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Determination of Arsenic Metabolic Complex Excreted in Human Urine after Administration of Sodium 2,3-Dimercapto-1-propane Sulfonate

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Sodium 2,3-dimercapto-1-propane sulfonate (DMPS) has been used to treat acute arsenic poisoning. Presumably DMPS functions by chelating some arsenic species to increase the excretion of arsenic from the body. However, the excreted complex of DMPS with arsenic has not been detected. Here we describe a DMPS complex with monomethylarsonous acid (MMA^{III}), a key trivalent arsenic in the arsenic methylation process, and show the presence of the DMPS−MMA^{III} complex in human urine after the administration of DMPS. The DMPS−MMA^{III} complex was characterized using electrospray tandem mass spectrometry and determined by using HPLC separation with hydride generation atomic fluorescence detection (HGAFD). The DMPS−MMA^{III} complex did not form a volatile hydride with borohydride treatment. On-line digestion with 0.1 M sodium hydroxide following HPLC separation decomposed the DMPS−MMA^{III} complex and allowed for the subsequent quantification by hydride generation atomic fluorescence. Arsenite (As^{III}), arsenate (As^{V}), monomethylarsonic acid (MMA^{V}), dimethylarsinic acid (DMA^{V}), MMA^{III}, and DMPS−MMA^{III} complex were analyzed in urine samples from human subjects collected after the ingestion of 300 mg of DMPS. The administration of DMPS resulted in a decrease of the DMA^{V} concentration and an increase of the MMA^{V} concentration excreted in the urine, confirming the previous results. The finding of the DMPS−MMA^{III} complex in human urine after DMPS treatment provides an explanation for the inhibition of arsenic methylation by DMPS. Because MMA^{III} is the substrate for the biomethylation of arsenic from MMA^{V} to DMA^{V}, the formation of DMPS−MMA^{III} complex would reduce the availability of MMA^{III} for the subsequent biomethylation.

Introduction

Human exposure to high levels of inorganic arsenic from drinking water has been associated with adverse health effects including cancer of skin, lung, bladder, and other noncancerous effects (*1*−*6*). The mechanism(s) of action responsible for arsenic toxicity remain(s) to be elucidated. There has been much effort directed toward obtaining a clearer understanding of arsenic metabolism and toxicity. Inorganic arsenic species undergo biomethylation in the human body, forming monomethylated and dimethylated arsenic species that are primarily excreted into the urine (*1*−*3, 7*−*10*). Biomethylation of arsenic has previously been regarded as a detoxification process (*11, 12*). Until recently, studies of arsenic metabolism largely relied on the analysis of pentavalent arsenic metabolites, monomethylarsonic acid (MMA^{V}) and dimethylarsinic acid (DMA^{V}), mainly because of the availability of a convenient analytical methodology. However, recent studies have demonstrated the presence of trivalent arsenic methylation metabolites, monomethylarsonous acid (MMA^{III}) and dimethylarsinous acid (DMA^{III}), in human urine of individuals who were chronically exposed to high levels of arsenic in drinking water (*13*−*18*). These intermediate arsenic metabolites (MMA^{III} and DMA^{III}) are more toxic to test animals (*19*) and to a variety of human and animal cells (*20*−*25*) than the inorganic arsenic species. MMA^{III} and DMA^{III} are also more potent than inorganic arsenic as inhibitors of various enzymes, including glutathione reductase (*26*−*28*), thioredoxin reductase (*29, 30*), and pyruvate dehydrogenase (*19*). Thus, the trivalent arsenic species, whether in the inorganic or organic form, appear to be generally more toxic than the pentavalent forms of arsenic.

2,3-Dimercapto-1-propane sulfonate (DMPS) is commonly used to treat acute arsenic poisoning (*31*−*33*). It is assumed that DMPS reacts with the trivalent arsenic species to form new species that are more easily excreted from the body into urine. However, the postulated complexes have not yet been detected.

Furthermore, urine analysis indicates that DMPS also interferes with arsenic methylation, resulting in a relatively low concentration of DMA^{V} and a high concentration of MMA^{V} (*13, 34*) . Two possible explanations have been proposed. First, DMPS may inhibit methylation enzyme activities. Several enzymes that catalyze arsenic methylation have been purified both from rabbit liver

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cytosol (*23, 35*) and recently from rat liver cytosol (*36*). Second, the formation of a complex between DMPS and MMA^{III} may reduce the availability of the MMA^{III} as a substrate for further methylation. However, the fact that the expected complex has not been previously detected supports the hypothesis.

We report here the development of a technique that allows for the direct speciation analyses of a DMPS−MMA^{III} complex, as well as MMA^{V}, DMA^{V}, and inorganic arsenite (As^{III}) and arsenate (As^{V}). We further demonstrate the presence of this complex in human urine collected from subjects after the administration of 300 mg of DMPS. The finding of a DMPS−MMA^{III} complex provides direct evidence in support of the postulate that its formation both increases the mobilization of arsenic from the body and inhibits arsenic methylation.

Experimental Section

Instrumentation. The HPLC separation system consisted of a Gilson model 307 pump (Middleton, WI), a Rheodyne six-port sample injector (model 7725i) with a 20 μL sample loop, and an appropriate column, as previously described (*16*). A short anion exchange column (Phenomenex Sphereclone 30 × 4.60 mm, 5 μm particle size; Phenomenex, Torrance, CA) was used for the separation of MMA^{III}, MMA^{V}, and DMPS−MMA^{III} complex. The mobile phase contained 5 mM tetrabutylammonium hydroxide and 3 mM phosphate. The pH was 4.0, and the flow rate was 1.2 mL/min. A strong anion exchange analytical column (Phenosphere 250 × 4.60 mm, 5 μm particle size; Phenomenex) was used to separate all the common arsenic metabolite and the DMPS−MMA^{III} complex in urine. The mobile phase contained 20 mM phosphate and 5% methanol at pH 6.0. All the HPLC separations were carried out at room temperature.

A hydride generation atomic fluorescence detection (HGAFD) system was used to detect arsenic compounds eluted from the HPLC columns. The HGAFD was similar to that described previously (*37, 38*) except that an on-line decomposition was incorporated between the HPLC and the HGAFD. This modification allowed for the decomposition of the DMPS−MMA^{III} complex by using a 0.1 M NaOH solution. The effluent from the HPLC column was mixed at a T-joint with a continuous flow of 0.1 M NaOH (2 mL/min) and was allowed to flow through a 30 cm coil (0.8 mm i.d.) to improve the decomposition efficiency. The output was then mixed at two T-joints with hydrochloric acid [20% (v/v), 4 mL/min] and sodium borohydroxide [1.6% (w/v), 2 mL/min] solutions that were introduced by using a peristaltic pump. Volatile arsenic hydrides produced in the reaction mixture were separated from liquid waste in a gas/liquid separator and were carried by a continuous flow of argon to an atomic fluorescence detector (PSA 10.055 Millennium Excalibur system, PS Analytical, Kent, U.K.). A pentium computer with Varian (Victoria, Australia) Star Workstation software and ADC board was used to acquire and process signals from the atomic fluorescence detector. Chromatograms were plotted using IgorPro software (WaveMetrics, Lake Oswego, OR).

A hybrid quadrupole time-of-flight QSTAR Pulsar I mass spectrometer (Applied Biosystem/MDS Sciex, Concord, Ontario, Canada) was equipped with a Turboionsray ion source (Applied Biosystem/MDS Sciex). Analyte solutions were introduced into the ion source by using an integrated syringe pump with an electrospray voltage of −4500 V. Mass spectra were acquired by using a first declustering potential (DP1) of 65 V, a second declustering potential (DP2) of 15 V, and a focusing potential (FP) of 215 V. In the MS/MS mode, the parent ion was selected by the first quadrupole (Q1) with low mass resolution and fragmented in the second quadrupole (Q2) by collision-induced dissociation (CID): a collision energy of 50 eV and collision gas (nitrogen) at a setting of 8 (arbitrary unit). The resulting daughter ions were analyzed by using a TOF analyzer. Analyst QS software was used for the spectrum acquisition and data analysis. Igor Pro software (WaveMetrics) was used to plot the spectra.

Reagents. As^{III}, As^{V}, and DMA^{V} were obtained from Sigma (St. Louis, MO) as arsenic trioxide [As_2O_3], sodium arsenate [$As(O)OH(ONa)_2 \cdot 7H_2O$)], and sodium cacodylate [$(CH_3)_2As(O)ONa$], respectively. MMA^{V} was obtained from Chem Service (West Chester, PA) as sodium monomethylarsonate [$CH_3As(O)OHONa$]. The source of MMA^{III} was the solid oxide (CH_3AsO) and was synthesized as described previously (*20, 39*). Standard solutions of these arsenic compounds were prepared in deionized water.

DMPS was obtained from Aldrich (Milwaukee, WI). Deionized water was used to make dilute solutions. Tetrabutylammonium hydroxide and NaH_2PO_4 were obtained from Aldrich. HPLC grade methanol was from Fisher (Pittsburgh, PA). The HPLC mobile phases were prepared in deionized water and filtered through a 0.45 μm membrane prior to use. Sodium borohydroxide (Aldrich) solutions (1.6% w/v) in 0.1 M sodium hydroxide (Fisher) were prepared fresh daily. All reagents used were of analytical grade or better.

Urine Samples. Urine samples were collected from 58 individuals in Romania who were exposed to high levels of arsenic in water as described previously (*14*). Arsenic levels in their well water that was used for consumption ranged up to 161 μg/L. They were asked to refrain from seafood consumption for 3 days prior to and during the urine sample collection period. They were fasted overnight and then orally administered 300 mg of sodium 2,3-dimercapto-1-propane sulfonate (DMPS). A urine sample was collected from each participant before the administration of DMPS. Four other urine samples were collected 0−2, 2−4, 4−6, and 6−8 h after the administration of DMPS. The urine samples were kept frozen at −20 °C. The samples that were previously shown to contain MMA^{III} were chosen for further analysis for the DMPS−MMA^{III} complex. The samples were thawed at room temperature, and an aliquot (20 μL) was analyzed by using HPLC−HGAFD.

To demonstrate the presence of the DMPS−MMA^{III} complex in some of these urine samples, we reanalyzed seven urine samples that were previously found to contain MMA^{III} (*14, 16*). These samples had been collected 1−8 h after the single oral administration of 300 mg of DMPS. We were able to detect the DMPS−MMA^{III} complex in six of these samples. The concentration of the DMPS−MMA^{III} complex in these samples ranged from 1 to 4.2 μg/L. In the one remaining sample, neither MMA^{III} nor the DMPS−MMA^{III} complex was detected, probably because of conversions of these relatively unstable arsenic species during sample storage.

Complexation of DMPS with MMA^{III}. Prior to the analyses of the urine samples, several experiments were carried out to study the complexation of DMPS with MMA^{III}. Because MMA^{III} can readily form a volatile hydride (CH_3AsH_2) and the DMPS−MMA^{III} complex does not, the free MMA^{III} and the DMPS−MMA^{III} complex can be distinguished by using hydride generation without HPLC separation. A sample (200 μL) containing MMA^{III} and DMPS was directly analyzed by using HGAFS for the unreacted MMA^{III} concentration.

DMPS and MMA^{III} were added to solutions of different pH (pH 1.0, 3.0, 5.0, 7.0, 9.0, and 11.0) to study the effect of the formation and stability of the DMPS−MMA^{III} complex. The initial concentration of DMPS was 100 μM, and that of MMA^{III} was 1 μM. The solutions were vortexed and analyzed by FIA-HGAFD immediately. They were subsequently kept at room temperature and analyzed every 40−60 min for a period of 5 h. The analysis of the residual amount of MMA^{III} provided information on the formation of the DMPS−MMA^{III} complex under the various pH conditions. In addition, these samples were kept at room-temperature overnight, and reanalyzed to study the stability of the complex. Because the DMPS−MMA^{III} complex does not form a volatile complex and cannot be detected by HGAFD, the decrease of the response of MMA^{III} after

addition of DMPS represents the increase of DMPS−MMA^{III} complex formation.

Chromatographic Separation of the DMPS−MMA^{III} Complex. The chromatographic retention behavior of the DMPS−MMA^{III} complex was studied by using several columns and under various conditions. Initially an ion-pairing separation was attempted by using a reversed phase ODS-3 column (Phenomenex Prodigy, 150 × 4.60 mm, 3 μm particle size) and a mobile phase (pH 5.8) containing 5 mM tetrabutylammonium hydroxide, 3 mM malonic acid, and 5% methanol. While good separations of As^{III}, As^{V}, MMA^{V}, DMA^{V}, MMA^{III}, and DMA^{III} were achieved, the DMPS−MMA^{III} complex could not be eluted from the column. Although a strong alkaline solution might be able to elute the complex, the extreme pH conditions could damage this column. We then used a polymer-based strong anion exchange column (Hamilton PRP X-100, 150 × 4.60 mm, 3 μm particle size, Reno, NV), which allowed for a working pH range of 1−13. When 0.1 M NaOH was used as the mobile phase, the DMPS−MMA^{III} complex could be eluted from the column and subsequently detected by hydride generation atomic fluorescence. However, under these conditions, the DMPS−MMA^{III} complex could not be separated from the other common arsenic species. So speciating the DMPS−MMA^{III} complex in the presence of other arsenic species was not possible. When sodium phosphate (5−35 mM) was used as the mobile phase, the DMPS−MMA^{III} complex could not be eluted from the column. Finally, we found that a silica-based anion exchange column (Phenosphere 250 × 4.60 mm, 5 μm particle size, Phenomenex) was suitable for the separation of the DMPS−MMA^{III} complex from the other common arsenic metabolites present in urine. Consequently, determination of MMA^{III}, the DMPS−MMA^{III} complex, and other arsenic species in urine was carried out by using this strong anion exchange column. The mobile phase contained 20 mM phosphate and 5% methanol (pH 7.0), and the flow rate was 1.0 mL/min. The separation was carried out at room temperature. Detection limits for MMA^{III} and the DMPS−MMA^{III} complex were ∼1 μg/L. They were measured as arsenic concentrations corresponding to 3 times the standard deviation of background.

Results and Discussion

We first tested the reaction between DMPS and MMA^{III} under various pH conditions to examine the formation of the DMPS−MMA^{III} complex. Aqueous reaction mixtures (pH 1, 3, 5, 7, 9, and 11) containing 100 μM DMPS and 1 μM MMA^{III} were analyzed for arsenic at different incubation intervals by using hydride generation atomic fluorescence. Figure 1 shows the concentration of arsenic in the reaction mixtures analyzed every 40−60 min over a period of 5 h. The results clearly show the rapid decrease of MMA^{III} concentration in the solutions. The decrease of MMA^{III} concentration is an indication of the formation of DMPS−MMA^{III} complex. The DMPS−MMA^{III} complex does not form a volatile hydride and cannot be detected by hydride generation atomic fluorescence.

To confirm the formation of a complex between DMPS and MMA^{III}, we further characterized the reaction mixture using electrospray ionization tandem mass spectrometry. The negative ion mode was used, resulting in $(M-H)^-$ as typical molecular ion species. Figure 2 shows mass spectra obtained from aqueous solutions of MMA^{III}, DMPS, and the mixture of MMA^{III} and DMPS. The major ion detected in the MMA^{III} solution is *m*/*z* 122.9425 (Figure 2a), corresponding to $CH_3As(OH)O^-$. The molecular species of DMPS is detected under the negative ion mode at *m*/*z* 186.9445 (Figure 2b). The mixture of DMPS and MMA^{III} (Figure 2c) clearly shows the presence of

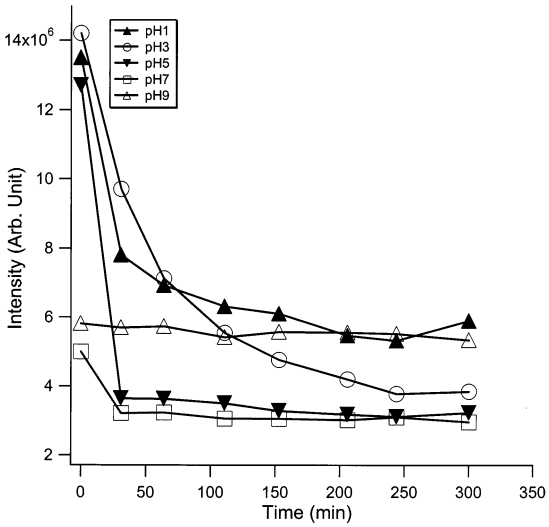

Figure 1. Analyses of MMA^{III} in solutions containing 1 μM MMA^{III} and 100 μM DMPS at various incubation intervals. The mixtures were prepared in 0.1 M NaH_2PO_4, and the pH was adjusted to 1.0, 3.0, 5.0, 7.0, or 9.0 with either 10% HNO_3 or 0.1 M NaOH. Flow injection with hydride generation atomic fluorescence was used for arsenic analysis.

DMPS−MMA^{III} complex (*m*/*z* 274.8633). A good match between the measured accurate mass and the theoretical mass of the DMPS−MMA^{III} complex (mass accuracy 80 ppm) confirms the identity of the DMPS−MMA^{III} complex. Furthermore, the tandem mass spectral analysis (Figure 2d) of the complex species (*m*/*z* 274.8633) provides additional supporting information corresponding to the characteristics of the DMPS−MMA^{III} complex.

Having confirmed the formation of the DMPS−MMA^{III} complex, we further developed a method for the analysis of trace levels of this complex that might be present in human urine. We initially examined whether the DMPS−MMA^{III} complex could be stable under various pH conditions. We found that under acidic, neutral, and weakly basic pH conditions, the complex does not form a volatile hydride upon treatment with sodium borohydride. Thus, the complex cannot be determined by using hydride generation. However, the complex is unstable at extreme alkaline conditions (pH >11). This is probably because the DMPS−MMA^{III} complex is hydrolyzed at the higher pH and the MMA^{III} is released and available for hydride generation. Therefore, we incorporated a postcolumn decomposition step with NaOH between the HPLC separation and hydride generation atomic fluorescence detection. Table 1 summarizes the decomposition efficiency using various concentrations of NaOH. A complete decomposition of the DMPS−MMA^{III} complex was achieved with 0.1−0.5 M NaOH.

Figure 3 shows chromatograms obtained from the analysis of a mixture of MMA^{III} and DMPS in an aqueous standard. The separation was carried out on a short strong anion exchange column (3 cm in length). MMA^{III}, MMA^{V}, and the DMPS−MMA^{III} complex are clearly resolved within 3 min. The DMPS−MMA^{III} complex was detected by hydride generation atomic fluorescence after a postcolumn decomposition with 0.1 M NaOH (trace a in Figure 3). Without the NaOH decomposition (trace b in Figure 3), the complex was not detected even though it was present in the sample. This HPLC separation followed by on-line decomposition with 0.1 M NaOH and hydride generation atomic fluorescence detection allowed

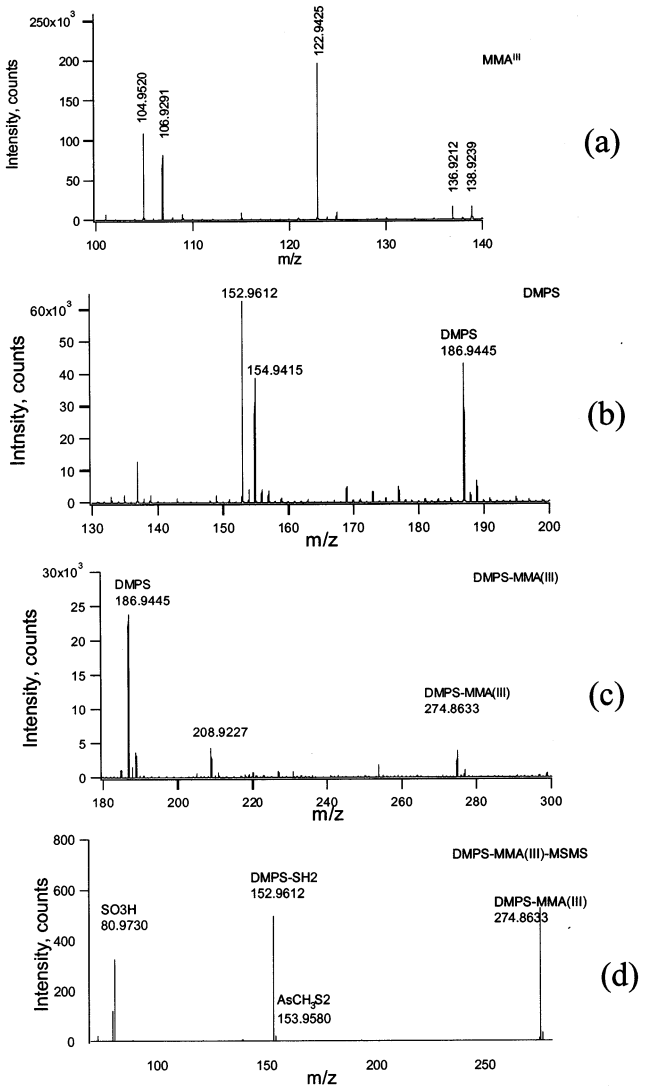

Figure 2. Typical mass spectra from the analysis of MMA^{III} (a), DMPS (b), and DMPS−MMA^{III} (c). The analyses were carried out using electrospray tandem mass spectrometry under the negative ion mode. The ion of *m*/*z* 274.8633 in (c) was selected to obtain the tandem mass spectrum shown in (d). Immediately prior to analysis, samples were diluted in methanol/water (50/50) and 0.5% ammonium hydroxide to facilitate electrospray ionization. Concentrations of MMA^{III} and DMPS were 100 μM.

Table 1. Effect of the Concentration of Sodium Hydroxide on the Decomposition Efficiency

	MMA^{III} (1 μM)	1 μM MMA^{III}+100 μM DMPS					
NaOH concn (M)		0	0.001	0.01	0.05	0.1	0.5
efficiency (%)	100	18.5	18.6	35.1	75.7	91.7	97.0

us to determine the free MMA^{III} and the DMPS−MMA^{III} complex in a single analysis.

To determine other arsenic metabolites that are usually present in urine, we further improved the HPLC separation by increasing the length of the separation column from 3 cm (Figure 3) to 25 cm (Figure 4) and optimizing the separation using the longer column. As a result of an increase in separation efficiency, we were able to resolve MMA^{III}, As^{III}, MMA^{V}, DMA^{V}, As^{V}, and the DMPS−MMA^{III} complex (Figure 4). Each analysis of the six arsenic species was complete within 14 min.

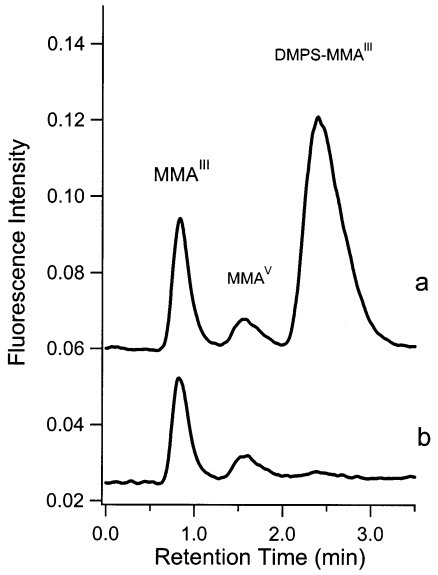

Figure 3. Typical chromatograms showing speciation analysis of MMA^{III}, MMA^{V}, and the DMPS−MMA^{III} complex. A Phenomenex Sphereclone strong anion exchange guard column (Phenomenex, 30 × 4.6 mm, 5 μm particle size) was used for separation with a mobile phase containing 5 mM tetrabutylammonium hydroxide and 3 mM phosphate (pH 4.0). The flow rate was 1.2 mL/min, and the separation was carried out at room temperature. Hydride generation atomic fluorescence was used for detection. For (a), 0.1 M NaOH was used to decompose the DMPS−MMA^{III} complex immediately after HPLC separation and before the hydride generation atomic fluorescence detection. For (b), the HPLC was directly connected to the hydride generation atomic fluorescence detector without using NaOH decomposition.

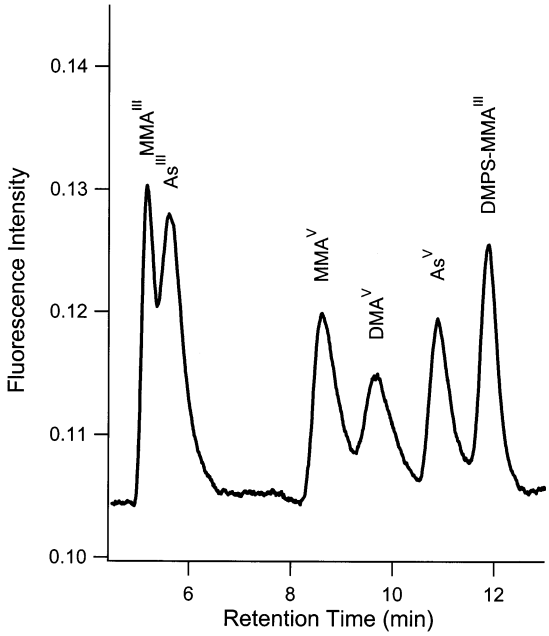

Figure 4. A chromatogram showing the HPLC−HGAFD analysis of MMA^{III}, As^{III}, MMA^{V}, DMA^{V}, As^{V}, and the DMPS−MMA^{III} complex. The HPLC separation was carried out by using a Phenomenex Phenosphere strong anion exchange column (Phenomenex, 250 × 4.6 mm, 5 μm particle size) with a mobile phase containing 20 mM phosphate and 5% methanol (pH 6.0). The flow rate was 0.8 mL/min, and the separation was carried out at room temperature. An on-line decomposition with 0.1 M NaOH was used between HPLC and HGAFD.

Having established a method for the analysis of arsenic species including the DMPS−MMA^{III} complex, we further

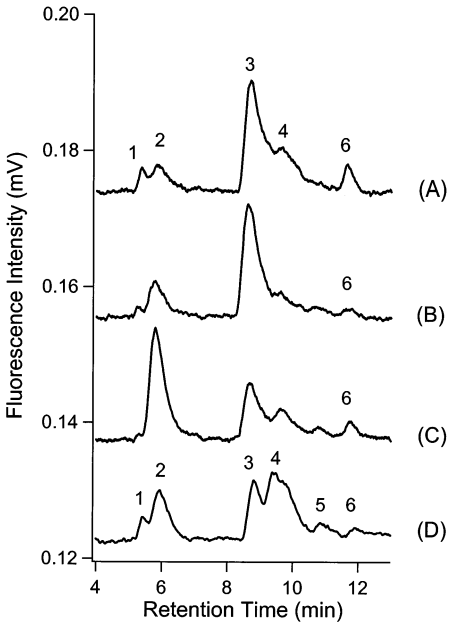

Figure 5. Typical chromatograms obtained from the analyses of selected urine samples collected from four individuals 2−8 h after a single oral administration of 300 mg of DMPS. The same HPLC/HGAFD conditions as shown in Figure 4 were used. The urine samples were from (A) subject 49 at 2 h, (B) subject 53 at 2 h, (C) subject 14 at 4 h, and (D) subject 50 at 8 h after the administration of DMPS. Peaks 1−6 represent MMA^{III}, As^{III}, MMA^{V}, DMA^{V}, As^{V}, and DMPS−MMA^{III} complex, respectively.

studied arsenic speciation in urine samples from subjects administered DMPS. Figure 5 shows typical chromatograms obtained from the analysis of urine samples collected 2−8 h after a single oral administration of 300 mg of DMPS. Both free MMA^{III} and the DMPS−MMA^{III} complex are clearly detected in these samples. Previously only MMA^{III} was identified (*14*) because no method was available for the determination of the DMPS−MMA^{III} complex. The present work, for the first time, demonstrates the presence of a DMPS−MMA^{III} complex in the urine samples. The concentrations of DMPS−MMA^{III} complex in these samples were 4.1 μg/L (sample A), ∼1 μg/L (sample B), 2.4 μg/L (sample C), and 1.2 μg/L (sample D). We did not observe a DMPS−As^{III} or DMPS-DMA^{III} complex in these samples.

It is interesting to note the relative concentrations of MMA^{V} and DMA^{V} in these urine samples. From the general population, the DMA^{V} concentration is usually dominant in urine samples, representing 60−80% of the total arsenic. MMA^{V} usually represents 10−15% of the total arsenic in urine. In the present study, the relative percentages of MMA^{V} and DMA^{V} in urine samples collected from the subjects before the administration of DMPS (*14, 16*) were consistent with those of other populations (*1, 7, 8, 40−42*). As expected, the DMPS−MMA^{III} complex was not detected in any urine samples collected before the administration of DMPS. However, after the administration of DMPS, there was a dramatic decrease of the percentage of DMA^{V} and an increase of MMA^{V} (*13, 34*). The percentage of DMA^{V} decreased to as much as one-tenth of the pre-DMPS value, while the percentage of MMA^{V} increased by almost 4-fold (*13*). The present study is consistent with the previous findings (*13, 14*).

The decrease of DMA^{V} and increase of MMA^{V} concentrations after ingestion of DMPS is probably the result of inhibition by DMPS of the methylation of MMA^{V} to DMA^{V}. The methylation of MMA^{V} to DMA^{V} requires a stepwise process of a two-electron reduction followed by oxidative addition of a methyl group.

$$\underset{MMA^{V}}{CH_3As^{V}O(OH)_2} \xrightarrow{2e^-} \underset{MMA^{III}}{CH_3As^{III}(OH)_2} \xrightarrow{CH_3^+} \underset{DMA^{V}}{(CH_3)_2As^{V}O(OH)}$$

We have demonstrated that DMPS binds to MMA^{III} to form the DMPS−MMA^{III} complex. The formation of the DMPS−MMA^{III} complex would reduce the availability of the "free" MMA^{III} as the substrate for the methylation, thereby inhibiting further methylation. This paper provides direct evidence of the DMPS−MMA^{III} complex in human urine after administration of DMPS, supporting the hypothesis that DMPS inhibits arsenic methylation by forming such a complex.

The urine samples analyzed in this study were obtained from individuals chronically exposed to inorganic arsenic in drinking water. The method described in this paper for the determination of the DMPS−MMA^{III} complex had a detection limit of ∼1 μg/L, and the method could be applied to urine samples from other populations. Two issues regarding the DMPS−MMA^{III} complex remain to be addressed: the kinetics of its formation in vivo and its stability during sample storage. Furthermore, studies of any complexes of As^{III}, MMA^{III}, and DMA^{III} with DMPS and other chelating agents (e.g., British anti-lewisite) will be very useful.

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