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Arsenic Speciation in the Blood of Arsenite-Treated F344 Rats

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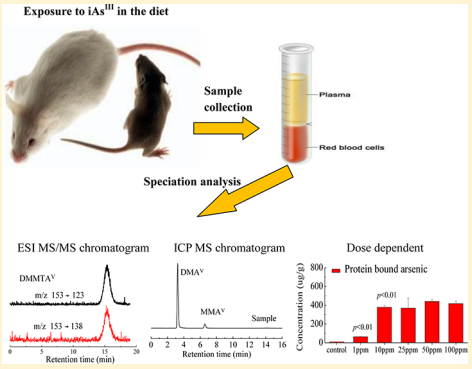
Supporting Information

ABSTRACT: Arsenic speciation in blood can improve understanding of the metabolism and toxicity of arsenic. In this study, arsenic species in the plasma and red blood cells (RBCs) of arsenite-treated female F344 rats were characterized using anion exchange and size exclusion chromatography separation with inductively coupled plasma mass spectrometry (ICPMS) and electrospray ionization tandem mass spectrometry (ESI MS/MS) detection. Arsenite (iAs^{III}), arsenate (iAs^{V}), monomethylarsonic acid (MMA^{V}), dimethylarsinic acid (DMA^{V}), trimethylarsine oxide ($TMAO^{V}$), monomethylmonothioarsonic acid ($MMMTA^{V}$), and dimethylmonothioarsinic acid ($DMMTA^{V}$) were detected in the plasma, with DMA^{V} being the predominant metabolite. Upon oxidative pretreatment with 5% hydrogen peroxide (H_2O_2), plasma proteins released bound arsenic in the form of DMA^{V} as the major species and MMA^{V} as the minor species. The ratio of protein-bound arsenic to total arsenic decreased with increasing dosage of iAs^{III} administered to the rats, suggesting a possible saturation of the binding capacity of the plasma proteins. The proportion of the protein-bound arsenic in the plasma varied among rats. In the H_2O_2-treated lysates of red blood cells of rats, DMA^{V} was consistently found as the predominant arsenic species, probably reflecting the preferential binding of dimethylarsinous acid (DMA^{III}) to rat hemoglobin. iAs^{V}, MMA^{V}, and trimethylarsine oxide ($TMAO^{V}$) were also detected in the hydrogen peroxide-treated lysates of red blood cells. Importantly, $DMMTA^{V}$ and $MMMTA^{V}$ have not been reported in rat blood, and the present finding of $DMMTA^{V}$ and $MMMTA^{V}$ in the rat plasma is toxicologically relevant because these pentavalent thioarsenicals are more toxic than their counterparts DMA^{V} and MMA^{V}. Identifying novel thiolated arsenicals and determining protein-bound arsenicals in the blood provide useful insights into the metabolism and toxicity of arsenic in animals.



INTRODUCTION

High levels of inorganic arsenic (iAs) in drinking water are a worldwide health concern, due to its prevalence and toxicity.[1] Epidemiological studies have demonstrated a significant association between arsenic ingestion and the risk of skin, lung, and bladder cancers.[1−4] Various noncancerous effects, including hypertension, diabetes, and cardiovascular disease, are also associated with exposure to high concentrations of arsenic.[1,2,5,6] However, the exact mechanism of action by which arsenic causes adverse health effects remains undefined.[2]

Rodent models have successfully been developed to help understand and evaluate arsenic carcinogenicity and toxicity.[7−9] For instance, rats (especially female rats) developed bladder tumors after long-term treatment with high doses (40−200 μg/g) of dimethylarsinic acid (DMA^{V}) in the diet[10] or drinking water,[11] in contrast to the negative results obtained from previous two-year bioassays with rats or mice administered inorganic arsenic.[12] Inorganic arsenic itself in drinking water or the diet has also been demonstrated to induce cytotoxicity and increased cell proliferation in the bladder epithelium of rats and mice, with a higher sensitivity of female rats.[13,14]

DMA^{V} is a major urinary metabolite of inorganic arsenic in humans and rodents. Generally, biotransformation of inorganic arsenic involves the reduction of pentavalent arsenicals and subsequent oxidative methylation of trivalent arsenicals. In rats, the biomethylation process is terminated at trimethylation mainly as trimethylarsine oxide ($TMAO^{V}$), whereas in humans dimethylated arsenicals are the end products.[15−17] Some sulfur analogues of methylated arsenic oxyanions are also formed during arsenic metabolism.[18−20] Detailed studies on arsenic speciation will help close the knowledge gap in understanding the possibility of As-induced bladder tumors in rat models.

Arsenic in blood is not generally recommended as an appropriate biomarker for arsenic exposure because of its short half-life (1−2 h), low concentration, and matrix complexity.[21−23] However, as the circulatory system for distribution, disposition, and elimination in the body, blood is a useful specimen to study arsenic speciation for a better understanding of arsenic metabolism and toxicity, including arsenic binding to proteins in blood. Arsenic in rat blood mainly accumulates in the erythrocytes (red blood cells) by binding with hemoglobin, and a higher affinity of rat hemoglobin to arsenicals leads to a longer retention of arsenic in rat blood than in human blood.[24−26] Arsenicals also showed the ability to bind with

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plasma proteins in animals, e.g., rabbit metallothionein[27] and the rat hemoglobin−haptoglobin complex.[28]

Although significant differences in the metabolism of arsenic exist between animals and humans and between different animal species, understanding these differences could contribute to elucidating the susceptibility of humans to arsenic toxicity. The animal experiments have already led to the improved understanding of the role of methylation in arsenic elimination[29] and arsenic accumulation.[24]

While many studies have dealt with common arsenic species, there is a need to characterize new arsenic metabolites and the protein-bound arsenic species. The objective of this study was to speciate free and protein-bound arsenicals in both the plasma and red blood cells of rats treated with iAs^{III}, which can improve our understanding of the metabolism of arsenic in animals. The present study for the first time reveals the presence of two thio-arsenicals, $DMMTA^{V}$ and $MMMTA^{V}$, in the plasma of rats. Detection and identification of $DMMTA^{V}$ and $MMMTA^{V}$ are meaningful because these pentavalent thio-arsenicals are more toxic than their oxygen-containing counterparts DMA^{V} and MMA^{V}.

MATERIALS AND METHODS

Caution: *Most arsenic compounds used in this study are highly toxic. These compounds should be handled with care.*

Reagents. All reagents used in this study were of analytical grade. The standards of $MMMTA^{V}$ and $DMMTA^{V}$ were synthesized according to a reported method[20] and kept at −20 °C. Diluted solutions were freshly prepared with deionized water. Stock solutions of other standards (1000 mg/L as As) were prepared by dissolving appropriate amounts of iAs^{III}, iAs^{V}, DMA^{V} (Aldrich), $TMAO^{V}$ (Tri Chemical Laboratories Inc.), and MMA^{V} (Chem Service) in deionized water. Working solutions were obtained daily by serial dilutions of stock solutions in deionized water.

Ammonium bicarbonate (Sigma), ammonium hydroxide (Fisher), and HPLC grade methanol (Fisher) were used to prepare HPLC mobile phases, which were filtered through a 0.45-μm membrane before use. Perchloric acid (Sigma) and hydrogen peroxide (CALEDON, Canada) were used for treatment of rat plasma and blood cells. A certified reference material (CRM No.18, Human Urine, Japan) was analyzed in parallel with samples for quality control purposes. Our replicate analyses of CRM No.18 urine showed the presence of DMA^{V} at 38 ± 4 μg/L, which is in good agreement with the reference value of 36 ± 9 μg/L in the form of DMA^{V}.

Rats Treated with iAs^{III}. Seven-week old female F344 rats were purchased from Charles River Breeding Laboratories (Raleigh, NC). The rats were housed in polycarbonate cages (five/cage) on dry corncob beddings in a room with a targeted temperature of 22 °C, humidity of 50%, and 12 h light/dark cycle, and fed a basal diet (Certified Purine 5002, Dyets Inc., Bethlehem, PA). Food and tap water were available ad libitum throughout the study. Rats were quarantined according to standard protocols before starting treatment.

After the quarantine period, the rats were randomized into 6 groups of 10 rats each: group 1 was fed a basal diet, and groups 2−6 were fed the basal diet containing 1, 10, 25, 50, or 100 ppm (μg/g) iAs^{III}, respectively. iAs^{III} was provided in the form of $NaAs^{III}O_2$ (99%), purchased from Sigma (St. Louis, MO) and mixed into the diet prior to pelleting (Dyets Inc.). Pelleted basal and iAs^{III}-supplemented diets were available ad libitum from cage lid hoppers throughout the treatment period. Forty-two days after treatment with the iAs^{III}-supplemented diets began, the rats were sacrificed, and blood samples were collected from the abdominal aorta into tubes containing lithium heparin. RBCs were separated from plasma by centrifugation at 4000 rpm (1500*g*) at 4 °C for 10 min using an IEC Microlite RF Refrigerated Microcentrifuge (Thermo Electron Corporation). The resultant packed RBCs and plasma from the rats were immediately frozen at −80 °C and kept on dry ice during shipping.

Quantification of Arsenic Species Using HPLC-ICPMS. An Agilent 1100 series HPLC system, including a pump, degasser, autosampler, and LC column, was used for HPLC separation. Separation of arsenic species was performed using anion exchange chromatography (AEC) or size exclusion chromatography (SEC). With respect to AEC, an analytical column (Hamilton, PRP-X100, 150 mm × 4.1 mm, 5 μm particle size) with a PRP-X100 guard column cartridge (20 mm × 2.3 mm) was operated with a mobile phase consisting of 35 mM ammonium bicarbonate and 5% methanol (pH 8.5). The column was equilibrated with the mobile phase for at least 0.5 h at a flow rate of 0.8 mL/min. The injection volume was 50 μL. A step-flow gradient program was used: 0.8 mL/min for the initial 4 min and 1.4 mL/min for the subsequent 11 min. Regarding SEC, an analytical column (Phenomenex, BioSep-SEC-S 2000, 300 mm × 4.6 mm, 5 μm particle size) was used along with a guard column cartridge (GFC-2000, 4 mm × 3.0 mm). The mobile phase was 10 mM ammonium bicarbonate (pH 7.55), and its flow rate was 1.0 mL/min. The sample injection volume was 20 μL. The protein-bound arsenicals were separated from the unbound arsenicals. The effluent from the HPLC column was directly introduced into the ICPMS (7500ce octopole reaction system, Agilent Technologies, Japan) using PEEK tubing.

The ICPMS was operated at a radio frequency power of 1550 W, and the argon carrier gas flow rate was 0.9−1.0 L/min. The octopole reaction system was operated with helium mode at a helium flow rate of 3.5 mL/min in the octopole reaction cell to reduce isobaric and polyatomic interferences. Arsenic was monitored at m/z 75. Chromatograms from HPLC separation and ICPMS detection were recorded using ChemStation (Agilent Technologies, Santa Clara, California).

Determination of $MMMTA^{V}$ and $DMMTA^{V}$ Using HPLC-ESI MS/MS. Initially for optimization of the operating conditions, 1−5 μM $MMMTA^{V}$ or $DMMTA^{V}$ in the solution of methanol and water (1:1) was infused into a triple-quadrupole mass spectrometer (ABI5000, AB Sciex, Concord, ON, Canada) equipped with an electrospray ionization source (ESI). The ESI MS/MS was operated in negative mode. Three characteristic MRM transitions of 155→107, 155→121, and 155→137 were used to detect $MMMTA^{V}$, and two MRM transitions of 153→123 and 153→138 were used to detect $DMMTA^{V}$. The optimal parameters were ionspray voltage (IS), −4500 V; temperature, 200 °C; and curtain gas, 10 L/min. For the detection of $DMMTA^{V}$, the declustering potential (DP) was −60 V. Collision energy (CE) was −28 V for the 153→123 transition and −20 V for the 153→138 transition. The cell exit potentials (CXP) were −11 V for the 153→123 transition and −13 V for the 153→138 transition. For the detection of $MMMTA^{V}$, the declustering potential (DP) was −75 V. Collision energies (CE) for three MRM transitions of 155→107, 155→121, and 155→137 were −34 V, −22 V, and −22 V, respectively. The CXPs were −13 V, −11 V, and −11 V, respectively, for the three MRM transitions.

An 1100 series HPLC system (Agilent, Santa Clara, CA) was used for the separation of arsenicals prior to ESI MS/MS detection. The HPLC system was equipped with a quaternary pump, degasser, column heater, temperature controlled autosampler, and a column (Hamilton PRP-X100, 50 mm × 4.6 mm, 10 μm). Five millimolar ammonium formate (pH 6.0) and 2 mM ammonium bicarbonate (pH 9.0) were used as aqueous mobile phases for the analysis of $MMMTA^{V}$ and $DMMTA^{V}$, respectively. Different mobile phases were selected for $MMMTA^{V}$ and $DMMTA^{V}$ in order for their retention times to be in an elution window free from matrix interference. The mobile phase was run at a flow rate of 1 mL/min. Following the separation, the effluent was split down to 100 μL/min. The 100 μL/min effluent flow was mixed with 100 μL/min of methanol containing 0.6% NH_4OH. The resulting 200 μL/min flow was introduced to the ESI MS/MS. Samples were placed on a temperature controlled (4 °C) autosampler, and the injection volume was 50 μL.

Pretreatment of Plasma for Arsenic Speciation Analysis. Plasma samples were thawed at room temperature and thoroughly vortex mixed. Twenty microliters of rat plasma was directly injected onto the SEC column for the separation of protein-bound and

unbound arsenicals. The chromatographic fractions containing protein-bound arsenicals were collected. A 100-μL fraction was mixed with 20 μL of hydrogen peroxide (30%) to release protein-bound arsenicals by cleaving the bonds between As and proteins. The solution was thoroughly vortex mixed and kept at room temperature overnight. Analysis using the same SEC column confirmed that bound arsenicals were thoroughly released from plasma proteins after this procedure. Quantification of arsenicals released from plasma proteins was performed on AEC coupled with ICPMS.

For the analysis of unbound arsenicals, plasma samples (180 μL) were mixed with 20 μL of perchloric acid (1 M) in a 1.5-mL vial for deproteinization, aiming to eliminate the interference of plasma proteins on arsenic analysis. The solution was then centrifuged at 15000 rpm for 10 min. After centrifugation, 100 μL aliquots of the supernatant were diluted appropriately with deionized water, and 50 μL of the resulting solutions was injected onto the AEC column for speciation analysis.

Pretreatment of RBCs for Arsenic Speciation Analysis. Approximately 20 mg of RBCs was lysed in 200 μL of deionized water plus 5 μL of ammonia (5%). Eight hundred microliters of the SEC mobile phase was added to the mixed solution followed by centrifugation at 12000 rpm (or 13400g) at 4 °C for 10 min. After the necessary dilution of the supernatants (lysates), an aliquot of 20 μL was injected onto the SEC column for the separation of protein-bound arsenicals.

The lysate from RBCs was further treated to release arsenic from hemoglobin in RBCs. An RBC lysate (100 μL aliquot) was mixed with 50 μL of hydrogen peroxide (30%) and 150 μL of the SEC mobile phase. The mixture was kept at room temperature overnight. Following appropriate dilution, an aliquot of 20 μL was injected onto the AEC column for arsenic analysis. The concentrations of DMA^V, iAs^V, MMA^V, and $TMAO^V$ represent the sum of the unbound DMA^V, iAs^V, MMA^V, and $TMAO^V$ and the DMA^{III}, iAs^{III}, and MMA^{III} that were oxidized and released from the proteins. Red blood cells were lysed and the lysates were treated with 10% H_2O_2. The trivalent arsenicals bound to the proteins (e.g., hemoglobin) in red blood cells were oxidized by H_2O_2 and released to the solution as the pentavalent arsenicals. Therefore, the solution contained the unbound DMA^V, iAs^V, MMA^V, and $TMAO^V$ species as well as any DMA^V, iAs^V, and MMA^V that might have been released from hemoglobin as a result of the oxidation of the bound DMA^{III}, iAs^{III}, and MMA^{III}. Note that the concentration of DMA^V is in microgram (μg) arsenic per gram (g) of red blood cells (wet weight), whereas the concentrations of iAs^V, MMA^V, and $TMAO^V$ are in nanogram (ng) arsenic per gram (g) of red blood cells (wet weight).

RESULTS

Arsenic Species in the Plasma. Figure 1 shows results from the analysis of seven arsenic species, including iAs^{III}, iAs^V, MMA^V, DMA^V, $TMAO^V$, $MMMTA^V$, and $DMMTA^V$, by using anion exchange chromatography with ICPMS detection. Comparisons of retention times between the seven authentic arsenic compounds (Figure 1A) and a plasma sample suggest the presence of iAs^{III}, iAs^V, MMA^V, DMA^V, $TMAO^V$, $MMMTA^V$, and $DMMTA^V$ species in the rat plasma sample (Figure 1B).

Because the presence of $MMMTA^V$ and $DMMTA^V$ in rat plasma was revealed for the first time, we conducted a number of additional experiments to confirm the identity of these new arsenic species. Figure S1 (Supporting Information) shows results from HPLC-ICPMS analyses of a rat plasma sample and the same sample spiked with authentic $MMMTA^V$ and $DMMTA^V$ standards. The identical retention times between the spiked standard arsenic species and the species of interest in the sample rule out the matrix effect and further support the identity of these two new arsenic species in rat plasma.

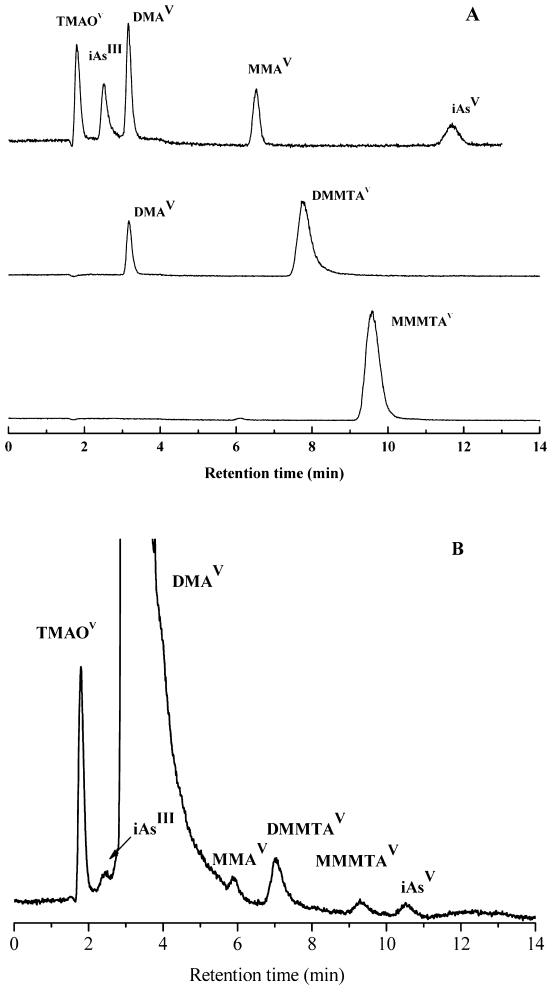

Figure 1. Representative anion exchange chromatograms of arsenic standards (A) and a rat plasma sample pretreated with 0.1 M perchloric acid (B). Separation of the arsenic species was achieved using anion exchange chromatography (Hamilton PRP-X100, 150 × 4.1 mm, 5 μm particle size). The mobile phase consisted of 35 mM ammonium bicarbonate and 5% methanol (pH 8.5) with a step-flow gradient of 0.8 mL/min for the initial 4 min and 1.4 mL/min for the subsequent 11 min. Injection volume was 50 μL. Arsenic was detected at m/z 75 using an Agilent 7500ce ICPMS.

Because the ESI MS/MS technique can provide useful molecular information of arsenic species, we further analyzed the plasma samples using HPLC-ESI MS/MS to confirm the identities of $MMMTA^V$ and $DMMTA^V$ in the rat plasma. Characteristic MRM transitions of 155→107, 155→121, and 155→137 were selected for $MMMTA^V$, and 153→123 and 153→138 for $DMMTA^V$. Different mobile phases chosen for the separation of $MMMTA^V$ and $DMMTA^V$ aimed to eliminate the matrix interferences from the plasma. Figure 2 shows results from the analyses of a plasma sample using anion exchange chromatography separation with ESI MS/MS detection of specific $MMMTA^V$ and $DMMTA^V$ transitions. The selective monitoring of $MMMTA^V$ and $DMMTA^V$ using multiple ion transitions further supports the presence of $MMMTA^V$ and $DMMTA^V$ in the rat plasma.

Table 1 summarizes the concentrations of arsenicals in the rat plasma from 24 rats that were given 0, 1, 10, 25, 50, and 100 ppm (μg/g) iAs^{III} in the diet. DMA^V was present as the major metabolite in rat plasma, and the other arsenicals, including

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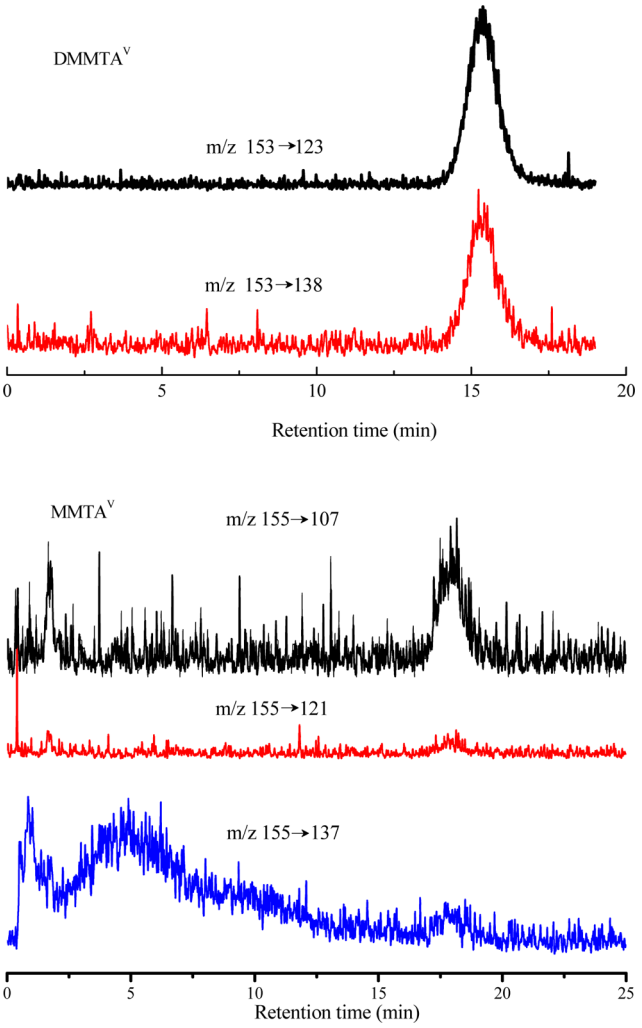

Figure 2. Anion exchange chromatograms from the ESI MS/MS analyses of rat plasma, showing the presence of $DMMTA^V$ and $MMMTA^V$. Separation of arsenic species was performed using an anion exchange chromatography column (Hamilton PRP-X100, 50 × 4.6 mm, 10 μm). The mobile phase for the analysis of $MMMTA^V$ was 5 mM ammonium formate (pH 6.0) and for the analysis of $DMMTA^V$ was 2 mM ammonium bicarbonate (pH 9.0). Optimized MRM transitions were used for the detection of $MMMTA^V$ (155→107, 155→121, and 155→137) and $DMMTA^V$ (153→123 and 153→138).

iAs^V, MMA^V, $TMAO^V$, $MMMTA^V$, and $DMMTA^V$, were detected as minor species. iAs^{III} was only detected in the plasma of rats treated with the highest dose (100 ppm). iAs^V, DMA^V, $DMMTA^V$, and $TMAO^V$ were detectable in the rat plasma of all exposed groups, while $MMMTA^V$ and MMA^V were only detected in the plasma of rats administered higher doses of iAs^{III} (50−100 ppm). In general, the concentrations of arsenicals in the rat plasma increased with the dosage of iAs^{III} administered to the rats (Figure 3). One-way ANOVA showed statistical significance in arsenic concentrations between the iAs^{III}-administered rats and the control rats. One-way ANOVA analyses comparing concentrations of individual arsenic species in rat plasma between the nearest adjacent dose groups showed statistical significance in a few cases, e.g., concentrations of $TMAO^V$ between the 1 ppm and the 10 ppm groups ($p < 0.01$) and between the 10 ppm and the 25 ppm groups ($p < 0.01$) (Figure 3).

Arsenicals bound to rat plasma protein were successfully separated from the unbound arsenicals in the rat plasma using SEC. ^{75}As and ^{34}S were simultaneously monitored using ICPMS to locate the retention times of the protein (by monitoring ^{34}S) and arsenic (by monitoring ^{75}As). Although in principle ^{34}S can be interfered with by polyatomic O_2, the use of helium gas in the collision cell of the octopole ICPMS system in the present study reduced this isobaric interference. A typical SEC chromatogram is shown in Figure S2A (in Supporting Information), in which the first peak overlaying the ^{75}As and ^{34}S signals corresponds to arsenicals that are associated with proteins.

Plasma samples from 10 rats across all six treatment groups were analyzed for quantitative concentrations of protein-bound arsenicals. The percentage of protein-bound arsenicals over the total arsenic declined with increasing dosages of iAs^{III} administration (Table 2). In the control and low-dose groups, more than 80% of the total arsenic was bound to the proteins in rat plasma. However, only half of the total arsenic existed in the protein-bound form in plasma samples from the high-dose (50 and 100 ppm) groups of rats.

The SEC fractions of protein-bound arsenicals were collected for further analyses. Figure S2B (Supporting Information) shows a typical SEC chromatogram from the reanalysis of such an SEC fraction (the early eluting peak). It shows that most of the unbound arsenicals (the later eluting peak) are removed and that the protein-bound arsenic fraction is purified. The purified protein-bound arsenic fraction from the first SEC was then treated with hydrogen peroxide (H_2O_2, 10%) to release arsenicals from plasma proteins, based on the experience that H_2O_2 at this concentration (10%) only changed the oxidation state but not the methylation status of arsenic.[28] Figure S2C (Supporting Information) shows that following H_2O_2 treatment, the peak of protein-bound arsenic disappeared and that the unbound (free) arsenic peak increased correspondingly. These results indicate that the protein-bound arsenic was completely released by the treatment with 10% H_2O_2. Under

Table 1. Concentrations of Arsenicals in Rat Plasma (ng/mL)[a]

treatment dosage of iAs^{III} (n)	iAs^{III}	iAs^V	MMA^V	DMA^V	$MMMTA^V$	$DMMTA^V$	$TMAO^V$
control (3)	N.D.	0.61 ± 0.59	N.D.	2.96 ± 1.34	N.D.	N.D.	0.95 ± 0.10
1 ppm (3)	N.D.	0.46 ± 0.33	N.D.	25.6 ± 4.4	N.D.	0.21 ± 0.27	1.23 ± 0.09
10 ppm (5)	N.D.	2.54 ± 1.86	N.D.	260 ± 193	N.D.	7.15 ± 4.75	3.12 ± 0.72
25 ppm (4)	N.D.	2.12 ± 1.60	N.D.	327 ± 134	1.90 ± 2.11	9.97 ± 5.62	9.20 ± 2.91
50 ppm (5)	N.D.	2.96 ± 1.37	4.15 ± 1.76	449 ± 113	4.61 ± 1.89	18.5 ± 7.9	25.8 ± 15.3
100 ppm (4)	2.95 ± 0.88	3.60 ± 2.26	7.72 ± 4.24	667 ± 115	11.4 ± 8.3	52.5 ± 39.9	26.2 ± 14.5

[a]The concentration unit is ng arsenic per mL plasma. n indicates the number of rats in each dosage group. In total, plasma samples were collected from 24 rats for arsenic speciation analysis. The concentration values represent the mean ± one standard deviation from triplicate analyses of 3−5 rat samples in each group (i.e., 9−15 analyses). N.D.: not detectable (below the detection limit of 0.1 ng/mL).

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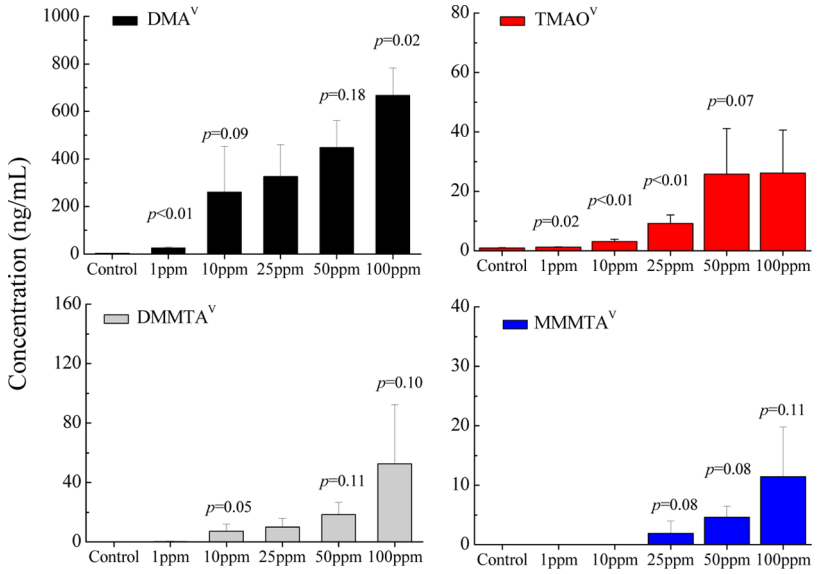

Figure 3. Concentrations of DMA^V, $TMAO^V$, $DMMTA^V$, and $MMMTA^V$ in rat plasma samples from the control rats and the rats administered 1, 10, 25, 50, and 100 ppm iAs^{III} in the diet. The same HPLC-ICPMS conditions as shown in Figure 1 were used. Error bars represent one standard deviation from triplicate analyses of plasma samples from 3 to 5 rats in each group. The *p* values were calculated using one-way ANOVA with mean comparison of Bonferroni using OriginPro 8.0 software (OriginLab Corporation). The *p* values shown were from one-way ANOVA analyses comparing concentrations of individual arsenic species in rat plasma between the nearest adjacent dose groups. For example, the *p* value from one-way ANOVA analyses comparing concentrations of DMA^V in the control and in the 1 ppm treated rats was <0.01, and it was 0.09 when comparing the 1 ppm and the 10 ppm treatment groups.

Table 2. Summary of Protein-Bound Arsenicals in Rat Plasma

treatment dosage of iAs^{III}	sample i.d.	bound arsenic[a] (ng/mL)	total arsenic[b] (ng/mL)	percentage of bound arsenic (%)	MMA^V after H_2O_2 treatment[c] (%)	DMA^V after H_2O_2 treatment[c] (%)
control	s34	3.6	4.4	80.5		
1 ppm	s42	65.6	66.9	96.7		
10 ppm	s55	175	209	82.7		
25 ppm	s61	678	814	82.2		
25 ppm	s63	321	476	66.5		
50 ppm	s71	812	1030	78.0	2.7	97.3
50 ppm	s73	372	622	59.0	5.9	94.1
50 ppm	s75	222	501	43.7	7.6	92.4
100 ppm	s81	397	740	52.9	14.3	85.7
100 ppm	s82	463	899	50.8	6.7	93.3

[a]The concentration of the protein-bound arsenic was determined by using SEC separation with ICPMS detection of As. There was no treatment of the plasma samples prior to analyses. [b]The total arsenic concentrations represent the sum of concentrations of all arsenic species determined by using anion exchange chromatography (AEC) separation with ICPMS detection. [c]MMA^V and DMA^V concentrations represent MMA^{III} and DMA^{III} that were oxidized and released from the proteins. Plasma samples were subjected to SEC separation, and the protein fraction was collected. H_2O_2 was added to the collected protein fraction to release trivalent arsenicals from the proteins by oxidizing the trivalent arsenicals to the pentavalent arsenicals. The H_2O_2-treated fractions were subjected to arsenic speciation analyses using anion exchange chromatography separation with ICPMS detection. MMA^V and DMA^V were the main arsenic species present in these treated fractions.

the size exclusion chromatographic conditions, only the molecules with sufficiently large size differences can be separated into two fractions, e.g., separating the protein-bound from the much smaller unbound arsenic species. Within the fraction of the unbound (free) arsenic species, individual arsenic species cannot be separated from one another because their size differences are too small to be distinguishable by SEC. However, these arsenic species can be determined using anion exchange chromatography with ICPMS detection.

Table 2 summarizes the results of protein-bound arsenic concentrations and released unbound arsenic concentrations. The protein-bound arsenic concentrations were obtained from SEC-ICPMS analyses of plasma samples from 10 rats across all six treatment groups. The percentage of protein-bound arsenicals over the total arsenic declined with increasing doses of iAs^{III} administration (Table 2). In the control and low-dose groups, more than 80% of the total arsenic was bound to the proteins in rat plasma. However, only half of the total arsenic existed in the protein-bound form in plasma samples from the high-dose (50 and 100 ppm) groups of rats.

The SEC fractions of protein-bound arsenicals were collected and treated with 10% H_2O_2. These were performed for five plasma samples originating from rats exposed to 50 and 100 ppm of iAs^{III}. These treated SEC fractions were analyzed for arsenic species using anion exchange chromatography separation with ICPMS detection. Figure 4 shows representative chromatograms from the analyses of a sample and a solution containing a mixture of arsenic standards. Only DMA^V and

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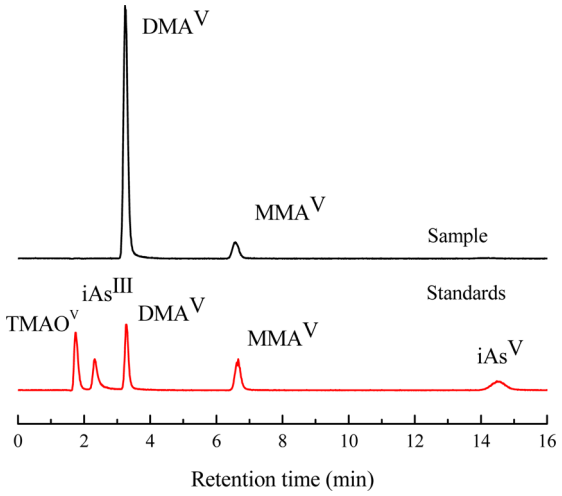

Figure 4. Representative anion exchange chromatograms from the analyses of the protein fraction of a plasma sample and a mixture of arsenic standards. The protein fraction was collected from size exclusion chromatography, and it was then treated with H_2O_2 before HPLC-ICPMS analysis.

MMA^V are detectable in the sample. Table 2 shows that DMA^V accounts for more than 90% and that MMA^V makes up less than 10% of the total arsenic released from the SEC fraction of all five rat plasma samples. Because these samples were the protein-bound fractions collected from SEC and because the collected fractions were treated with H_2O_2, which released trivalent arsenicals from the proteins by oxidizing the trivalent arsenicals to the pentavalent arsenicals, the detected DMA^V and MMA^V represent the protein-bound DMA^{III} and MMA^{III} that were oxidized and released from the proteins. These results suggest that DMA^{III} and MMA^{III} were the major arsenic species originally bound to the rat plasma proteins.

Arsenic Species in the Red Blood Cells. Figure S3A (in Supporting Information) shows typical SEC-ICPMS chromatograms from the analysis of a lysate of red blood cells (lysed with aqueous ammonia solution). Because the major protein in red blood cells is hemoglobin and because hemoglobin contains iron, detection of ^{57}Fe can serve as a retention time marker for hemoglobin. Figure S3A (Supporting Information) shows that the major peak in the ^{75}As chromatogram (top trace) overlays with the peak of ^{57}Fe (bottom trace), suggesting that the majority of arsenic in the red blood cell sample is bound to hemoglobin. A small peak in the ^{75}As chromatogram corresponds to the unbound arsenic species. The concentrations of the protein-bound arsenic in the red blood cells show a good dose-dependent relationship with the concentrations of iAs^{III} administered to the rats (first two columns in Table 3).

Figure S3B (in Supporting Information) shows the results from the analyses of the lysate pretreated with 10% H_2O_2. A single peak in the ^{75}As chromatogram (top trace) corresponds to the unbound arsenicals (small molecule fraction). There is no detectable ^{75}As peak corresponding to the hemoglobin peak in the ^{57}Fe chromatogram. These results indicate again that arsenicals are efficiently released from the proteins by the H_2O_2 treatment.

Figure 5 shows representative chromatograms from the analyses of a sample and a solution containing a mixture of arsenic standards. Red blood cells were lysed, and the lysates were treated with 10% H_2O_2. The separation of individual arsenic species were carried out using anion exchange chromatography. The trivalent arsenicals bound to the proteins (e.g., hemoglobin) in red blood cells were oxidized by H_2O_2 and released to the solution as the pentavalent arsenicals. As a result, only pentavalent arsenic species are detected in the H_2O_2-treated lysates of red blood cells. Among the arsenic species detected, DMA^V is the major arsenic species. The detected DMA^V is the sum of the unbound DMA^V and the hemoglobin-bound DMA^{III} that was released and oxidized by the H_2O_2 treatment. Three other smaller peaks detected in the H_2O_2-treated red blood cells sample correspond to TMAO^V, MMA^V, and iAs^V. Trimethylarsine (TMA^{III}) and trimethylarsine oxide (TMAO^V) are not known to bind to proteins. The presence of TMAO^V species in the red blood cells sample is probably due to the unbound TMAO^V in the sample. MMA^V and iAs^V in the H_2O_2-treated red blood cells sample are likely due to the combination of the unbound MMA^V and iAs^V and the hemoglobin-bound MMA^{III} and iAs^{III} that were released and oxidized by the H_2O_2 treatment.

Table 3 summarizes the concentrations of DMA^V, iAs^V, MMA^V, and TMAO^V in the lysed and H_2O_2-treated red blood cell samples. The samples were not fractionated using SEC. Different from the analysis of plasma samples (Table 2) where

Table 3. Concentrations of the Protein-Bound Arsenicals in the Red Blood Cells (μg/g) and the Arsenic Species in the Lysates of Red Blood Cells after Treatment of the Lysates with H_2O_2[a]

treatment dosage of iAs^{III} (n)	bound arsenic (μg/g)	DMA^V (μg/g)	iAs^V (ng/g)	MMA^V (ng/g)	TMAO^V (ng/g)
control (3)	10 ± 1	8.1 ± 0.8	212 ± 92	49 ± 8	N.D.
1 ppm (3)	64 ± 2	59 ± 1	180 ± 47	227 ± 34	14 ± 2
10 ppm (5)	378 ± 18	324 ± 58	187 ± 102	969 ± 156	64 ± 12
25 ppm (4)	370 ± 105	333 ± 99	212 ± 52	980 ± 325	87 ± 38
50 ppm (5)	440 ± 22	420 ± 28	392 ± 80	1050 ± 150	112 ± 14
100 ppm (4)	417 ± 28	427 ± 27	515 ± 112	1260 ± 100	100 ± 12

[a] n indicates the number of samples. In total, red blood cell samples were collected from 24 rats of six treatment groups. The concentrations of the protein-bound arsenic were determined in lysed red blood cell samples by using SEC separation with ICPMS detection. The concentration unit is in microgram (μg) arsenic per gram (g) of red blood cells (wet weight). The concentrations of DMA^V, iAs^V, MMA^V, and TMAO^V represent the sum of the unbound pentavalent arsenicals and the protein-bound arsenicals that were oxidized and released from the proteins. Red blood cells were lysed, and the lysates were treated with H_2O_2. The arsenicals bound to the proteins in red blood cells were oxidized by H_2O_2 and released to the solution as the pentavalent arsenicals. Note that the concentration of DMA^V is in microgram (μg) arsenic per gram (g) of red blood cells (wet weight), whereas the concentrations of iAs^V, MMA^V, and TMAO^V are in nanogram (ng) arsenic per gram (g) of red blood cells (wet weight). N.D.: not detectable (below the detection limit of 5 ng/g). The concentration values represent the mean ± one standard deviation from triplicate analyses of 3–5 rat samples in each group (i.e., 9–15 analyses).

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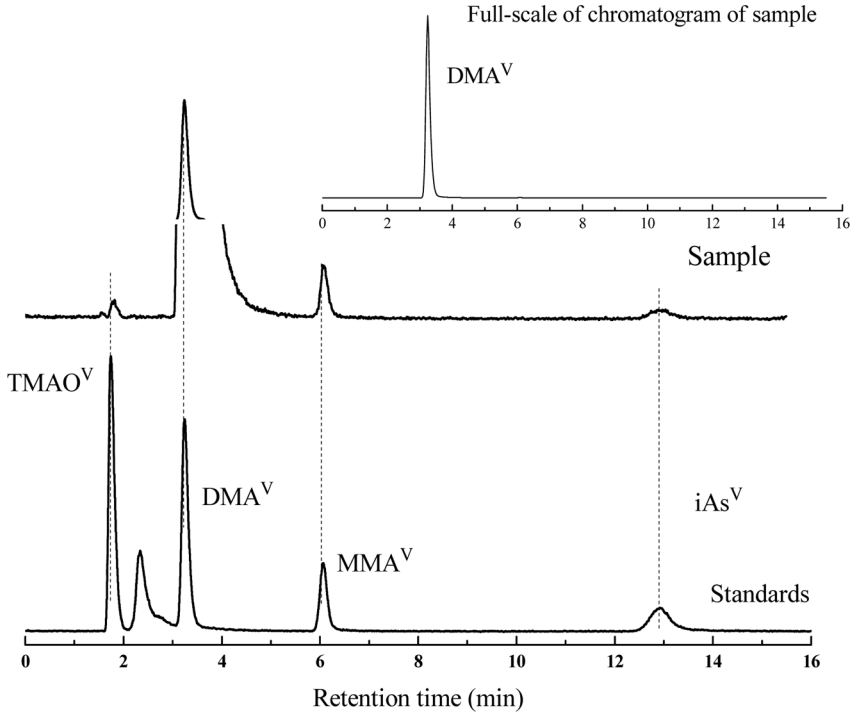

Figure 5. Representative anion exchange chromatograms from the analyses of a red blood cell lysate and a mixture of arsenic standards. The lysate of red blood cells was treated with H_2O_2 before HPLC-ICPMS analysis. The inset shows the full-scale chromatogram from the analysis of the lysate sample. The chromatogram with the DMA^V peak truncated is to show the presence of $TMAO^V$, MMA^V, and iAs^V that are present at much lower concentrations than DMA^V in the sample.

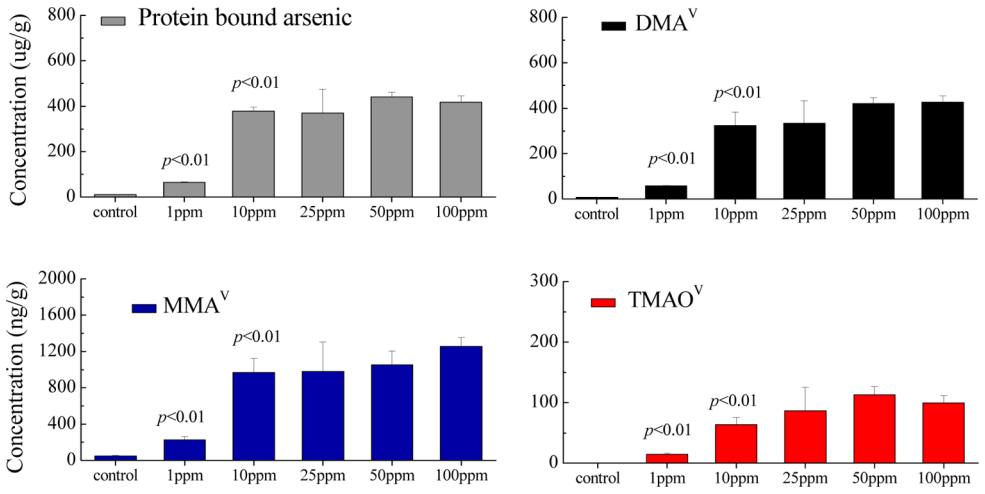

Figure 6. Concentrations of the protein-bound arsenic, DMA^V, MMA^V, and $TMAO^V$ in rat red blood cell samples from the control rats and the rats administered 1, 10, 25, 50, and 100 ppm iAs^{III} in the diet. The concentrations of the protein-bound arsenic in the lysates of red blood cells were determined using size exclusion chromatography separation with ICPMS detection. Concentrations of DMA^V, MMA^V, and $TMAO^V$ species in the H_2O_2-treated lysates were determined using anion exchange chromatography separation with ICPMS detection. Error bars represent one standard deviation from triplicate analyses of the samples from 3 to 5 rats in each group. The *p* values were from one-way ANOVA analyses comparing concentrations of arsenic species in samples between the nearest adjacent dose groups. For example, the *p* value from one-way ANOVA analyses comparing concentrations of protein-bound arsenic in the control and in the 1-ppm-treated rats was <0.01, and it was <0.01 when comparing the 1 ppm and the 10 ppm treatment groups.

DMA^V and MMA^V were released from the protein fraction only, the red blood cells were not fractionated, and the results of arsenic species in red blood cells (Table 3) represent oxidation products of both protein-bound and the unbound arsenic species, i.e., the sum of both the initially unbound pentavalent arsenic species and the protein-bound trivalent arsenic species. During the treatment with H_2O_2, the trivalent arsenicals that were initially bound to the proteins (e.g., hemoglobin) in red blood cells were oxidized by H_2O_2 and released to the solution as pentavalent arsenicals (Figure 6). Therefore, the H_2O_2-treated red blood cell samples contained the unbound DMA^V, iAs^V, MMA^V, and $TMAO^V$ species as well as any arsenic species that were released from hemoglobin as a result of the oxidation of the bound DMA^{III}, iAs^{III}, and MMA^{III}. Note that the concentration of DMA^V is in microgram (μg) of arsenic per gram (g) of red blood cells (wet weight), whereas

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the concentrations of iAs^V, MMA^V, and $TMAO^V$ are in nanogram (ng) of arsenic per gram (g) of red blood cells (wet weight). The concentrations of the dimethylarsenicals are at least 2 orders of magnitude higher than the inorganic, monomethyl, and trimethyl arsenicals. These results are consistent with previous findings that rat hemoglobin preferentially binds to DMA^{III}.

DISCUSSION

Arsenic occurs naturally in the environment.[1,30−32] An estimated 140 million people around the world are exposed to high concentrations of arsenic in drinking water (mainly groundwater).[1,2] Epidemiological data have shown that risks of skin, lung, and bladder cancers significantly increase in humans exposed to high concentrations of arsenic in drinking water.[1,3,4,33] The mechanism or mode of action of inorganic arsenic causing cancers in humans has not been definitively elucidated,[34,35] but evidence is accumulating to suggest that cytotoxicity and regenerative proliferation are involved.[36,37] The rat has been developed as an animal model for scientists to understand the toxicological effect of arsenic on humans.[7−9,12−14]

After being ingested, inorganic arsenic is easily absorbed and transported to the liver where it is transformed into methylated arsenic metabolites.[29] The rat liver methyltransferase As3MT (previously also known as Cyt19) was believed to catalyze the conversion of inorganic arsenic to methylated arsenicals, using glutathione, thioredoxin, and NADPH as reducing agents, and *S*-adenosylmethionine (SAM) as the methyl donor.[38−40] The information on arsenic speciation in the blood is useful for understanding arsenic disposition, metabolism, and toxicity.

In the present study, arsenite (iAs^{III}) was administered to rats in the diet. However, iAs^{III} was only detectable in the plasma of rats treated with iAs^{III} at the highest dose (100 ppm). Most of the iAs^{III} was biotransformed into methylated arsenicals, and a small portion was present as iAs^V (the oxidation product). In the RBCs, the concentration of inorganic arsenic was almost 70−400 times higher than that in the plasma. Methylated arsenic metabolites including MMA^V, DMA^V, and $TMAO^V$ were detected in both the plasma and RBCs from iAs^{III}-treated rats. Concentrations of methylated arsenic metabolites were much higher in the RBCs than in the plasma, which supports the notion that the RBCs serve as the arsenic storing reservoir in blood.[24,26] DMA^V was the predominant species both in plasma and RBCs. The methylation capacity of arsenic in rats is believed to be substantially higher than that in humans, and the synthesis of DMA^V in humans is inhibited by iAs^{III} in a concentration-dependent manner.[24,41]

Orally administered DMA^V has been demonstrated to induce tumors in the urothelial epithelium of the rat urinary bladder.[10,11,42] However, it is questionable whether DMA^V produced from endogenous metabolism of inorganic arsenic has the same metabolic fate as DMA^V exogenously applied.[21,43] In contrast to DMA^V, MMA^V and $TMAO^V$ were detectable at far lower concentrations in both the plasma and RBCs of iAs^{III}-treated rats. The concentration of MMA^V was lower than that of $TMAO^V$ in plasma, but the case was reversed in RBCs. MMA^V is generally not a primary metabolite in most mammals, except for humans in which significant amounts of MMA^V are present in urine.[21,44] MMA^V has not been shown to have any tumorigenic effects on the rat bladder or other tissues in previous studies.[45,46] Different from previous reports that $TMAO^V$ was undetectable in the blood of DMA^V- or iAs^V-treated rats,[43] our present study showed that $TMAO^V$ could be detected in both the plasma and RBCs of iAs^{III}-treated rats. $TMAO^V$ was identified as a possible carcinogen in rat liver.[47] It is possible that its formation in the rats contributes to carcinogenicity induced by inorganic arsenic.

Methylated thioarsenicals have also been identified as arsenic metabolites. Thio-DMA^V was shown to be present in the urine of sheep or humans exposed to arsenosugars.[48,49] $DMMTA^V$ and $DMDTA^V$ were also identified in the urine of hamsters or rats exposed to iAs^{III}[20,50] and $DMMTA^V$ and trimethylarsine sulfide ($TMAS^V$) in the urine of rats exposed to DMA^V or iAs^V.[43] Likewise, $DMMTA^V$ was also found to be a significant metabolite in the urine of women exposed to arsenic via drinking water in Bangladesh.[19] Our present study for the first time demonstrated the presence of thio-arsenicals ($DMMTA^V$ and $MMMTA^V$) in the plasma of the rats administered iAs^{III}. The finding of $MMMTA^V$ and $DMMTA^V$ in rat plasma is meaningful and may imply that urinary thio-arsenicals previously detected could originate from blood via renal elimination.

Cytotoxicity testing with various cell lines indicated that $MMMTA^V$ and $DMMTA^V$ were much more toxic than their oxygen-containing counterparts.[19,50−54] It was also observed [55] that $DMMTA^V$ was taken up and distributed in organs or tissues in a manner similar to that of DMA^{III}, whereas $DMDTA^V$ behaved in a manner more similar to that of DMA^V. Rapid cellular uptake of $DMMTA^V$ is followed by conversion to DMA^{III}, the most likely explanation for the cytotoxicity.[50] Formation of these thio-arsenicals in rats could produce more toxic species as the initiating stimuli in arsenic-induced urinary bladder cancer.[43]

Binding of arsenicals with blood proteins can be a critical issue in arsenic speciation in blood. It may alter arsenic metabolism and distribution, and consequently lead to changes in arsenic bioavailability and the kinetics of accumulation and excretion, as well as the function of proteins.[56] For example, transferrin in plasma can reportedly bind to inorganic arsenic instead of methylated arsenic.[57,58] A ternary dimethylarsinous-hemoglobin−haptoglobin complex was also detected in rat plasma.[59] In the present study, DMA^V as the major species and MMA^V as the minor species were released from plasma proteins upon treatment with H_2O_2. Previous studies have demonstrated that MMA^{III} can bind to some plasma proteins, e.g., thioredoxin[60] and metallothionein.[27] However, MMA^{III}-bound proteomes in the plasma have not been studied. Moreover, 44−97% of the total arsenic in rat plasma was found to bind to proteins, varying with the dosage of iAs^{III}. High doses of iAs^{III} may lead to the saturation of the binding capacity of plasma proteins to arsenicals. Individual variations within the same dose group possibly reflected the phenotypic polymorphism of plasma proteins in rats in addition to experimental variability. In the RBCs, most of the arsenic was bound to hemoglobin, the predominant protein in RBC. Rat hemoglobin contains a highly reactive Cys-13 in the α chain, serving as the major binding site responsible for long retention of arsenic in rat blood.[25]

In conclusion, arsenicals in the plasma and RBCs of the female F344 rat treated with iAs^{III} have been speciated using HPLC-ICPMS and HPLC-ESI MS/MS. The concentrations of arsenic species were dosage-dependent in both plasma and RBCs. Two thiol-arsenicals, $DMMTA^V$ and $MMMTA^V$, were detected in rat blood for the first time. Protein−DMA^{III} complexes were implicated as the predominant arsenic species

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in both the plasma and RBCs of rats, in accordance with previous findings.[24,25] Further targeted proteomic studies identifying the specific blood proteins that may bind to arsenic[61,62] could improve the understanding of arsenic distribution, metabolism, and elimination.

■ ASSOCIATED CONTENT

Supporting Information

Representative anion exchange chromatograms from the ICPMS analyses of a rat plasma sample and the sample spiked with $MMMTA^V$ and $DMMA^V$ standards; size exclusion chromatograms from the ICPMS analyses of a rat plasma sample, SEC fraction, and the H_2O_2-treated SEC fraction; and representative size exclusion chromatograms from the analyses of the lysate of red blood cells from a rat before and after H_2O_2-treatment of the lysate. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

As, arsenic; AEC, anion exchange chromatography; As3MT, arsenic (+3 oxidation state) methyltransferase; CRM, certified reference material; DMA^V, dimethylarsinic acid; DMA^{III}, dimethylarsinous acid; $DMDTA^V$, dimethyldithioarsinic acid; $DMMTA^V$, dimethylmonothioarsinic acid; ESI MS/MS, electrospray ionization tandem mass spectrometry; Hb, hemoglobin; HPLC, high performance liquid chromatography; ICPMS, inductively coupled plasma mass spectrometry; iAs^{III}, inorganic arsenite; iAs^V, inorganic arsenate; MMA^V, monomethylarsonic acid; MMA^{III}, monomethylarsonous acid; $MMMTA^V$, monomethylmonothioarsonic acid; MRM, multiple reaction monitoring; RBC, red blood cell; SAM, *S*-adenosylmethionine; SEC, size exclusion chromatography; TMA^{III}, trimethylarsine; $TMAO^V$, trimethylarsine oxide; $TMAS^V$, trimethylarsine sulfide

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