantitative, oxidation state specific analysis of As species, assuming that these samples are properly handled and stored prior to the analysis. Thus, while urine analysis helps to estimate the levels of exposure to iAs and to evaluate the efficiency of iAs methylation, the analysis of tissues or cells may provide important information about the internal dose and chemical species of As, including highly toxic but unstable MAs^{III} and DMAs^{III}, in target tissues.

CHAPTER IV

Comparative Oxidation State Specific Analysis of Arsenic by High-Performance Liquid

Chromatography-Inductively Coupled Plasma-Mass Spectrometry and Hydride

Generation-Cryotrapping-Atomic Absorption Spectrometry[3]

4.1 Overview

The formation of methylarsonous acid (MAs^{III}) and dimethylarsinous acid (DMAs^{III}) in the course of inorganic arsenic (iAs) metabolism play an important role in the adverse effects of chronic exposure to iAs. High-performance liquid chromatography-inductively coupled plasma-mass spectrometry (HPLC-ICP-MS) and hydride generation-cryotrapping-atomic absorption spectrometry (HG-CT-AAS) have been frequently used for the analysis of MAs^{III} and DMAs^{III} in biological samples. While HG-CT-AAS has consistently detected MAs^{III} and DMAs^{III}, HPLC-ICP-MS analyses have provided inconsistent and contradictory results. This study compares the capacities of both methods to detect and quantify MAs^{III} and DMAs^{III} in an *in vitro* methylation system consisting of recombinant human arsenic (+3 oxidation state) methyltransferase (AS3MT), S-adenosylmethionine as a methyl donor, a non-thiol reductant tris(2-carboxyethyl)phosphine, and arsenite (iAs^{III}) or MAs^{III} as substrate. The results show that reversed-phase HPLC-ICP-MS can identify and quantify MAs^{III} and DMAs^{III} in aqueous mixtures of biologically relevant arsenical standards. However, HPLC separation of the *in vitro* methylation mixture resulted in significant losses of MAs^{III}, and particularly

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[3] Jenna M. Currier, R. Jesse Saunders, Lan Ding, Wanda Bodnar, Peter Cable, Tomáš Matoušek, John T. Creed, and Miroslav Stýblo. (2013) *J. Anal. At. Spectrom.* Reproduced by permission of The Royal Society of Chemistry. http://pubs.rsc.org/en/content/articlelanding/2013/ja/c3ja30380b

DMAs^{III} with total arsenic recoveries below 25%. Further analyses showed that MAs^{III} and DMAs^{III} bind to AS3MT or interact with other components of the methylation mixture, forming complexes that do not elute from the column. Oxidation of the mixture with H_2O_2, which converted trivalent arsenicals to their pentavalent analogs prior to HPLC separation, increased total arsenic (As) recoveries to ~95%. In contrast, HG-CT-AAS analysis found large quantities of methylated trivalent arsenicals in mixtures incubated with either iAs^{III} or MAs^{III} and provided high (>72%) As recoveries. These data suggest that an HPLC-based analysis of biological samples can underestimate MAs^{III} and DMAs^{III} concentrations and that controlling for As species recovery is essential to avoid artifacts.

4.2 Introduction

Arsenic (As) is one of the most prevalent elements in the Earth's crust. Inorganic As (iAs) species, arsenite (iAs^{III}) and arsenate (iAs^{V}) are common ground and surface water contaminants (16). Millions of people worldwide who drink iAs-contaminated water are at risk of developing cancer and non-cancerous diseases, including hypertension, peripheral neuropathy, or diabetes (1-4). However, the susceptibility to chronic iAs toxicity varies among individuals and depends, in part, on the efficiency and pattern of iAs metabolism. Thus, the analysis of iAs metabolites in biological matrices has become an essential tool for population studies examining the inter-individual differences in responses to iAs exposure, as well as for laboratory studies using animal or *in vitro* models for iAs toxicity and metabolism.

Once ingested, iAs is enzymatically methylated by As (+3 oxidation state) methyltransferase (AS3MT) in a sequence of *S*-adenosylmethionine-dependent reactions

(53). Both tri- and pentavalent methylated oxoarsenicals are generated in this pathway, including methylarsonic acid (MAs^V), methylarsonous acid (MAs^{III}), dimethylarsinic acid (DMAs^V), and dimethylarsinous acid (DMAs^{III}) with trimethylarsine oxide (TMAs^{V}O) being the final metabolite in some mammalian species (59, 60). All these tri- and pentavalent oxoarsenicals have been detected in human urine and in biological samples collected in laboratory experiments (70, 148, 156, 167, 173-177). Recent studies have suggested that in addition to oxoarsenicals, a variety of sulfur-containing As species (thioarsenicals) can be produced in the course of iAs metabolism, possibly by intestinal bacteria or by reactions of oxoarsenicals with hydrogen sulfide in tissues (178-180). To date, only one of these thioarsenicals, dimethylthioarsinic acid (DMTA), has been detected in urine of people exposed to iAs in drinking water (181, 182). A growing body of evidence suggests that among all known metabolites of iAs, the methylated trivalent oxoarsenicals (MAs^{III} and DMAs^{III}) are the most biologically active and toxic species (8-11).

The methods used for speciation analysis of As in biological samples have been discussed in detail in several recent reviews (23, 151, 175, 183, 184). Most of these methods use high-performance liquid chromatography (HPLC) for separation of As species and various spectrometric techniques, including inductively coupled plasma-mass spectrometry (ICP-MS) for quantification of As in the chromatographic fractions. An alternative approach uses hydride generation (HG) to convert iAs and methylated As species in analyzed samples to gaseous arsine (AsH$_3$) with boiling point (b.p.) of -55°C and methyl-substituted arsines: methylarsine (CH$_3$AsH$_2$, b.p. 2 °C), dimethylarsine ((CH$_3$)$_2$AsH, b.p. 36 °C) and trimethylarsine ((CH$_3$)$_3$As, b.p. 52 °C). The generation of

arsines by reaction with sodium borohydride is then followed by a cryotrapping (CT) step and separation of arsines by their boiling points. The HG-CT techniques typically use atomic absorption spectrometry (AAS), AFS or ICP-MS for the detection and quantification of As in the separated arsines (151, 175). In general, the HPLC-based methods are highly specific and can identify a wide spectrum of As species, including species that originate in foods and are not products of iAs metabolism in human tissues, e.g., arsenobetaine (AsB), arsenocholine (AsC), or arsenosugars (184). The HG-based techniques are characterized by low detection limits, but have only limited specificity. A thorough method development and validation using appropriate standards and HG conditions are required for these methods to reliably identify and quantify As species and to avoid artifacts associated with generation of a single form of arsine from two or more As species present in the analyzed samples.

A variety of HPLC-ICP-MS techniques have been used for separation of iAs metabolites and other As species in aqueous solutions and biological samples, mainly urine. Anion-exchange HPLC has been shown in some studies to separate tri- and pentavalent metabolites of iAs and As species originating in seafood, including AsB and AsC (159, 173, 184-186). Other studies used a combination of anion- and cation-exchange chromatography to achieve the same goal (148). On the other hand, reversed-phase HPLC has successfully separated AsC and the tri- and pentavalent metabolites of iAs, including MAs[III] and DMAs[III] with the use of a single column (12, 14, 147, 187, 188), but could not fully resolve AsB (189). Notably, while some researchers using HPLC for As speciation analysis found MAs[III] and/or DMAs[III] in biological samples, including human urine (12, 13, 44, 147, 148, 173, 186-188, 190, 191), others failed to

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detect these metabolites (183, 192). What causes these inconsistent results is unclear but the oxidation of MAs^{III} and DMAs^{III} in aqueous solutions and in human urine (14, 70, 193) could play a major role. In addition, HPLC-based analysis typically involves sample preparation (extraction, filtration, etc.) which could produce oxidation state specific conversions and/or provide opportunities for unwanted analyte interaction with the matrix, chemicals, or surfaces inherent within the analysis procedure (143). Because surprisingly many of the published studies did not employ oxidation state specific procedural spikes or control for As-based mass balance to assess the impact of these undesirable artifacts, it is difficult to determine whether a procedure element was responsible for the analyte loss or conversion.

Unlike HPLC, the HG-CT-based methods, specifically HG-CT-AAS and HG-CT-ICP-MS, have consistently detected MAs^{III} and DMAs^{III} in biological samples, including urine of individuals exposed to iAs in drinking water (69, 70, 143, 156), tissues from mice exposed chronically to iAs (167, 193), mammalian cells or tissue cultures treated *in vitro* with iAs (136, 143, 149), and *in vitro* systems in which iAs was methylated by recombinant rat As3mt or human AS3MT (149, 154). However, because of a limited specificity of the HG-based techniques, results of these analyses have often been questioned, and the detection of DMAs^{III} has been viewed by some as an artifact (183, 194). The present study was designed to address these concerns. This study compares the capacities of previously described reversed-phase HPLC-ICP-MS (14, 147, 147, 187, 188) and HG-CT-AAS (136, 195) techniques to detect and quantify MAs^{III} and DMAs^{III} in an *in vitro* system in which the methylated As metabolites are produced in reactions catalyzed by recombinant human AS3MT and which, because of its simplicity, limits

possible artifacts. Results of this study suggest that analysis of biological samples by HPLC-based techniques can underestimate MAs^{III} or $DMAs^{III}$ content due formation of stable complexes between these arsenicals and proteins or other endogenous substances and due to retention of these complexes on the chromatographic column.

4.3 Experimental

4.3.1 Arsenicals

The following arsenicals were used for method optimization and calibration: iAs^{III} ($NaAs^{III}O_2$) and iAs^V ($Na_2HAs^VO_4$), both >99% pure from Sigma-Aldrich (St. Louis, MO, USA); MAs^V ($CH_3As^VO(ONa)_2$) and $DMAs^V$ ($(CH_3)_2As^VO(OH)$), both >98% pure from Chem Service (West Chester, PA); oxomethylarsine (CH_3AsO, $MAs^{III}O_4$) and iododimethylarsine ($(CH_3)_2AsI$, $DMAs^{III}I$) provided by Dr. William Cullen (University of British Columbia, Vancouver, Canada). In aqueous solutions, $MAs^{III}O$ and $DMAs^{III}I$ form the corresponding MAs^{III} and $DMAs^{III}$ oxoanions (196). $TMAs^VO$, AsC and AsB were also provided by Dr. William Cullen. DMTA was synthesized in Dr. Creed's laboratory as previously described (195). Stock solutions of the pentavalent arsenicals, DMTA, AsC and AsB were prepared in deionized water (DIW) and stored at -80°C. Stock solutions of the methylated trivalent arsenicals were prepared in ice-cold DIW immediately before each experiment to limit oxidation.

4.3.2 Instrumentation

4.3.2.1 HG-CT-AAS

The speciation analysis of As by HG-CT-AAS was performed as previously described (146, 149). The instrumentation, reagents, and operating conditions are

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summarized in **Table 4.1**. Briefly, the HG-CT-AAS system consisted of custom made HG and CT units controlled by FIAS 400 flow injection accessory (Perkin-Elmer, Norwalk, CT) and coupled with AAnalyst 800 spectrometer (Perkin-Elmer) that was equipped with a multiple microflame quartz tube atomizer (multiatomizer) (146, 149). Arsine and the methyl-substituted arsines from trivalent arsenicals (iAs^{III}, MAs^{III}, and DMAs^{III}) and from TMAs^{V}O were generated directly in a buffered reaction mixture containing 0.75 M Tris (pH 6) and 1% NaBH$_4$ in 0.1 % KOH (all from Sigma-Aldrich). To generate arsines from both tri- and pentavalent arsenicals (iAs^{III+V}, MAs^{III+V} and DMAs^{III+V}) at pH 6, standards or samples were pre-reduced with 2% L-cysteine hydrochloride (EMD Chemicals Inc., Gibbstown, NJ) for 1 hour prior to the HG step. Arsines were cryotrapped in a capillary U-tube filled with Chromosorb WAW-DCMS 45/60 (15% OV-3) (Sigma-Aldrich) and submerged in liquid N$_2$, and then separated by their boiling points prior to detection by the AA spectrometer. Concentrations of iAs^V, MAs^V, and DMAs^V were calculated as the differences between species concentrations obtained for the cysteine-treated and untreated sample aliquots.

4.3.2.2 HPLC-ICP-MS

The HPLC-ICP-MS analysis followed a previously described isocratic reversed-phase HPLC protocol (147). The operating conditions are summarized in **Table 4.1**. The Prodigy ODS(3) C$_{18}$ column (150 x 4.6 mm, Phenomenex, Torrance, CA) was heated to 30°C and eluted with a mobile phase containing tetrabutylammonium hydroxide (TBAH) (Acros Organic, Morris Plains, NJ), malonic acid (Sigma-Aldrich), and methanol (JT Baker Chemical Co., Phillipsburg, NJ). Ultrapure nitric acid (Fisher Scientific Co., Fair Lawn, NJ) was used to adjust pH of the mobile phase for optimal arsenical separation.

The HPLC column was connected with PTFE tubing to the nebulizer of a 7500cx ICP-MS (Agilent Technologies, Palo Alto, CA).

4.3.3 *In vitro* methylation mixture

Recombinant human wild-type AS3MT which has methionine in position 287 was expressed and purified as previously described (154). Methylated arsenicals were generated enzymatically in a reaction mixture containing 100 mM TRIS-HCl buffer (pH 7.4, Mediatech, Inc., Manassas, VA), recombinant AS3MT (60 or 80 μg/mL), 1 mM tris(2-carboxyethyl)phosphine (TCEP, Sigma), 1 mM S-adenosylmethionine (SAM, Sigma), and 1 μM iAs^{III} or 1 μM MAs^{III} as substrates. The final volume of the mixture was 150 μL. The *in vitro* methylation mixtures were incubated at 37°C for up to 2 hours. The mixture was then chilled on ice and split into aliquots that were analyzed concurrently by HG-CT-AAS and HPLC-ICP-MS. Some of these aliquots were treated with 2% L-cysteine (for 1 hour) or 3% H_2O_2 (for 4 hours) prior to analysis as described in Results and Discussion. Sums of As species detected by HG-CT-AAS or HPLC-ICP-MS analyses were used to calculate As recovery as percentage of the known amount of As added into the *in vitro* methylation mixture. Spikes of arsenical standards into complete or incomplete reaction mixtures were used to assess interactions between the components of the mixture and the metabolites of iAs^{III} or MAs^{III} and effects of these interactions on results of the HG-CT-AAS and HPLC-ICP-MS analyses.

Table 4.1: HG-CT-AAS and HPLC-ICP-MS operating conditions

HG-CT	Perkin-Elmer FIAS 400
Sample volume	500 μL
Buffer	0.75 M TRIS-HCl (pH 6.0)
Reducing agent	1% NaBH$_4$ in 0.1% KOH
Carrier gases	He (75 ml/min); H$_2$ (15 ml/min)

Column packing	Chromosorb WAW-DCMS 45/60 (15% OV-3)
Column heating	Ni80/Cr20 wire, 20 Ω
AAS	Perkin-Elmer AAnalyst 800
Lamp	As electrodeless discharge (390 mA)
Wavelength	193.7 nm
Slit width	0.7 nm
Atomizer	Multiatomizer (900°C)
Outer gas	Air (35 ml/min)
HPLC	Agilent 1260 Infinity Series
Column	Phenomenex Prodigy 3µ ODS(3) 100A, 150x4.60 mm
Temperature	30 °C
Mobile phase	4.7 mM tetrabutylammonium hydroxide + 2 mM malonic acid + 4% methanol (pH 5.85)
Flow rate	1.5 mL/min
Injection volume	20 µL
ICP-MS	Agilent 7500cx Series
Masses	75 (As), 77 (ArCl)
Integration time	0.1 s
RF power	1550 W
Skimmer cone	Ni
Spray chamber	Double-pass Scott-type
Sample depth	8 mm
S/C temperature	2 °C
Plasma gas (Ar)	15 L/min
Carrier gas (Ar)	0.95 L/min
Make-up gas (Ar)	0.25 L/min
Cell gas (He)	4.0 L/min
Nebulizer	Micromist

4.3.4 Ultrafiltration

To examine binding of As species to AS3MT, the reaction mixtures with or without recombinant AS3MT were spiked with iAs[III], MAs[III], or DMAs[III] (1 µM each) and incubated on ice for 5 minutes. After incubation, the reaction mixtures were filtered using Nanosep Omega spin columns with 3 kDa cut-off (Pall Life Sciences, Ann Arbor, MI) at 4°C and 14,000 x g for 15 minutes. To ensure maximum As recovery, the columns

were washed with 100 μL of 100 mM TRIS-HCl buffer (pH 7.4) and centrifuged for an additional 15 minutes at 4°C. The filtrate and wash containing unbound arsenicals were analyzed by HG-CT-AAS after cysteine pre-treatment.

4.3.5 Statistical Analysis

All statistical analyses were performed using the GraphPad Instat software package (GraphPad Software Inc., San Diego, CA). Linear regression and correlation analyses were employed to characterize the calibration curves. ANOVA followed by Bonferroni's multiple comparison posttest was used to determine significant differences in As concentration between the analytical methods and the effects of protein binding on As recovery. The differences with $p < 0.05$ were considered statistically significant.

4.4 Results and discussion

4.4.1 Method optimization

The method optimization focused on tri- and pentavalent As species that are known to be produced in the course of iAs methylation by human AS3MT, including iAs^{III}, iAs^{V}, MAs^{III}, MAs^{V}, $DMAs^{III}$, $DMAs^{V}$ (59, 60). The following As species that are not products of AS3MT-catalyzed methylation of iAs but are commonly found in human urine were also included: $TMAs^{V}O$, DMTA, AsC and AsB.

4.4.1.1 HG-CT-AAS

The HG-CT-AAS technique has been previously optimized and used for analysis of As species in biological systems as complex as tissue homogenates (167, 193) or as simple as *in vitro* methylation systems in which iAs was methylated by recombinant rat As3mt or human AS3MT (149, 154). The oxidation state specific generation of arsine

and methyl-substituted arsines from tri- and pentavalent iAs and methylated arsenicals in a buffered system at pH 6 has been described in details in our previous reports (146, 149). In absence of cysteine pretreatment, arsines were generated almost exclusively from trivalent arsenicals (iAs^{III}, MAs^{III}, and DMAs^{III}) and from TMAs^{V}O. Only small portions of DMAs^V (up to 3.5%) and DMTA (up to 11.7%) were converted to dimethylarsine under these conditions (149). However, DMTA cannot be present in the *in vitro* methylation system used in this study because the reaction mixture does not contain H$_2$S or other donors of sulfur needed for DMTA formation. Thus, arsine and methyl-substituted arsines generated from the *in vitro* methylation mixture can only represent iAs^{III}, iAs^V, MAs^{III}, MAs^V, DMAs^{III}, DMAs^V, or TMAs^{V}O. Notably, neither AsC nor AsB produced arsines in the reaction with 1% NaBH$_4$ at pH 6 regardless of cysteine treatment (data not shown).

4.4.1.2 HPLC-ICP-MS

The reversed-phase C$_{18}$ column and mobile phases consisting of TBAH, malonic acid, and methanol have been used by other laboratories for the HPLC-ICP-MS analysis of As species in aqueous solutions, human urine, saliva or cells (147, 187, 188, 197). This method was tested in the present study using a 150 x 4.6 mm C$_{18}$ column heated to 30°C. A base-line separation of six oxoarsenicals (iAs^{III}, iAs^V, MAs^{III}, MAs^V, DMAs^{III}, and DMAs^V) and DMTA was achieved with the mobile phase consisting of 4.7 mM TBAH, 2 mM malonic acid, and 4% methanol at pH 5.85 and with a flow rate of 1.5 mL/min (**Figure 4.1**). In this study, pH lower or higher than 5.85 resulted in an incomplete separation of iAs^V, DMAs^{III}, and DMTA (data not shown). The optimized mobile phase was also used to examine elution profiles of TMAs^{V}O, AsC and AsB. The retention time
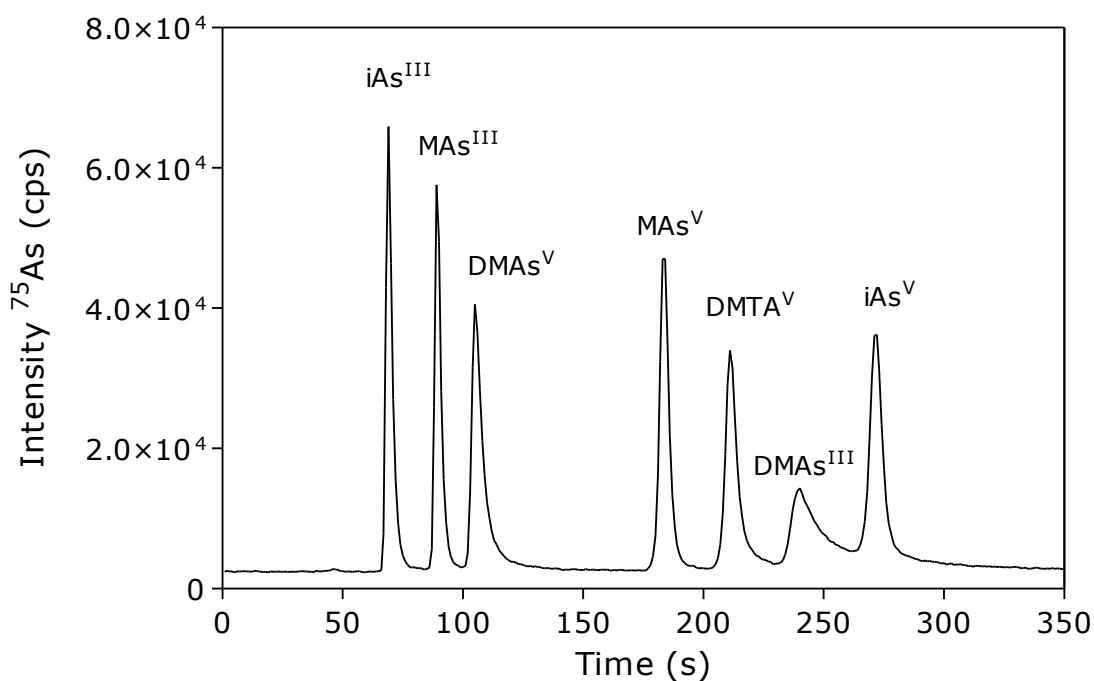
Figure 4.1: The separation and detection of six oxoarsenicals and DMTA by optimized HPLC-ICP-MS. The operating conditions are described in **Table 4.1**. The standards (250 pg As each) were injected in 20 µL of DIW.

for AsC was shorter than those for all the other As species; however, $TMAs^VO$, and AsB co-eluted with iAs^{III} (**Figure 4.2**). The co-elution of $TMAs^VO$ and AsB with iAs^{III} did not represent a problem for the current study because neither $TMAs^VO$ nor AsB are products of the AS3MT-catalyzed methylation of iAs. However, without further optimization, this HPLC technique could produce artifacts if used for analysis of biological samples that contain all three As species, e.g., human urine.

4.4.2 Calibration

Calibration curves were prepared only for the tri- and pentavalent oxoarsenicals that are products of As methylation by human AS3MT.
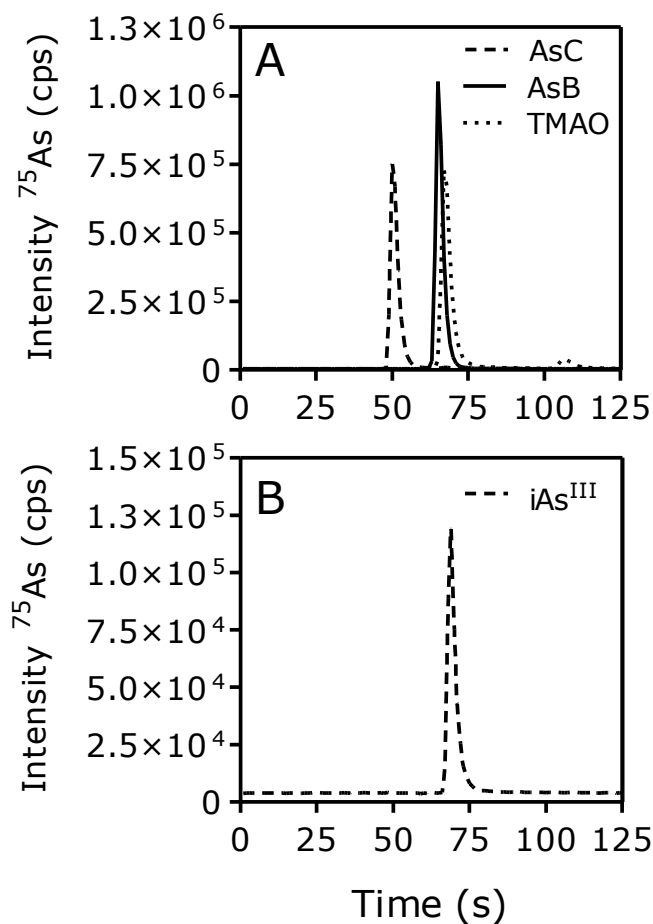
Figure 4.2: The HPLC-ICP-MS profiles of As standards in DIW, including AsC, AsB, TMAO (A, 2,000 pg each) and iAs^{III} (B, 280 pg). Detailed operating conditions are described in **Table 4.1**.

4.4.2.1 HG-CT-AAS

Aqueous solutions of the pentavalent As standards (iAs^V, MAs^V, and DMAs^V) pre-reduced with L-cysteine were used to generate 5-point calibration curves. The calibration procedure, including validation using the trivalent As standards has been described in our previous reports (146, 149). Slopes of the calibration curves are provided in (**Table 4.2**). We have previously shown that the slopes of calibration curves for As standards prepared in DIW and for As standards prepared in complex biological matrices (e.g., cell lysates or tissue homogenates) do not significantly differ (143, 149, 167). Thus,

the calibration curves for As standards prepared in DIW were used in the present study for quantification of As metabolites in the *in vitro* methylation mixtures analyzed by HG-CT-AAS.

Table 4.2: The slopes of calibration curves generated by HG-CT-AAS (10-3s/ng) and HPLC-ICP-MS (cps*s/pg) for As standards

	HG-CT-AAS [a]					
Solvents	iAs^{III+V}		MAs^{III+V}		$DMAs^{III+V}$	
DIW	814		808		788	
	HPLC-ICP-MS [b]					
	iAs^{III}	iAs^{V}	MAs^{III}	MAs^{V}	$DMAs^{III}$	$DMAs^{V}$
DIW	1503	1461	1152	1543	632	1425
Reaction Mixture	1550	707	1145	1643	811	1486

[a] Standards were prepared in DIW and were reduced with 2% cysteine prior to HG-CT-AAS analysis.

[b] Standards were prepared in DIW or in the *in vitro* reaction mixture containing Tris-HCl buffer, SAM and TCEP, but not AS3MT.

4.4.2.2 HPLC-ICP-MS

Six-point calibration curves were prepared using solutions of tri- and pentavalent As standards in DIW or in the *in vitro* methylation mixture containing Tris-HCl buffer, SAM and TCEP, but not AS3MT. Consistent with previously published data (14, 188), slopes of the calibration curves for individual As standards varied (**Table 4.2**). Specifically, the calibration slopes for MAs^{III} and $DMAs^{III}$ in either solvent were lower than those for the other As standards, suggesting that these methylated trivalent arsenicals are partially retained on the column. A lower calibration slope was also found for iAs^{V} standard prepared in the *in vitro* methylation mixture lacking AS3MT. Thus, the calibration curves for As standards prepared in this mixture were used in the present study to quantify As metabolites detected by HPLC-ICP-MS.

4.4.3 Limits of detection (LODs)

Standard deviations (SDs) of the integrated peak areas corresponding to the retention times of As standards were determined for 10 injections of 500 μL of 2% L-cysteine in DIW into the HG-CT-AAS system or 20 μl of DIW into the HPLC-ICP-MS system. These injection volumes are consistent with the volumes of samples analyzed by HG-CT-AAS and HPLC-ICP-MS in this study. The LOD value was calculated for each As species as 3*SD/slope*injection volume. LOD values for HG-CT-AAS ranged from 24 to 57 pg As mL^{-1}; LODs for HPLC-ICP-MS were generally higher, ranging from 31 pg As mL^{-1} for MAs^{III} to 295 pg As mL-1 for iAs^{V} (**Table 4.3**).

Table 4.3: Limits of detection (LOD) for the analysis of As species by HPLC-ICP-MS and HG-CT-AAS

Method	LOD (pg/mL)								
	iAs^{III}	iAs^{V}	iAs^{III+V}	MAs^{III}	MAs^{V}	MAs^{III+V}	DMAs^{III}	DMAs^{V}	DMAs^{III+V}
HPLC-ICP-MS [a]	32	295		31	57		88	98	
HG-CT-AAS [b]	36	54	40	32	57	46	24	44	38

[a] LODs were calculated as 3*SD/slope for 10 injections of DIW (20 μl each).

[b] LODs were calculated as 3*SD/slope for 10 injections of DIW or 2% cysteine in DIW (0.5 ml each).

4.4.4 Speciation analysis of As in the *in vitro* methylation mixture

The *in vitro* methylation was carried out in reaction mixtures containing either 1 μM iAs^{III} or 1 μM MAs^{III} as substrate (i.e., 11.25 ng As in 150 μl of the mixture). The reaction mixtures with iAs^{III} contained 80 μg/mL of recombinant AS3MT and were incubated for 2 hours at 37°C. MAs^{III} is methylated by AS3MT more efficiently than iAs^{III} (154). Therefore, the mixtures containing MAs^{III} were incubated with 60 μg/mL of AS3MT for only 40 minutes. Two aliquots from each reaction mixture were immediately and simultaneously analyzed by HG-CT-AAS and HPLC-ICP-MS. Here, one aliquot (20

μL of the mixture) was diluted to 500 mL with ice-cold DIW and analyzed by HG-CT-AAS. The other 20-μl aliquot was injected directly into the HPLC-ICP-MS system. Two additional aliquots (20 μL each) were treated either with L-cysteine for determination of As^{III+V} species by HG-CT-AAS or with H_2O_2 for analysis of As^{III+V} species by HPLC-ICP-MS.

4.4.4.1 Analysis of the reaction mixture incubated with iAs^{III}

Representative chromatograms and amounts of As species detected in the mixture after incubation with iAs^{III} are shown in **Figure 4.3**. HG-CT-AAS identified two methylated metabolites, $DMAs^{III}$ and $DMAs^{V}$ (**Figure 4.3A**), which accounted for 43% and 16% of As added as iAs^{III} into the mixture prior to incubation; 13% of iAs^{III} remained unmethylated (**Figure 4.3C**). The average sum of As species detected by HG-CT-AAS in the mixtures treated with cysteine was 7.5 ng, i.e., 72.1% of the added As (**Figure 4.3D**). In contrast, two major peaks recorded by the direct HPLC-ICP-MS analysis represented $DMAs^{V}$ and iAs^{III} (**Figure 4.3B**) and accounted for only 12.3% and

9.4% of the added As, respectively (**Figure 4.3C**). DMAs^{III} was a minor metabolite
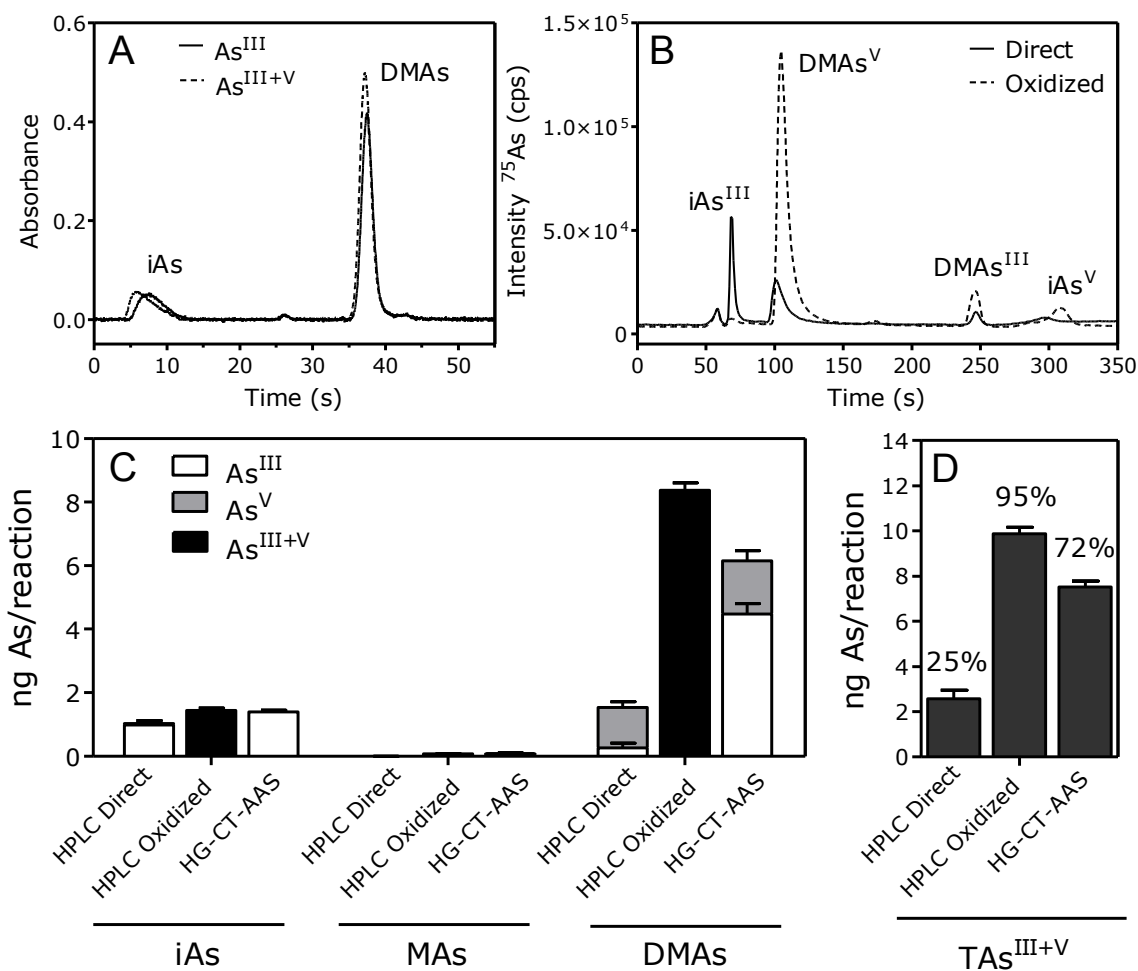
Figure 4.3: Analyses of the complete *in vitro* methylation mixtures containing recombinant AS3MT (80 μg/mL) incubated with 1 μM iAs^{III} (11.25 ng) for 2 hours at 37°C. Representative HG-CT-AAS (A) and HPLC-ICP-MS (B) chromatograms and amounts of As species detected (C) are shown. The recovery of As by either method is expressed in ng As and as percentage of the amount of As added into the reaction mixture prior to incubation (D). The mixtures were analyzed by HPLC-ICP-MS directly (HPLC direct) or after oxidation with 3% H_2O_2 (HPLC oxidized). HG-CT-AAS analysis was performed directly and after 2% L-cysteine pretreatment to measure As^{III} and As^{III+V} species, respectively. Pentavalent As species were calculated as the difference between these two analyses. Values are expressed as mean ± SD for four separate reaction mixtures.

(2.5% of the added As). Only 25% of the added As was recovered by the direct HPLC-

ICP-MS analysis (**Figure 4.3D**). However, As recovery by HPLC-ICP-MS increased

dramatically after oxidation of the reaction mixture with H_2O_2, reaching on average 95%. Here, $DMAs^V$ was the major As species detected, representing on average 7.1 ng As, i.e., 68% of the added As. Notably, the amount of $DMAs^V$ detected in the oxidized mixtures exceeded by 5.4 folds sums of $DMAs^{III}$ + $DMAs^V$ found by the direct HPLC-ICP-MS analysis but was in a good agreement with the sum of $DMAs^{III}$ + $DMAs^V$ detected by HG-CT-AAS.

4.4.4.2 Analysis of the reaction mixture incubated with MAs^{III}

Results of this analysis are summarized in **Figure 4.4**. The HG-CT-AAS analysis found $DMAs^{III}$ to be the major product of MAs^{III} methylation, accounting on average for 52% of the added As; $DMAs^V$ represented 10% of the added As. About 21.5% of the substrate remained unmethylated and was partially oxidized during the incubation to MAs^V (**Figure 4.4A,C**). The recovery of As in the L-cysteine-treated mixtures was 83.4% (**Figure 4.4D**). In comparison, $DMAs^V$ was the only major species detected by the direct HPLC-ICP-MS analysis, representing 17% of added As. No $DMAs^{III}$ and only traces of the unmethylated MAs^{III} were found. In the reaction mixtures oxidized with H_2O_2, $DMAs^V$ and MAs^V were the major species, accounting for 74% and 23% of added As. Here again, the total amounts of MAs^V and $DMAs^V$ in the oxidized mixtures analyzed by HPLC-ICP-MS compared well with the sums of MAs^{III} + MAs^V and $DMAs^{III}$ + $DMAs^V$ detected by HG-CT-AAS. The average As recovery by HPLC-ICP-MS was 19.3% and 99% for the reaction mixtures before and after oxidation with H_2O_2.

Taken together, these results suggest that $DMAs^{III}$ is the major product of iAs^{III} and MAs^{III} methylation by recombinant AS3MT. However, HPLC-ICP-MS analysis underestimates $DMAs^{III}$ yields because a major portion of this metabolite either alone or

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in complexes with AS3MT protein or other components of the reaction mixture is lost on the chromatographic column. The interactions of DMAs^{III} with the components of the reaction mixture may also be responsible for the incomplete As recoveries during the HG-CT-AAS analysis.
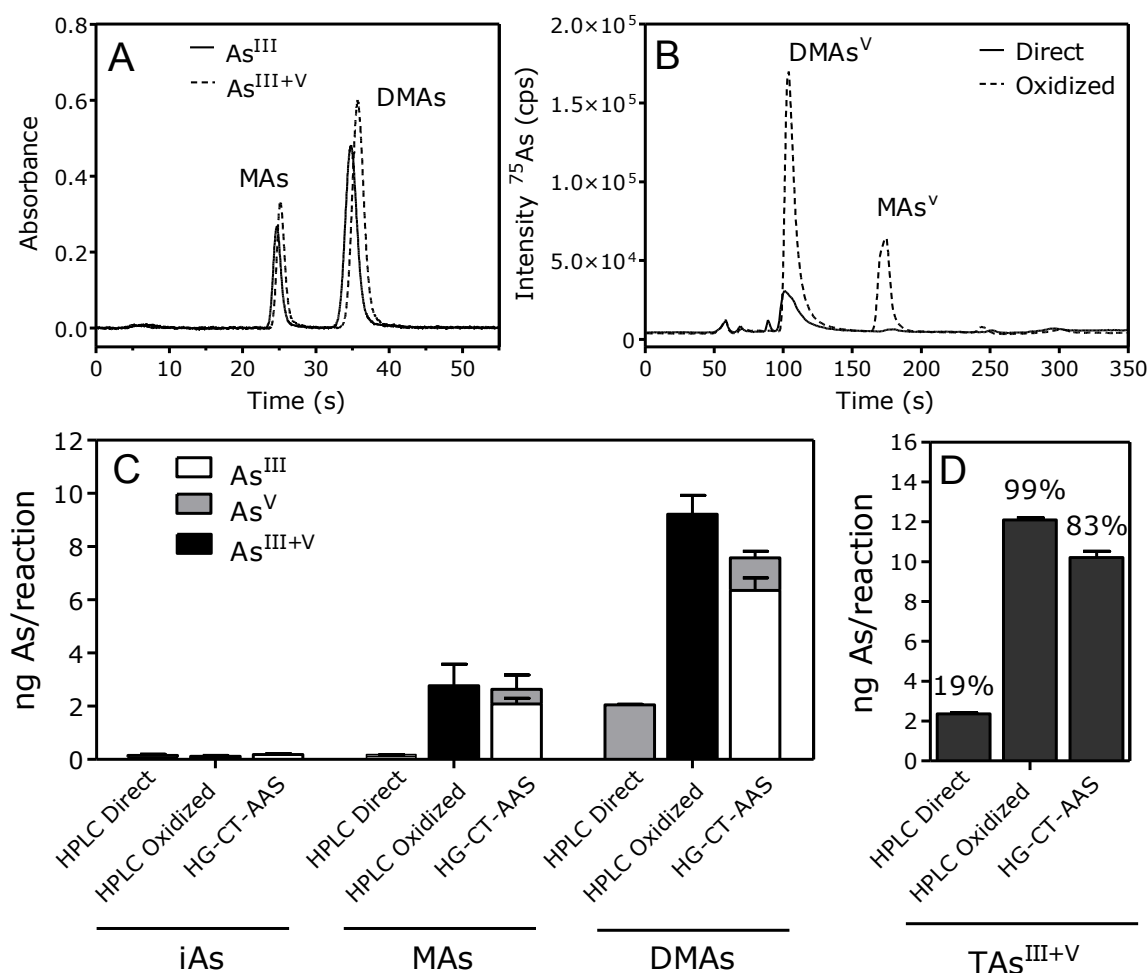
Figure 4.4: Analyses of the complete *in vitro* methylation mixtures containing recombinant AS3MT (60 μg/mL) incubated with 1 μM MAs^{III} (11.25 ng) for 40 min at 37°C. Representative HG-CT-AAS (A) and HPLC-ICP-MS (B) chromatograms and amounts of As species detected (C) are shown. The recovery of As by either method is expressed in ng As and as percentage of the amount of As added into the reaction mixture prior to incubation (D). The mixtures were analyzed by HPLC-ICP-MS directly (HPLC direct) or after oxidation with 3% H$_2$O$_2$ (HPLC oxidized). HG-CT-AAS analysis was performed directly and after 2% L-cysteine pretreatment to measure As^{III} and As^{III+V} species, respectively. Pentavalent As species were calculated as the difference between these two analyses. Values are expressed as mean ± SD for four separate reaction mixtures.

4.4.5 Interactions of As^{III} species with the components of the methylation mixture

To investigate the interactions of $DMAs^{III}$ and other trivalent oxoarsenicals with AS3MT, we compared HPLC profiles for the iAs^{III}, MAs^{III} and $DMAs^{III}$ standards (0.5 µM each) that were incubated for 5 minutes in the complete reaction mixture (1 mM TCEP and 1 mM SAM in 100 mM Tris-HCl buffer) or in the Tris-HCl buffer in the presence or absence of recombinant AS3MT (60 µg/mL). The incubation was carried out at $0^{o}C$ to suppress the enzymatic activity of AS3MT. The presence of the recombinant protein in the complete reaction mixture dramatically changed the HPLC profiles and recoveries for all three arsenicals (**Figure 4.5**). Only 47% of iAs^{III} and 6% of MAs^{III} were recovered from the mixtures containing AS3MT as compared to the mixtures that lacked the protein (**Figure 4.5B,C**). However, presence of AS3MT had no significant effects on HPLC profiles and recoveries of the iAs^{III} or MAs^{III} standards that were incubated in the Tris-HCl buffer (**Figure 4.6A,B**), suggesting that the presence of SAM or TCEP facilitates the interactions of these arsenicals with the enzyme. In contrast, $DMAs^{III}$ was detected only in the mixture with Tris-HCl buffer, but not in the complete reaction mixtures incubated either in the presence or absence of AS3MT (**Figure 4.5D**). Incubation of $DMAs^{III}$ with AS3MT in the Tris-HCl buffer in absence of SAM and TCEP decreased the area of $DMAs^{III}$ to 21% (**Figure 4.6C**). Incubation of $DMAs^{III}$ in the Tris-HCl buffer with either 1 mM SAM or 1 mM TCEP in the absence of AS3MT also decreased the $DMAs^{III}$ signal, and thus recovery during the HPLC separation (**Figure 4.6C**). These results suggest that, unlike iAs^{III} or MAs^{III}, $DMAs^{III}$ interacts not only with AS3MT, but also with SAM and TCEP, making a quantitative HPLC-ICP-MS analysis of this metabolite in the complete reaction mixture practically impossible.
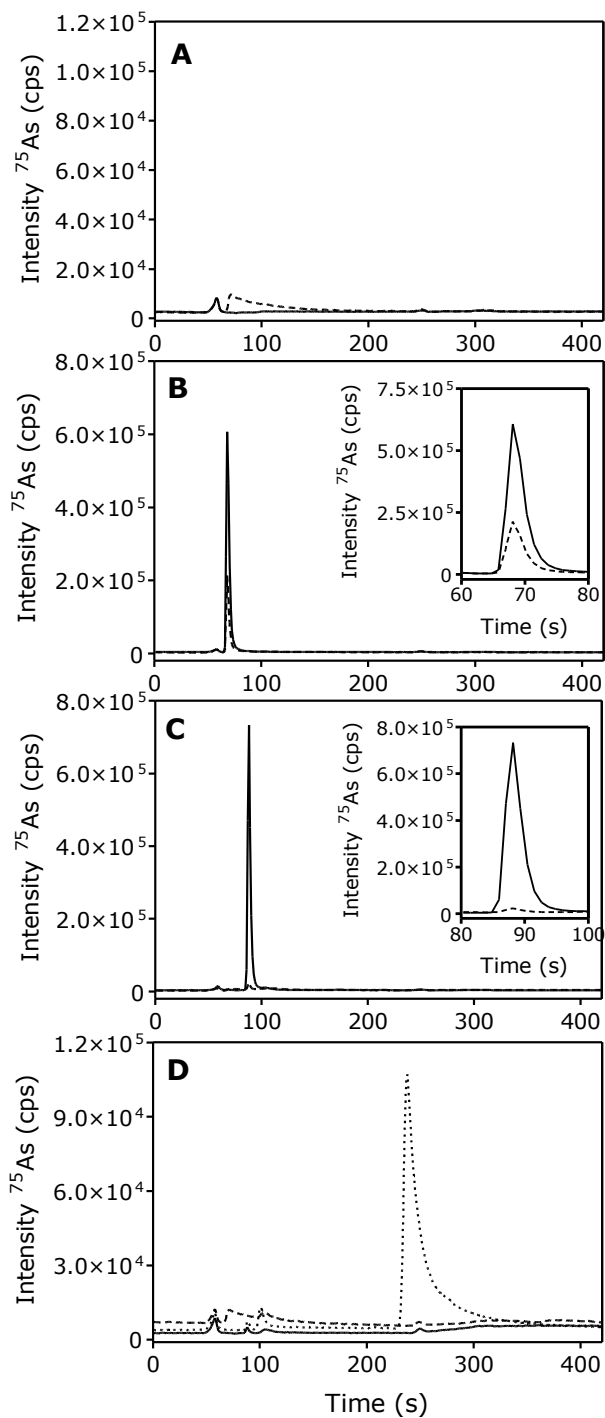
Figure 4.5: The HPLC-ICP-MS profiles for trivalent arsenical standards spiked into the complete *in vitro* methylation mixture (1 mM TCEP, 1 mM SAM in 100 mM TRIS-HCl buffer, pH 7.4) in absence (full line) or presence (dashed line) of recombinant AS3MT (60 μg/mL). Representative chromatograms for the methylation mixtures without As standards (A) and for the mixtures incubated at 0°C for 5 minutes with 0.5 μM iAs[III] (B), MAs[III] (C), or DMAs[III] (D) are shown. The dotted line in panel D represents 0.5 μM DMAs[III] incubated in the TRIS-HCl buffer alone.

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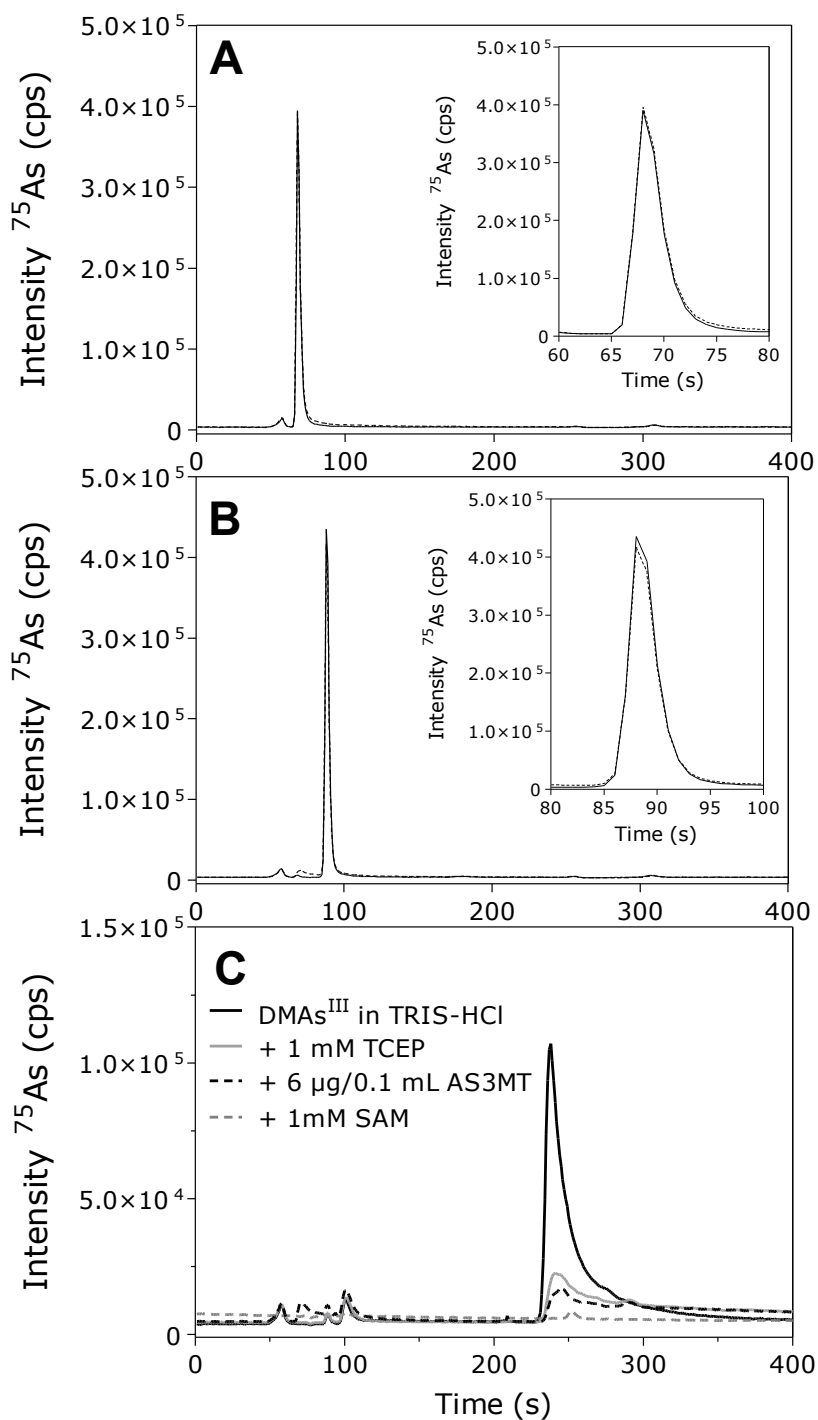
Figure 4.6: Comparison of the HPLC-ICP-MS profiles for As standards prepared in solutions containing the components of the *in vitro* methylation mixture: 0.5 μM iAs[III] (A) and 0.5 μM MAs[III] (B) in the 100 mM TRIS-HCl buffer (pH 7.4) in absence (—) and presence (---) of AS3MT (60 μg/mL). 0.5 μM DMAs[III] (C) in a mixture with TRIS-HCl buffer (—), 1 mM TCEP (—), AS3MT (60 μg/mL; ---) or 1 mM SAM (---).

4.4.6 Binding of As^{III} species to AS3MT

The interactions of As species with AS3MT were further examined using ultrafiltration. Here, iAs^{III}, MAs^{III}, or DMAs^{III} (1 µM each) were incubated in the complete *in vitro* reaction mixture in presence or absence of recombinant AS3MT (60 µg/mL) at 0°C for 5 minutes. After incubation, an aliquot of each reaction mixture was treated with L-cysteine and analyzed by HG-CT-AAS for As^{III+V} species. A second aliquot was ultrafiltered using a Pall Omega 3k spin column with a 3 kDa cut-off filter membrane. The filtrate and wash (i.e., the low-molecular weight fraction) were analyzed by HG-CT-AAS for As^{III+V} species. **Figure 4.7** summarizes results of the analyses of the unfiltered aliquots and the ultrafiltrates. The amounts of arsenicals detected by HG-CT-AAS in the unfiltered aliquots of the mixtures containing AS3MT were lower as compared to the unfiltered aliquots of mixtures that did not contain the recombinant protein. However, this difference was statistically significant only for mixtures incubated with MAs^{III} or DMAs^{III} (**Figure 4.7B,C**), suggesting that interactions of these arsenicals with the recombinant protein limit the efficiency of hydride generation. The HG-CT-AAS analysis of ultrafiltrates confirmed that significant amounts of MAs^{III} or DMAs^{III} are bound to AS3MT. Specifically, the amounts of MAs^{III} or DMAs^{III} were significantly lower (by 38 and 40%, respectively) in ultrafiltrates from the AS3MT-containing mixtures as compared to the unfiltered AS3MT-containing mixtures. In contrast, ultrafiltration had no significant effects on the amount of iAs^{III} suggesting that iAs^{III} binds to AS3MT with lower affinity than the methylated trivalent species (**Figure 4.7A**). These findings are consistent with results of a recent study by Marapakala and associates
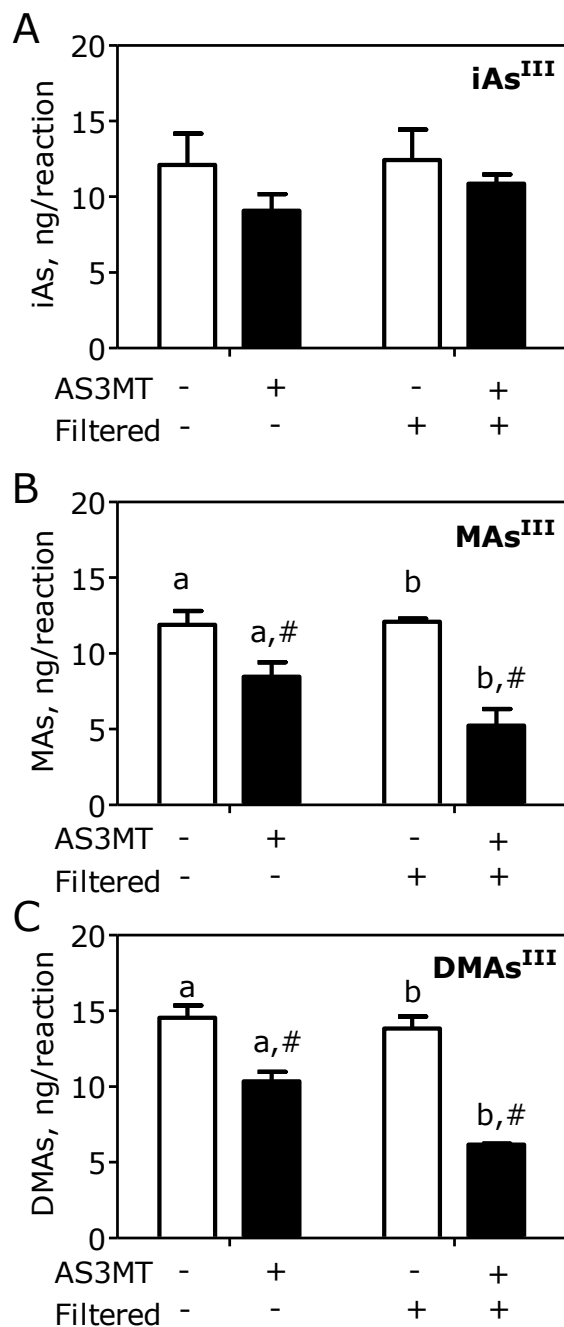
Figure 4.7: The amounts of trivalent arsenical standards in the complete reaction mixture before and after ultrafiltration. The mixture (1 mM TCEP, 1 mM SAM in 100 mM TRIS-HCl buffer, pH 7.4) was incubated at 0°C for 5 minutes with 1 μM iAs^{III} (A), MAs^{III} (B), or DMAs^{III} (C) in presence or absence of recombinant AS3MT (60 μg/mL). The As^{III+V} species were analyzed by HG-CT-AAS in the mixtures before and after ultrafiltration using a 3 kDa cutoff membrane. Values are expressed as mean ± SD for 3 separate mixtures. The effect of AS3MT protein (a,b) and of ultrafiltration (#) on the recovery of As were examined. Values labeled with the same letter or symbol are significantly different ($p < 0.05$).

who showed that MAs^{III} binds faster than iAs^{III} to CmArsM, a bacterial orthologue of AS3MT (binding of DMAs^{III} was not examined) (65). Notably, the losses of MAs^{III} or DMAs^{III} after ultrafiltration were not as high as those observed during the separation of the *in vitro* methylation mixtures by reversed-phase HPLC (**Figure 4.3 and 4.4**), suggesting that that the AS3MT-bound MAs^{III} or DMAs^{III} partially oxidized and detach from the recombinant protein during the ultrafiltration or that small complexes of arsenicals with TCEP and SAM which are not eluted from the HPLC column can pass through the filter.

AS3MT is a cysteine-rich protein (60). Some of its cysteine residues have been shown to play the catalytic role in the process of iAs methylation (198-200), possibly providing binding sites for the substrate or reaction intermediates. However, other cysteine-containing proteins and endogenous low-molecular weight thiols, like glutathione have been shown to bind As (45, 201-205). Results of the present study suggest that by binding trivalent arsenicals, these proteins and thiols can interfere with quantitative speciation analysis of As by HPLC-based techniques. These results also show that DMAs^{III} interacts not only with AS3MT, but also with other components of the *in vitro* methylation mixture – SAM and TCEP. Thus, it is possible that interactions with these or other compounds may interfere with DMAs^{III} analysis in biological samples, including urine. To test this hypothesis, we compared HPLC-ICP-MS profiles for DMAs^{III} spiked into an ice-cold TRIS-HCl buffer or human urine at final concentration of 0.5 μM (**Figure 4.8**). Notably, the peak area for DMAs^{III} in urine represented only 27% of the peak area in the buffer, suggesting that the urine matrix significantly impairs the ability of HPLC-ICP-MS to detect DMAs^{III} and that calibrating for aqueous standards

when analyzing As species in urine could result in a substantial underestimation of DMAs^{III} content.
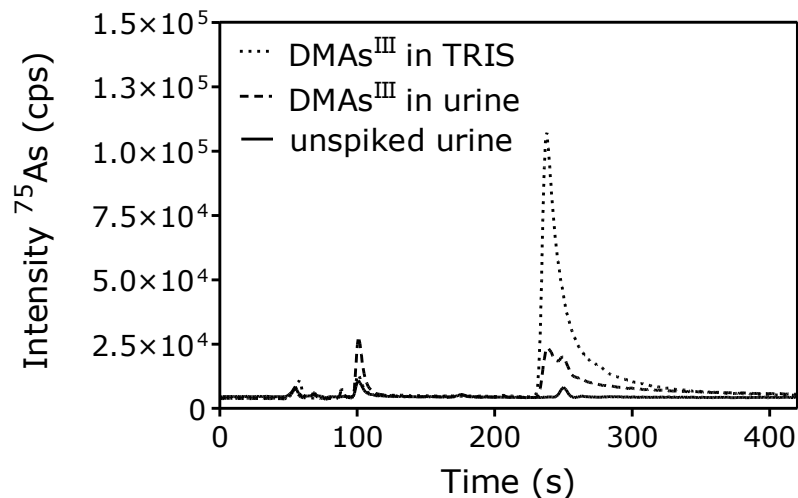
Figure 4.8: The HPLC-ICP-MS profiles for 0.5 μM DMAs^{III} standard spiked into 100 mM TRIS-HCl buffer (pH 7.4) or into human urine from an unexposed subject.

4.5 Conclusions

Results of this study suggest that reversed phase HPLC-ICP-MS and HG-CT-AAS are almost equally effective when used for analysis of tri- and pentavalent iAs, MAs and DMAs in standard solutions prepared in DIW or TRIS-HCl buffer. However, when these techniques are utilized for analysis of a simple biological system in which the methylated arsenicals are generated in enzymatic reactions, HG-CT-AAS produces much better As recoveries (mass balances) than does HPLC-ICP-MS. This difference in performance is not detector based but rather is associated with losses of the methylated trivalent arsenicals during chromatographic separation. Spiking of the reaction mixture with standards provides evidence that MAs^{III} and DMAs^{III} interact with AS3MT and/or other components of the mixture to produce complexes that do not elute from the

chromatographic column. These findings are consistent with results of Šlejkovec and associates who reported an on-column binding and losses of As species during analyses using an ion-exchange HPLC-ICP-MS (192). The higher recoveries associated with HG-CT-AAS indicate that either the MAs^{III}- and $DMAs^{III}$-complexes are unstable in the chemical environment associated with the HG step or the complexes produce the same hydrides as do free MAs^{III} and $DMAs^{III}$. In either case, if quantifying MAs^{III} and/or $DMAs^{III}$ as a biomarker is an essential part of the study objectives then the existing HG-CT-AAS technique provides far less negative bias as compared to the existing HPLC-ICP-MS approach. This negative bias is not only present in the *in vitro* system used here to illustrate the problem but it should also be expected in other biological matrices which provide binding sites for MAs^{III} and $DMAs^{III}$, including human urine. The need to assess the losses of trivalent methylated arsenicals during HPLC-based analyses is clearly demonstrated in this study by almost complete recoveries of As from the reaction mixtures treated with H_2O_2. Thus, laboratories that do not attempt to quantify species specific recoveries or quantify the chromatographic mass balance within the matrix are choosing not to estimate a source of uncertainty that may undermine the reliability and utility of the data set.

4.6 Acknowledgements

CHAPTER V

Oxidation State Specific Analysis of Arsenic Species in Tissues of Wild-type and Arsenic

(+3 oxidation state) Methyltransferase (As3mt) Knockout Mice[4]

5.1 Overview

Arsenic (+3 oxidation state) methyltransferase (As3mt) catalyzes the conversion

of inorganic arsenic (iAs) to methylated metabolites, including methylarsonite (MAs^{III})

and dimethylarsinite (DMAs^{III}). While this enzyme is critical for the detoxification of

iAs, MAs^{III} and DMAs^{III} are more toxic than iAs. *As3mt*-knock out (KO) mice can be

used to explore the role of MAs^{III} and DMAs^{III} in the adverse effects of iAs exposure.

However, this mouse strain retains significantly more iAs than wild-type (WT) mice and

are more susceptible to iAs-induced toxicity. Previously, WT C57BL/6 mice exposed to

50 ppm As as arsenite (iAs^{III}) in drinking water developed diabetes characterized by

impaired glucose tolerance without insulin resistance. Methylated arsenicals were

detected in tissues maintaining glucose homeostasis, including liver, pancreas, skeletal

muscle and adipose tissue, but the oxidation state of As was not determined. In this study,

a recently developed hydride generation-cryotrapping-atomic absorption spectrometry

(HG-CT-AAS) method for the oxidation state specific speciation of As in complex

biological matrices was used to compare retention of tri- and pentavalent As species in

tissues of WT and *As3mt*-KO mice, and to determine doses in *As3mt*-KO mice that

produce equivalent internal dose of total As in tissues critical for glucose homeostasis

and in other tissues. WT mice were exposed to 50 ppm and *As3mt*-KO mice were

exposed to 0, 15, 20, 25 or 30 ppm As as iAs^{III} in drinking water for 4 weeks. *As3mt*-KO mice retained almost exclusively iAs; iAs^{III} was the most prevalent species in liver, pancreas, adipose, lung, heart, and kidney, ranging from 53 to 74% of total As. Methylated arsenicals did not exceed 10% of total As in any tissue. Tissues of WT mice retained iAs and methylated arsenicals; iAs^{III}, MAs^{III} and DMAs^{III} represented 55-68% of the total As in the liver, pancreas, and brain. High levels of MAs^{III} were found in the intestine and intestinal content of WT, but not *As3mt*-KO mice, suggesting that intestinal bacteria are not a major source of methylated As species. Significantly more total As was detected in plasma and blood cells of WT mice exposed to 50 ppm As as compared to all *As3mt*-KO treatment groups. These results indicate that internal total As doses in tissues critical to glucose homeostasis (liver, pancreas, skeletal muscle, adipose) equivalent to WT mice can be achieved in *As3mt*-KO mice after exposure to 25 and 30 ppm As. These results will help to design future studies comparing the diabetogenic and other adverse effects of iAs exposure in WT and *As3mt*-KO mice lacking the capacity to methylate iAs.

5.2 Introduction

Inorganic arsenic (iAs), a potent human carcinogen, is ubiquitous in the environment and accumulates in aquifers naturally and through anthropogenic activities. The ingestion of iAs through contaminated drinking water, most commonly as arsenite (iAs^{III}) and arsenate (iAs^{V}), has been associated with several adverse effects, including peripheral vascular disease, hypertension, and cancer of the lungs, liver, and bladder (1, 3, 163). A recent National Toxicology Program workshop examining the effects of environmental chemicals on the development of diabetes and obesity concluded that there

was sufficient evidence to link iAs exposures to an increased prevalence of diabetes in populations exposed to levels of iAs in drinking water greater than 150 ppb (5).

The enzyme, arsenic (3+ oxidation state) methyltransferase (AS3MT) mediates the S-adenosylmethionine (SAM) dependent biomethylation of As^{III} species to tri- and pentavalent methylated metabolites (52). AS3MT mRNA has been found in several human and rodent tissues, including, liver, kidney, urinary bladder, heart, lung, testes, and adrenal gland (53). Once ingested, iAs is sequentially methylated by AS3MT producing methylarsonite (MAs^{III}), methylarsonate (MAs^{V}), dimethylarsinite ($DMAs^{III}$), and dimethylarsinate ($DMAs^{V}$). Increasing evidence suggest that the methylated trivalent As (As^{III}) species, MAs^{III} and $DMAs^{III}$, produced in the course of iAs metabolism, are more toxic than iAs or their pentavalent counterparts (8-11, 136).

Laboratory-based studies have shown that As exposure alters glucose homeostasis and several mechanisms responsible for glucose metabolism. *In vitro* studies implicate diminished pancreatic β-cell function and glucose uptake in peripheral tissues as targets for iAs-induced diabetes. Our laboratory has recently shown that in murine pancreatic islets exposure to subtoxic concentrations of iAs^{III}, MAs^{III} or, $DMAs^{III}$ inhibited glucose-stimulated insulin secretion without affecting basal insulin secretion or insulin content and expression, suggesting that As^{III} species inhibit insulin transport vesicle packaging or translocation to the plasma membrane (136). In β-cell cultures, diminished glucose-stimulated insulin secretion has been associated with reduced insulin mRNA levels (131), alterations in free Ca^{2+} oscillations (132), and the induction of a an Nrf2-mediated antioxidant response suppressing endogenous reactive oxygen species (ROS) (133, 134). Because ROS produced in the course of glucose metabolism may act as intracellular

regulators of insulin secretion, an Nrf2-mediated antioxidant response would hinder ROS signaling, and thus, the secretion of insulin (135). In cell culture models of peripheral tissues, iAs[III] been shown to inhibit differentiation of adipocytes (137, 138) and myoblasts (139). Moreover, in adipocytes, As[III] species have been shown to inhibit insulin-stimulated glucose uptake by interfering with GLUT4 translocation to the plasma membrane (140, 141).

We have previously shown that in C57/BL6 mice exposure to 50 ppm As as iAs[III] in drinking water for as little as 8 weeks resulted in impaired glucose tolerance (86, 87, 123). Moreover, mice chronically exposed to iAs[III] in combination with high-fat diet produced a unique diabetic phenotype characterized by impaired glucose tolerance in the absence of significant obesity and fasting hyperinsulinemia, but not insulin resistance, suggesting an atypical As-induced diabetic phenotype (87).

Genetically altered, *As3mt*-knockout (KO) mice lacking the ability to methylate iAs have been recently developed (55). These mice retain significantly higher total levels of As (55, 90, 91) and exhibit increased sensitivity to iAs-induced toxicity compared to WT mice (56, 92). In *As3mt*-KO mice, the toxic effects of iAs exposure have been observed at concentrations as low as 25 ppm As, with significant lethality at concentrations greater than 50 ppm (56, 92). While iAs was the predominant species in tissues from *As3mt*-KO mice exposed to iAs, methylated metabolites have been detected in liver and plasma, suggesting the methylation of As by other methyltransferases or intestinal microbiota (55, 93). Nonetheless, because the *As3mt* genotype produces significantly different As methylation phenotypes, *As3mt*-KO mice can be used as a

model to explore the role of iAs methylation, and specifically the formation of trivalent methylated arsenicals in the development of iAs-induced diseases.

Hydride generation-cryotrapping-atomic absorption spectrometry (HG-CT-AAS) is uniquely suited for analysis of complex biological matrices because no sample pretreatments or extractions are required, thus preserving the methylation state of unstable MAs^{III} and $DMAs^{III}$ (146, 149, 167, 193). This method has successfully detected the methylated trivalent arsenicals, MAs^{III} and $DMAs^{III}$, after iAs exposure in human urine (69, 70, 143, 156), mouse tissues (167, 193), and *in vitro* cell cultures (136, 143, 149) or *in vitro* mixtures for methylation of iAs by recombinant AS3MT (149, 154).

In this study, we aimed to characterize the retention of tri- and pentavalent arsenicals in target organs of wild-type (WT) C57/BL6 and *As3mt*-KO mice after chronic iAs^{III} exposure and to determine exposure levels that produce equivalent internal doses of total As in tissues critical for regulating glucose homeostasis, as well as in other tissues. Our results indicate that 4-week exposure to 25 or 30 ppm As as iAs^{III} in *As3mt*-KO mice produces equivalent internal doses of As in liver, pancreas, skeletal muscle, and adipose tissue compared to WT mice treated with 50 ppm As. These doses will be used in follow up studies examining the effects of methylated As species in a mouse model of As-induced diabetes.

5.3 Methods

5.3.1 Arsenicals

The following pentavalent arsenicals were used for mouse exposure in drinking water and for determining calibration slopes by HG-CT-AAS: sodium arsenite ($NaAs^{III}O_2$) and sodium arsenate ($Na_2HAs^VO_4$) (99% pure) were purchased from Sigma-

Aldrich (St. Louis, MO). Methylarsonic acid, disodium salt ($CH_3As^VO(ONa)_2$), and dimethylarsinic acid (($CH_3)_2As^VO(OH)$)) both better than 98% pure were purchased from Chem Service (West Chester, PA). The As content in each of the standards was determined by graphite furnace-AAS (146).

5.3.2 Mice and Treatments

All procedures involving mice were approved by the University of North Carolina Institutional Animal and Use Committee. *As3mt*-KO mice were developed and maintained as previously described (55). In this study, 13- to 18-week old male *As3mt*-KO mice were bred by brother-sister matings at the University of North Carolina Animal Facility. Male C57BL/6 mice (WT) between 13 and 17 weeks were obtained from Jackson Laboratory (Bar Harbor, ME, USA). All mice were housed in polycarbonate cages (5 per cage) with corn cob bedding at the University of North Carolina Animal Facility (12 h light/dark cycle, $22 \pm 1°C$ and humidity $50 \pm 10\%$). Mice were fed Lab Diet 5058 (Nutrition International, Brentwood, MO) and drank pure deionized water (DIW) or DIW containing iAs^{III} *ad libitum* for the 4-week study period. *As3mt*-KO mice were exposed to 0, 15, 20, 25, or 30 ppm As as iAs^{III} (5 mice per exposure group). WT mice were exposed to 50 ppm As (n = 10), the dose that previously produced a diabetic phenotype (86). The concentration of iAs in water in all treatment groups was verified by HG-CT-AAS analysis (data not shown). Mice were euthanized by cervical dislocation. Freshly dissected tissues were processed as previously described, and tissue homogenates were prepared in ice cold DIW (10% w/v) using an electrical overhead stirrer equipped with a Teflon pestle tissue homogenizer (Wheaton Industries, Inc., Millville, NJ) (167).

5.3.3 Speciation analysis of As by HG-CT-AAS

As species were analyzed by an optimized method for oxidation state specific analysis of As species in biological matrices (146, 149, 167, 193). Briefly, HG-CT-AAS was performed using a FIAS 400 flow injection accessory (Perkin-Elmer, Norwalk, CT, USA) coupled to a cryotrapping unit and an AAnalyst 800 spectrometer (Perkin-Elmer) equipped with a multiple microflame quartz tube atomizer (multiatomizer) (146, 149). To measure the oxidation state of As species, two aliquots of each sample are analyzed; arsine gases from trivalent arsenicals (iAs^{III}, MAs^{III} and $DMAs^{III}$) are generated directly at pH 6, while arsines from both tri- and pentavalent arsenicals (iAs^{III+V}, MAs^{III+V} and $DMAs^{III+V}$) are measured after pre-reduction for 1 hour at room temperature with 2% L-cysteine (EMD Chemicals Inc., Gibbstown, NJ) (146, 149). The concentrations of the pentavalent arsenicals, iAs^{V}, MAs^{V}, and $DMAs^{V}$, are calculated by subtracting the AAS peak area signals obtained for L-cysteine-treated and directly analyzed, untreated sample aliquots.

Calibration curves for quantification of tri- and pentavalent As species were generated using aqueous solutions of the pentavalent standards (iAs^{V}, MAs^{V}, and $DMAs^{V}$) pre-treated with 2% L-cysteine. To determine the recovery of speciated As during the direct analyses, aliquots of tissue homogenates were acid digested in ultrapure phosphoric acid, using a MARS Microwave Reaction System equipped with MarsXpress Temperature Control (CAM, Matthew, NC), as previously described (167). This procedure oxidizes trivalent arsenicals to pentavalency, but does not convert methylated arsenicals to iAs.

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5.3.5 Statistical Analysis

All statistical analyses were performed using GraphPad Instat software package (GraphPad Software Inc., San Diego, CA). Linear regression and correlation analyses were employed to characterize the calibration curves of aqueous pentavalent As standards. ANOVA followed by Bonferroni's multiple comparison posttest was used to determine significant differences between total speciated As in tissues from *As3mt*-KO and WT mice. Statistical significance was considered at the level of $p < 0.05$.

5.4 Results and Discussion

5.4.1 Water consumption and body weights

The consumption of water for each exposure group and individual body weights were measured weekly throughout the study. **Figure 5.1** depicts the estimated daily water consumption, calculated from group weekly consumption, and the corresponding iAs intake for mice in each exposure group over the 4-week study period. Water intake rose after the first week and then plateaued for the remaining study period except in *As3mt*-KO mice exposed to 25 ppm As, which exhibited decreased water consumption in weeks 3 and 4 (**Figure 5.1A**). **Figure 5.1B** depicts the average daily water intake for mice within each group; *As3mt*-KO mice exposed to pure DIW consumed an average of 3.9 mL/day, which is significantly more water than all iAs-treated groups ranging from 1.4 to 2.2 mL/day. The average daily iAs intake per mouse was estimated from the average daily water consumption. *As3mt*-KO mice exposed to 15, 20, 25, or 30 ppm As ingested approximately 27.1, 34.9, 43.4, and 42.7 µg of iAs/day, respectively, while WT mice exposed to 50 ppm As ingested approximately 107.6 µg of iAs/day (**Figure 5.1C**). There

was no significant difference in iAs/day ingestion among the *As3mt*-KO groups, but the

WT group ingested significantly more iAs than all other groups.
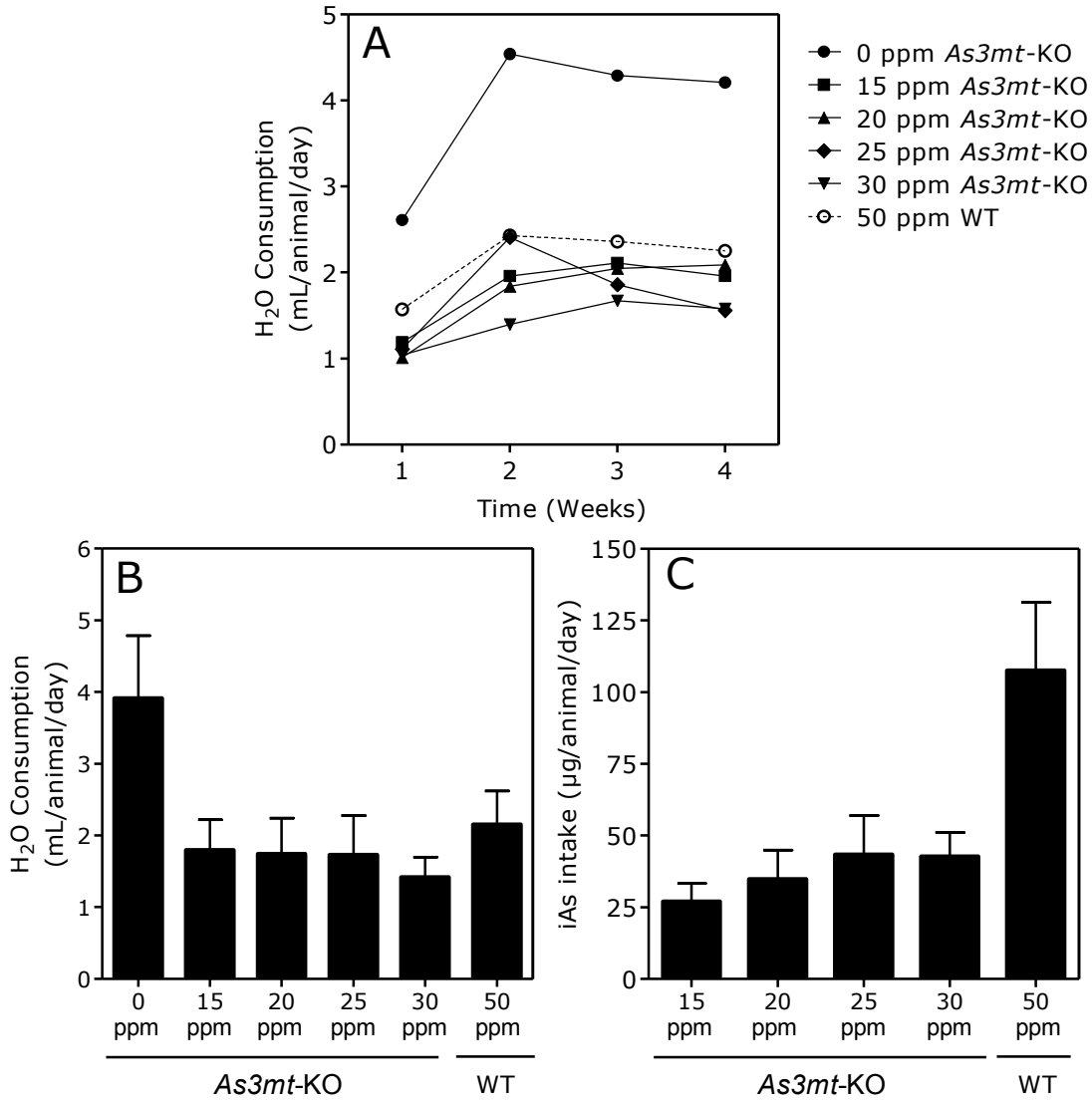
Figure 5.1: Average daily water consumption and estimated daily iAs intake for *As3mt*-KO and WT mice. (A) Variations in daily water consumption by week for *As3mt*-KO mice exposed to 0 (●), 15 (■), 20 (▲), 25 (♦), or 30 (▼) ppm As and WT mice exposed to 50 ppm As (○). (B) Average daily water consumption per mouse for each treatment group. (C) Estimated daily iAs intake per mouse for each treatment group. Mean and SD, n = 5 for each *As3mt*-KO group and n = 10 for WT group.

The body weight of each mouse was measured prior to iAs exposure and weekly throughout the study (**Figure 5.2**). On average, all mice gained weight except for *As3mt*-KO mice exposed to 30 ppm As (**Figure 5.2**). Unexposed *As3mt*-KO mice gained an average of 3.2 g over the 4-week study period, while *As3mt*-KO mice exposed to 15, 20, or 25 ppm As gained an average of 1.2, 1.6, and 1.1 g, respectively. *As3mt*-KO mice exposed to 30 ppm As lost an average of 1.2 g over the study period. However, no signs of toxicity were observed for any treatment group during the study or tissue dissection. This is in contrast with previous studies reporting lethality and histopathological abnormalities in *As3mt*-KO mice exposed to 25 ppm for 4 weeks (91, 92).
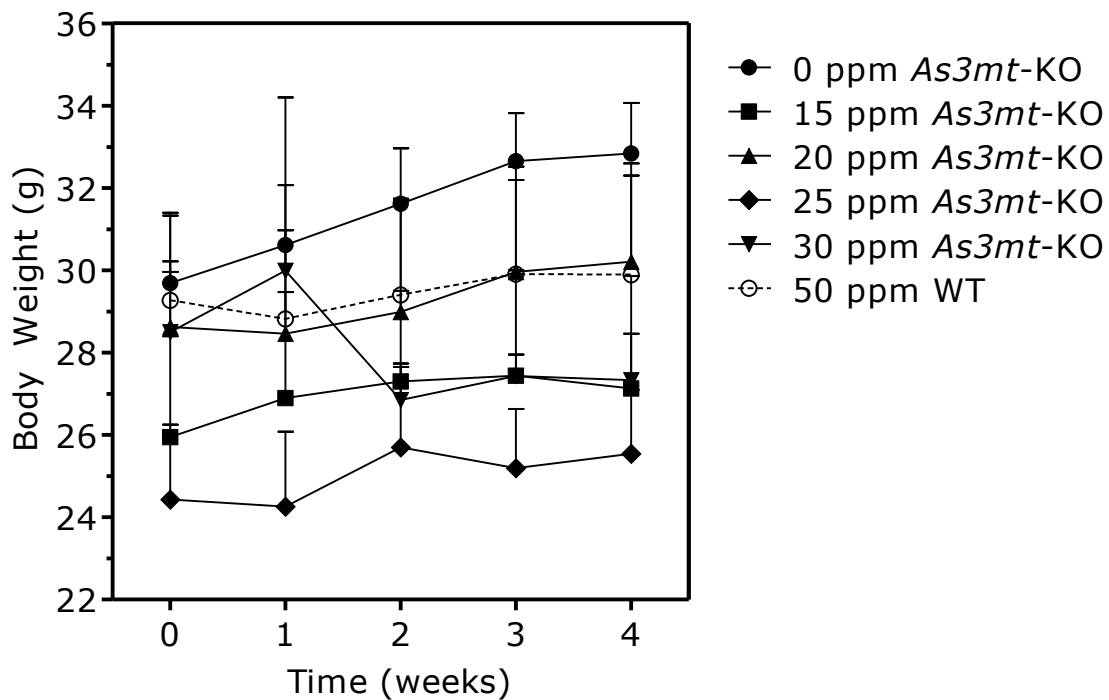
Figure 5.2: Change in body weights of *As3mt*-KO mice exposed to 0 (●), 15 (■), 20 (▲), 25 (♦), or 30 (▼) ppm As and WT mice exposed to 50 ppm As (○). Mean and SD, n = 5 for each *As3mt*-KO group and n = 10 for the WT group. There were no significant differences in weekly average weights determined by two-way ANOVA.

5.4.2 Effect of genotype on As speciation in tissues

The speciation of As in tissues of *As3mt*-KO mice has previously been examined. However, the analysis was limited to plasma, red blood cells, liver, kidney, lung, and bladder and did not determine the oxidation state of As in the detected As species (55, 90, 91). Only recent optimization of the HG-CT-AAS method has allowed for the analysis of tri-and pentavalent As species in tissue homogenates without sample extractions or digestions. This method was used in the present study to characterize retention of As^{III} and As^V species retained in tissues of *As3mt*-KO mice at various levels of exposure and to compare the internal doses to that in WT mice exposed to 50 ppm As, the concentration previously used in this laboratory to examine the diabetogenic effects of iAs.

As expected, iAs^{III} and iAs^V were the main species retained in tissues of *As3mt*-KO mice, while methylated As metabolites predominated in most tissues from WT mice. **Figure 5.3** displays As retention in tissues involved in regulation of glucose homeostasis (i.e., liver, adipose tissue, pancreas, and skeletal muscle). *As3mt*-KO mice exposed to 0 ppm As (pure DIW) retained only small amounts of iAs, likely due to the presence of iAs in standard rodent chow (86). In *As3mt*-KO mice, iAs^{III} was the predominate species retained in liver, adipose tissue, and pancreas, representing 57 to 74% of total speciated As (**Figure 5.3A,B,C**). In skeletal muscle, iAs^{III} and iAs^V ranged from 42 to 53% and 45 to 57% of total speciated As, respectively (**Figure 5.3D**). The concentration of As species in *As3mt*-KO mice exposed to 15, 20, 25, or 30 ppm As increased in a dose-dependent

manner in liver, skeletal muscle and pancreas. In adipose tissue, the amount of iAs increased only between 15 and 25 ppm iAs, but decreased at 30 ppm.
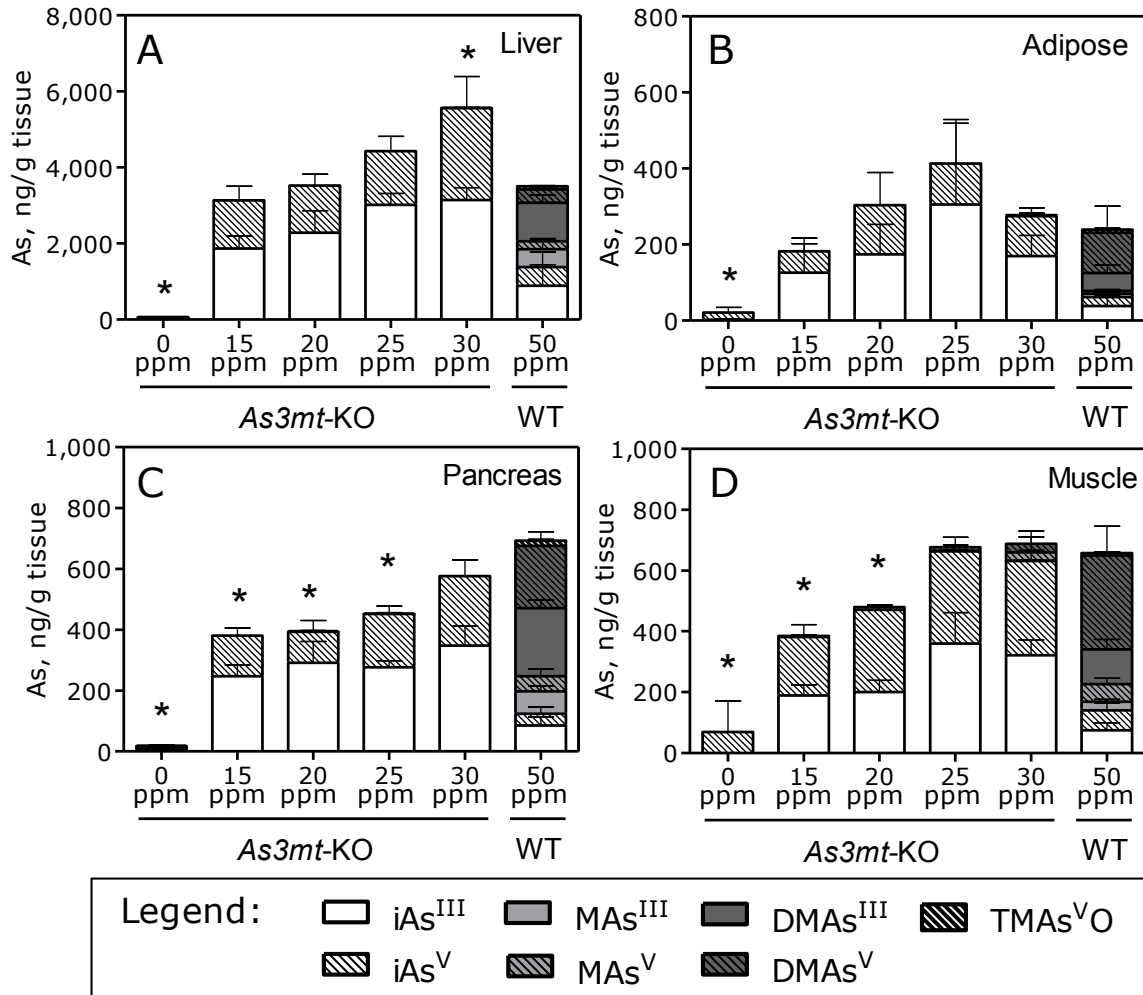
Figure 5.3: Oxidation state specific analysis of As in tissues critical for glucose homeostasis. The concentration (ng As/g wet tissue) of seven As species in liver (A), adipose tissue (B), pancreas (C), and skeletal muscle (D) of *As3mt*-KO mice exposed to 0, 15, 20, 25, and 30 ppm and WT mice exposed to 50 ppm As as iAs[III]. Homogenates (10% w/v) were prepared in ice-cold DIW. All tissues and homogenates were stored at -80°C prior to analysis by HG-CT-AAS. One aliquot was measured directly to determine As[III] species and another was measured after 2% L-cysteine pretreatment to determine As[III+V] species. Pentavalent arsenicals were determined as the difference between the peak areas of each arsenical for the two aliquots. Data is represented as means with SD for each arsenical (*As3mt*-KO, n = 5; WT, n =10). (*) Statistically significant difference in total speciated As compared to 50 ppm WT group ($p > 0.05$).

This decrease was associated with the overall loss of body mass of the *As3mt*-KO mice exposed to 30 ppm As, implying toxicity of the exposure. DMAs^{III} predominated in the liver and pancreas of WT mice, accounting for 29% and 32% of total speciated As respectively. DMAs^{V} represented 45% and 47% of As in adipose and skeletal muscle, respectively. Notably, the sum of trivalent species (iAs^{III} + MAs^{III} + DMAs^{III}) accounted for 55% and 68% of As in pancreas and liver of WT mice, respectively.

In the previous report, methylated species accounted for 28 to 32% of total As in liver and 22 to 28% in urine of *As3mt*-KO mice exposed to a single dose of iAs^{V}, suggesting the role of alternative As methylation mechanisms (55). However, a more recent study in *As3mt*-KO mice exposed to iAs^{III} through drinking water support our finding that iAs^{III} and iAs^{V} were exclusively retained in liver (91). Methylated metabolites generated in *As3mt*-KO mice may be quickly cleared, and the observed differences are likely explained by the dosing (single vs. subchronic) or by the fact that mice were exposed to iAs^{III} in one study and to iAs^{V} in the other. Notably, in the present study, the methylated arsenicals in tissues of *As3mt*-KO mice never exceeded 10% of the total speciated As and no methylated As species were detected in liver, pancreas, or adipose tissue.

Figure 5.4 displays the oxidation state specific speciation of As in blood plasma and blood cells of WT and *As3mt*-KO mice. Significantly more total As was detected in plasma and blood cells of WT mice exposed to 50 ppm As as compared to all *As3mt*-KO treatment groups. In plasma and erythrocytes of *As3mt*-KO mice, iAs^{III} and iAs^{V} were present at approximately equal concentrations. An average of 348 ng As/mL was present in plasma of WT mice exposed to 50 ppm As compared with only 6, 17, 21,33, and 22 ng

As/mL in *As3mt*-KO mice exposed to 0, 15, 20, 25 and 30 ppm As, a greater than 10-fold difference (**Figure 5.4A**). In blood cells of *As3mt*-KO mice, a dose-dependent increase in
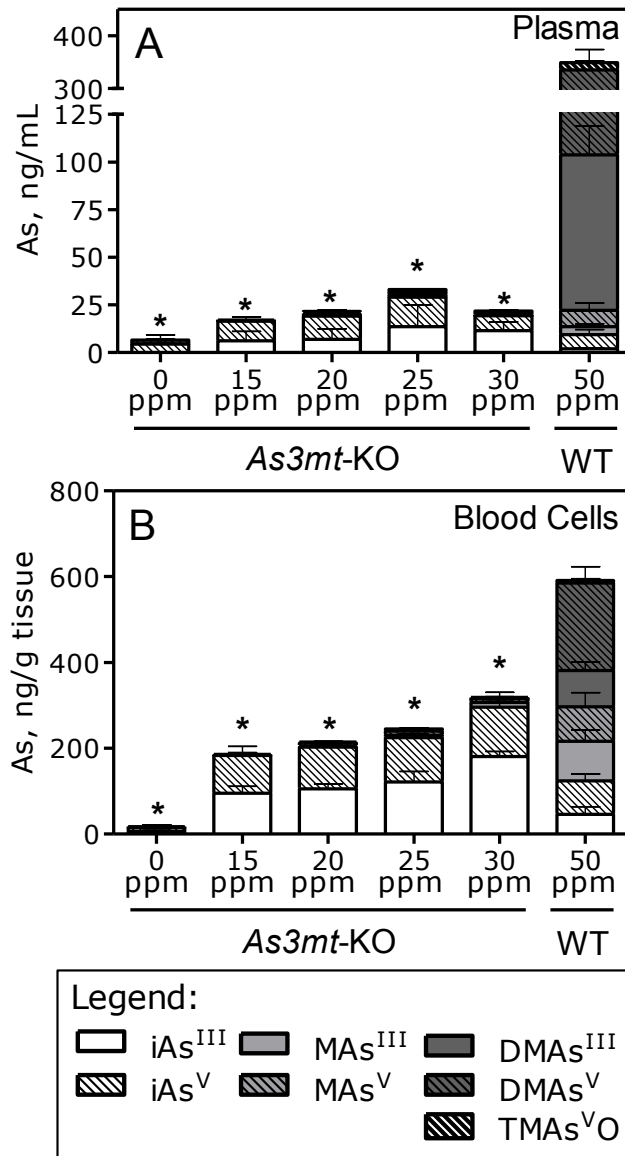
Figure 5.4: Oxidation state specific analysis of circulating As in *As3mt*-KO mice exposed to 0, 15, 20, 25, and 30 ppm As and WT mice exposed to 50 ppm As as iAs^{III}. The concentration of seven As species in plasma (A, ng As/mL) and blood cells (B, ng As/g wet tissue). Analysis was performed as described in **Figure 5.3**. Data is represented as means with SD for each arsenical. (*As3mt*-KO, n = 5; WT, n =10). (*) Statistically significant difference in total speciated As compared to 50 ppm WT group ($p > 0.05$).

As from 15 to 317 ng As/g tissue was observed, while an average of 506 ng As/g tissue was retained in blood cells of WT mice (**Figure 5.4B**). Our finding that WT mice retain more total As in blood cells than *As3mt*-KO mice conflicts with a previous study where WT and *As3mt*-KO mice were exposed to 1, 10, and 25 ppm As as iAs^{III} in drinking water for 33 days (91). Here, the authors reported that greater levels of total As were retained by erythrocytes of *As3mt*-KO mice than WT mice. This difference could be associated with differences in sample preparation, analytical speciation technique, or with higher exposures in the present study for WT mice. Notably, the total As level in blood cells from *As3mt*-KO exposed to 25 ppm As in our study (245 ng As/g tissue) is in good agreement with the level reported by Chen and associates (~ 300 ppb As).

The concentrations of As species in several other tissues from WT and *As3mt*-KO mice, including intestine with intestinal content, kidney, lung, heart, brain, and testes were also examined (**Figure 5.5**). Notably, MAs^{III} accounted for 62% of As in the intestine (with intestinal content included) of WT mice exposed to 50 ppm As, while iAs^{III} predominated in the intestine from *As3mt*-KO mice (**Figure 5.5A**). Additionally, total speciated As in the intestinal tissue averaged between 4,040 and 9,273 ng As/g tissue in *As3mt*-KO mice, but 24,879 ng As/g tissue in WT mice. It has been previously suggested that other methyltransferases or bacterial-mediated As methylation contribute to the formation of methylated As species in *As3mt*-KO mice (55); however, our results indicate that functional As3mt enzyme is necessary for the methylated As species to be found in the intestine and intestinal content. Moreover, the greater than 2-fold increase in total speciated As retained in the intestine of WT mice suggests that methylation increases excretion of ingested As in feces. In a previous study where WT C57/BL6 and *As3mt*-KO

mice were repeatedly dosed with 0.5 mg/kg iAs^V, whole body As burden and daily excretion analysis revealed that WT mice excreted As greater than 10 to 20 times faster than *As3mt*-KO mice (90). Thus, it is possible that in addition to urine, excretion in feces contributes significantly to the clearance of methylated As species from the mouse body.

In the kidney, lung, brain, and testes of *As3mt*-KO mice, iAs^{III} accounted for 49 to 81% of total speciated As. In kidney, heart, lung of WT mice, $DMAs^V$ accounted for 36, 40, and 63% of speciated As, respectively, while $DMAs^{III}$ predominated in brain (61%) and testes (67%). Notably, total speciated As in lung from WT mice exposed to 50 ppm As was significantly higher than in *As3mt*-KO mice in any treatment group. A recent report indicates significantly higher retention in lungs of *As3mt*-KO mice exposed to 1 and 10 ppm iAs^{III} compared to WT mice at the same exposures, but not 25 ppm (91). Our results suggest that at higher exposures, methylated arsenicals are more preferentially retained in lung tissue. Overall, these data indicate that trivalent As species are highly retained in most tissues of WT and *As3mt*-KO mice exposed to As and likely play a significant role in the development of iAs-induced diseases.
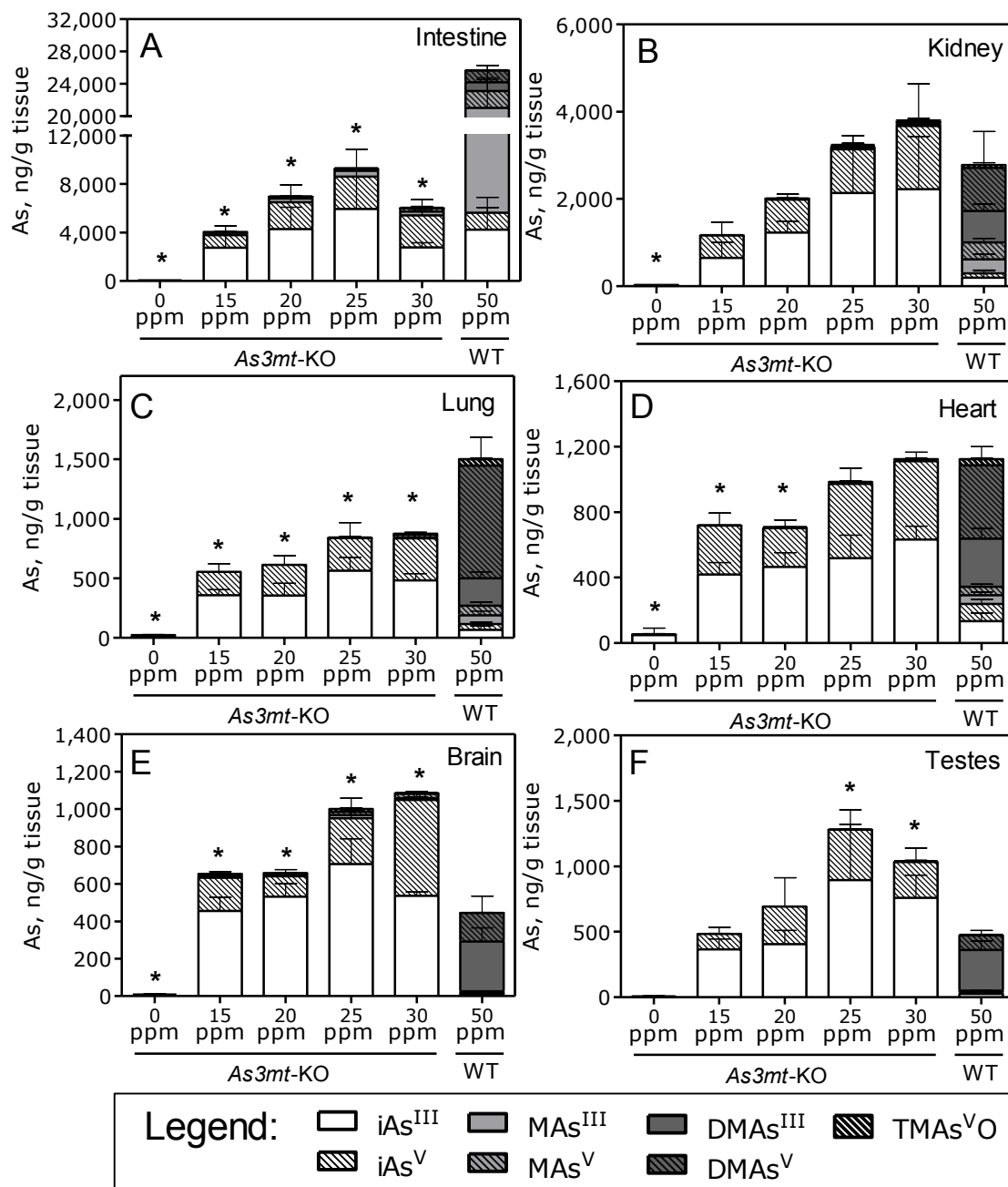
Figure 5.5: Oxidation state specific analysis of As in tissues from *As3mt*-KO mice exposed to 0, 15, 20, 25, and 30 ppm As and WT mice exposed to 50 ppm As as iAs[III]. The concentration of seven As species (ng As/g wet tissue) in intestine with intestinal content (A), kidney (B), lung (C), heart (D), brain (E), and testes (F). Analysis was performed as described in **Figure 5.3**. Data is represented as means with SD for each arsenical. (*As3mt*-KO: n = 5; WT, n =10).

5.4.3 Recovery of speciated As during direct analysis

The %recovery of As during was determined by comparing the amount of total speciated As found in tissue homogenates by direct analysis and the amount of total speciated As determined in tissue homogenates after digestion in phosphoric acid (**Table 5.1**). Plasma was not acid digested due to sample volume constraints. This digestion oxidizes all trivalent As species to pentavalency, and thus, only the total iAs (iAs^{III+V}), MAs (MAs^{III+V}), and DMAs ($DMAs^{III+V}$) can be measured. Our laboratory has previously shown that in liver homogenates from WT mice exposed to 50 ppm As as iAs^{III} for 9 days, near 100% recovery of MAs and DMAs were achieved by direct HG-CT-AAS analysis; however, the recovery of iAs by direct analysis was only ~83% (167). A portion of iAs^{III} retained in this tissue is likely bound to high-affinity targets and is unavailable for hydride generation. A follow-up study revealed that the recovery of iAs in mouse liver increased to approximately 100% after 3 weeks of storage at 0°C or -80°C, indicating that prolonged storage may result in the release and subsequent oxidation of iAs^{III} from the high-affinity thiol binding sites (193).

In the present study, As recovery during direct analysis of all treatment groups was greater than 80% for pancreas liver, lungs, intestine, brain, adipose tissue, and blood cells. As recoveries lower than 80% were observed for three tissues collected from *As3mt*-KO mice. In heart and skeletal muscle of *As3mt*-KO mice, the recovery of iAs ranged from 52 to 89%, while in testes of *As3mt*-KO mice treated with 30 ppm As, 66% of iAs was recovered during direct analysis. In WT mice, total As recoveries were low (63%-85%) in kidney and skeletal muscle. In adipose tissue of WT mice, 89% of iAs was recovered. Notably, only 25% of MAs in kidney and 85% of DMAs in lung in WT mice

was recovered by direct analysis. High levels of MAs in kidneys of C57/BL6 mice treated with iAs^V have been previously reported (158). As accumulation has been observed in the renal cortex of rats treated with iAs, but As speciation and potential binding targets have not been identified (206).

Table 5.1: Recovery of speciated As during direct analysis

| | %recovery of direct analysis[a] | | | | |
| | *As3mt*-KO[b] | | | | WT[c] |
	15 ppm	20 ppm	25 ppm	30 ppm	50 ppm
Pancreas	106 (19)	89 (1)	90 (7)	92 (7)	103 (11)
Liver	119 (18)	112 (27)	106 (13)	108 (17)	119 (38)
Skeletal Muscle	52 (10)	66 (20)	60 (6)	63 (12)	85 (15)
Adipose Tissue	92 (50)	100 (45)	115 (96)	104 (14)	109 (47)
Blood Cells	133 (10)	139 (19)	142 (18)	148 (22)	128 (23)
Intestine	106 (21)	162 (31)	179 (121)	125 (27)	114 (49)
Kidney	123 (23)	81 (4)	112 (59)	98 (50)	63 (17)
Lung	95 (14)	121 (32)	128 (24)	131 (31)	102 (45)
Heart	71 (8)	78 (6)	77 (7)	89 (7)	124 (23)
Brain	83 (11)	80 (8)	83 (10)	101 (14)	120 (16)
Testes	114 (49)	178 (43)	123 (55)	66 (9)	156 (66)

[a] Mean (SD), *As3mt*-KO, n = 5 and WT, n = 10

[b] For *As3mt*-KO treatment groups, %recovery is for iAs^{III+V}

[c] For WT mice treated with 50 ppm As, %recovery is for total speciated As

5.4.4 Comparison of the internal of As

Previous studies in our laboratory have shown the development of diabetes in WT mice exposed to 50 ppm As (86, 87). While methylated As metabolites and toxic trivalent species have been detected in tissues critical for glucose homeostasis, the effects of specific As species on disease development have yet to be examined. The *As3mt*-KO mouse model provides a unique platform to study the adverse effects of individual arsenicals because little internal methylation occurs. However, the *As3mt* genotype

affects As retention and susceptibility to As-induced toxicity; studies in *As3mt*-KO mice have reported lethality in 50 ppm As exposures and toxic effects at exposure as low as 25 ppm, concentrations that are well tolerated in WT mice (56, 92). Thus, to determine which As exposures in *As3mt*-KO mice yield equivalent internal As doses compared to WT mice, As retention in tissues critical for glucose homeostasis (i.e., pancreas, liver, skeletal muscle, and adipose tissue) was examined (**Figure 5.3**). Here, statistical difference between the sum of iAs^{III+V}, MAs^{III+V}, $DMAs^{III+V}$ and $TMAs^{V}O$ for each *As3mt*-KO group and the WT group exposed to 50 ppm As were determined by one-way ANOVA and Bonferroni's multiple comparison test (**Table 5.2**). Total As values for *As3mt*-KO exposure groups that were not significantly different from the 50 ppm WT group were considered to produce equivalent internal doses. In the pancreas, equivalent internal doses to WT mice were only achieved in *As3mt*-KO mice exposed to 30 ppm As. Equivalent internal As doses in the liver were seen in *As3mt*-KO mice exposed to 15, 20, and 25 ppm As and for all exposures in adipose tissue. In skeletal muscle, 25 and 30 ppm As exposure in *As3mt*-KO mice resulted in equivalent internal As doses compared to WT mice exposed to 50 ppm As. Because recoveries of the direct analysis method determined after acid digestion were low (50-75%) for skeletal muscle in all treatment groups, statistical analysis was repeated with the digested sums of iAs, MAs, and DMAs (data not shown). Reanalysis revealed that all *As3mt*-KO treatment groups (15, 20, 25, and 30 ppm As) produced equivalent internal As doses in skeletal muscle compared to the 50 ppm WT group. However, the equivalent internal As doses determined for pancreas, liver or adipose tissue of *As3mt*-KO mice did not change.

While exposure to 30 ppm in *As3mt*-KO mice for 4 weeks produced equivalent total As tissue retention in the pancreas, skeletal muscle and adipose, decreased body weights were observed in this treatment group after 4 weeks of exposure (**Figure 5.2**). However, in contrast to other studies, As-induced toxicity after 4 weeks of exposure was not observed in any of the *As3mt*-KO treatment groups (56, 92). Nonetheless, study duration and potential toxicity should be carefully considered when planning experiments with *As3mt*-KO mice. Therefore, exposure to both 25 and 30 ppm would produce equivalent internal As doses in tissues critical for glucose homeostasis and will be used in future studies examining the effects of As on the development of diabetes.

Table 5.2: Determination of equivalent internal As doses in tissues critical for glucose homeostasis

| Treatment Group | Total speciated As[a], ng As/g tissue | | | |
	Pancreas	Liver	Skeletal Muscle	Adipose
0 ppm *As3mt*-KO	16 (7)	67 (10)	69 (102)	21 (14)
15 ppm *As3mt*-KO	381 (38)	3,137 (476)#	386 (55)	181 (107)#
20 ppm *As3mt*-KO	394 (86)	3,525 (787)#	479 (49)	304 (112)#
25 ppm *As3mt*-KO	452 (26)	4,497 (300)#	677 (114)#	413 (329)#
30 ppm *As3mt*-KO	576 (83)#	5,564 (1,024)	688 (147)#	277 (61)#
50 ppm WT	693 (141)	3,511 (1,174)	658 (185)	239 (118)

[a] Total speciated As includes the sum of iAs^{III+V}, MAs^{III+V}, $DMAs^{III+V}$ and $TMAs^{V}O$ determined by HG-CT-AAS. Mean (SD); *As3mt*-KO, n = 5 and WT, n = 10

Statistically non-significant difference in total speciated As compared to 50 ppm WT group ($p > 0.05$).

5.5 Conclusions

In A*s3mt*-KO mice, iAs^{III} is the most prevalent species in liver, pancreas, and adipose exposed to 15, 20, 25, or 30 ppm As. Moreover, the majority of species retained in liver and pancreas of WT mice exposed to 50 ppm As are in the trivalent form. $DMAs^{V}$ is the most prevalent species retained in skeletal muscle and adipose of WT

mice. For tissues critical to glucose homeostasis, doses of 25 and 30 ppm As as iAs^{III} in *As3mt*-KO mice will produce equivalent total As retention to that of WT mice. Ongoing studies will examine the effects of As methylation phenotype on the development of iAs-induced diabetes.

CHAPTER VI

Retention of Trivalent Arsenic Metabolites in Urothelial Cells is Associated with

Markers of iAs Exposure and Diabetes[5]

6.1 Overview

Chronic exposure to inorganic arsenic (iAs) in drinking water has been linked to an increased prevalence of diabetes. Laboratory evidence suggests that trivalent arsenicals formed in the course of iAs metabolism contribute to the diabetogenic effects of iAs exposure. To date, risk assessment of iAs-associated diabetes has relied almost entirely on the measure of As in drinking water or urine. This study examined whether trivalent arsenical retained in tissues can provide better markers of the diabetogenic effects of iAs exposure. We determined the concentrations of iAs in urine and in urinary bladder exfoliated cells (BECs) isolated from urine of 378 residents of Chihuahua, Mexico who drink water contaminated with iAs (0.01 - 400 ppb). Diabetes was diagnosed using fasting plasma glucose (FPG), two-hour plasma glucose (2HPG) after oral glucose tolerance test (OGTT), self-reported doctor diagnosis, or use of anti-diabetic medications. As species in BEC and in urine were measured by hydride generation (HG)-cryotrapping (CT)-inductively coupled plasma-mass spectrometry and HG-CT-atomic absorption spectrometry, respectively. Associations between diabetes and As species retained in BECs or found in urine were analyzed by logistic and linear regression with adjustment for age, sex, and body mass index. iAs was the major species retained in

[5] Jenna M. Currier, María C. Ishida, Carmen González-Horta, Luz M. Del Razo, Blanca Sánchez-Ramírez, Lourdes Ballinas-Casarrubias, Gonzalo García-Vargas, Michelle A. Mendez, John B. Buse, Daniela Guttierez, R. Jesse Saunders, Zuzana Drobna, Dana Loomis, and Miroslav Stýblo

BECs (~66% of total As). The log-transformed sum of As species and individual As metabolites retained in BECs were positively correlated with As metabolites in urine (ß = 0.31 – 0.79, r^2 = 0.06 – 0.55, p < 0.01) and iAs in drinking water (ß = 0.21 – 0.43, r^2 = 0.07 – 0.54, p < 0.01). There was significant positive correlation between FPG and 2HPG with iAs in drinking water, trivalent As species retained in BECs and urinary As species (ß = 0.041 – 0.078, r^2 = 0.12 - 0.21, p < 0.01). Moreover, the risk of diabetes was positively associated with iAs^{III} and MAs^{III} in BECs (OR = 1.57 and 1.63, 95% CI 1.19 – 2.07 and 1.24 – 2.15 per inter-quartile range, respectively, p < 0.01) and with the ratio of DMAs/MAs in urine (OR 1.37 per inter-quartile range, p = 0.03). Significantly negative correlations between DMAs/MAs and DMAs/iAs ratios in BECs and the risk of diabetes were observed (OR 0.62 and 0.72 per inter-quartile range, respectively, p < 0.02). These data suggest that As metabolites retained in BECs are sensitive biomarkers of iAs exposure and the risk of diabetes associated with this exposure.

6.2 Introduction

Growing evidence suggests that chronic exposure to inorganic arsenic (iAs) increases risk of developing diabetes. A recent National Toxicology Program (NTP) workshop on the diabetogenic and obesogenic effects of environmental chemicals concluded that there was sufficient evidence to link iAs exposures to an increased prevalence of diabetes in populations exposed to levels of iAs in drinking water greater than 150 ppb (5). Strong associations between iAs exposure and diabetes were found in areas of Taiwan (99, 101-103) and Bangladesh (100, 104) where individuals are exposed to iAs in drinking water at levels greater than 500 ppb. However, results from epidemiological studies in population exposed to lower levels of iAs are inconsistent.

The World Health Organization and American Diabetes Association define the diagnostic criteria for diabetes as individuals presenting with fasting plasma glucose levels (FPG) ≥ 126 mg/dL (based on two independent examinations) or two-hour plasma glucose levels (2HPG) ≥ 200 mg/dL after oral glucose tolerance test (OGTT) (97, 207). Additionally, levels of glycosylated hemoglobin (HbA1c), an estimate of average plasma glucose level over a 2 to 3 month period, ≥ 6.5% are sufficient for a diabetes diagnosis (98). Recently, concerns regarding diabetes diagnosis and exposure assessment have been raised for earlier epidemiological studies assessing the diabetogenic effects of iAs mainly in high exposure areas (5, 105). Results from a recent study of individuals in Bangladesh primarily exposed to < 300 ppb As in drinking water showed no associations between time weighted As exposure or urinary As and self-reported diabetes or glycosuria (106). However, glucosuria is not an accepted diagnostic marker of diabetes (208).

Several recent studies provide consistent associations between As exposure and diabetes (70, 111, 112) or gestational diabetes (113) in populations exposed to less than 150 ppb As. In these studies, diabetes was diagnosed by preferred methods, including FPG, OGTT, or HbA1c levels. Notably, in an As-endemic region of Mexico, urinary DMAs[III] concentration was significantly associated with the prevalence of diabetes (70). Additional epidemiological studies with improvements in study design, including increasing population size, conducting more accurate exposure assessment, and using consistent diagnostic criteria for diabetes are likely to strengthen the evidence for an As-induced diabetes even at low levels of exposure.

Assessment of iAs exposure often relies on urinary As excretion or estimates of As ingestion and varies widely among epidemiological studies examining the toxicity of

iAs. Because iAs levels in drinking water sources and individual consumption patterns vary, measurements of exposure using these variables may not accurately reflect actual internal doses. As metabolites in biological samples, including blood, hair, nails or urine, can be used as biomarkers of exposure. Although, hair and nail matrices are rich in sulfhydryl moieties that accumulate As and are not recommended for As exposure analysis (115). Moreover, As concentration in urine can be affected by hydration status and normalization techniques have limitations. Recent reports indicate that urinary creatinine is associated with total urinary As (116) and increased urinary DMAs excretion (117). Furthermore, creatinine adjustment in diabetic individuals was shown to overestimate urinary As levels (118). Specific gravity may also be used for adjusting urinary As levels to hydration status. However, abnormal urinary glucose and albumin excretion associated with certain disease states, including diabetes, would result in the overestimation of urine density (119, 120).

Measurement of As concentrations in target tissues or body liquids other than urine may more accurately reflect iAs exposure. While these biomarkers have not been widely researched, exposure assessments have been performed in bladder exfoliated cells (BECs) (85) and saliva (121, 122). In BECs from 21 individuals living in an As-endemic region of Mexico, the retention of speciated As did not correlated with urinary As (85); however, As species in saliva samples from individuals exposed to As in China correlated with iAs concentrations in drinking water (121). Our optimization of an analytical technique for oxidation state specific As speciation can be used to examine As retention in target human tissues and assess the adverse effects of iAs exposure.

The hydride generation-cryotrapping (HG-CT) methods for the oxidation state specific analysis of As metabolites are uniquely suited for complex biological matrices because no sample pretreatments or extractions are required, thus limiting loses of the unstable methylated trivalent species (146, 149). In a pilot study conducted using BECs collected from 21 individuals residing in Zimapan, Mexico, As metabolites were analyzed using HG-CT-AAS, but oxidation state specific speciation was not performed (85). Recent optimization of a HG-CT-inductively coupled plasma-mass spectrometry (ICP-MS) technique provides limits of detection (LOD) between 0.04 and 2.0 pg As compared to 10 and 26 pg for HG-CT-AAS. This system has been successfully used to quantify iAs metabolites in murine pancreatic islets exposed *ex vivo* (136). Here, we aimed to examine the retention of tri- and pentavalent metabolites of iAs in BECs isolated from residents of an As-endemic region of Mexico and determine the associations between diabetes and markers of As exposure in BECs and urine. Our findings indicate that As metabolites retained in BECs are sensitive biomarkers of the diabetogenic effects of iAs exposure.

6.3 Methods

6.3.1 Study Population

Study subjects were recruited among residents living in Chihuahua, Mexico for at least two years. The study area is located in eastern and north-eastern regions of Chihuahua state where As levels in drinking water range from <10 to 800 ppb. Recruitment was carried out in collaboration with local public health and municipal authorities. Residents were initially approached in their homes and invited for a brief screening interview. The interview informed participants about the purpose of the study

and determined eligibility to participate. Pregnant women and subjects with kidney or urinary tract infection or those using medication for this type of infection were excluded because these conditions could affect the urinary pattern of iAs metabolites. Individuals with previous occupational exposure to As were also excluded. Subjects enrolled were transported to Chihuahua University to undergo a thorough evaluation, including a basic physical exam and a medical exam focusing on skin lesions and other pathologies associated with exposure to iAs. The body weight, height, body mass index (BMI), waist-to-hip ratio, and blood pressure for each subject were recorded.

6.3.2 Sample Collection

Individuals provided drinking water samples for As analysis and spot urine samples were collected at Universidad Autónoma de Chihuahua between 8 and 10 am. The containers were immediately stored on ice and divided into two 50 mL polyethylene tubes. BECs were isolated by centrifugation at $700 \times g$ for 10 min at 4°C. Cells from each donor were then transferred into a single conical 1.5-mL Eppendorf tube, washed with 1 mL ice-cold phosphate-buffered saline (PBS), and centrifuged at $700 \times g$ for 10 min at 4°C. Cells were resuspended in 1 mL of PBS, and 10 µL was removed to perform cell counts. This aliquot was incubated with an equal volume of 0.4% trypan blue exclusion dye for 3 minutes and cells were counted using a hemocytometer. Urines contaminated with yeast or bacteria were excluded from the study. The remaining urine aliquot was pelleted by centrifugation and stored at -80°C. The BEC pellets were packed in dry ice and air-shipped to UNC-Chapel Hill once per month. Here, the pellets were analyzed within three days for tri- and pentavalent As species. Aliquots of urine were stored at -80°C and shipped with the BECs to UNC-Chapel Hill for analysis of total tri- plus

118

pentavalent species. To assess hydration status, urinary creatinine and specific gravity measurements were performed. A multi-well colorimetric assay kit was used to determine urinary creatinine concentration (Cayman Chemical Company, Ann Arbor, MI), and urine density was measured using a digital Atago PAL refractometer (Atago USA, Bellevue, WA).

MAs^{III} and DMAs^{III} are not stable in urine; however, we have shown that in the cellular environment, these trivalent arsenicals in cell lysates from an As methylating urothelial cell line are stable for at least 3 weeks at -80°C (193). Moreover, in a simulated shipping experiment, DMAs^{III} in cell lysates was stable for at least two days when packed in dry ice (**Figure 3.9**). Based on these results, the concentrations of MAs^{III} and DMAs^{III} in exfoliated urothelial cells isolated in Chihuahua should remain unchanged if the cells are frozen at -80°C immediately after collection and shipped in dry ice to UNC-Chapel Hill.

6.3.3 Diagnosis of Diabetes

Fasting venous blood was collected during the medical exam, followed by an OGTT where individuals were administered 75 g of glucose and samples of venous blood were collected 30 minutes and 2 hours into the test. All three blood samples were collected in EDTA-vacutainers. Plasma was isolated from freshly collected blood by centrifugation at 4°C and stored at -80°C. Plasma glucose levels were measured by Prestige 24i Chemistry Analyzer (Tokyo Boeki, Tokyo, Japan). To assure accuracy, the analyzer was calibrated prior to analysis of plasma samples and reference human sera with normal and elevated glucose levels were used for quality control. FPG and 2HPG levels were used to identify diabetic subjects. Study participants were classified as

diabetic if they presented with a FPG level $\geq$ 126 mg/dL, 2HPG $\geq$ 200 mg/dL, or if they reported a doctor's diagnosis or use of anti-diabetic medication. In participants not taking anti-diabetic medication, FPG and 2HPG levels correlated well ($r^2 = 0.42$, $p < 0.01$).

6.3.4 Analyses of As in Urine and BECs

As species in BECs were analyzed at UNC-CH by HG-CT-ICP-MS using the previously described method (136). Briefly, iAs and the methylated arsenicals in the BECs are reduced to their corresponding arsines (arsine, methylarsine, and dimethylarsine) in a reaction mixture containing 1% sodium borohydride ($NaBH_4$; Sigma-Aldrich, St. Louis, MO) and a 0.75 M Tris(hydroxymethyl)aminomethane (TRIS)-HCl buffer (pH 6; Sigma-Aldrich). The gaseous phase with generated arsines is separated from the liquid phase by gas-liquid separator and dried by a polyethylene cartridge (100 mm long, 17 mm i.d.) containing approximately 25 g of NaOH pellets (Sigma-Aldrich) (172). The arsines are cryotrapped in a Ni80/Cr20 wire wrapped capillary U-tube (total resistance of 15 Ω, Omega Engineering, Inc., Stanford, CT) filled with approximately 0.9 g of Chromosorb WAW-DMCS 45/60, 15 % OV-3 (Supelco, Bellefonte, USA) and submerged in a Dewar flask filled with liquid N_2. After sufficient time for arsine generation and cryotrapping, the Dewar flask is removed from the U-tube and upon electrical heating, arsines are separated by their boiling points. The CT outlet is connected between the spray chamber and the torch of the Agilent 7500cx ICP-MS spectrometer (Agilent Technologies, Santa Clara, CA). The HG and CT steps were controlled by a FIAS 400 flow injection accessory (Perkin-Elmer, Norwalk, CT).

To determine both tri- and pentavalent As (As^{III+V}) species, one aliquot of each sample is measured directly to determine As^{III} species and an additional aliquot is

measured after pretreatment for one hour at room temperature with 2% L-cysteine (biochemistry grade, EMD Chemicals, Inc., Gibbstown, NJ) to determine As^{III+V} species. At pH 6, trivalent arsenicals (iAs^{III}, MAs^{III} and $DMAs^{III}$) selectively generate arsines directly without sample extraction or pretreatment, while L-cysteine reduces the pentavalent arsenicals to trivalency, allowing determination of both As^{III} and As^{V} species. The concentrations of iAs^{V}, MAs^{V}, and $DMAs^{V}$ are determined as a difference between signals obtained for cysteine-treated and untreated sample aliquots. Aliquots of urine from individuals providing BECs were also shipped to UNC-CH for analysis. Here, samples were measured only for As^{III+V} species by HG-CT-AAS after 2% L-cysteine pretreatment due to the rapid oxidation of MAs^{III} and $DMAs^{III}$ in this matrix (70).

Calibration curves for the quantification of tri- and pentavalent As species in this study were generated using aqueous solutions of the pentavalent standards, (iAs^{V}, MAs^{V}, and $DMAs^{V}$) treated with L-cysteine. We have previously shown that the slopes of the calibration curves for the L-cysteine-treated As^{V} standards prepared in DIW are identical with the slopes of curves generated for the trivalent arsenicals in the absence of cysteine (149). We have also shown that the slopes of the calibration curves for the L-cysteine-treated aqueous solutions of As standards do not significantly differ from the slopes of curves prepared for liver homogenates spiked with these standards and treated with L-cysteine (149). Moreover, our results indicate that trivalent arsenicals are stable in these matrices when stored at -80°C for at least 3 weeks and when shipped in containers packed on dry ice for at least 2 days (167, 193). The quantification of individual As species was made by determining peak areas for arsine and the methyl-substituted arsines.

6.3.5 Statistical Analysis

Continuous variables were analyzed using means and standard deviations, and their distributions were assessed graphically. Categorical variables were described using frequencies. For As species below the limit of detection (LOD), LOD/√2 values were used for statistical analysis. The LOD ranged from 10 to 26 pg for urinary As measurements by HG-CT-AAS and 0.04 to 2.0 pg for BEC As measurements by HG-CT-ICP-MS. The associations of the diabetic phenotype with iAs in water and As species in BEC and urine were assessed using logistic regression to estimate odds ratios (ORs) and 95% confidence intervals (CIs). To control for potential confounding, age, sex, and BMI were included as covariates. Associations of FPG and 2HPG with measures of iAs metabolites and the sum of speciated As were analyzed by linear regression. Age, sex, and BMI were included as covariates in these models. The assumption of linear exposure-response was assessed graphically and found to be adequate. ORs, regression coefficients, and CIs are reported for a one interquartile range (IQR) increment of exposure to facilitate comparison because of the different concentration ranges of As in BECs, urine, and water. Analyses of urinary metabolites of iAs were conducted both with and without urinary creatinine concentration and specific gravity adjustment to assess for possible effects of variation in hydration status. Chromatograms from HG-CT-AAS and HG-CT-ICP-MS analyses were exported and peak areas were integrated in Microsoft Excel. All statistical analyses were performed in Epi Info 7 version 1.0.6 (Centers for Disease Control and Prevention, Atlanta, GA) and graphical representations were generated using GraphPad Instat software package (GraphPad Software Inc., San Diego, CA). Statistical significance was considered at the level of $p < 0.05$.

6.4 Results and Discussion

6.4.1 Study Population Characteristics

We isolated BECs from a cohort within the NIH population study, "Environmental arsenic and diabetes mellitus," (n = 378). Individuals participating in this study lived in As-endemic regions of Chihuahua, Mexico for at least the previous two years. General characteristics of this cohort are presented in **Table 6.1**. Enrolled subjects included both males (n = 123) and females (n = 255) ages 18 to 90. The average BMI of the population was 29.2 ± 6.1 with 41% presenting as clinically obese (BMI > 30). FPG and 2HPG levels were measured in 374 individuals. Samples of drinking water collected from 300 individuals contained iAs concentrations ranging from 0.01 to 275 ppb.

The prevalence of diabetes in the cohort examined in this study was 18% (n = 66), and consisted of 44 females and 22 males. Glucose tolerance was within normal or pre-diabetic ranges (FPG < 126 and 2HPG < 200) for six individuals that reported taking anti-diabetic medications. Four subjects reported a previous doctor diagnosis but had normal FPG or 2HPG. Moreover, one individual reported a previous diabetes diagnosis, but FPG or 2HPG data was unavailable. Logistic regression analysis of the associations between diabetes and markers of iAs exposure were performed with and without these 11 subjects.

Table 6.1: Characteristics of the study population

	Population		Diabetic[a]		Non-diabetic	
	Mean (N)	SD (%)	Mean (N)	SD (%)	Mean (N)	SD (%)
Population (N, %)	(378)	(100)	(66)	(18)	(308)	(82)
Female (N, %)	(255)	(67)	(44)	(67)	(211)	(67)

Age (years)	49.0	16.0	56*	12.0	48*	16.0
iAs in drinking water (ppb)	54.9	52.7	60.0	50.9	53.7	53.2
BMI	29.2	6.1	30.8*	5.4	28.9*	6.2
BMI > 30	(155)	(41)	(31)	(47)	(124)	(40)
FPG (mg/dL)	95.9	39.5	155.7*	62.8	83.2*	12.1
2HPG (mg/dL)	118.6	60.4	204.9*	86.0	100.4*	31.1
Sum As Urine (ng/mL)	73.6	73.3	82.0	74.9	71.8	72.9
Sum As BECs (pg/10,000 cells)	126.4	357.9	90.3	202.1	134.0	382.6
Creatinine (mg/dL)	128.4	90.1	127.1	85.7	128.7	91.1
Sum As Urine (ng/mg creatinine)	65.8	66.8	73.1	77.9	64.2	64.3
Specific Gravity[b]	1.014	0.007	1.017*	0.008	1.014*	0.007
Sum As Urine (ng/SG unit)	109.5	102.2	92.6	62	113.1	108.5

[a] Diabetes classified by either FPG $\geq$ 126 mg/dL, 2HPG $\geq$ 200 mg/dL, self-report of doctor diagnosis or use of anti-diabetic medication. Previous diabetes diagnosis, FPG or OGTT data are not available for four individuals and are excluded from the diabetes stratification analysis.

[b] Specific gravity was measured in 377 samples.

* For continuous variables, a significant different between diabetics and non-diabetics by Student's t-test, $p < 0.05$.

For some variables, significant differences between diabetics and individuals free of diabetes (non-diabetic) were observed (**Table 6.1**). The average age of diabetic individuals was 56, while non-diabetics were an average of 48 years old. However, those classified as diabetic ranged in age from 24 to 84. Moreover, individuals presenting with diabetes exhibited a significantly higher ($p < 0.05$) average BMI (30.8) compared with of non-diabetics (28.9). The average urinary specific gravity was significantly higher ($p < 0.05$) in diabetic individuals (1.017) compared with non-diabetics (1.014). Impaired kidney function associated with the diabetic phenotype can result in the excretion of glucose or protein in the urine, which will overestimate urine density, leading to the underestimation of urinary arsenicals in these individuals when adjusted for specific gravity.

6.4.2 Markers of iAs Exposure

The concentration of iAs in drinking water and metabolites of iAs exposure in BECs and urine of individuals participating in this study were measured and are presented in **Table 6.2**. The levels of iAs in drinking water obtained from 300 study participants ranged from 0.01 to 275 ppb. Urinary As was measured for total tri- and pentavalent As species, iAs^{III+V}, MAs^{III+V}, and $DMAs^{III+V}$. Trimethylarsine oxide ($TMAs^{V}O$) was not measured because this arsenical is lost during 2% L-cysteine pretreatment (146). MAs^{III+V} and $DMAs^{III+V}$ were measured in all 378 urine samples, while iAs^{III+V} was below the LOD in five samples. $DMAs^{III+V}$ was the predominant species present in urine, accounting for an average of 76% of speciated As. iAs^{III+V} and MAs^{III+V} represented approximately 9% and 15% of speciated As, respectively.

In BECs, oxidation state specific analysis of As species was performed and the following six arsenicals were quantified: iAs^{III}, iAs^{V}, MAs^{III}, MAs^{V}, $DMAs^{III}$, and $DMAs^{V}$. For individual As species, concentrations greater than the LOD were observed in 94% to 99% of samples. All BEC samples contained measurable quantities of As for at least three As species. Notably, iAs^{III} was the predominant species retained in BECs, representing on average 36% of the sum of the total speciated As. Notably, only small amounts of $DMAs^{III}$ were retained in BECs, representing on average 2.4% of the sum of As species. $DMAs^{III}$ is the least stable trivalent arsenical in aqueous (14) or cellular matrices (193) and under these HG conditions, up to 5.9% of $DMAs^{V}$ has been shown to form dimethylated arsine in this matrix (unpublished data). This suggests that some, if not all $DMAs^{III}$ measured in BECs is likely in the oxidized form.

Table 6.2: Descriptive statistics for exposure to iAs in drinking water and iAs metabolites in BECs and urine, n = 378.

BECs (pg As/10,000 cells)	Min.	25th	Median	75th	Max.	Mean	SD
iAs^{III}	0.04	2.05	8.12	17.65	1807	23.82	102
MAs^{III}	0.01	0.44	1.76	4.02	151.7	4.14	11.16
DMAs^{III}	0.01	0.16	0.40	1.47	141.3	2.7	9.53
iAs^{V}	0.001	1.27	4.52	22.46	728.7	34.45	85.86
MAs^{V}	0.0003	0.19	0.85	4.87	776.2	12.98	52.21
DMAs^{V}	0.001	0.66	1.86	13.67	2303	48.67	199.7
Sum As^{III+V}	0.78	9.94	24.91	74.5	3773	126.4	357.9
MAs/iAs ratio	0.01	0.15	0.2	0.28	3.64	0.26	0.28
DMAs/MAs ratio	0.04	0.55	1.10	2.87	51.47	2.14	3.63
DMAs/iAs ratio	0.004	0.10	0.20	0.50	35.0	0.77	2.5
MAs+DMAs/iAs ratio	0.02	0.29	0.40	0.78	35.63	1.03	2.66
Urine (ng As/mL)							
iAs^{III+V}	0.02	0.92	4.61	10.21	119.2	7.4	10.4
MAs^{III+V}	0.03	2.21	7.31	15.97	131.1	11.1	13.0
DMAs^{III+V}	0.36	12.24	40.38	82.73	307.2	55.12	53.8
Sum As^{III+V}	0.52	15.62	53.23	108.4	492.5	73.61	73.27
MAs/iAs ratio	0.10	1.28	1.64	2.12	199.4	4.90	19.1
DMAs/MAs ratio	1.73	4.09	5.18	7.05	86.2	6.22	5.25
DMAs/iAs ratio	0.41	6.43	9.25	12.45	2117	31.0	144.4
MAs+DMAs/iAs ratio	0.51	7.96	10.94	14.59	2317	35.94	162.6
Drinking Water$^{\#}$	0.01	5.88	48.41	83.72	275.4	54.9	52.7

[#] iAs in drinking water (ng iAs/mL) was measured in 300 samples.

The As speciation profiles for urine and BECs are depicted in **Figure 6.1** and suggest that the urinary profiles of iAs metabolites are not an accurate reflection of actual As retention in target tissues. iAs^{III+V} represented 65% and 9% of sum of As species in BECs and urine, respectively. DMAs^{III+V} was highly excreted in urine, representing an average of 76% of total speciated As, but it only represented an average of 22% of total speciated As in BECs. These results are in agreement with a previous study examining the effects of iAs^{III} on urinary bladder cell proliferation in mice. The authors report accumulation of iAs^{III} in bladder tissue with primary urinary excretion in the form of DMAs (209). High affinity binding of iAs^{III} species to intracellular sulfhydryls, including

glutathione (210) and protein thiols (211) may drive the retention of this As species in BECs.
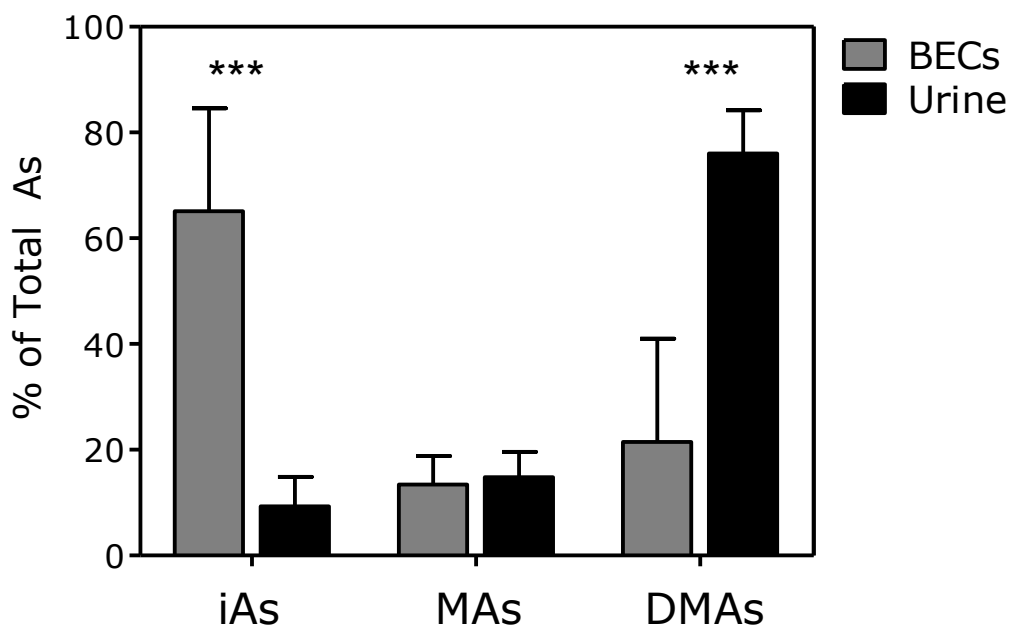
Figure 6.1: Differences in composition of As species in BECs and urine. (***) Significant difference in percent of total speciated As between BECs and urine performed by one-way ANOVA, $p < 0.05$.

To assess the sensitivity of As species retained in BECs as biomarkers of iAs exposure, associations between log-transformed arsenicals in BECs and urine were compared. While a pilot study examining the retention of As in BECs of 21 individuals residing in Zimapan, Mexico showed no correlation between arsenicals measured in BECs and urine, results of this larger population study show significant positive associations (**Figure 6.2**). The trivalent As species, iAs^{III} and MAs^{III} retained in BECs were highly correlated with iAs^{III+V} and MAs^{III+V} in urine ($\beta = 0.67$ and 0.79, $r^2 = 0.45$ and 0.49, respectively). The strongest association was observed for sum of As^{III} species in BECs and sum of As species in urine ($\beta = 0.88$, $r^2 = 0.55$). For the 300 individuals that provided drinking water samples, iAs concentrations were compared to urinary excretion

and BEC retention of As species. **Figure 6.3** and **Figure 6.4** display scatter plots of log-transformed iAs in water and As species in urine or BECs, respectively. Significant positive associations with iAs in water were observed for all As species in urine regardless of adjustment for hydration status (ß = 0.32 to 0.41, r^2 = 0.41 to 0.75) and for As species retained in BECs (ß = 0.21 to 0.43, r^2 = 0.07 to 0.54). The strongest associations between iAs in drinking water were for sum of As^{III+V} species in urine (ß = 0.40, r^2 = 0.68) and sum of As^{III} species in BECs (ß = 0.42, r^2 = 0.54).

The positive associations between arsenicals in BEC, urine, and drinking water are in agreement with previous assessments of the relationship between iAs exposure and population-derived biomarkers, including As in urine (212), blood (213), saliva (121), hair (214), and toenails (215). However, unlike previous reports, the present study directly measures As species in cells originated in a target tissue.
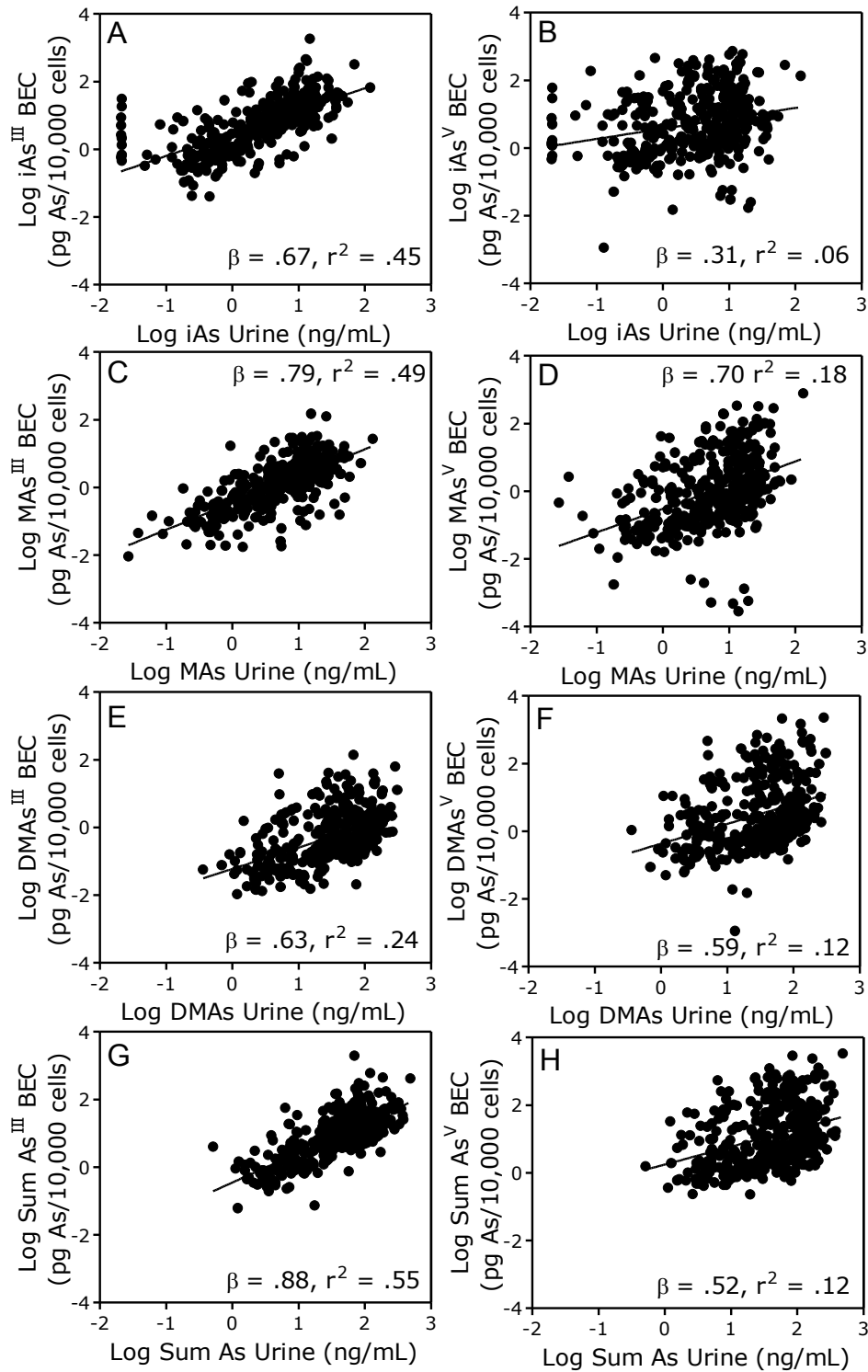
Figure 6.2: The correlations between logarithmically transformed concentrations of iAs (A,B), MAs (C,D), DMAs (E,F) and sum of As species (G,H) in urine and BECs. Results of linear regression analysis are presented as slopes (ß) and coefficient of determination (r^2). All slopes are significantly non-zero, $p < 0.05$.
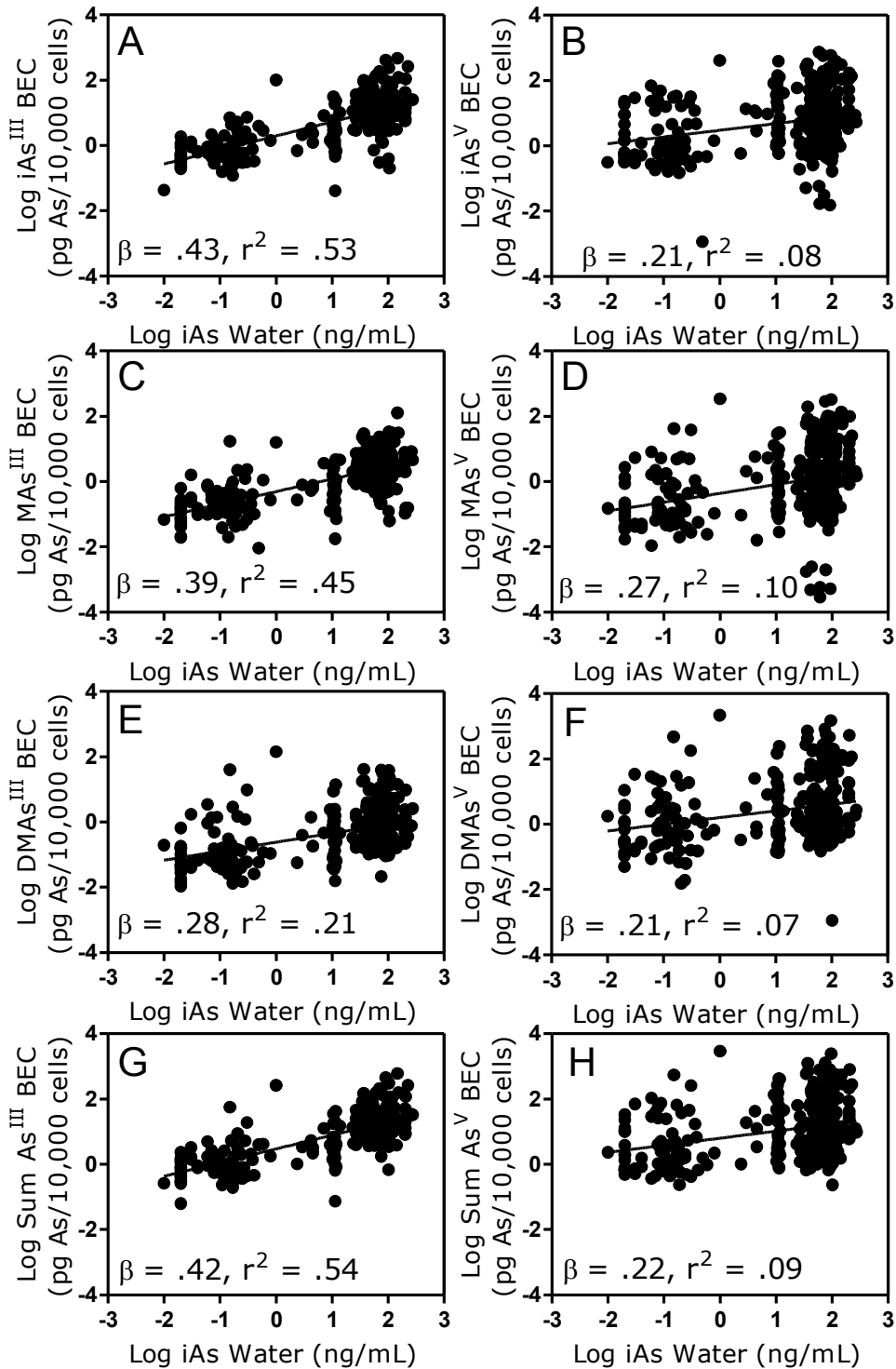
Figure 6.3: The correlations between logarithmically transformed concentrations of iAs in water and iAs (A,B), MAs (C,D), DMAs (E,F) and sum of As species (G,H) in BECs. Results of linear regression analysis are presented as slopes (ß) and coefficient of determination (r^2). All slopes are significantly non-zero, $p < 0.05$.
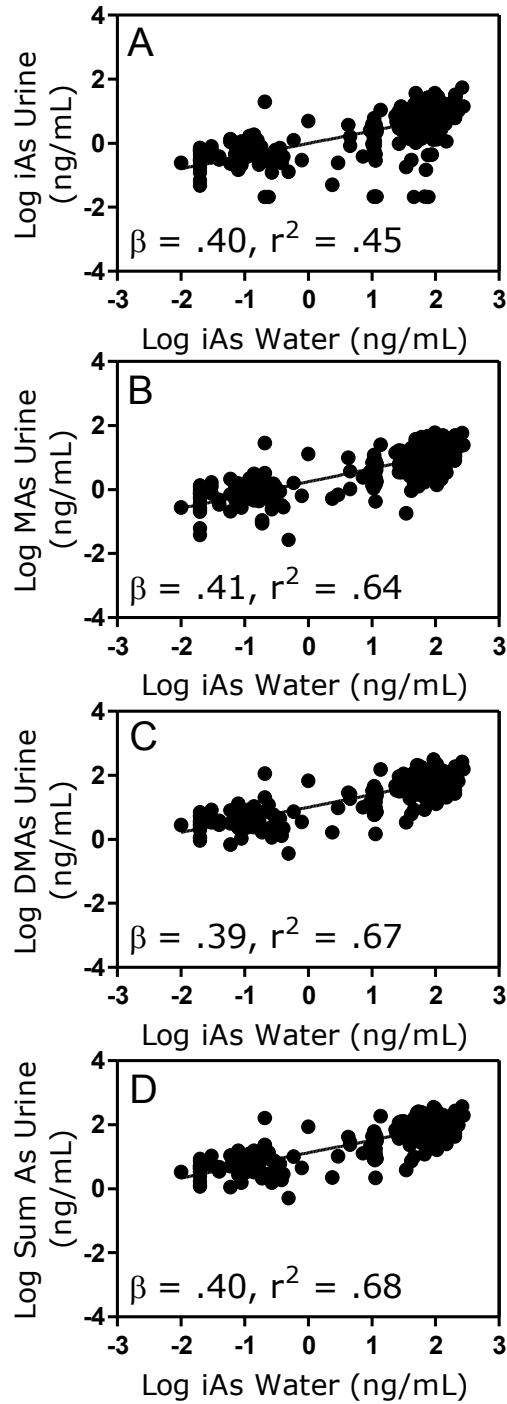
Figure 6.4: The correlations between logarithmically transformed concentrations of iAs in drinking water and As metabolites in urine. Results of linear regression analysis for iAs (A), MAs (B), DMAs (C), and sum of speciated As (D) in urine compared with iAs in drinking water are presented as slopes (ß) and coefficient of determination (r^2). All slopes are significantly non-zero, $p < 0.05$.

6.4.3 Gender Differences in BECs As Retention

Significant differences in numbers of bladder cells exfoliated and As retention were observed between males and females in this study (**Figure 6.5**). Cell counts from BEC samples obtained from males ranged from 450 to 2,128,000 cells, while samples from females ranged from 1,800 to 9,717,000 cells. BECs from females contained on average 10-fold more cells than samples from males (**Figure 6.5A**). Notably, when As retention in BECs was normalized for cell counts, significant differences in the sum of As species between males and females are observed. The sum of speciated As in BECs from males and females ranged from 0.78 to 3773 pg/10,000 cells and 0.83 to 2367 pg/10,000 cells, respectively. However, average As retention was significantly lower in BECs from females (39 ± 162 pg/10,000 cells) than males (307 ± 541 pg/10,000 cells) (**Figure 6.5B**). Anatomical differences in the urinary and genital tracts of females may contribute to the presence of vaginal epithelial cell contamination. Moreover, the vesical trigone area of bladders from adult female bladders is increasingly susceptible to squamous metaplasia, a phenomenon rarely observed in males; this finding in women decreases with age (216). Notably, regression analysis reveals a significantly negative association between age and BEC counts from females in this study (β = -11,287, r^2 = 0.04).
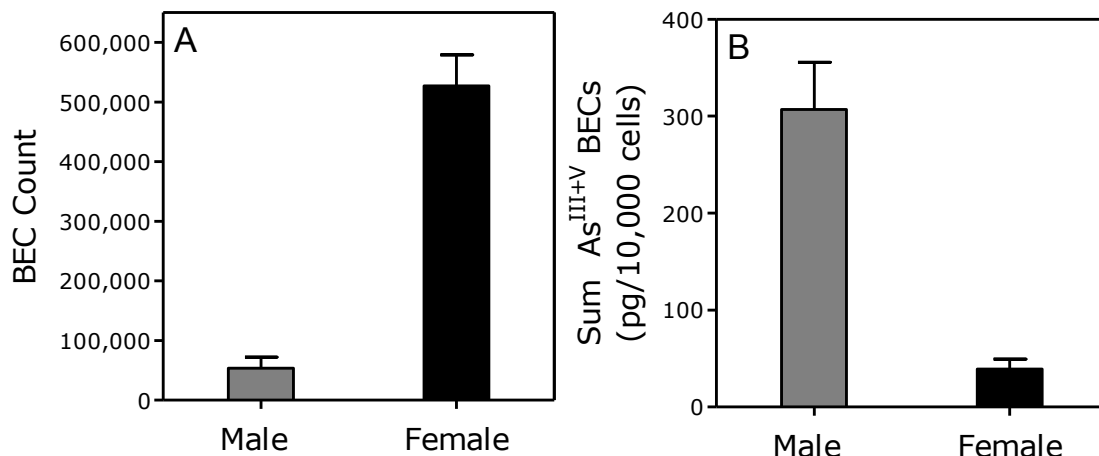
Figure 6.5: Gender differences in cells counts (A) and As retention (B) in BECs. Values are presented as mean ± SEM, n = 378.

While cells were observed microscopically and assessed for viability by dye exclusion during cell counting, cell types were not characterized. Because distinguishing between epithelial cells of urothelial or squamous origin is not easily achieved using routine visual or staining techniques, immunohistochemistry has been used to characterize squamous metaplasia in bladder cancer (217) and interstitial cystitis (218). This approach was not possible in our study due to only limited numbers of cells collected from subject urines. The lower As retention for female BEC samples could be a dilution effect; however, log-transformed scatter plots of cell counts and sum of As species in BECs for males and females presented in **Figure 6.6** suggest a more prominent dilution effect for males (ß = -0.70, r^2 = 0.54) than females (ß = -0.35, r^2 = 0.12). Additional differences in As retention and cell exfoliated counts may arise from differences in the characteristics of urination frequency (219) and urine concentration (220) between genders.
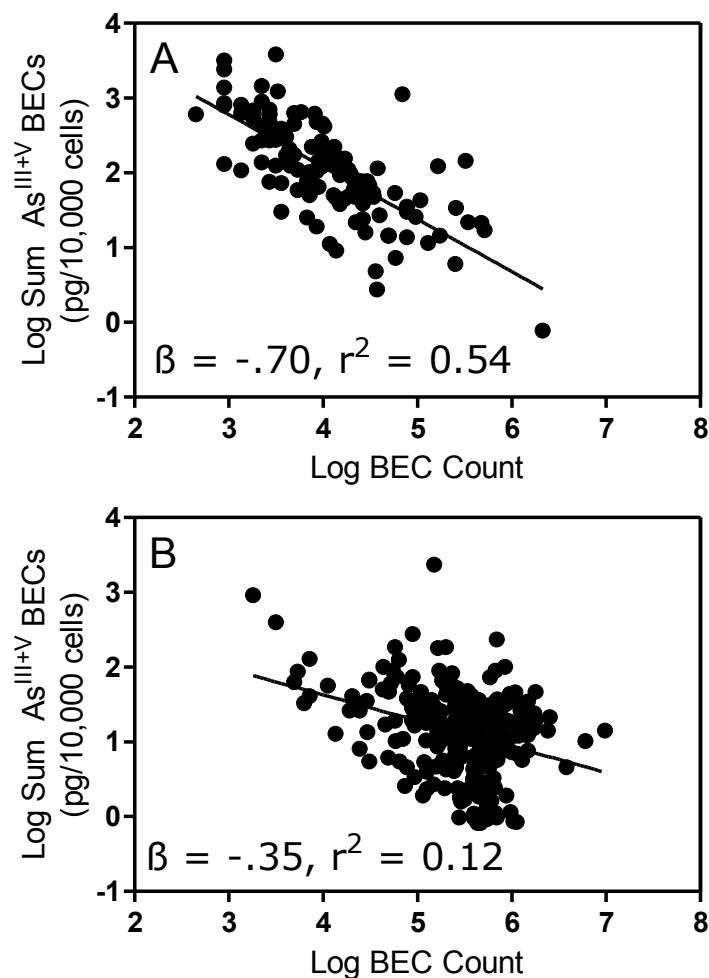
Figure 6.9: The associations of cell counts with sum of As species retained in BECs for samples obtained from males (A) and females (B). Results from linear regression analysis are presented as slopes (ß) and coefficient of determination (r^2). All slopes are significantly non-zero, $p < 0.05$.

The percent composition of As species retained in BECs or excreted in urine of males and females were compared (**Figure 6.7**). In BECs, significant differences between males and females were observed for all As species retained in BECs except DMAs[III]. iAs[III] and MAs[III] represented on average 43% and 10% of total speciated As in BECs from females, but only 18% and 4% in BECs from males. These data further suggest the cell type composition differs between genders and that iAs
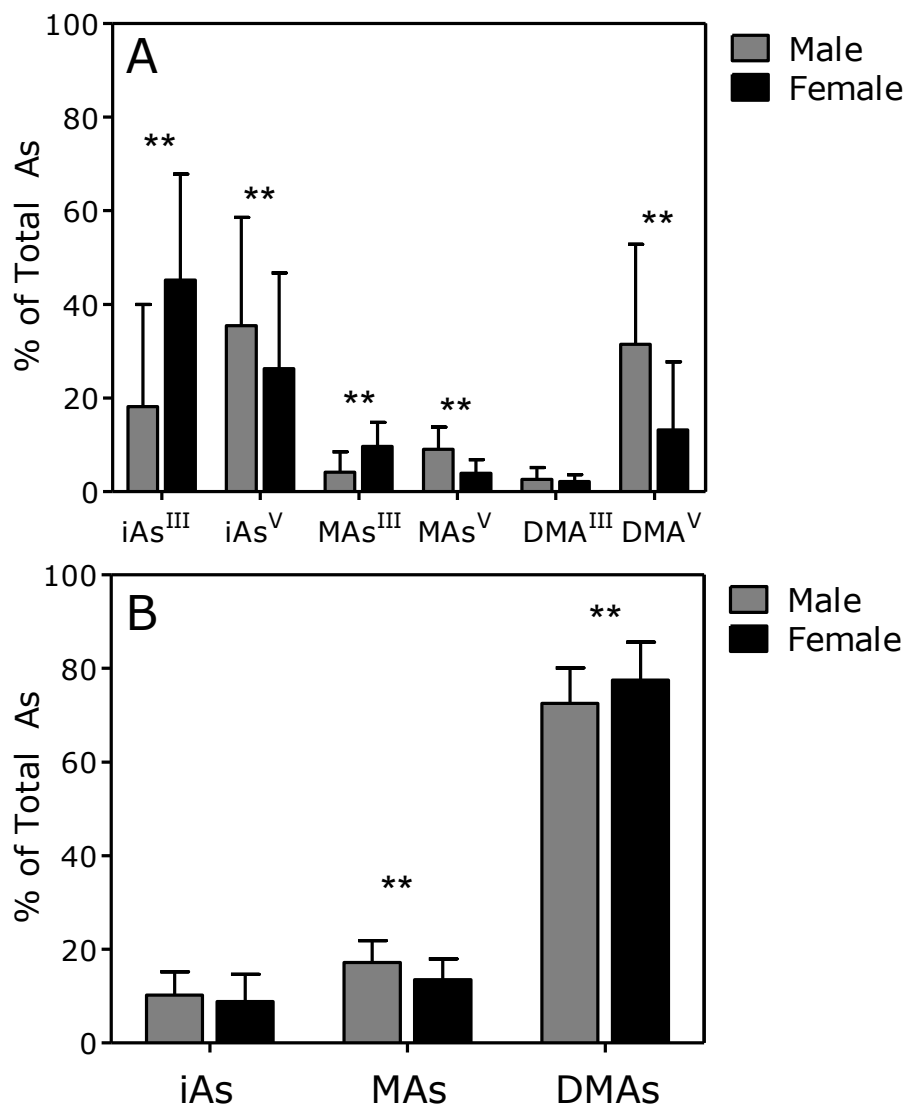
Figure 6.7: Gender differences in percent composition of As metabolites retained in BECs (A) and excreted in urine (B). Values for each arsenical represent percent of the sum of speciated As (mean $\pm$ SD, n = 378). (**) Significant difference between male and female percent composition determined by one-way ANOVA with Bonferroni's posttest ($p < 0.05$).

metabolism and retention are dependent on the cell type. Males excreted significantly more MAs^{III+V} in urine than females (17% vs. 14%), but less $DMAs^{III+V}$ (73% vs. 78%). Differences in urinary As excretion between genders have been previously reported. Notably, Basu and associates found that in a population exposed to iAs through drinking

water in West Bengal, India, males excreted lower iAs and higher MAs in urine compared to female and suggest that animal fat consumption is associated with increased urinary MAs excretion (117).

6.4.4 Associations of iAs Exposure and Diabetes

Diabetes classified by FPG, 2HPG, reported doctor diagnosis, or use of anti-diabetic medications was used to assess the associations between iAs in drinking water and As metabolites in BECs and urine. To adjust for potential confounding, age, sex, and BMI were included as covariates in each model. The logistic regression analyses presented in **Table 6.3** were performed for all individuals diagnosed with diabetes using the above criteria (Model 1, n = 374) and then repeated after exclusion of individuals reporting diagnosis or use of anti-diabetic medications, but not exhibiting diabetic phenotype based on both FPG and 2HPG measurements (Model 2, n = 363). To facilitate comparison between iAs exposure markers, ORs and CIs were standardized to an increment of one IQR. Concentration ranges corresponding to the IQR for each marker are presented in **Table 6.2**.

6.4.4.1 Associations of Diabetes with iAs in Drinking Water

While, ORs for diabetes classified by FPG $\geq$ 126 mg/dl, self-reported diabetes diagnosis or medication, were significantly higher for subjects exposed to high levels of iAs in drinking water (185 – 420 ppb As) in the larger cohort, no significant association was found between risk of diabetes diagnosis and current iAs concentration in drinking water (OR 1.27, 95% CI 0.96 – 1.68, p = 0.10) when standardized to IQR in this smaller cohort. The association did not change after restricting the classification criteria for diabetes to subjects with diabetic phenotype (i.e., FPG $\geq$ 126 and 2HPG $\geq$ 200) or

standardizing the OR to 10 ppb increments. Although, linear exposure response assessment presented in **Figure 6.8** reveals significant associations between individuals with iAs concentration is drinking water in the 25th percentile (0.01 – 7.21 ppb; OR = 1.00) compared to either the 50th (7.22 – 48.69 ppb; OR 4.50, 95% CI 1.63 – 12.43, $p <$ 0.01), 75th (48.70 – 83.72 ppb; OR 3.49, 95% CI 1.20 – 10.13, p = 0.02), or 100th percentile (83.73 – 275.36 ppb; OR 3.12, 95% CI 1.09 – 8.98, p = 0.10). The absence of a significant linear dose-response is likely due to variation in individual water consumption habits, which was not accounted for by the analysis. Furthermore, data on As in drinking water were available only for a subset of the cohort (n = 298).

These findings are in agreement with recent epidemiological studies conducted in As-endemic regions of Mexico (70) and Bangladesh (221) where diabetes diagnosis was based on criteria currently recommended by the WHO. Islam and associates reported an OR of 2.1 for individuals drinking water with iAs concentrations greater than 50 ppb; however, IQR analysis revealed significant associations for the highest quartile of iAs in drinking water (> 262 ppb) compared to the lowest quartile (10 – 22 ppb) (221). Notably, even the individuals in the lowest quartile drinking water iAs exposure ingest levels greater than 10 ppb, the current recommended maximum contaminate level set by the US EPA and WHO. In contrast, the lowest quartile of the present study contains individuals with iAs concentrations in drinking water less than 7.21 ppb. A pilot study conducted by our group in the Zimapan and Lagunera regions of Mexico, where individuals are exposed to similar concentration of iAs in drinking water, revealed a 13% increase in OR per 10 ppb increase in drinking water iAs concentration (OR 1.13, 95% CI 1.05 – 1.22, p < 0.01) (70).
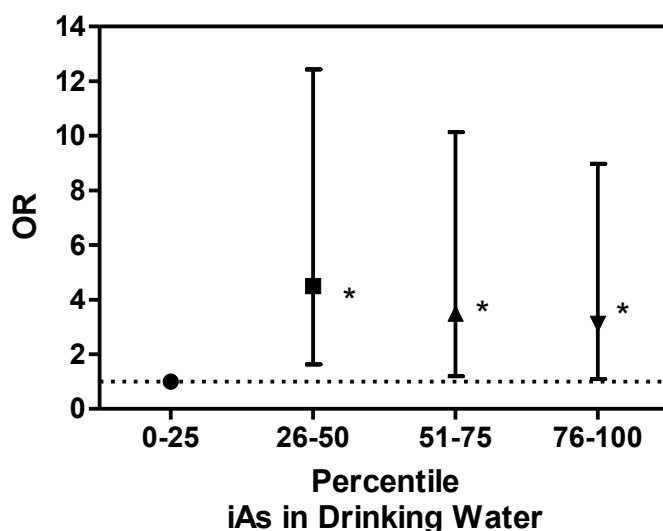
Figure 6.8: Association of diabetes with exposure to iAs in drinking water adjusted for age, sex, and BMI. Diabetes is classified by either FPG $\geq$ 126 mg/dL, 2HPG $\geq$ 200 mg/dL, self-report of doctor diagnosis or use of anti-diabetic medication, n = 374. (*) $p <$ 0.05 for the comparison of cases to non-diabetic individuals. The values for each IQR are presented in **Table 6.2**.

6.4.4.2 Associations of Diabetes with Metabolites of iAs in BECs and Urine

Diabetes was associated with trivalent As species retained in BECs (**Table 6.3**). For iAs^{III} in BECs, a 57% increase in OR for each IQR was observed (OR 1.57, 95% CI 1.19 $-$ 2.07, $p <$ 0.01). The strongest positive association was observed for MAs^{III} in BECs, with the OR increasing 63% per IQR (OR 1.63, 95% CI 1.24 $-$ 2.15, $p <$ 0.01). Notably, the ratios of DMAs/MAs (OR 0.62, 95% CI 0.47 $-$ 0.83, $p <$ 0.01) and DMAs/iAs in BECs were negatively associated with the risk of diabetes (OR 0.72, 95% CI 0.55 $-$ 0.96, $p =$ 0.02). When diabetes classification was restricted to subjects with the diabetic phenotype (Model 2), associations for As metabolites in BECs strengthened. Here, a significant positive association was found with DMAs^{III} (OR 1.49, 95% CI 1.04 $-$ 2.13, $p =$ 0.03) and the sum of As^{III+V} species (OR 1.41, 95% CI 1.01 $-$ 1.97, $p =$ 0.04) retained in BECs.

In urine, a significant positive association with risk of diabetes was only observed for DMAs/MAs ratio (OR 1.37, 95% CI 1.03 – 1.84, p = 0.03). However, when diabetes classification was limited only to subjects with diabetes phenotype, significant positive associations were also found for unadjusted $DMAs^{III+V}$ concentration (OR 1.34, 95% CI 1.02 – 1.76, p = 0.04). Here, significant associations were found for all As species in urine adjusted for urinary creatinine (**Table 6.3**). Notably, specific gravity was positively associated with diabetes when classified by FPG or 2HPG only (OR 1.42, 95% CI 1.07 – 1.89, p = 0.02). This suggests that adjusting urinary As concentration for urine density may mask potential associations because this variable is itself associated with diabetes.

Previously published OR values for the associations between As exposure and diabetes varied widely depending on the population, statistical model and method of exposure assessment (5). While no studies have used the concentrations of As species retained in BECs, in a study of gestational diabetes, women in the highest quartile of circulating As concentration in blood were at increased odds of having diabetes compared to women in the lowest quartile of blood As concentrations (OR 2.79, 95% CI 1.13 – 6.87, p = 0.01) (113). A reexamination of the concentrations of As species in BECs comparing individuals in the highest quartile with those in the lowest quartiles or exposure also reveals significant associations between iAs^{III} (OR 5.63, 95% CI 2.02 – 15.70, p = 0.001) and MAs^{III} (OR 4.99, 95% CI 2.01 – 12.37, p = 0.001), which are much stronger than those reported by Ettinger, et al. For urine, several studies have reported ORs examining the relationship between As exposure and the development of diabetes (107, 108, 110, 111). In the present study, comparing individuals in the highest to those in the lowest quartile of urinary speciated As concentrations (> 108.4 vs. < 15.6 ppb As)

also reveals significantly increased odds of exhibiting the diabetic phenotype (OR 3.44, 95% CI 1.35 – 8.85, p = 0.01). In a population study exposed to similar concentrations of As through drinking water, Coronado-Gonzalez and associates reported an OR of 2.84 (CI 95% 1.64 – 4.92, p = 0.01) when comparing individuals with urinary As concentrations > 105 ppb with those having < 63.5 ppb (111).These results are in agreement and suggest that even levels below 63.5 ppb may contribute to the development of diabetes.

6.4.4.3 Associations between Diabetes and iAs Exposure Based on Linear Regression Analysis

To further examine the relationships between iAs exposure and diabetes, linear regression analysis for log-transformed FPG and 2HPG and markers of iAs exposure was performed (**Table 6.4**). In contrast to the results of logistic regression analysis, FPG and 2HPG were positively associated with iAs in drinking water (r^2 = 0.21 and 0.17, p < 0.01). FPG and 2HPG were also positively associated with concentrations of iAs^{III}, MAs^{III}, DMAs^{III}, and the sum of As^{III+V} species retained in BECs (r^2 = 0.11 – 0.17, p < 0.01). In agreement with the logistic regression analysis, the ratios of DMAs/MAs and DMAs/iAs in BECs were negatively associated with FPG and 2HPG levels (r^2 = 0.08 – 0.12, p < 0.04). These finding suggest that increased methylation capacity as indicated by higher DMAs/MAs and DMAs/iAs protects against the risk of diabetes.

Table 6.3: Association of diabetes with exposure to iAs in drinking water and iAs metabolites in BECs and urine adjusted for age, sex, and BMI

	Model 1[a]				Model 2[b]			
	OR[c]	95% CI		p[d]	OR[c]	95% CI		p[d]
iAs in Water	1.27	0.96	1.68	0.10	1.30	0.96	1.75	0.09
BECs								
iAs^{III}	1.57	1.19	2.07	<0.01	1.75	1.29	2.39	<0.01
MAs^{III}	1.63	1.24	2.15	<0.01	2.02	1.48	2.77	<0.01
DMAs^{III}	1.13	0.96	1.84	0.09	1.49	1.04	2.13	0.03
iAs^{V}	1.23	0.90	1.67	0.20	1.41	1.00	1.98	0.05
MAs^{V}	1.09	0.79	1.50	0.61	1.26	0.89	1.78	0.20
DMAs^{V}	0.97	0.71	1.33	0.85	0.99	0.70	1.38	0.94
Sum As^{III+V}	1.24	0.91	1.68	0.17	1.41	1.01	1.97	0.04
MAs/iAs	1.06	0.83	1.36	0.63	1.09	0.83	1.42	0.54
DMAs/MAs	0.62	0.47	0.83	<0.01	0.53	0.38	0.73	<0.01
DMAs/iAs	0.72	0.55	0.96	0.02	0.65	0.48	0.89	0.01
(DMAs+MAs)/iAs	0.77	0.56	1.04	0.08	0.78	0.56	1.05	0.09
Urine								
iAs^{III+V}	1.18	0.91	1.53	0.22	1.34	1.00	1.79	0.05
MAs^{III+V}	1.13	0.87	1.46	0.36	1.23	0.93	1.63	0.14
DMAs^{III+V}	1.24	0.96	1.60	0.10	1.34	1.02	1.76	0.04
Sum As^{III+V}	1.19	0.93	1.54	0.17	1.31	0.99	1.72	0.06
MAs/iAs	0.86	0.67	1.11	0.25	0.77	0.58	1.02	0.07
DMAs/MAs	1.37	1.03	1.84	0.03	1.38	1.00	1.89	0.05
DMAs/iAs	1.12	0.86	1.46	0.38	1.05	0.79	1.40	0.74
(DMAs+MAs)/iAs	0.99	0.75	1.30	0.95	1.02	0.77	1.34	0.91
Creatinine adj.								
iAs^{III+V}	1.19	0.92	1.54	0.19	1.38	1.04	1.83	0.03
MAs^{III+V}	1.17	0.91	1.51	0.23	1.35	1.01	1.79	0.04
DMAs^{III+V}	1.26	0.98	1.62	0.08	1.39	1.05	1.84	0.02
Sum As^{III+V}	1.24	0.96	1.60	0.10	1.39	1.05	1.84	0.02
Creatinine	1.01	0.78	1.31	0.93	1.00	0.75	1.32	0.98
SG adj.								
iAs^{III+V}	0.98	0.76	1.27	0.87	1.08	0.81	1.42	0.61
MAs^{III+V}	0.94	0.73	1.22	0.65	0.99	0.75	1.30	0.92
DMAs^{III+V}	1.04	0.81	1.33	0.78	1.10	0.84	1.44	0.51
Sum As^{III+V}	1.00	0.78	1.29	0.99	1.04	0.79	1.36	0.79
SG	1.32	1.01	1.71	0.07	1.42	1.07	1.89	0.02

Abbreviations: OR, odds ratio; CI, confidence interval; FPG, fasting plasma glucose; 2HPG, 2-hour plasma glucose; SG, specific gravity

[a] Diabetes classified by either FPG ≥ 126 mg/dL, 2HPG ≥ 200 mg/dL, self-report of doctor diagnosis or use of anti-diabetic medication, n = 374.

[b] Diabetes classified by either FPG ≥ 126 mg/dL or 2HPG ≥ 200 mg/dL. Individuals self-reporting a doctor diagnosis or taking anti-diabetic medication but not classified as diabetic by FPG or 2HPG are excluded, n = 363.

[c] Units of OR and CI are standardized to an increment of one inter-quartile range. Descriptive statistics for iAs in drinking water and metabolites of As in BECs and urine are presented in **Table 6.2**.

[d] *p*-value for comparison of cases to non-diabetic individuals.

The linear regression analysis also found positive associations between FPG or 2HPG and each of the As species in urine regardless of adjustment for hydration status. Notably, positive associations between urinary specific gravity and FPG or 2HPG levels were observed (r^2 = 0.13 and 0.11, respectively, $p < 0.01$). These results are in agreement with logistic regression analysis and highlight the limitation of using creatinine or specific gravity to adjust for hydration status in studies examining diabetes. Taken together, these data present clear differences in the composition of As species retained in BECs and those present in urine. Combined results of the logistic and linear regression analyses suggest that As species retained in BECs are sensitive biomarkers of the diabetic phenotype and provide additional evidence for the important role of the trivalent arsenicals in the development of disease in subjects chronically exposed to iAs.

Table 6.4: Association of log-transformed FPG and 2HPG with iAs in drinking water and iAs metabolites in BECs and urine adjusted for age, sex, and BMI.

	FPG				2HPG			
	β^a	SE	p^b	r^2	β^a	SE	p^b	r^2
iAs in Water	0.041	0.006	<0.01	0.21	0.031	0.006	<0.01	0.17
BECs								
iAs^{III}	0.056	0.009	<0.01	0.16	0.042	0.009	<0.01	0.14
MAs^{III}	0.062	0.009	<0.01	0.17	0.050	0.009	<0.01	0.15
DMAs^{III}	0.050	0.011	<0.01	0.12	0.039	0.011	<0.01	0.12
iAs^{V}	0.013	0.009	0.15	0.07	0.007	0.009	0.45	0.09
MAs^{V}	0.015	0.008	0.08	0.08	0.010	0.008	0.21	0.09
DMAs^{V}	0.010	0.009	0.25	0.07	0.007	0.009	0.40	0.09
Sum As^{III+V}	0.045	0.011	<0.01	0.11	0.035	0.011	<0.01	0.11
MAs/iAs	0.046	0.022	0.04	0.08	0.039	0.022	0.08	0.09
DMAs/MAs	-0.072	0.015	<0.01	0.12	-0.057	0.016	<0.01	0.12
DMAs/iAs	-0.032	0.013	0.01	0.08	-0.033	0.016	0.04	0.10
(DMAs+MAs)/iAs	-0.014	0.016	0.40	0.07	-0.009	0.017	0.60	0.09
Urine								
iAs^{III+V}	0.048	0.009	<0.01	0.13	0.043	0.009	<0.01	0.14
MAs^{III+V}	0.062	0.010	<0.01	0.15	0.045	0.011	<0.01	0.13
DMAs^{III+V}	0.078	0.011	<0.01	0.18	0.061	0.011	<0.01	0.15
Sum As^{III+V}	0.076	0.011	<0.01	0.17	0.059	0.011	<0.01	0.15
MAs/iAs ratio	-0.001	0.019	0.95	0.02	-0.032	0.019	0.05	0.09
DMAs/MAs ratio	0.072	0.036	0.05	0.08	0.100	0.036	<0.01	0.11
DMAs/iAs ratio	0.016	0.018	0.37	0.07	-0.005	0.018	0.80	0.09
(DMAs+MAs)/iAs	0.014	0.018	0.45	0.07	-0.009	0.018	0.62	0.09
Creatinine Adj.								
iAs^{III+V}	0.052	0.011	<0.01	0.12	0.053	0.011	<0.01	0.15
MAs^{III+V}	0.072	0.012	<0.01	0.15	0.060	0.013	<0.01	0.14
DMAs^{III+V}	0.093	0.031	<0.01	0.18	0.083	0.013	<0.01	0.18
Sum As^{III+V}	0.091	0.013	<0.01	0.18	0.081	0.013	<0.01	0.17
Creatinine	0.033	0.020	0.09	0.07	0.005	0.020	0.79	0.09
SG Adj.								
iAs^{III+V}	0.040	0.011	<0.01	0.10	0.042	0.011	<0.01	0.13
MAs^{III+V}	0.055	0.120	<0.01	0.12	0.042	0.011	<0.01	0.13
DMAs^{III+V}	0.073	0.013	<0.01	0.14	0.065	0.013	<0.01	0.14
Sum As^{III+V}	0.070	0.013	<0.01	0.14	0.062	0.013	<0.01	0.14
SG	10.314	2.070	<0.01	0.13	5.995	2.130	<0.01	0.11

Abbreviations: Adj., adjusted; FPG, fasting plasma glucose; 2HPG, 2-hour plasma glucose; β, regression coefficient; SE, standard error; SG, specific gravity

[a] Coefficients are standardized to an increment of one inter-quartile range.

[b] p-value for test of β = 0.

6.5 Conclusions

In this report, we have shown that exposure to iAs through contaminated drinking water is associated with an increased risk of diabetes characterized by widely accepted diagnostic criteria. Moreover, the retention of the toxic trivalent As species in BECs was found to be a sensitive biomarker of both iAs exposure and the risk of diabetes associated with this exposure. The compositional differences in the As metabolite profiles of urine and BECs indicate that the analysis of urinary As metabolites does not reflect As retention in a target tissue. Finally, this study provides further evidence for the diabetogenic effects of iAs exposure.

CHAPTER VII

SYNTHESIS

7.1 Introduction

Studies described in this dissertation sought to explore whether the quantitative analysis of trivalent As metabolites, produced in the course of iAs metabolism, are (i) suitable biomarkers for iAs exposure and (ii) can be used to inform the risk assessment of iAs-induced diabetes. The analysis of these metabolites in biological samples and thus, the use of these results to assess the risk associated with As exposure, are difficult endeavors because DMAs^{III} and, to a lesser extent, MAs^{III} rapidly oxidize in the presence of oxygen. Thus, this work sought to answer the following two questions:

1) Can HG-CT-AAS be used to quantify trivalent As species reliably in biological samples?

2) Does the concentration of trivalent As species in biological specimens predict susceptibility to the diabetogenic effects of As exposure?

The oxidation state specific analysis of As species by HG-CT-based techniques was validated in cells and tissues. The technique was then applied to both *in vivo* mouse and human population studies examining the retention of trivalent arsenicals in target tissues. The findings of these studies contributed a robust HG-CT-based technique for the measurement and quantification of As^{III} species in a variety of biological samples that

can serve as sensitive biomarkers for the risk assessment of diseases associated with exposure to environmental iAs. This chapter recapitulates the major findings, discusses their implications, and provides a critical analysis of future directions for this research.

7.2 Findings and Implications

Establishing methods for quantifying trivalent arsenicals in biological samples is an important step for improving the risk assessment of environmental iAs exposure. While previous studies used HG-CT-AAS to detect MAs^{III} and DMAs^{III} in cell culture lysates with minimal sample handling and pretreatment (149), the stability of these As species in biological matrices was not characterized. Furthermore, no studies have quantitatively analyzed MAs^{III} and DMAs^{III} in cells or tissues without performing pretreatment or extractions that may facilitate the oxidation of trivalent species. Because these toxic trivalent metabolites have been identified in the urine of individuals exposed to As in drinking water, it is likely that target organ retention contributes to development of As-related diseases and thus, potentially be used as a biomarker to inform the risk assessment of As exposure. Using cells and tissues, we validated a HG-CT-AAS technique for the quantification of trivalent arsenicals and demonstrated that MAs^{III} and DMAs^{III} can be quantified and are stable in this reductive environment. Our results describe, for the first time, the quantification of trivalent arsenicals in mouse liver homogenates after exposure to iAs through drinking water (Chapter 2). Moreover, MAs^{III} and DMAs^{III} were found to be stable for at least 3 weeks in cells and tissues when stored at -80°C (Chapter 3). These results support the theory that high concentrations of endogenous reductants, such as glutathione or protein thiols may preserve these trivalent species in biological samples. These studies confirm the feasibility of designing

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experiments that analyze As^{III} metabolites systematically in laboratory- and population-based studies.

Our analytical method represents a novel approach that has the advantage of avoiding harsh pretreatments or extractions during the analysis. However, it has been argued that HG-based separations alone are not selective because they quantify arsine and methyl-substituted arsines as opposed to the As species present in the sample. In response, we compared HG-CT-AAS with a commonly used HPLC-based technique for the analysis of trivalent arsenicals using a simple *in vitro* reaction mixture. Recent developments in reversed-phase HPLC have successfully separated six As species in an aqueous solution (147), but MAs^{III} and DMAs^{III} have only been analyzed in biological samples using pretreatments or extractions that produce low yield and potentially result in oxidation of the As species (188). Our data demonstrate that protein binding and other reagent reactions interfere with the HPLC separation and detection of trivalent methylated species (Chapter 4). In contrast, the HG-CT method for separation produced high recoveries of these species regardless of biological matrix. These results strengthen the validity of HG-CT-AAS for the oxidation state speciation of iAs metabolites in biological samples that can be applied to a variety of studies assessing susceptibility to iAs-induced disease.

To our knowledge, no reports have used the quantification of trivalent arsenicals present in biological specimens as a biomarker of diabetes associated with iAs exposure. Traditionally, analysis of human samples has been limited to measuring the excretion profiles of iAs metabolites present in urine. Moreover, *in vivo* studies in mice (157, 158)

and humans (85) suggest that urinary excretion of iAs and its metabolites does not correspond well with their tissue distribution.

Our laboratory has previously developed a mouse model for As-induced diabetes and shown accumulation of methylated As species in target organs (86). However, this study did not characterize the retention of trivalent methylated arsenicals. Our new *in vitro* studies are novel because they assess retention of tri- and pentavalent species in several target tissues, including those critical for glucose homeostasis (liver, pancreas, adipose tissue, and skeletal muscle). Moreover, the recently developed *As3mt*-KO mouse model represents a novel approach for characterizing the role of methylation in the development of disease. We used HG-CT-AAS to compare As speciation in tissues of WT and A*s3mt*-KO mice exposed to iAs^{III} though drinking water. MAs^{III} and DMAs^{III} are extensively retained in tissues regulating glucose homeostasis in WT mice. iAs^{III} is highly retained in tissues of A*s3mt*-KO mice, , and these mice retain more total As than WT mice. We demonstrate that exposing A*s3mt*-KO mice to 25 or 30 ppm As produces equivalent internal As doses in tissues critical to glucose homeostasis compared with WT mice that develop the diabetic phenotype after 8 weeks of exposure to 50 ppm As in drinking water. Our research analyzing the metabolites of iAs, including MAs^{III} and DMAs^{III}, in tissues involved in insulin production and glucose homeostasis is a crucial step for validating these biomarkers for the risk assessment of environmental As exposure.

To determine whether trivalent As species in a target human tissue could predict susceptibility to the diabetogenic effects of As exposure, we characterized the retention of trivalent As species in BECs and assessed associations between other biomarkers of

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exposure, including urinary As species and iAs in drinking water, and the development of diabetes. Our work represents a novel approach because our previously described HG-CT technique was coupled with an ICP-MS, providing better LOD. The results from this study indicate that the composition of As species retained in BECs is significantly different than the urinary As excretion profile, but also that the concentrations of these species in BECs are positively associated with both As in urine and drinking water. Most importantly, we report that the retention of iAs^{III} and MAs^{III} in BECs is associated with the risk of iAs-induced diabetes. Taken together, these results indicate that the concentrations of trivalent arsenicals in BECs are highly sensitive biomarkers of iAs exposure and predict the development of As-induced diabetes. This ground-breaking study paves the way for examining the adverse effects of iAs exposure in future epidemiological-based studies and highlights the need and feasibility of considering As retention in target tissues.

7.3 Future Studies

Future analytical method development and optimization should focus on developing an HPLC-based reference method to further support the HG-CT separation methods presented in this dissertation. While the body of evidence supporting the analysis of trivalent arsenicals by HG-CT-AAS and HG-CT-ICP-MS are mounting, critics still argue that the method is not selective and question its use for the quantification of methylated trivalent arsenicals. While our efforts to optimize a reversed-phase HPLC-ICP-MS reference method have revealed some critical shortcomings, additional studies should explore other mobile phases for this column type or anion-exchange chromatography methods. Furthermore, future studies should utilize updated

equipment comprised of chemically inert parts, including those made from polyether ether ketone (PEEK), that do not adsorb metals or proteins. Plans in our laboratory are already under way to optimize an anion-exchange HPLC-ICP-MS method for As speciation using new instrumentation containing only chemically inert parts.

Additional concerns have been made regarding the formation of thiol analogues of methylated As species, including dimethylthioarsenic acid (DMTA), and their contributions to the toxic effects of iAs exposure (181). While our optimization of a reversed-phase HPLC-ICP-MS method successfully separated seven As species, including DMTA from DMAs[III], in an aqueous mixture, no research has successfully quantified both DMTA and DMAs[III] in biological samples. Future work should optimize an HPLC-based method for the separation of tri- and pentavalent oxoarsenicals and DMTA in biological samples. Quantitatively determining both oxo- and thioarsenicals will enhance the risk assessment of the adverse effects associated with chronic As exposure.

Our *in vivo* studies characterizing the retention of trivalent arsenicals in tissues of WT and *As3mt*-KO mice clearly demonstrate the need for additional studies that correlate the retention of these toxic As species to disease development. We have shown that trivalent arsenicals are extensively retained in several target tissues, including those critical for glucose homeostasis. No studies report the oxidation state specific speciation of As in an *in vivo* model of chronic iAs toxicity. While our data demonstrate the plausibility of such experiments, future studies should assess whether *As3mt*-KO mice are less sensitive to the diabetogenic effects of iAs exposure and characterize the role of methylation in the development of the diabetic phenotype. Using *As3mt*-KO mice and the

doses we determined to produce equivalent internal As exposures compared with WT mice, we hypothesize that the methylated trivalent arsenicals, MAs^{III} and $DMAs^{III}$ are responsible for the impaired glucose tolerance observed in WT mice exposed to 50 ppm As is iAs^{III}.

The biomarker development and validation presented in this dissertation paves the way for future studies characterizing internal retention of iAs metabolites and disease development. Our results demonstrate the feasibility of measuring As retention in BECs and suggest they may serve as more sensitive biomarkers than commonly collected human samples, e.g., urine. However, gaps still exist in the epidemiological literature examining the associations between low iAs exposures and development of diabetes. Recent cross-sectional studies and results presented in this dissertation are unable to determining the temporal relationship between iAs exposure and incidence of diabetes. Future prospective epidemiological studies should be designed to address this association, as characterizing the relationship between duration of exposure and onset of diabetes is crucial for informing environmental policy and protecting public health.

While biomarkers of iAs metabolite retention in target human tissues can help elucidate the roles of inter-individual variation in iAs metabolism and its impact on disease development, additional investigations should explore the mechanisms of these adverse effects. Epigenetic information, including promotor region DNA methylation patterns, can provide mechanistic insight into the pathways involved in the diabetic phenotype. Studies are already underway assessing the associations between DNA methylation and As retention in BECs of individuals with diabetes. The information on

pathways involved in As-induced diabetes can then be used to design *in vitro* and *in vivo* studies examining mechanisms of action.

7.4 Conclusions

The aim of this dissertation has been to develop novel biomarkers to better inform the risk assessment of the diabetogenic effects associated with iAs exposure. We demonstrated that trivalent metabolites of iAs can be measured in a variety of biological samples and that these species play an important role in the development of iAs-induced diabetes. The contributions of these novel studies include:

a) A robust HG-CT-based technique for the quantification of trivalent arsenicals in a variety of biological samples.

b) The use of trivalent arsenical quantification in biological systems as sensitive biomarkers for the risk assessment of iAs-associated diseases, including diabetes mellitus.

The results presented here support the growing body of evidence that the measurement of trivalent arsenic metabolites in biological systems is critical for assessing the adverse effects of iAs exposure and have contributed to my desire as a scientist to conduct meaningful research that impacts human health.

REFERENCES

(1) IARC Working Group on the Evaluation of Carcinogenic Risks to Humans **(2004)** Some drinking-water disinfectants and contaminants, including arsenic. *IARC Monogr. Eval. Carcinog. Risks Hum. 84*, 1-477.

(2) Tseng, C. H. **(2007)** Arsenic methylation, urinary arsenic metabolites and human diseases: current perspective. *J. Environ. Sci. Health. C. Environ. Carcinog. Ecotoxicol. Rev. 25*, 1-22.

(3) Wang, C. H.; Hsiao, C. K.; Chen, C. L.; Hsu, L. I.; Chiou, H. Y.; Chen, S. Y.; Hsueh, Y. M.; Wu, M. M.; Chen, C. J. **(2007)** A review of the epidemiologic literature on the role of environmental arsenic exposure and cardiovascular diseases. *Toxicol. Appl. Pharmacol. 222*, 315-326.

(4) Thayer, K. A.; Heindel, J. J.; Bucher, J. R.; Gallo, M. A. **(2012)** Role of Environmental Chemicals in Diabetes and Obesity: A National Toxicology Program Workshop Review. *Environ. Health Perspect. 120*, 779-789.

(5) Maull, E. A.; Ahsan, H.; Edwards, J.; Longnecker, M. P.; Navas-Acien, A.; Pi, J.; Silbergeld, E. K.; Styblo, M.; Tseng, C. H.; Thayer, K. A.; Loomis, D. **(2012)** Evaluation of the Association between Arsenic and Diabetes: A National Toxicology Program Workshop Review. *Environ. Health Perspect. 120*, 1658-1670.

(6) USEPA **(2001)** National Drinking Water Regulations; Arsenic and Clarification to Compliance and New Source Contaminants Monitoring: Final Rule. *Fed. Regist. 66*, 6975-7066.

(7) The World Bank **(2005)** Towards a more effective operational response. Arsenic contamination of groundwater in South and East Asian Countries. *Volume 1, Policy Report, No. 31303*.

(8) Thomas, D. J.; Styblo, M.; Lin, S. **(2001)** The cellular metabolism and systemic toxicity of arsenic. *Toxicol. Appl. Pharmacol. 176*, 127-144.

(9) Lin, S.; Del Razo, L. M.; Styblo, M.; Wang, C.; Cullen, W. R.; Thomas, D. J. **(2001)** Arsenicals inhibit thioredoxin reductase in cultured rat hepatocytes. *Chem. Res. Toxicol. 14*, 305-311.

(10) Drobna, Z.; Jaspers, I.; Thomas, D. J.; Styblo, M. **(2003)** Differential activation of AP-1 in human bladder epithelial cells by inorganic and methylated arsenicals. *FASEB J. 17*, 67-69.

(11) Wang, T. C.; Jan, K. Y.; Wang, A. S.; Gurr, J. R. **(2007)** Trivalent arsenicals induce lipid peroxidation, protein carbonylation, and oxidative DNA damage in human urothelial cells. *Mutat. Res. 615*, 75-86.

(12) Le, X.; Lu, X.; Ma, M.; Cullen, W.; Aposhian, H.; Zheng, B. **(2000)** Speciation of key arsenic metabolic intermediates in human urine. *Anal. Chem. 72*, 5172-5177.

(13) Le, X.; Ma, M.; Lu, X.; Cullen, W.; Aposhian, H.; Zheng, B. **(2000)** Determination of monomethylarsonous acid, a key arsenic methylation intermediate, in human urine. *Environ. Health Perspect. 108*, 1015-1018.

(14) Gong, Z. L.; Lu, X. F.; Cullen, W. R.; Le, X. C. **(2001)** Unstable trivalent arsenic metabolites, monomethylarsonous acid and dimethylarsinous acid. *J. Anal. At. Spectrom. 16*, 1409-1413.

(15) McDonough, W. **(2003)** Compositional model for the Earth's core. *Treatise on geochemistry 2*, 547-568.

(16) Smedley, P. L.; Kinniburgh, D. G. **(2002)** A review of the source, behaviour and distribution of arsenic in natural waters. *Appl. Geochem. 17*, 517-568.

(17) Walsh, P.; Duce, R.; Fasching, J. **(1979)** Considerations of the enrichment, sources, and flux of arsenic in the troposphere. *Journal of Geophysical Research 84*, 1719-1726.

(18) ATSDR **(2007)** Toxicological profile for Arsenic. *U. S. Department of Health and Human Services* .

(19) Welch, A.; Westjohn, D.; Helsel, D.; Wanty, R. **(2000)** Arsenic in ground water of the United States: Occurrence and geochemistry. *Ground Water 38*, 589-604.

(20) Yudovich, Y.; Ketris, M. **(2005)** Arsenic in coal: a review. *Int. J. Coal Geol. 61*, 141-196.

(21) Webster, J.; Nordstrom, D. **(2003)** Geothermal arsenic. *Arsenic in ground water* , 101-125.

(22) Khan, B. I.; Solo-Gabriele, H. M.; Townsend, T. G.; Cai, Y. **(2006)** Release of arsenic to the environment from CCA-treated wood. 1. Leaching and speciation during service. *Environ. Sci. Technol. 40*, 988-993.

(23) Komorowicz, I.; Baralkiewicz, D. **(2011)** Arsenic and its speciation in water samples by high performance liquid chromatography inductively coupled plasma mass spectrometry--last decade review. *Talanta 84*, 247-261.

(24) Garelick, H.; Dybowska, A.; Valsami-Jones, E.; Priest, N. **(2005)** Remediation technologies for arsenic contaminated drinking waters. *J. Soils Sediments 5*, 182-190.

(25) Ryker, S. **(2001)** Mapping arsenic in groundwater. *Geotimes 46*, 34-36.

(26) Borak, J.; Hosgood, H. D. **(2007)** Seafood arsenic: implications for human risk assessment. *Regul. Toxicol. Pharmacol. 47*, 204-212.

(27) Rose, M.; Lewis, J.; Langford, N.; Baxter, M.; Origgi, S.; Barber, M.; MacBain, H.; Thomas, K. **(2007)** Arsenic in seaweed—Forms, concentration and dietary exposure. *Food and Chemical Toxicology 45*, 1263-1267.

(28) Kawalek, J.; Carson, M.; Conklin, S.; Lancaster, V.; Howard, K.; Ward, J.; Farrell, D.; Myers, M.; Swain, H.; Jeanettes, P.; Frobish, S.; Matthews, S.; McDonald, M. **(2011)** Final Report on Study 275.30. Provide Data on Various Arsenic Species Present in Broilers Treated with Roxarsone: Comparison with Untreated Birds. *Laurel, Rockville, and College Park, MD:Center for Veterinary Medicine/Office of Regulatory Science, U. S. Food and Drug Administration* .

(29) Park, D.; Yang, H.; Jeong, J.; Ha, K.; Choi, S.; Kim, C.; Yoon, C.; Park, D.; Paek, D. **(2010)** A Comprehensive Review of Arsenic Levels in the Semiconductor Manufacturing Industry. *Ann. Occup. Hyg. 54*, 869-879.

(30) Lazarevic, K.; Nikolic, D.; Stosic, L.; Milutinovic, S.; Videnovic, J.; Bogdanovic, D. **(2012)** Determination of Lead and Arsenic in Tobacco and Cigarettes: an Important Issue of Public Health. *Cent. Eur. J. Public Health 20*, 62-66.

(31) Saad, A.; Hassanien, M. **(2001)** Assessment of arsenic level in the hair of the nonoccupational Egyptian population: Pilot study. *Environ. Int. 27*, 471-478.

(32) Lindberg, A.; Goessler, W.; Gurzau, E.; Koppova, K.; Rudnai, P.; Kumar, R.; Fletcher, T.; Leonardi, G.; Slotova, K.; Gheorghiu, E.; Vahter, M. **(2006)** Arsenic exposure in Hungary, Romania and Slovakia. *J. Environ. Monit. 8*, 203-208.

(33) Thorburn, A. L. **(1983)** Paul Ehrlich: pioneer of chemotherapy and cure by arsenic (1854-1915). *Br. J. Vener. Dis. 59*, 404-405.

(34) Dilda, P. J.; Hogg, P. J. **(2007)** Arsenical-based cancer drugs. *Cancer Treat. Rev. 33*, 542-564.

(35) Antman, K. H. **(2001)** Introduction: the history of arsenic trioxide in cancer therapy. *Oncologist 6*, 1-2.

(36) Juhasz, A. L.; Smith, E.; Weber, J.; Rees, M.; Rofe, A.; Kuchel, T.; Sansom, L.; Naidu, R. **(2006)** In vivo assessment of arsenic bioavailability in rice and its significance for human health risk assessment. *Environ. Health Perspect. 114*, 1826.

(37) Freeman, G.; Schoof, R.; Ruby, M.; Davis, A.; Dill, J.; Liao, S.; Lapin, C.; Bergstrom, P. **(1995)** Bioavailability of arsenic in soil and house dust impacted by smelter activities following oral administration in cynomolgus monkeys. *Toxicological Sciences 28*, 215-222.

(38) Calatayud, M.; Barrios, J. A.; Velez, D.; Devesa, V. **(2012)** In vitro study of transporters involved in intestinal absorption of inorganic arsenic. *Chem. Res. Toxicol. 25*, 446-453.

(39) Jiang, X.; McDermott, J. R.; Ajees, A. A.; Rosen, B. P.; Liu, Z. **(2010)** Trivalent arsenicals and glucose use different translocation pathways in mammalian GLUT1. *Metallomics 2*, 211-219.

(40) Drobna, Z.; Walton, F. S.; Paul, D. S.; Xing, W.; Thomas, D. J.; Styblo, M. **(2010)** Metabolism of arsenic in human liver: the role of membrane transporters. *Arch. Toxicol. 84*, 3-16.

(41) Liu, Z.; Sanchez, M. A.; Jiang, X.; Boles, E.; Landfear, S. M.; Rosen, B. P. **(2006)** Mammalian glucose permease GLUT1 facilitates transport of arsenic trioxide and methylarsonous acid. *Biochem. Biophys. Res. Commun. 351*, 424-430.

(42) Carbrey, J. M.; Song, L.; Zhou, Y.; Yoshinaga, M.; Rojek, A.; Wang, Y.; Liu, Y.; Lujan, H. L.; DiCarlo, S. E.; Nielsen, S.; Rosen, B. P.; Agre, P.; Mukhopadhyay, R. **(2009)** Reduced arsenic clearance and increased toxicity in aquaglyceroporin-9-null mice. *Proc. Natl. Acad. Sci. U. S. A. 106*, 15956-15960.

(43) Leslie, E. M. **(2012)** Arsenic-glutathione conjugate transport by the human multidrug resistance proteins (MRPs/ABCCs). *J. Inorg. Biochem. 108*, 141-149.

(44) Suzuki, K. T.; Tomita, T.; Ogra, Y.; Ohmichi, M. **(2001)** Glutathione-conjugated arsenics in the potential hepato-enteric circulation in rats. *Chem. Res. Toxicol. 14*, 1604-1611.

(45) Cui, X.; Kobayashi, Y.; Hayakawa, T.; Hirano, S. **(2004)** Arsenic speciation in bile and urine following oral and intravenous exposure to inorganic and organic arsenics in rats. *Toxicol. Sci. 82*, 478-487.

(46) Braman, R. S.; Foreback, C. C. **(1973)** Methylated Forms of Arsenic in Environment. *Science 182*, 1247-1249.

(47) Smith, T. J.; Crecelius, E. A.; Reading, J. C. **(1977)** Airborne arsenic exposure and excretion of methylated arsenic compounds. *Environ. Health Perspect. 19*, 89-93.

(48) Crecelius, E. A. **(1977)** Changes in the chemical speciation of arsenic following ingestion by man. *Environ. Health Perspect. 19*, 147-150.

(49) Healy, S. M.; Zakharyan, R. A.; Vasken Aposhian, H. **(1997)** Enzymatic methylation of arsenic compounds: IV. In vitro and in vivo deficiency of the methylation of arsenite and monomethylarsonic acid in the guinea pig. *Mutation Research/Reviews in Mutation Research 386*, 229-239.

(50) Vahter, M.; Couch, R.; Nermell, B.; Nilsson, R. **(1995)** Lack of methylation of inorganic arsenic in the chimpanzee. *Toxicol. Appl. Pharmacol. 133*, 262-268.

(51) Zakharyan, R. A.; Wildfang, E.; Aposhian, H. V. **(1996)** Enzymatic methylation of arsenic compounds: III. The marmoset and tamarin, but not the rhesus, monkeys are deficient in methyltransferases that methylate inorganic arsenic. *Toxicol. Appl. Pharmacol. 140*, 77-84.

(52) Thomas, D.; Waters, S.; Styblo, M. **(2004)** Elucidating the pathway for arsenic methylation. *Toxicol. Appl. Pharmacol. 198*, 319-326.

(53) Lin, S.; Shi, Q.; Nix, F. B.; Styblo, M.; Beck, M. A.; Herbin-Davis, K. M.; Hall, L. L.; Simeonsson, J. B.; Thomas, D. J. **(2002)** A novel S-adenosyl-L-methionine:arsenic(III) methyltransferase from rat liver cytosol. *J. Biol. Chem. 277*, 10795-10803.

(54) Drobna, Z.; Waters, S. B.; Devesa, V.; Harmon, A. W.; Thomas, D. J.; Styblo, M. **(2005)** Metabolism and toxicity of arsenic in human urothelial cells expressing rat arsenic (+3 oxidation state)-methyltransferase. *Toxicol. Appl. Pharmacol. 207*, 147-159.

(55) Drobna, Z.; Naranmandura, H.; Kubachka, K. M.; Edwards, B. C.; Herbin-Davis, K.; Styblo, M.; Le, X. C.; Creed, J. T.; Maeda, N.; Hughes, M. F.; Thomas, D. J. **(2009)** Disruption of the arsenic (+3 oxidation state) methyltransferase gene in the mouse alters the phenotype for methylation of arsenic and affects distribution and retention of orally administered arsenate. *Chem. Res. Toxicol. 22*, 1713-1720.

(56) Yokohira, M.; Arnold, L. L.; Pennington, K. L.; Suzuki, S.; Kakiuchi-Kiyota, S.; Herbin-Davis, K.; Thomas, D. J.; Cohen, S. M. **(2010)** Severe systemic toxicity and urinary bladder cytotoxicity and regenerative hyperplasia induced by arsenite in arsenic (+3 oxidation state) methyltransferase knockout mice. A preliminary report. *Toxicol. Appl. Pharmacol. 246*, 1-7.

(57) Challenger, F. **(1945)** Biological Methylation. *Chem. Rev. 36*, 315-361.

(58) Cullen, W. R.; McBride, B. C.; Reglinski, J. **(1984)** The Reduction of Trimethylarsine Oxide to Trimethylarsine by Thiols - a Mechanistic Model for the Biological Reduction of Arsenicals. *J. Inorg. Biochem. 21*, 45-60.

(59) Waters, S. B.; Devesa, V.; Del Razo, L. M.; Styblo, M.; Thomas, D. J. **(2004)** Endogenous reductants support the catalytic function of recombinant rat cyt19, an arsenic methyltransferase. *Chem. Res. Toxicol. 17*, 404-409.

(60) Thomas, D. J.; Li, J.; Waters, S. B.; Xing, W.; Adair, B. M.; Drobna, Z.; Devesa, V.; Styblo, M. **(2007)** Arsenic (+3 oxidation state) methyltransferase and the methylation of arsenicals. *Exp. Biol. Med. (Maywood) 232*, 3-13.

(61) Waters, S.; Devesa, V.; Fricke, M.; Creed, J.; Styblo, M.; Thomas, D. **(2004)** Glutathione modulates recombinant rat arsenic (+3 oxidation state) methyltransferase-catalyzed formation of trimethylarsine oxide and trimethylarsine. *Chem. Res. Toxicol. 17*, 1621-1629.

(62) Naranmandura, H.; Suzuki, N.; Suzuki, K. T. **(2006)** Trivalent arsenicals are bound to proteins during reductive methylation. *Chem. Res. Toxicol. 19*, 1010-1018.

(63) Hayakawa, T.; Kobayashi, Y.; Cui, X.; Hirano, S. **(2005)** A new metabolic pathway of arsenite: arsenic-glutathione complexes are substrates for human arsenic methyltransferase Cyt19. *Arch. Toxicol. 79*, 183-191.

(64) Ajees, A. A.; Marapakala, K.; Packianathan, C.; Sankaran, B.; Rosen, B. P. **(2012)** Structure of an As(III) S-Adenosylmethionine Methyltransferase: Insights into the Mechanism of Arsenic Biotransformation. *Biochemistry* .

(65) Marapakala, K.; Qin, J.; Rosen, B. P. **(2012)** Identification of catalytic residues in the As(III) S-adenosylmethionine methyltransferase. *Biochemistry 51*, 944-951.

(66) Drobna, Z.; Styblo, M.; Thomas, D. J. **(2009)** An overview of arsenic metabolism and toxicity. *Current Protocols in Toxicology* , 4.31. 1-4.31. 6.

(67) Drobna, Z.; Waters, S. B.; Walton, F. S.; LeCluyse, E. L.; Thomas, D. J.; Styblo, M. **(2004)** Interindividual variation in the metabolism of arsenic in cultured primary human hepatocytes. *Toxicol. Appl. Pharmacol. 201*, 166-177.

(68) Agusa T., Fujihara J., Takeshita H., Iwata H. **(2011)** Individual Variations in Inorganic Arsenic Metabolism Associated with *AS3MT* Genetic Polymorphisms. *International Journal of Molecular Sciences 12*, 2351-2382.

(69) Valenzuela, O. L.; Drobna, Z.; Hernandez-Castellanos, E.; Sanchez-Pena, L. C.; Garcia-Vargas, G. G.; Borja-Aburto, V. H.; Styblo, M.; Del Razo, L. M. **(2009)** Association of AS3MT polymorphisms and the risk of premalignant arsenic skin lesions. *Toxicol. Appl. Pharmacol. 239*, 200-207.

(70) Del Razo, L. M.; Garcia-Vargas, G. G.; Valenzuela, O. L.; Hernandez Castellanos, E.; Sanchez-Pena, L. C.; Currier, J. M.; Drobna, Z.; Loomis, D.; Styblo, M. **(2011)** Exposure to arsenic in drinking water is associated with increased prevalence of diabetes: a cross-sectional study in the Zimapan and Lagunera regions in Mexico. *Environ. Health 10*, 73.

(71) Lindberg, A. L.; Kumar, R.; Goessler, W.; Thirumaran, R.; Gurzau, E.; Koppova, K.; Rudnai, P.; Leonardi, G.; Fletcher, T.; Vahter, M. **(2007)** Metabolism of low-dose inorganic arsenic in a central European population: influence of sex and genetic polymorphisms. *Environ. Health Perspect. 115*, 1081-1086.

(72) Agusa, T.; Iwata, H.; Fujihara, J.; Kunito, T.; Takeshita, H.; Minh, T. B.; Trang, P. T.; Viet, P. H.; Tanabe, S. **(2009)** Genetic polymorphisms in AS3MT and arsenic metabolism in residents of the Red River Delta, Vietnam. *Toxicol. Appl. Pharmacol. 236*, 131-141.

(73) Chen, J.; Wang, S.; Wang, Y.; Sun, C.; Huang, Y.; Chen, C.; Li, W. **(2012)** Arsenic methylation, GSTO1 polymorphisms, and metabolic syndrome in an arseniasis endemic area of southwestern Taiwan. *Chemosphere 88*, 432-438.

(74) Porter, K. E.; Basu, A.; Hubbard, A. E.; Bates, M. N.; Kalman, D.; Rey, O.; Smith, A.; Smith, M. T.; Steinmaus, C.; Skibola, C. F. **(2010)** Association of genetic variation in cystathionine-beta-synthase and arsenic metabolism. *Environ. Res. 110*, 580-587.

(75) Steinmaus, C.; Carrigan, K.; Kalman, D.; Atallah, R.; Yuan, Y.; Smith, A. **(2005)** Dietary intake and arsenic methylation in a US population. *Environ. Health Perspect. 113*, 1153-1159.

(76) Heck, J. E.; Nieves, J. W.; Chen, Y.; Parvez, F.; Brandt-Rauf, P. W.; Graziano, J. H.; Slavkovich, V.; Howe, G. R.; Ahsan, H. **(2009)** Dietary Intake of Methionine, Cysteine, and Protein and Urinary Arsenic Excretion in Bangladesh. *Environ. Health Perspect. 117*, 99-104.

(77) Gamble, M. V.; Liu, X.; Ahsan, H.; Pilsner, J. R.; Ilievski, V.; Slavkovich, V.; Parvez, F.; Chen, Y.; Levy, D.; Factor-Litvak, P.; Graziano, J. H. **(2006)** Folate and arsenic metabolism: a double-blind, placebo-controlled folic acid-supplementation trial in Bangladesh. *Am. J. Clin. Nutr. 84*, 1093-1101.

(78) Buchet, J. P.; Lauwerys, R.; Roels, H. **(1981)** Urinary excretion of inorganic arsenic and its metabolites after repeated ingestion of sodium metaarsenite by volunteers. *Int. Arch. Occup. Environ. Health 48*, 111-118.

(79) Del Razo, L. M.; Garcia-Vargas, G. G.; Vargas, H.; Albores, A.; Gonsebatt, M. E.; Montero, R.; Ostrosky-Wegman, P.; Kelsh, M.; Cebrian, M. E. **(1997)** Altered profile of urinary arsenic metabolites in adults with chronic arsenicism. A pilot study. *Arch. Toxicol. 71*, 211-217.

(80) Yu, R. C.; Hsu, K. H.; Chen, C. J.; Froines, J. R. **(2000)** Arsenic methylation capacity and skin cancer. *Cancer Epidemiol. Biomarkers Prev. 9*, 1259-1262.

(81) Steinmaus, C.; Bates, M. N.; Yuan, Y.; Kalman, D.; Atallah, R.; Rey, O. A.; Biggs, M. L.; Hopenhayn, C.; Moore, L. E.; Hoang, B. K.; Smith, A. H. **(2006)** Arsenic methylation and bladder cancer risk in case-control studies in Argentina and the United States. *J. Occup. Environ. Med. 48*, 478-488.

(82) Wu, M. M.; Chiou, H. Y.; Hsueh, Y. M.; Hong, C. T.; Su, C. L.; Chang, S. F.; Huang, W. L.; Wang, H. T.; Wang, Y. H.; Hsieh, Y. C.; Chen, C. J. **(2006)** Effect of plasma homocysteine level and urinary monomethylarsonic acid on the risk of arsenic-associated carotid atherosclerosis. *Toxicol. Appl. Pharmacol. 216*, 168-175.

(83) Yamauchi, H.; Yamamura, Y. **(1983)** Concentration and chemical species of arsenic in human tissue. *Bull. Environ. Contam. Toxicol. 31*, 267-270.

(84) Concha, G.; Vogler, G.; Lezcano, D.; Nermell, B.; Vahter, M. **(1998)** Exposure to inorganic arsenic metabolites during early human development. *Toxicol. Sci. 44*, 185-190.

(85) Hernandez-Zavala, A.; Valenzuela, O. L.; Matousek, T.; Drobna, Z.; Dedina, J.; Garcia-Vargas, G. G.; Thomas, D. J.; Del Razo, L. M.; Styblo, M. **(2008)** Speciation of arsenic in exfoliated urinary bladder epithelial cells from individuals exposed to arsenic in drinking water. *Environ. Health Perspect. 116*, 1656-1660.

(86) Paul, D. S.; Hernandez-Zavala, A.; Walton, F. S.; Adair, B. M.; Dedina, J.; Matousek, T.; Styblo, M. **(2007)** Examination of the effects of arsenic on glucose homeostasis in cell culture and animal studies: development of a mouse model for arsenic-induced diabetes. *Toxicol. Appl. Pharmacol. 222*, 305-314.

(87) Paul, D. S.; Walton, F. S.; Saunders, R. J.; Styblo, M. **(2011)** Characterization of the impaired glucose homeostasis produced in C57BL/6 mice by chronic exposure to arsenic and high-fat diet. *Environ. Health Perspect. 119*, 1104-1109.

(88) Kenyon, E. M.; Hughes, M. F.; Adair, B. M.; Highfill, J. H.; Crecelius, E. A.; Clewell, H. J.; Yager, J. W. **(2008)** Tissue distribution and urinary excretion of inorganic arsenic and its methylated metabolites in C57BL6 mice following subchronic exposure to arsenate in drinking water. *Toxicol. Appl. Pharmacol. 232*, 448-455.

(89) Devesa, V.; Adair, B. M.; Liu, J.; Waalkes, M. P.; Diwan, B. A.; Styblo, M.; Thomas, D. J. **(2006)** Arsenicals in maternal and fetal mouse tissues after gestational exposure to arsenite. *Toxicology 224*, 147-155.

(90) Hughes, M. F.; Edwards, B. C.; Herbin-Davis, K. M.; Saunders, J.; Styblo, M.; Thomas, D. J. **(2010)** Arsenic (+3 oxidation state) methyltransferase genotype affects steady-state distribution and clearance of arsenic in arsenate-treated mice. *Toxicol. Appl. Pharmacol. .*

(91) Chen, B.; Arnold, L. L.; Cohen, S. M.; Thomas, D. J.; Le, X. C. **(2011)** Mouse Arsenic (+3 Oxidation State) Methyltransferase Genotype Affects Metabolism and Tissue Dosimetry of Arsenicals after Arsenite Administration in Drinking Water. *Toxicol. Sci. 124*, 320-326.

(92) Yokohira, M.; Arnold, L. L.; Pennington, K. L.; Suzuki, S.; Kakiuchi-Kiyota, S.; Herbin-Davis, K.; Thomas, D. J.; Cohen, S. M. **(2011)** Effect of sodium arsenite dose administered in the drinking water on the urinary bladder epithelium of female arsenic (+3 oxidation state) methyltransferase knockout mice. *Toxicol. Sci. 121*, 257-266.

(93) Naranmandura, H.; Rehman, K.; Le, X. C.; Thomas, D. J. **(2012)** Formation of methylated oxyarsenicals and thioarsenicals in wild-type and arsenic (+3 oxidation state) methyltransferase knockout mice exposed to arsenate. *Anal. Bioanal Chem. .*

(94) Shaw, J.; Sicree, R.; Zimmet, P. **(2010)** Global estimates of the prevalence of diabetes for 2010 and 2030. *Diabetes Res. Clin. Pract. 87*, 4-14.

(95) Centers for Disease Control and Prevention **(2011)** National diabetes fact sheet: national estimates and general information on diabetes and prediabetes in the United States, 2011.

(96) Prentki, M.; Nolan, C. J. **(2006)** Islet beta cell failure in type 2 diabetes. *J. Clin. Invest. 116*, 1802-1812.

(97) World Health Organization **(2006)** International Diabetes Federation. *Definition and diagnosis of diabetes mellitus and intermediate hyperglycemia: report of a WHO/IDF consultation.Geneva (CH): World Health Organization .*

(98) World Health Organization **(2011)** Use of glycated haemoglobin (HbA1c) in the diagnosis of diabetes mellitus. Abbreviated report of a WHO consultation. *Geneva, WHO ,* 1-25.

(99) Lai, M. S.; Hsueh, Y. M.; Chen, C. J.; Shyu, M. P.; Chen, S. Y.; Kuo, T. L.; Wu, M. M.; Tai, T. Y. **(1994)** Ingested inorganic arsenic and prevalence of diabetes mellitus. *Am. J. Epidemiol. 139*, 484-492.

(100) Rahman, M.; Tondel, M.; Ahmad, S. A.; Axelson, O. **(1998)** Diabetes mellitus associated with arsenic exposure in Bangladesh. *Am. J. Epidemiol. 148*, 198-203.

(101) Tsai, S. M.; Wang, T. N.; Ko, Y. C. **(1999)** Mortality for certain diseases in areas with high levels of arsenic in drinking water. *Arch. Environ. Health 54*, 186-193.

(102) Tseng, C. H.; Tai, T. Y.; Chong, C. K.; Tseng, C. P.; Lai, M. S.; Lin, B. J.; Chiou, H. Y.; Hsueh, Y. M.; Hsu, K. H.; Chen, C. J. **(2000)** Long-term arsenic exposure and incidence of non-insulin-dependent diabetes mellitus: a cohort study in arseniasis-hyperendemic villages in Taiwan. *Environ. Health Perspect. 108*, 847-851.

(103) Wang, S. L.; Chiou, J. M.; Chen, C. J.; Tseng, C. H.; Chou, W. L.; Wang, C. C.; Wu, T. N.; Chang, L. W. **(2003)** Prevalence of non-insulin-dependent diabetes

mellitus and related vascular diseases in southwestern arseniasis-endemic and nonendemic areas in Taiwan. *Environ. Health Perspect. 111*, 155-159.

(104) Nabi, A. H.; Rahman, M. M.; Islam, L. N. **(2005)** Evaluation of biochemical changes in chronic arsenic poisoning among Bangladeshi patients. *Int. J. Environ. Res. Public. Health. 2*, 385-393.

(105) Chen, C. J.; Wang, S. L.; Chiou, J. M.; Tseng, C. H.; Chiou, H. Y.; Hsueh, Y. M.; Chen, S. Y.; Wu, M. M.; Lai, M. S. **(2007)** Arsenic and diabetes and hypertension in human populations: a review. *Toxicol. Appl. Pharmacol. 222*, 298-304.

(106) Chen, Y.; Ahsan, H.; Slavkovich, V.; Peltier, G. L.; Gluskin, R. T.; Parvez, F.; Liu, X.; Graziano, J. H. **(2010)** No association between arsenic exposure from drinking water and diabetes mellitus: a cross-sectional study in Bangladesh. *Environ. Health Perspect. 118*, 1299-1305.

(107) Navas-Acien, A.; Silbergeld, E. K.; Pastor-Barriuso, R.; Guallar, E. **(2008)** Arsenic exposure and prevalence of type 2 diabetes in US adults. *JAMA 300*, 814-822.

(108) Steinmaus, C.; Yuan, Y.; Liaw, J.; Smith, A. H. **(2009)** Low-level population exposure to inorganic arsenic in the United States and diabetes mellitus: a reanalysis. *Epidemiology 20*, 807-815.

(109) Ma, M.; Le, X. C. **(1998)** Effect of arsenosugar ingestion on urinary arsenic speciation. *Clin. Chem. 44*, 539-550.

(110) Navas-Acien, A.; Silbergeld, E. K.; Pastor-Barriuso, R.; Guallar, E. **(2009)** Arsenic exposure and Prevalence of Type 2 Diabetes: Updated Findings from the National Health Nutrition and Examination Survey, 2003-2006. *Epidemiology 20*, 816.

(111) Coronado-Gonzalez, J. A.; Del Razo, L. M.; Garcia-Vargas, G.; Sanmiguel-Salazar, F.; Escobedo-de la Pena, J. **(2007)** Inorganic arsenic exposure and type 2 diabetes mellitus in Mexico. *Environ. Res. 104*, 383-389.

(112) Gribble, M. O.; Howard, B. V.; Umans, J. G.; Shara, N. M.; Francesconi, K. A.; Goessler, W.; Crainiceanu, C. M.; Silbergeld, E. K.; Guallar, E.; Navas-Acien, A. **(2012)** Arsenic exposure, diabetes prevalence, and diabetes control in the strong heart study. *Am. J. Epidemiol. 176*, 865-874.

(113) Ettinger, A. S.; Zota, A. R.; Amarasiriwardena, C. J.; Hopkins, M. R.; Schwartz, J.; Hu, H.; Wright, R. O. **(2009)** Maternal arsenic exposure and impaired glucose tolerance during pregnancy. *Environ. Health Perspect. 117*, 1059-1064.

(114) Drobna, Z.; Del Razo, L. M.; Garcia-Vargas, G. G.; Sanchez-Pena, L. C.; Barrera-Hernandez, A.; Styblo, M.; Loomis, D. **(2012)** Environmental exposure to arsenic,

AS3MT polymorphism and prevalence of diabetes in Mexico. *J. Expo. Sci. Environ. Epidemiol.* .

(115) National Research Council (US). Subcommittee on Arsenic in Drinking Water In *Arsenic in drinking water;* National Academies Press: 1999; .

(116) Nermell, B.; Lindberg, A. L.; Rahman, M.; Berglund, M.; Persson, L. A.; El Arifeen, S.; Vahter, M. **(2008)** Urinary arsenic concentration adjustment factors and malnutrition. *Environ. Res. 106*, 212-218.

(117) Basu, A.; Mitra, S.; Chung, J.; Mazumder, D. N. G.; Ghosh, N.; Kalman, D.; von Ehrenstein, O. S.; Steinmaus, C.; Liaw, J.; Smith, A. H. **(2011)** Creatinine, Diet, Micronutrients, and Arsenic Methylation in West Bengal, India. *Environ. Health Perspect. 119*, 1308-1313.

(118) Yassine, H.; Kimzey, M. J.; Galligan, M. A.; Gandolfi, A. J.; Stump, C. S.; Lau, S. S. **(2012)** Adjusting for Urinary Creatinine Overestimates Arsenic Concentrations in Diabetics. *Cardiorenal Med. 2*, 26-32.

(119) Chadha, V.; Garg, U.; Alon, U. S. **(2001)** Measurement of urinary concentration: a critical appraisal of methodologies. *Pediatr. Nephrol. 16*, 374-382.

(120) Voinescu, G. C.; Shoemaker, M.; Moore, H.; Khanna, R.; Nolph, K. D. **(2002)** The relationship between urine osmolality and specific gravity. *Am. J. Med. Sci. 323*, 39-42.

(121) Yuan, C.; Lu, X.; Oro, N.; Wang, Z.; Xia, Y.; Wade, T. J.; Mumford, J.; Le, X. C. **(2008)** Arsenic speciation analysis in human saliva. *Clin. Chem. 54*, 163-171.

(122) Lew, K.; Acker, J. P.; Gabos, S.; Le, X. C. **(2010)** Biomonitoring of arsenic in urine and saliva of children playing on playgrounds constructed from chromated copper arsenate-treated wood. *Environ. Sci. Technol. 44*, 3986-3991.

(123) Paul, D. S.; Devesa, V.; Hernandez-Zavala, A.; Adair, B. M.; Walton, F. S.; Drobnâ, Z.; Thomas, D. J.; Styblo, M. In *In Environmental arsenic as a disruptor of insulin signaling;* Metal ions in biology and medicine: proceedings of the... International Symposium on Metal Ions in Biology and Medicine held...= Les ions metalliques en biologie et en medecine:... Symposium international sur les ions metalliques. NIH Public Access: 2008; Vol. 10, pp 1.

(124) Hill, D. S.; Wlodarczyk, B. J.; Mitchell, L. E.; Finnell, R. H. **(2009)** Arsenate-induced maternal glucose intolerance and neural tube defects in a mouse model. *Toxicol. Appl. Pharmacol. 239*, 29-36.

(125) Pal, S.; Chatterjee, A. K. **(2005)** Prospective protective role of melatonin against arsenic-induced metabolic toxicity in Wistar rats. *Toxicology 208*, 25-33.

(126) Izquierdo-Vega, J. A.; Soto, C. A.; Sanchez-Pena, L. C.; De Vizcaya-Ruiz, A.; Del Razo, L. M. **(2006)** Diabetogenic effects and pancreatic oxidative damage in rats subchronically exposed to arsenite. *Toxicol. Lett. 160*, 135-142.

(127) Singh, N.; Rana, S. V. **(2009)** Effect of insulin on arsenic toxicity in diabetic rats-liver function studies. *Biol. Trace Elem. Res. 132*, 215-226.

(128) Wang, J. P.; Wang, S. L.; Lin, Q.; Zhang, L.; Huang, D.; Ng, J. C. **(2009)** Association of arsenic and kidney dysfunction in people with diabetes and validation of its effects in rats. *Environ. Int. 35*, 507-511.

(129) Lu, M.; Wang, H.; Li, X. F.; Arnold, L. L.; Cohen, S. M.; Le, X. C. **(2007)** Binding of dimethylarsinous acid to Cys-13α of rat hemoglobin is responsible for the retention of arsenic in rat blood. *Chem. Res. Toxicol. 20*, 27-37.

(130) Huang, C. F.; Chen, Y. W.; Yang, C. Y.; Tsai, K. S.; Yang, R. S.; Liu, S. H. **(2011)** Arsenic and diabetes: Current perspectives. *Kaohsiung J. Med. Sci. 27*, 402-410.

(131) Diaz-Villasenor, A.; Sanchez-Soto, M. C.; Cebrian, M. E.; Ostrosky-Wegman, P.; Hiriart, M. **(2006)** Sodium arsenite impairs insulin secretion and transcription in pancreatic beta-cells. *Toxicol. Appl. Pharmacol. 214*, 30-34.

(132) Diaz-Villasenor, A.; Burns, A. L.; Salazar, A. M.; Sordo, M.; Hiriart, M.; Cebrian, M. E.; Ostrosky-Wegman, P. **(2008)** Arsenite reduces insulin secretion in rat pancreatic beta-cells by decreasing the calcium-dependent calpain-10 proteolysis of SNAP-25. *Toxicol. Appl. Pharmacol. 231*, 291-299.

(133) Yen, C. C.; Lu, F. J.; Huang, C. F.; Chen, W. K.; Liu, S. H.; Lin-Shiau, S. Y. **(2007)** The diabetogenic effects of the combination of humic acid and arsenic: in vitro and in vivo studies. *Toxicol. Lett. 172*, 91-105.

(134) Fu, J.; Woods, C. G.; Yehuda-Shnaidman, E.; Zhang, Q.; Wong, V.; Collins, S.; Sun, G.; Andersen, M. E.; Pi, J. **(2010)** Low-level arsenic impairs glucose-stimulated insulin secretion in pancreatic beta cells: involvement of cellular adaptive response to oxidative stress. *Environ. Health Perspect. 118*, 864-870.

(135) Pi, J.; Collins, S. **(2010)** Reactive oxygen species and uncoupling protein 2 in pancreatic beta-cell function. *Diabetes Obes. Metab. 12 Suppl 2*, 141-148.

(136) Douillet, C.; Currier, J.; Saunders, R. J.; Bodnar, W. M.; Matoušek, T.; Stýblo, M. **(2013)** Methylated Trivalent Arsenicals are Potent Inhibitors of Glucose Stimulated Insulin Secretion by Murine Pancreatic Islets. *Toxicol. Appl. Pharmacol. 267*, 11-15.

(137) Trouba, K. J.; Wauson, E. M.; Vorce, R. L. **(2000)** Sodium arsenite inhibits terminal differentiation of murine C3H 10T1/2 preadipocytes. *Toxicol. Appl. Pharmacol. 168*, 25-35.

(138) Wauson, E. M.; Langan, A. S.; Vorce, R. L. **(2002)** Sodium arsenite inhibits and reverses expression of adipogenic and fat cell-specific genes during in vitro adipogenesis. *Toxicol. Sci. 65*, 211-219.

(139) Steffens, A. A.; Hong, G. M.; Bain, L. J. **(2011)** Sodium arsenite delays the differentiation of C2C12 mouse myoblast cells and alters methylation patterns on the transcription factor myogenin. *Toxicol. Appl. Pharmacol. 250*, 154-161.

(140) Walton, F. S.; Harmon, A. W.; Paul, D. S.; Drobna, Z.; Patel, Y. M.; Styblo, M. **(2004)** Inhibition of insulin-dependent glucose uptake by trivalent arsenicals: possible mechanism of arsenic-induced diabetes. *Toxicol. Appl. Pharmacol. 198*, 424-433.

(141) Paul, D. S.; Harmon, A. W.; Devesa, V.; Thomas, D. J.; Styblo, M. **(2007)** Molecular mechanisms of the diabetogenic effects of arsenic: inhibition of insulin signaling by arsenite and methylarsonous acid. *Environ. Health Perspect. 115*, 734-742.

(142) Marsh, J. **(1836)** Account of a method of separating small quantities of arsenic from substances with which it may be mixed. *Edinburgh New Philosophical Journal 21*, 229-236.

(143) Del Razo, L. M.; Styblo, M.; Cullen, W. R.; Thomas, D. J. **(2001)** Determination of trivalent methylated arsenicals in biological matrices. *Toxicol. Appl. Pharmacol. 174*, 282-293.

(144) Devesa, V.; Maria, D. R.; Adair, B.; Drobna, Z.; Waters, S. B.; Hughes, M. F.; Styblo, M.; Thomas, D. J. **(2004)** Comprehensive analysis of arsenic metabolites by pH-specific hydride generation atomic absorption spectrometry. *J. Anal. At. Spectrom. 19*, 1460-1467.

(145) Musil, S.; Matoušek, T. **(2008)** On-line pre-reduction of pentavalent arsenicals by thioglycolic acid for speciation analysis by selective hydride generation–cryotrapping–atomic absorption spectrometry. *Spectrochimica Acta Part B: Atomic Spectroscopy 63*, 685-691.

(146) Matousek, T.; Hernandez-Zavala, A.; Svoboda, M.; Langrova, L.; Adair, B. M.; Drobna, Z.; Thomas, D. J.; Styblo, M.; Dedina, J. **(2008)** Oxidation State Specific Generation of Arsines from Methylated Arsenicals Based on L- Cysteine Treatment in Buffered Media for Speciation Analysis by Hydride Generation - Automated Cryotrapping - Gas Chromatography-Atomic Absorption Spectrometry with the Multiatomizer. *Spectrochim. Acta Part B At. Spectrosc. 63*, 396-406.

(147) Rabieh, S.; Hirner, A. V.; Matschullat, J. **(2008)** Determination of arsenic species in human urine using high performance liquid chromatography (HPLC) coupled with inductively coupled plasma mass spectrometry (ICP-MS). *J. Anal. At. Spectrom. 23*, 544-549.

(148) Xie, R.; Johnson, W.; Spayd, S.; Hall, G. S.; Buckley, B. **(2006)** Arsenic speciation analysis of human urine using ion exchange chromatography coupled to inductively coupled plasma mass spectrometry. *Anal. Chim. Acta 578*, 186-194.

(149) Hernandez-Zavala, A.; Matousek, T.; Drobna, Z.; Paul, D. S.; Walton, F.; Adair, B. M.; Dedina, J.; Thomas, D. J.; Styblo, M. **(2008)** Speciation analysis of arsenic in biological matrices by automated hydride generation-cryotrapping-atomic absorption spectrometry with multiple microflame quartz tube atomizer (multiatomizer). *J. Anal. At. Spectrom. 23*, 342-351.

(150) Heilier, J. F.; Buchet, J. P.; Haufroid, V.; Lison, D. **(2005)** Comparison of atomic absorption and fluorescence spectroscopic methods for the routine determination of urinary arsenic. *Int. Arch. Occup. Environ. Health 78*, 51-59.

(151) Lindberg, A. L.; Goessler, W.; Grander, M.; Nermell, B.; Vahter, M. **(2007)** Evaluation of the three most commonly used analytical methods for determination of inorganic arsenic and its metabolites in urine. *Toxicol. Lett. 168*, 310-318.

(152) Sanchez-Rodas, D.; Corns, W. T.; Chen, B.; Stockwell, P. B. **(2010)** Atomic Fluorescence Spectrometry: a suitable detection technique in speciation studies for arsenic, selenium, antimony and mercury. *J. Anal. At. Spectrom. 25*, 933-946.

(153) Styblo, M.; Thomas, D. J. **(1997)** Binding of arsenicals to proteins in an in vitro methylation system. *Toxicol. Appl. Pharmacol. 147*, 1-8.

(154) Ding, L.; Saunders, R. J.; Drobna, Z.; Walton, F. S.; Xun, P.; Thomas, D. J.; Styblo, M. **(2012)** Methylation of arsenic by recombinant human wild-type arsenic (+3 oxidation state) methyltransferase and its methionine 287 threonine (M287T) polymorph: Role of glutathione. *Toxicol. Appl. Pharmacol. 264*, 121-130.

(155) Navas-Acien, A.; Silbergeld, E. K.; Streeter, R. A.; Clark, J. M.; Burke, T. A.; Guallar, E. **(2006)** Arsenic exposure and type 2 diabetes: a systematic review of the experimental and epidemiological evidence. *Environ. Health Perspect. 114*, 641-648.

(156) Valenzuela, O. L.; Borja-Aburto, V. H.; Garcia-Vargas, G. G.; Cruz-Gonzalez, M. B.; Garcia-Montalvo, E. A.; Calderon-Aranda, E. S.; Del Razo, L. M. **(2005)** Urinary trivalent methylated arsenic species in a population chronically exposed to inorganic arsenic. *Environ. Health Perspect. 113*, 250-254.

(157) Kenyon, E. M.; Del Razo, L. M.; Hughes, M. F.; Kitchin, K. T. **(2005)** An integrated pharmacokinetic and pharmacodynamic study of arsenite action 2. Heme oxygenase induction in mice. *Toxicology 206*, 389-401.

(158) Kenyon, E. M.; Del Razo, L. M.; Hughes, M. F. **(2005)** Tissue distribution and urinary excretion of inorganic arsenic and its methylated metabolites in mice following acute oral administration of arsenate. *Toxicol. Sci. 85*, 468-475.

(159) Suzuki, K. T.; Mandal, B. K.; Ogra, Y. **(2002)** Speciation of arsenic in body fluids. *Talanta 58*, 111-119.

(160) Sampayo-Reyes, A.; Zakharyan, R. A.; Healy, S. M.; Aposhian, H. V. **(2000)** Monomethylarsonic acid reductase and monomethylarsonous acid in hamster tissue. *Chem. Res. Toxicol. 13*, 1181-1186.

(161) Raab, A.; Meharg, A. A.; Jaspars, M.; Genney, D. R.; Feldmann, J. **(2004)** Arsenic-glutathione complexes-their stability in solution and during separation by different HPLC modes. *J. Anal. At. Spectrom. 19*, 183-190.

(162) Hughes, M. F.; Devesa, V.; Adair, B. M.; Styblo, M.; Kenyon, E. M.; Thomas, D. J. **(2005)** Tissue dosimetry, metabolism and excretion of pentavalent and trivalent monomethylated arsenic in mice after oral administration. *Toxicol. Appl. Pharmacol. 208*, 186-197.

(163) Tseng, C. H.; Chong, C. K.; Tseng, C. P.; Centeno, J. A. **(2007)** Blackfoot disease in Taiwan: its link with inorganic arsenic exposure from drinking water. *Ambio 36*, 82-84.

(164) Saha, K. C. **(2003)** Diagnosis of arsenicosis. *J. Environ. Sci. Health. A. Tox. Hazard. Subst. Environ. Eng. 38*, 255-272.

(165) Buchet, J. P.; Lauwerys, R.; Roels, H. **(1981)** Comparison of the urinary excretion of arsenic metabolites after a single oral dose of sodium arsenite, monomethylarsonate, or dimethylarsinate in man. *Int. Arch. Occup. Environ. Health 48*, 71-79.

(166) Delnomdedieu, M.; Basti, M. M.; Styblo, M.; Otvos, J. D.; Thomas, D. J. **(1994)** Complexation of arsenic species in rabbit erythrocytes. *Chem. Res. Toxicol. 7*, 621-627.

(167) Currier, J. M.; Svoboda, M.; de Moraes, D. P.; Matousek, T.; Dedina, J.; Styblo, M. **(2011)** Direct Analysis of Methylated Trivalent Arsenicals in Mouse Liver by Hydride Generation-Cryotrapping-Atomic Absorption Spectrometry. *Chem. Res. Toxicol. 24*, 478-480.

(168) Rahman, I.; Kode, A.; Biswas, S. K. **(2006)** Assay for quantitative determination of glutathione and glutathione disulfide levels using enzymatic recycling method. *Nat. Protoc. 1*, 3159-3165.

(169) Hopenhayn-Rich, C.; Biggs, M. L.; Smith, A. H.; Kalman, D. A.; Moore, L. E. **(1996)** Methylation study of a population environmentally exposed to arsenic in drinking water. *Environ. Health Perspect. 104*, 620-628.

(170) Vahter, M. **(2002)** Mechanisms of arsenic biotransformation. *Toxicology 181-182*, 211-217.

(171) Yan, H.; Wang, N.; Weinfeld, M.; Cullen, W. R.; Le, X. C. **(2009)** Identification of arsenic-binding proteins in human cells by affinity chromatography and mass spectrometry. *Anal. Chem. 81*, 4144-4152.

(172) Taurkova, P.; Svoboda, M.; Musil, S.; Matousek, T. **(2011)** Loss of di- and trimethylarsine on Nafion membrane dryers following hydride generation. *J. Anal. At. Spectrom. 26*, 220-223.

(173) Mandal, B.; Ogra, Y.; Suzuki, K. **(2001)** Identification of dimethylarsinous and monomethylarsonous acids in human urine of the arsenic-affected areas in West Bengal, India. *Chem. Res. Toxicol. 14*, 371-378.

(174) Cohen, S. M.; Arnold, L. L.; Uzvolgyi, E.; Cano, M.; St John, M.; Yamamoto, S.; Lu, X.; Le, X. C. **(2002)** Possible role of dimethylarsinous acid in dimethylarsinic acid-induced urothelial toxicity and regeneration in the rat. *Chem. Res. Toxicol. 15*, 1150-1157.

(175) Gong, Z.; Lu, X.; Ma, M.; Watt, C.; Le, X. **(2002)** Arsenic speciation analysis. *Talanta 58*, 77-96.

(176) Mandal, B.; Ogra, Y.; Anzai, K.; Suzuki, K. **(2004)** Speciation of arsenic in biological samples. *Toxicol. Appl. Pharmacol. 198*, 307-318.

(177) Okina, M.; Yoshida, K.; Kuroda, K.; Wanibuchi, H.; Fukushima, S.; Endo, G. **(2004)** Determination of trivalent methylated arsenicals in rat urine by liquid chromatography-inductively coupled plasma mass spectrometry after solvent extraction. *J. Chromatogr. B. Analyt Technol. Biomed. Life. Sci. 799*, 209-215.

(178) Naranmandura, H.; Suzuki, N.; Iwata, K.; Hirano, S.; Suzuki, K. T. **(2007)** Arsenic metabolism and thioarsenicals in hamsters and rats. *Chem. Res. Toxicol. 20*, 616-624.

(179) Kubachka, K. M.; Kohan, M. C.; Conklin, S. D.; Herbin-Davis, K.; Creed, J. T.; Thomas, D. J. **(2009)** In vitro biotransformation of dimethylarsinic acid and

trimethylarsine oxide by anaerobic microflora of mouse cecum analyzed by HPLC-ICP-MS and HPLC-ESI-MS. *J. Anal. At. Spectrom. 24*, 1062-1068.

(180) Pinyayev, T. S.; Kohan, M. J.; Herbin-Davis, K.; Creed, J. T.; Thomas, D. J. **(2011)** Preabsorptive Metabolism of Sodium Arsenate by Anaerobic Microbiota of Mouse Cecum Forms a Variety of Methylated and Thiolated Arsenicals. *Chem. Res. Toxicol.* , 475-477.

(181) Raml, R.; Rumpler, A.; Goessler, W.; Vahter, M.; Li, L.; Ochi, T.; Francesconi, K. A. **(2007)** Thio-dimethylarsinate is a common metabolite in urine samples from arsenic-exposed women in Bangladesh. *Toxicol. Appl. Pharmacol. 222*, 374-380.

(182) Cubadda, F.; Aureli, F.; D'Amato, M.; Raggi, A.; Turco, A. C.; Mantovani, A. **(2012)** Speciated urinary arsenic as a biomarker of dietary exposure to inorganic arsenic in residents living in high-arsenic areas in Latium, Italy. *Pure Appl. Chem. 84*, 203-214.

(183) Francesconi, K.; Kuehnelt, D. **(2004)** Determination of arsenic species: A critical review of methods and applications, 2000-2003. *Analyst 129*, 373-395.

(184) Hsu, K.; Sun, C.; Huang, Y. **(2011)** Arsenic speciation in biomedical sciences: Recent advances and applications. *Kaohsiung J. Med. Sci. 27*, 382-389.

(185) B'Hymer, C.; Caruso, J. **(2004)** Arsenic and its speciation analysis using high-performance liquid chromatography and inductively coupled plasma mass spectrometry. *J. Chromatogr. A 1045*, 1-13.

(186) Raab, A.; Feldmann, J. **(2005)** Arsenic speciation in hair extracts. *Anal. Bioanal. Chem. 381*, 332-338.

(187) Wang, Z.; Zhou, J.; Lu, X.; Gong, Z.; Le, X. **(2004)** Arsenic speciation in urine from acute promyelocytic leukemia patients undergoing arsenic trioxide treatment. *Chem. Res. Toxicol. 17*, 95-103.

(188) Hippler, J.; Zdrenka, R.; Reichel, R. A. D.; Weber, D. G.; Rozynek, P.; Johnen, G.; Dopp, E.; Hirner, A. V. **(2011)** Intracellular, time-resolved speciation and quantification of arsenic compounds in human urothelial and hepatoma cells. *J. Anal. At. Spectrom. 26*, 2396-2403.

(189) Pan, F.; Tyson, J. F.; Uden, P. C. **(2007)** Simultaneous speciation of arsenic and selenium in human urine by high-performance liquid chromatography inductively coupled plasma mass spectrometry. *J. Anal. At. Spectrom. 22*, 931-937.

(190) Aposhian, H., et al **(2000)** Occurrence of monomethylarsonous acid in urine of humans exposed to inorganic arsenic. *Chem. Res. Toxicol. 13*, 693-697.

(191) Aposhian, H., et al **(2000)** DMPS - Arsenic Challenge Test II. Modulation of arsenic species, including monomethylarsonous acid (MMA(III)), excreted in human urine. *Toxicol. Appl. Pharmacol. 165*, 74-83.

(192) Slejkovec, Z.; Falnoga, I.; Goessler, W.; van Elteren, J. T.; Raml, R.; Podgornik, H.; Cernelc, P. **(2008)** Analytical artefacts in the speciation of arsenic in clinical samples. *Anal. Chim. Acta 607*, 83-91.

(193) Currier, J. M.; Svoboda, M.; Matousek, T.; Dedina, J.; Styblo, M. **(2011)** Direct analysis and stability of methylated trivalent arsenic metabolites in cells and tissues. *Metallomics 3*, 1347-1354.

(194) Hansen, H. R.; Raab, A.; Jaspars, M.; Milne, B. F.; Feldmann, J. **(2004)** Sulfur-containing arsenical mistaken for dimethylarsinous acid [DMA(III)] and identified as a natural metabolite in urine: major implications for studies on arsenic metabolism and toxicity. *Chem. Res. Toxicol. 17*, 1086-1091.

(195) Fricke, M.; Zeller, M.; Cullen, W.; Witkowski, M.; Creed, J. **(2007)** Dimethylthioarsinic anhydride: a standard for arsenic speciation. *Anal. Chim. Acta 583*, 78-83.

(196) Styblo, M.; Serves, S. V.; Cullen, W. R.; Thomas, D. J. **(1997)** Comparative inhibition of yeast glutathione reductase by arsenicals and arsenothiols. *Chem. Res. Toxicol. 10*, 27-33.

(197) Shraim, A.; Sekaran, N.; Anuradha, C.; Hirano, S. **(2002)** Speciation of arsenic in tube-well water samples collected from West Bengal, India, by high-performance liquid chromatography-inductively coupled plasma mass spectrometry. *Appl. Organomet. Chem. 16*, 202-209.

(198) Fomenko, D. E.; Marino, S. M.; Gladyshev, V. N. **(2008)** Functional diversity of cysteine residues in proteins and unique features of catalytic redox-active cysteines in thiol oxidoreductases. *Mol. Cells 26*, 228.

(199) Song, X.; Geng, Z.; Zhu, J.; Li, C.; Hu, X.; Bian, N.; Zhang, X.; Wang, Z. **(2009)** Structure-function roles of four cysteine residues in the human arsenic (+3 oxidation state) methyltransferase (hAS3MT) by site-directed mutagenesis. *Chem. Biol. Interact. 179*, 321-328.

(200) Song, X.; Geng, Z.; Li, X.; Zhao, Q.; Hu, X.; Zhang, X.; Wang, Z. **(2011)** Functional and structural evaluation of cysteine residues in the human arsenic (3 oxidation state) methyltransferase (hAS3MT). *Biochimie 93*, 369-375.

(201) Gailer, J.; George, G. N.; Pickering, I. J.; Prince, R. C.; Younis, H. S.; Winzerling, J. J. **(2002)** Biliary excretion of [(GS)(2)AsSe](-) after intravenous injection of rabbits with arsenite and selenate. *Chem. Res. Toxicol. 15*, 1466-1471.

(202) Kala, S. V.; Kala, G.; Prater, C. I.; Sartorelli, A. C.; Lieberman, M. W. **(2004)** Formation and urinary excretion of arsenic triglutathione and methylarsenic diglutathione. *Chem. Res. Toxicol. 17*, 243-249.

(203) Rey, N. A.; Howarth, O. W.; Pereira-Maia, E. C. **(2004)** Equilibrium characterization of the As (III)–cysteine and the As (III)–glutathione systems in aqueous solution. *J. Inorg. Biochem. 98*, 1151-1159.

(204) Spuches, A. M.; Kruszyna, H. G.; Rich, A. M.; Wilcox, D. E. **(2005)** Thermodynamics of the As(III)-thiol interaction: arsenite and monomethylarsenite complexes with glutathione, dihydrolipoic acid, and other thiol ligands. *Inorg. Chem. 44*, 2964-2972.

(205) Percy, A. J.; Gailer, J. **(2008)** Methylated trivalent arsenic-glutathione complexes are more stable than their arsenite Analog. *Bioinorganic chemistry and applications 2008*.

(206) Rubatto Birri, P. N.; Perez, R. D.; Cremonezzi, D.; Perez, C. A.; Rubio, M.; Bongiovanni, G. A. **(2010)** Association between As and Cu renal cortex accumulation and physiological and histological alterations after chronic arsenic intake. *Environ. Res. 110*, 417-423.

(207) [Anonymous] **(2012)** Standards of Medical Care in Diabetes-2012. *Diabetes Care 35*, S11-S63.

(208) Goldstein, D. E.; Little, R. R.; Lorenz, R. A.; Malone, J. I.; Nathan, D.; Peterson, C. M.; Sacks, D. B. **(2004)** Tests of glycemia in diabetes. *Diabetes Care 27*, 1761-1773.

(209) Simeonova, P. P.; Wang, S.; Toriuma, W.; Kommineni, V.; Matheson, J.; Unimye, N.; Kayama, F.; Harki, D.; Ding, M.; Vallyathan, V.; Luster, M. I. **(2000)** Arsenic mediates cell proliferation and gene expression in the bladder epithelium: association with activating protein-1 transactivation. *Cancer Res. 60*, 3445-3453.

(210) Scott, N.; Hatlelid, K. M.; MacKenzie, N. E.; Carter, D. E. **(1993)** Reactions of arsenic(III) and arsenic(V) species with glutathione. *Chem. Res. Toxicol. 6*, 102-106.

(211) Chang, Y. Y.; Kuo, T. C.; Hsu, C. H.; Hou, D. R.; Kao, Y. H.; Huang, R. N. **(2012)** Characterization of the role of protein-cysteine residues in the binding with sodium arsenite. *Arch. Toxicol. 86*, 911-922.

(212) Calderon, R. L.; Hudgens, E.; Le, X. C.; Schreinemachers, D.; Thomas, D. J. **(1999)** Excretion of arsenic in urine as a function of exposure to arsenic in drinking water. *Environ. Health Perspect. 107*, 663-667.

(213) Hall, M.; Chen, Y.; Ahsan, H.; Slavkovich, V.; van Geen, A.; Parvez, F.; Graziano, J. **(2006)** Blood arsenic as a biomarker of arsenic exposure: results from a prospective study. *Toxicology 225*, 225-233.

(214) Kurttio, P.; Komulainen, H.; Hakala, E.; Kahelin, H.; Pekkanen, J. **(1998)** Urinary excretion of arsenic species after exposure to arsenic present in drinking water. *Arch. Environ. Contam. Toxicol. 34*, 297-305.

(215) Slotnick, M. J.; Meliker, J. R.; AvRuskin, G. A.; Ghosh, D.; Nriagu, J. O. **(2007)** Toenails as a biomarker of inorganic arsenic intake from drinking water and foods. *J. Toxicol. Env. Health Part A 70*, 148-158.

(216) TYLER, D. **(1962)** Stratified Squamous Epithelium in Vesical Trigone and Urethra - Findings Correlated with Menstrual Cycle and Age. *Am. J. Anat. 111*, 319-&.

(217) Gaisa, N. T.; Braunschweig, T.; Reimer, N.; Bornemann, J.; Eltze, E.; Siegert, S.; Toma, M.; Villa, L.; Hartmann, A.; Knuechel, R. **(2011)** Different immunohistochemical and ultrastructural phenotypes of squamous differentiation in bladder cancer. *Virchows Arch. 458*, 301-312.

(218) Laguna, P.; Smedts, F.; Nordling, J.; Horn, T.; Bouchelouche, K.; Hopman, A.; de la Rosette, J. **(2006)** Keratin expression profiling of transitional epithelium in the painful bladder syndrome/interstitial cystitis. *Am. J. Clin. Pathol. 125*, 105-110.

(219) Mueller, E.; Latini, J.; Lux, M.; Stablein, U.; Brubaker, L.; Kreder, K.; Fitzgerald, M. **(2005)** Gender differences in 24-hour urinary diaries of asymptomatic North American adults. *J. Urol. 173*, 490-492.

(220) Perucca, J.; Bouby, N.; Valeix, P.; Bankir, L. **(2007)** Sex difference in urine concentration across differing ages, sodium intake, and level of kidney disease. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 292*, R700-R705.

(221) Islam, M. R.; Khan, I.; Hassan, S. M. N.; McEvoy, M.; D'Este, C.; Attia, J.; Peel, R.; Sultana, M.; Akter, S.; Milton, A. H. **(2012)** Association between type 2 diabetes and chronic arsenic exposure in drinking water: A cross sectional study in Bangladesh. *Environ. Health 11*, 38.