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Arsenic Speciation in Urine from Acute Promyelocytic Leukemia Patients undergoing Arsenic Trioxide Treatment

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Arsenic has been used successfully in clinical trials for treating acute promyelocytic leukemia (APL). Although sublethal doses of inorganic arsenic are used, little is known about the pharmacokinetics and metabolism of the high levels of arsenic in APL patients. To fill this important gap, this study describes the speciation of arsenic in urine from four APL patients treated with arsenic. Each patient was injected daily with an arsenite (As^{III}) solution that contained 10 mg of As_2O_3 precursor. Speciation analysis of the patient urine samples collected consecutively for 48 h, encompassing two intravenous injections of arsenic, revealed the presence of monomethylarsonous acid (MMA^{III}), dimethylarsinous acid (DMA^{III}), monomethylarsonic acid (MMA^{V}), and dimethylarsinic acid (DMA^{V}). The intermediate methyl arsenic metabolites, MMA^{III} and DMA^{III}, were detected in most urine samples from all of the patients when a preservative, diethyldithiocarbomate, was added to the urine samples to stabilize these trivalent arsenic species. The major arsenic species detected in the urine samples from the patients were As^{III}, MMA^{V}, and DMA^{V}, accounting for >95% of the total arsenic excreted. The relative proportions of As^{III}, As^{V}, MMA^{V}, and DMA^{V} in urine samples collected 24 h after the injections of As^{III} were 27.6 ± 6.1, 2.8 ± 2.0, 22.8 ± 8.1, and 43.7 ± 13.3%, respectively. The relatively lower fraction of the methylated arsenic species in these APL patients under arsenic treatment as compared with that from the general population exposed to much lower levels of arsenic suggests that the high levels of As^{III} inhibit the methylation of arsenic (inhibits the formation of methyl arsenic metabolites). The arsenic species excreted into the urine accounted for 32−65% of the total arsenic injected. These results suggest that other pathways of excretion, such as through the bile, may play an important role in eliminating (removing) arsenic from the human body when challenged by high levels of As^{III}.

Introduction

A number of recent clinical trials showing the success of treating APL[1] with arsenic have attracted much attention (*1*−*8*). Typically, relapsed or refractory APL patients were administered high doses (sublethal) of arsenic. Usually, an aqueous saline solution containing 10 mg of As_2O_3 was injected intravenously once a day (over 2−3 h) for a period of up to 60 days (*1*−*6*). An alternative treatment involved oral administration of solid As_4S_4 four times a day at a dose of ∼750 mg for each oral ingestion (*7*). These clinical trials involving treatment of APL with arsenic compounds have shown that the majority of the APL patients achieved complete clinical remission (*1*−*7*). Despite the overwhelming success, at least three fatalities of APL patients under arsenic treatment have been reported (*5*, *9*), and potential long-term side effects have not been studied. Data from a phase I/II study indicated that "though actively against APL, arsenic trioxide might be significantly, perhaps fatally, toxic at doses currently in use" (*9*). To maximize the treatment efficacy and to minimize side effects, there have been extensive studies to understand mechanisms of action responsible for the treatment (see ref *8* for review). Most of these studies focus on effects of arsenic on cell apoptosis, differentiation, and signaling transduction (*10*−*16*). Little attention has been paid to studies of arsenic metabolism and excretion by APL patients.

Inorganic arsenic can be extensively metabolized in biological systems (*17*−*22*). Human metabolism of inorganic arsenic results in the formation of the methyl arsenic metabolites, MMA^{III}, DMA^{III}, MMA^{V}, and DMA^{V}, which can be monitored in the urine (*17*−*22*). Although MMA^{V} and DMA^{V} have been frequently detected in human urine, MMA^{III} and DMA^{III} have only been detected recently (*23*−*29*). These trivalent methyl arsenic metabolites are more reactive and more toxic than the pentavalent arsenic species (*30*−*38*). Because of the different toxicities of the various arsenic metabolites, understanding of arsenic metabolism in APL patients is necessary in order to implement proper doses and duration of arsenic treatment and to optimize the treatment

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[1] Abbreviations: APL, acute promyelocytic leukemia; As^{III}, arsenite; As^{V}, arsenate; DDDC, diethylammonium diethyldithiocarbamate; DMA^{V}, dimethylarsinic acid; DMA^{III}, dimethylarsinous acid; ICPMS, inductively coupled plasma mass spectrometry; MMA^{V}, monomethylarsonic acid; MMA^{III}, monomethylarsonous acid; TMA^{III}, trimethylarsine; TMAO, trimethylarsine oxide.

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Table 1. Summary of APL Patients Participated in the Study

patient	agent (year)	weight (kg)	height (cm)	past days of arsenic trioxide treatment (days)
1	41	90	170	7
2	51	50	160	10
3	36	54	175	32
4	61	61	176	31

outcome. However, in APL patients who are administered sublethal doses of arsenic trioxide, the metabolism and excretion of arsenic species have not been studied in detail. How the individual differences in metabolism and excretion of arsenic might influence the treatment is unclear. Such information would be very useful for the safe use of arsenic compounds to treat APL.

The objective of this work was to study arsenic metabolites in urine samples collected from APL patients who were under treatment with arsenic trioxide. We report in this paper the presence of MMA^{III} and DMA^{III} in urine samples of APL patients after treatment with arsenic trioxide, arsenic speciation profiles in patient urine during the course of treatment, and the accumulative urinary excretion of arsenic species over a 48 h period. Determination of arsenic species in patient urine samples revealed the presence of the intermediate trivalent methyl arsenic metabolites. Speciation results also showed interpatient differences in metabolism and excretion of arsenic species.

Materials and Methods

Caution: *These arsenic compounds are toxic and should be handled with care.*

Reagents. The source of MMA^{III} was the solid oxide (CH_3AsO), and that of DMA^{III} was the iodide [$(CH_3)_2AsI$]. The precursors were prepared following literature procedures (*39*, *40*) and were kept at 4 and −20 °C. Dilute solutions of the precursors were freshly prepared in deionized water to form $CH_3As(OH)_2$ (MMA^{III}) and $(CH_3)_2AsOH$ (DMA^{III}), respectively. Solutions of other standard arsenic compounds, As_2O_3, $Na_2HAsO_4{\cdot}7H_2O$, $(CH_3)_2As(O)OH$ (Aldrich, Milwaukee, WI), and $CH_3As(O)OHONa$ (Chem Service, West Chester, PA) were prepared by appropriate dilution with deionized water from 1000 mg/L stock solutions, as described previously (*41*−*44*). The concentrations of arsenic in these arsenic compounds were standardized against a primary standard As^{III} solution (Aldrich).

Tetrabutylammonium hydroxide (Aldrich), malonic acid (Aldrich), and HPLC grade methanol (Fisher) were used for preparation of the mobile phase for HPLC separation. The mobile phase was prepared in deionized water and was filtered through a 0.45 μm membrane. DDDC was obtained from Aldrich. All reagents used were of analytical reagent grade or better.

A standard reference material, Toxic metals in freeze-dried human urine (CRM no.18 from National Institute for Environmental Studies, Japan Environment Agency) was used for quality control. The freeze-dried urine was reconstituted by the addition of 20.0 mL of deionized water, as recommended by the supplier. The certified value for total arsenic concentration was 134 ± 11 μg/L and for DMA^V was 36 ± 9 μg/L.

APL Patients and Urine Samples. At the time of this study, four APL patients (all males) were under arsenic trioxide treatment in the Harbin Medical University Hospital (Table 1). They all agreed to participate in this study. Patient 1 had been treated with arsenic trioxide 2 years earlier. He returned for additional treatment because of a relapse. The other three patients were newly diagnosed with APL. All of the four patients had been treated with arsenic trioxide continuously on a daily basis since the admission to the Harbin Medical University Hospital (China). Thus, the four patients had been treated with arsenic trioxide for 7, 10, 32, and 31 days, respectively, prior to the first collection of urine samples for the present study.

Qualified hospital physicians implemented the following standard treatment protocols that had been previously established by the hospital. An aqueous solution (10 mL) containing 10 mg of As_2O_3 was the approved drug for clinical trial, and it was obtained from Yida Pharmaceutical (Harbin, China). Dissolution of As_2O_3 in water presumably leads to the formation of As^{III} in the form of $As^{III}(OH)_3$. This solution was added into 500 mL of 5% glucose normal saline solution for intravenous injection that was administered over a 2−3 h period. The injection of this As^{III} solution usually took place at 8:00 am. It was administered under the supervision of medical specialists. Patients' health was monitored. White blood cell counts and routine blood analysis were carried out. The present study did not alter any scheduled treatment or any patient care protocol. It only involved the collection of patient urine samples. All procedures of sample collection and analysis were in compliance with the guidelines and regulations of both the Ethical Review Board of the University of Alberta and Harbin Medical University Hospital.

A urine sample was collected from each patient immediately before the injection of As^{III} solution on the day of experiment (8:00 am, December 16). Each patient was then injected intravenously the same dose of As^{III} (10 mg of As_2O_3). This took typically 2−3 h. All urine samples from each of the patients were collected separately for the subsequent 24 h, including a urine sample from each patient immediately prior to the next injection of As^{III} solution the following morning (8:00 am, December 17). Typically, 4−8 samples were obtained from each patient during the 24 h period. Following the injection of As^{III} solution, urine sample collection was repeated for another 24 h. The last urine samples were collected from each patient immediately prior to another scheduled injection of As^{III} solution the following morning (8:00 am, December 18).

Immediately after each urine sample collection, urine volume was measured. Aliquots (1 mL each) were transferred to 1.5 mL vials, and the vials were placed in a portable icebox containing dry ice. To preserve the trivalent methyl arsenic species, 8 μmol of DDDC was added to each sample vial so that the final concentration of DDDC in the urine sample was approximately 8 mM. For comparison, an aliquot of urine samples was also stored in sample vials that did not contain DDDC. The urine samples in both types of vials were kept frozen on dry ice during transportation and were stored at −50 °C until before the speciation analysis when the samples were thawed at room temperature. Before analysis, urines samples were filtered through 0.45 μm membrane and diluted to an appropriate concentration, if necessary, with deionized water. Filtration removed any precipitate in the urine sample so that the filtrate was suitable for HPLC analysis. ICPMS analysis of total arsenic concentration in the well-mixed, unfiltered urine samples (before filtration) and the filtrate of the same samples showed that there was no significant loss of arsenic concentration by the filtration.

Instrumentation. The HPLC separation system consisted of a Perkin-Elmer 200 Series pump, an autosampler, a column oven (Perkin-Elmer, Norwalk, CT), and a reversed-phase C18 column (ODS-3, 150 mm × 4.6 mm, 3 μm particle size; Phenomenex, Torrance, CA). An ODS guard cartridge (4 mm × 3 mm, Phenomenex) was mounted before the analytical column. The analytical column and the cartridge were placed in a column oven, and the temperature was maintained at 50 °C.

A Perkin-Elmer 6100 DRC^{plus} ICPMS (Perkin-Elmer/Sciex, Concord, Ontario, Canada) was used as the HPLC detector. ICP was operated at a radio frequency power of 1100 W. Argon flow as plasma gas, auxiliary gas, and nebulizer gas were 15, 1.2, and 0.9 L/min, respectively. Arsenic was monitored at *m*/*z* 75. The standard liquid sample introduction system consisted of a Meinhard nebulizer coupled to a Cyclonic spray chamber. The HPLC eluent outlet was directly connected to the inlet of the

ICP nebulizer with a small PTFE tubing and appropriate fittings.

Turbochrom workstation software (Perkin-Elmer) and Igro-Pro software (WaveMetrics, Lake Oswego, OR) were used to convert ICPMS data and to plot HPLC chromatograms.

Urinary Arsenic Speciation Analysis. Speciation of arsenic was carried out using HPLC separation with ICPMS detection. The frozen urine samples were thawed at room temperature. They were filtered through a 0.45 μm membrane. A 20 μL aliquot of the filtered urine was injected onto the HPLC column (ODS-3, 150 mm × 4.6 mm, 3 μm particle size) for separation of arsenic species. A mobile phase (pH 5.9−6.2) contained 5 mM tetrabutylammonium hydroxide, 3 mM malonic acid, and 5% (v/v) methanol. The flow rate of the mobile phase was 1.2 mL/min, and the column temperature was maintained at 50 °C. Each chromatographic analysis of As^{III}, MMA^{III}, DMA^{III}, MMA^{V}, DMA^{V}, and As^{V} took approximately 6 min (*25*). Analysis of the standard reference material CRM no. 18 human urine showed that the concentration of DMA^{V} was 42.9 ± 0.1 μg/L (n = 3), in good agreement with the certified value (36 ± 9 μg/L). There was no certified value for other arsenic species in any urine standard reference materials.

The frozen urine samples containing DDDC were thawed at room temperature. They were acidified with acetic acid to a final concentration of 20 mM (adding 200 μL of 1 M acetic acid to 1 mL of urine sample) immediately prior to HPLC−ICPMS analysis.

Total Urinary Arsenic Analysis. For comparison, the concentration of total arsenic in each sample was determined by ICPMS directly after appropriate dilution of the urine samples. Each sample was analyzed in duplicate, and the standard reference material CRM no.18 human urine was also analyzed. The measured value of total arsenic in CRM no.18 human urine was 127 ± 3 μg/L, which is in good agreement with the certified value (134 ± 11 μg/L).

Creatinine Analysis. The creatinine concentration of each urine sample was determined by using a colorimetric procedure (Stanbio Creatinine Procedure 0400, Sigma-Aldrich, St. Louis, MO). The total amounts of creatinine detected in the urine samples collected for 48 h from the four patients were 2.09, 1.24, 2.26, and 1.92 g, respectively.

Results

Trivalent Methyl Arsenic Species, MMA^{III}, and DMA^{III}. Figure 1 shows typical chromatograms from the analysis of urine samples from the four APL patients. These samples were collected from the patients approximately 24 h after the previous injection of arsenic and immediately before the next injection of arsenic. The arsenic species detected in these urine samples include inorganic As^{III}, MMA^{III}, DMA^{V}, MMA^{V}, DMA^{III}, and inorganic As^{V}. The predominant arsenic species are As^{III}, MMA^{V}, and DMA^{V}, because As^{III} is the initial arsenic species administered to the patients and MMA^{V} and DMA^{V} are the major metabolites of inorganic arsenic methylation in humans.

Figure 1 also indicates the presence of the trivalent methyl arsenic metabolites, MMA^{III} and DMA^{III}. These are key intermediate metabolites of the arsenic biomethylation process, as shown in Scheme 1. Biomethylation of arsenic is believed to involve alternating steps of reduction and oxidative addition of a methyl group, leading to the formation of MMA^{V}, MMA^{III}, DMA^{V}, DMA^{III}, TMAO, and TMA^{III}. The metabolic pathway usually does not proceed beyond the dimethylarsenic species stage for humans.

Analysis of urine samples spiked with authentic MMA^{III} and DMA^{III} standard further supports the presence of these arsenic species in urine samples. Figure 2 shows representative chromatograms obtained from the analyses of a urine sample (Figure 2a) and the same urine sample spiked with DMA^{III} (Figure 2b). Coelution of the spiked DMA^{III} standard with the suspected compound in the urine sample demonstrates their same chromatographic behavior. Coelution of the spiked MMA^{III} standard with the suspected MMA^{III} in the urine sample was also observed (data not shown).

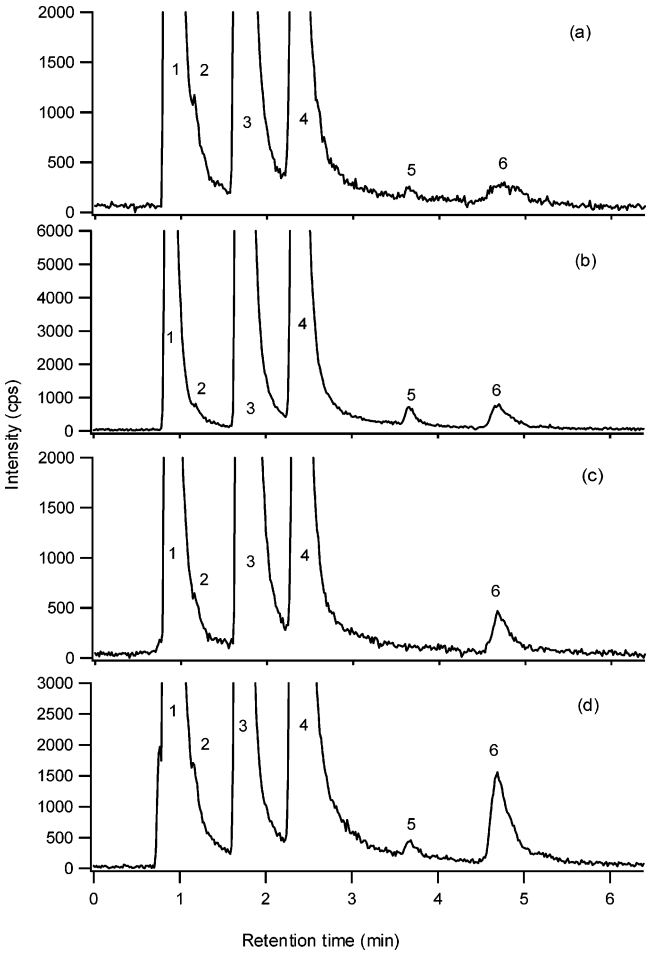
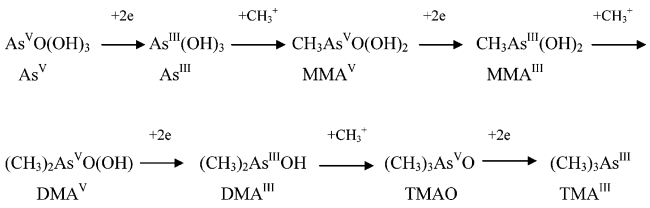


Figure 1. Chromatograms showing typical arsenic species in urine samples from four APL patients. The samples were collected 24 h after the previous administration of As^{III} and immediately prior to the next administration of As^{III}. Separation was carried out on an ODS-3 column with a mobile phase containing 5 mM tetrabutylammonium hydroxide, 3 mM malonic acid, and 5% methanol at pH 6.0. The HPLC column (150 mm × 4.6 mm, 3 μm particle size) was maintained at 50 °C. Arsenic was detected at m/z 75 using Perkin-Elmer 6100 DRC^{plus} ICPMS. Peaks 1−6 correspond to As^{III}, MMA^{III}, DMA^{V}, MMA^{V}, DMA^{III}, and As^{V}, respectively. The chromatograms a, b, c, and d were obtained from urine samples from patients 1, 2, 3, and 4, respectively. Chromatograms showing full intensity scales are included in the Supporting Information section.

Scheme 1. Pathway of Arsenic Methylation, Showing Alternate Steps of Two-Electron Reduction (2e−) and Oxidative Addition of a Methyl Group (CH_3^+)



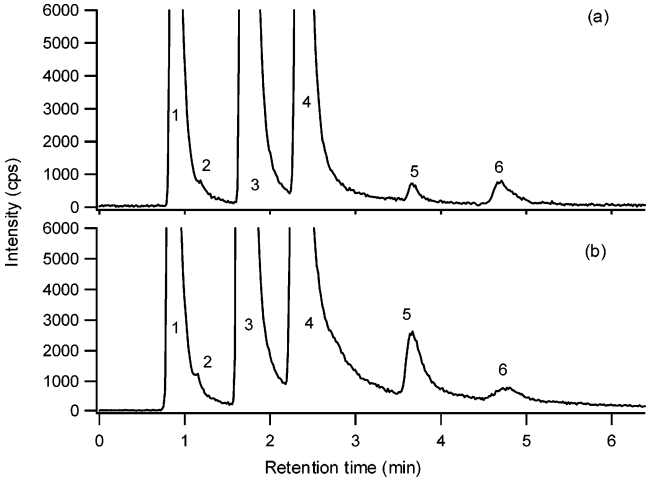


Figure 2. Chromatograms showing arsenic species in a urine sample (a) and the same sample spiked with DMA^{III} (b). The urine sample (a) was collected from patient two at 24 h after administration of As^{III}. Peak identities and HPLC−ICPMS conditions were the same as those in Figure 1. Chromatograms showing full intensity scales are included in the Supporting Information section.

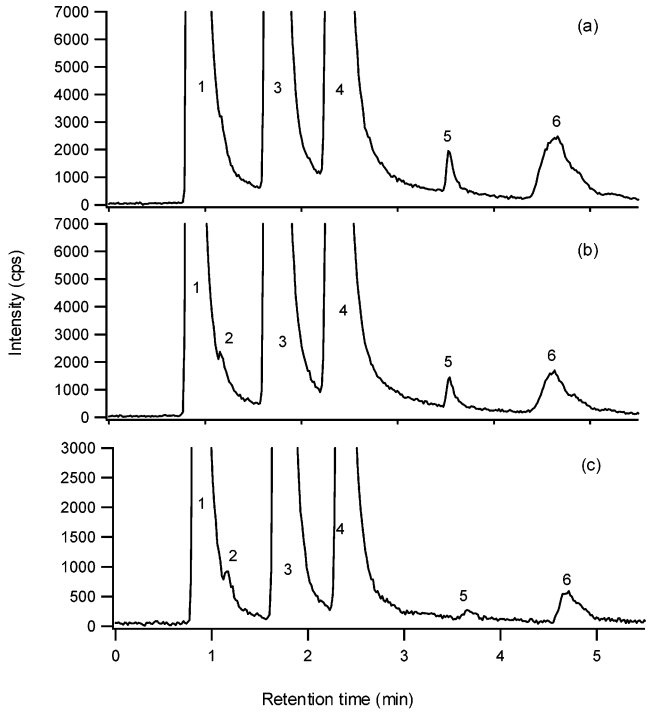


Figure 3. Typical chromatograms obtained from speciation analysis of urine samples collected from patient two at 12 (a), 24 (b), and 47.5 h (c) after the injection of As^{III} on day 8. Peak identities and HPLC−ICPMS conditions were the same as those in Figure 1. Chromatograms showing full intensity scales are included in the Supporting Information section.

Analyses of urine samples collected consecutively from the patients following the injections of As^{III} repeatedly show the presence of MMA^{III} and DMA^{III}. Chromatograms from the analysis of selected urine samples from patient 2 are shown in Figure 3. This patient had been treated with As^{III} for 7 days immediately prior to the start of this study. Urine samples were collected beginning on day 8 immediately prior to the injection of 10 mg of As_2O_3 (i.e., 7.5 mg As^{III}) and continued for 48 h. On day 9, another injection of 10 mg of As_2O_3 was administered, 24 h after the injection of As^{III} on day 8. Chromatograms in Figure 3 represent urine samples collected 12 h after

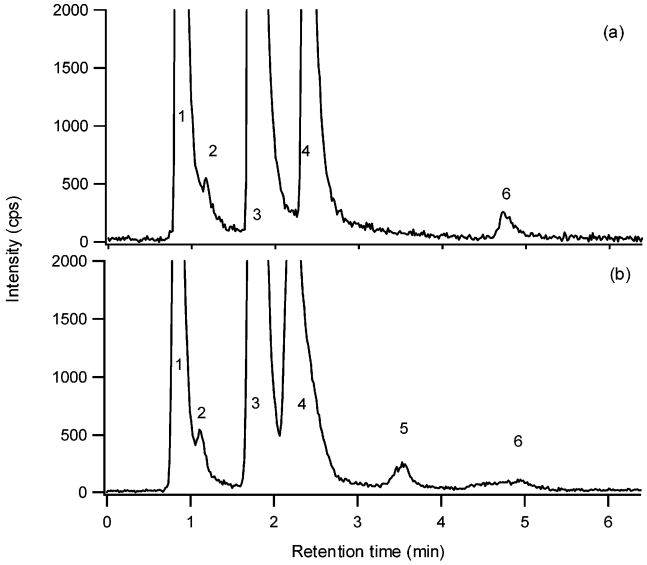


Figure 4. Chromatograms comparing arsenic species in a urine sample in the absence (a) and presence (b) of DDDC as a preservative. The sample was collected from patient three at 14.5 h after an injection of As^{III}. Two aliquots of the sample were stored at −50 °C, one without DDDC (a) and the other with 8 mM DDDC (b). The samples were analyzed 2 months after collection. Peak identities and HPLC−ICPMS conditions were the same as those in Figure 1. Chromatograms showing full intensity scales are included in the Supporting Information section.

(Figure 3a), 24 h after (Figure 3b), and 47.5 h after the injection on day 8 (Figure 3c). DMA^{III} is clearly observed. Although the MMA^{III} peak overlaps with that of higher concentrations of As^{III} in the sample, a small peak at the tail of As^{III} is an indication of the presence of MMA^{III}.

Because MMA^{III} and DMA^{III} can be readily oxidized to MMA^{V} and DMA^{V} during sample transport and storage prior to speciation analysis (*45*), we have developed a method for the preservation of these arsenic species. The method involves the use of a chelating agent, DDDC, which binds with the trivalent arsenic species, making them less prone to oxidation. Figure 4 compares the analysis of two aliquots of the same urine sample, with or without the presence of DDDC as a preservative. The sample was collected from patient 3, 14.5 h after the injection of As^{III}, and was analyzed 2 months after collection. In the absence of DDDC (Figure 4a), no DMA^{III} was detected. When another aliquot of the same sample was stored under the same conditions except with the presence of DDDC, the presence of MMA^{III} and DMA^{III} is clearly demonstrated (Figure 4b). Experiments were carried out to confirm that DDDC (<10 mM) did not reduce the pentavalent arsenic species to the trivalent arsenic species.

We have analyzed arsenic species in patient urine samples containing DDDC. Results of MMA^{III} and DMA^{III} concentrations are summarized in Table 2. With the use of DDDC as a preservative, most samples show detectable concentrations of MMA^{III} and DMA^{III}. In the absence of DDDC preservative, few samples showed detectable DMA^{III}. This is consistent with previous observations; DMA^{III} spiked in human urine samples was readily oxidized within 17 h at −20 °C (*45*).

Pentavalent Methyl Arsenic Metabolites, MMA^{V}, and DMA^{V}. In addition to the determination of MMA^{III} and DMA^{III} species in the urine samples, we have also quantified the other major arsenic metabolites. Figure 5

Table 2. Summary of MMA^{III} and DMA^{III} Concentration in Urine Samples from the Four APL Patients[a]

		MMA^{III}					DMA^{III}				
		no. of detectable MMA^{III} samples	mean concn (μg/L)	range (μg/L)	percent over total arsenic (%)		no. of detectable DMA^{III} samples	mean concn (μg/L)	range (μg/L)	percent over total arsenic (%)	
patient	no. of urine samples				mean	range				mean	range
1	9	4	22	5.6−35	1.9	0.7−3.6	7	29	2.6−125	1.5	0.3−4.7
2	7	7	52	15−95	1.4	0.2−2.8	5	65	8.1−171	1.2	0.3−2.1
3	14	14	25	4.2−54	0.9	0.7−2.1	7	33	4.1−64	0.6	0.1−0.9
4	12	7	19	9.2−46	0.8	0.1−1.6	3	14	4.9−23	0.8	0.2−2.0

[a] The samples contained 8 mM DDDC and had been stored at −50 °C for 2 months prior to analysis. HPLC conditions were the same as in Figure 1. Mean values were calculated from those urine samples that contained MMA^{III} and DMA^{III}.

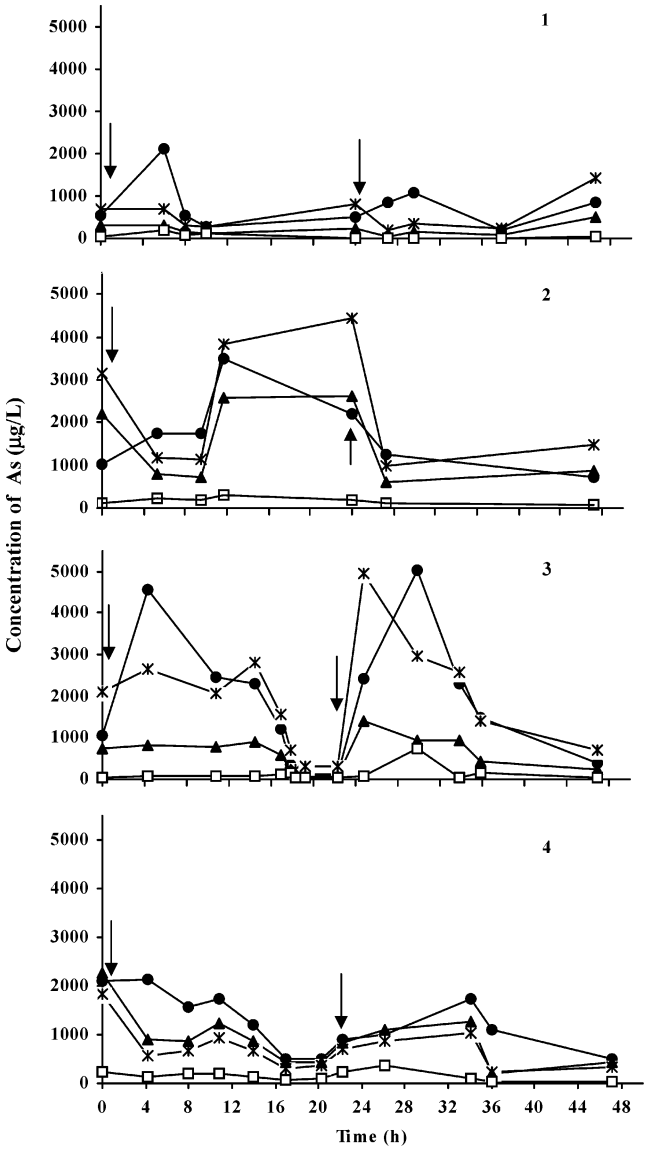


Figure 5. Concentration of arsenic species in urine samples from four APL patients. The patients were administered daily 7.5 mg of As^{III} by intravenous injection. The arrows indicate the time of injection of As^{III}. Urine samples were collected consecutively for 48 h, during which period two As^{III} doses were administered. Each urine sample was analyzed for arsenic speciation using the same method and conditions as shown in Figure 1. Graphs 1, 2, 3, and 4 represent arsenic speciation results for patients 1, 2, 3, and 4, respectively; As^{III} (●), DMA^{V} (*), MMA^{V} (▲), and As^{V} (□).

shows profiles of major arsenic species in patient urine samples collected over a 48 h period. Two injections of As^{III} were administered during the 48 h period; one was shortly (within 30 min) after time 0, and the other was shortly after 24 h, as indicated by the arrows in the figure. Consistent with previous observations, arsenic species are rapidly excreted into the urine. In the present study, As^{III} was injected into the blood stream. This may be the reason for the observed no lag time after injection of As^{III}.

Results in Figure 5 demonstrate interindividual differences. Although all of the four patients excreted arsenic species rapidly into the urine, the excretion profiles and the relative concentrations of major arsenic species excreted are different among the patients. For example, there is a clear maximum of As^{III} concentration in urine samples of patients 1 and 3, approximately 4 h after each injection of As^{III}. However, patients 2 and 4 did not show a similar profile. The exact reason for this apparent difference is not known, except to point out that both patients 2 and 4 are >10 years older than patients 1 and 3. Kurttio et al. (*46*) reported that older persons were better methylators of inorganic arsenic than younger individuals.

Results in Figure 5 also show the urinary excretion of arsenic metabolites as a consequence of methylation of the injected As^{III}. Immediately after the injection of As^{III}, the major arsenic species excreted is the unmetabolized As^{III}. However, the metabolism of As^{III} leads to decreases in As^{III} concentrations and increases in MMA^{V} and DMA^{V} concentrations. Thus, 24 h after each injection of As^{III}, DMA^{V} became the major arsenic species.

Daily Excretion of Arsenic Species. The accumulative excretion of arsenic species into urine is shown in Figure 6. During the 48 h period, a total of 15 mg of As^{III} was administered to each patient from the two injections (10 mg of As_2O_3 each). The total arsenic excreted over the 48 h was 6.3 (patient 1), 4.8 (patient 2), 9.8 (patient 3), and 8.0 mg (patient 4). These accounted for 42, 32, 65, and 53%, respectively, of the total arsenic administered during the 48 h by intravenous injections.

To examine whether the collection of urine samples over the 48 h was complete, we determined creatinine concentrations in each urine sample. Our results showed that the amounts of creatinine excreted were 1.05 (patient 1), 0.62 (patient 2), 1.13 (patient 3), and 0.96 g/day (patient 4). A typical normal range of creatinine excretion is 14−26 mg/kg body weight/day (*47*). Thus, if the collection of the urine sample was complete, the creatinine amounts would be 1.3−2.3 (patient 1, 90 kg), 0.7−1.3 (patient 2, 50 kg), 0.76−1.5 (patient 3, 54 kg), and 0.85−1.6 g/day (patient 4, 61 kg). The measured amounts of creatinine for patients 3 and 4 were within the normal range, suggesting that the 24 h urine sample collection was complete. Their arsenic excretion amounted to 65 and 53% of the injected dose, consistent with previous findings from human volunteers who repeatedly

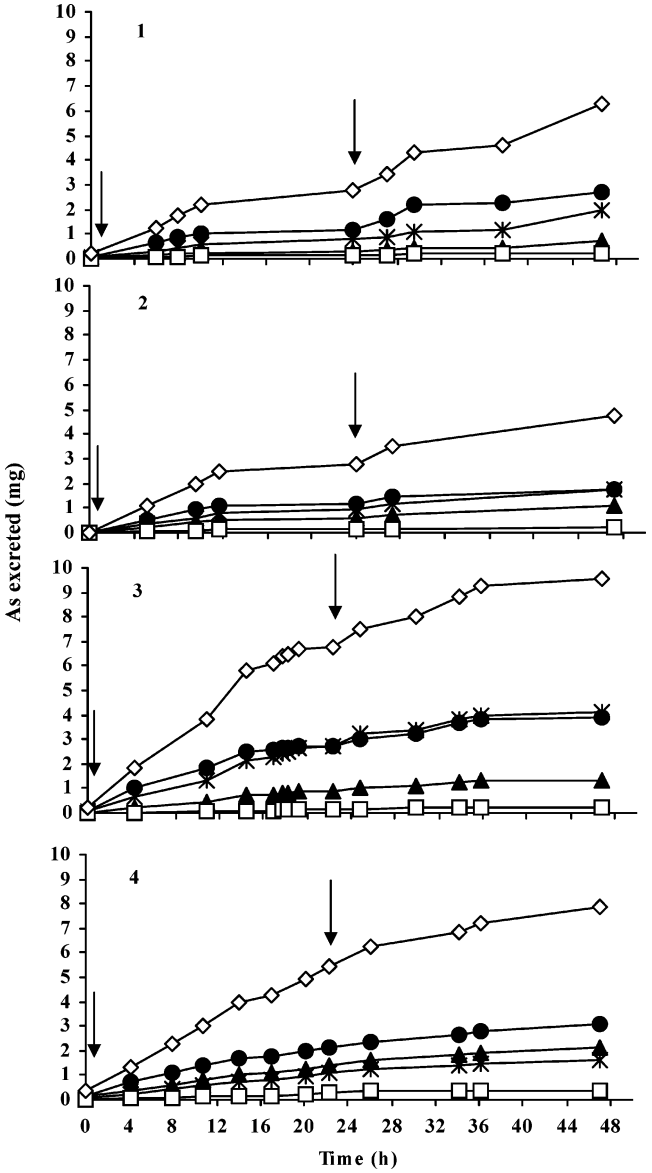


Figure 6. Accumulative excretion of arsenic into the urine by four APL patients who were administered daily 7.5 mg of As^{III} by intravenous injection. Urine samples were collected consecutively for 48 h, during which period two As^{III} doses were administered. The arrows indicate the time of injection of As^{III}. The volume of each urine sample was measured. Data on excretion of each arsenic species were obtained from speciation analysis of arsenic concentrations in each urine sample using HPLC−ICPMS and the measurement of the urine sample volume. Data on total arsenic excretion were obtained from separate analysis of total arsenic concentration using ICPMS. The sums of DMA^V, MMA^V, As^{III}, and As^V were in good agreement with total arsenic concentration. Graphs 1, 2, 3, and 4 represent urinary arsenic excretion results for patients 1, 2, 3, and 4, respectively; As^{III} (●), DMA^V (*), MMA^V (▲), As^V (□), and total arsenic (◇).

ingested As^{III} (*48*). The measured amounts of creatinine for patients 1 and 2 were lower than the normal range, suggesting that the 24 h urine sample collection from these patients might not be complete. Incomplete collection of urine samples would underestimate the amounts of arsenic excreted over the 24 h period, consistent with the observed lower values, 42 and 32% of the injected dose.

Relative Concentration of the Methylated Arsenic Species. As shown in Figures 5 and 6, the relative concentrations of arsenic species change following the administration of As^{III}. The major arsenic species were As^{III}, DMA^V, MMA^V, and As^V. This was confirmed by comparing the total arsenic obtained from direct ICPMS analysis with the sum of As^{III}, DMA^V, MMA^V, and As^V obtained from speciation analysis using HPLC−ICPMS. There was a good agreement between the total arsenic and the sum of As^{III}, DMA^V, MMA^V, and As^V.

Table 3. Percent (%) of Each Arsenic Species over the Total Arsenic Excreted in Urine Samples Collected 24 h after the Injection of As^{III}

patient	DMA^V	MMA^V	As^{III}	As^V	MMA^{III}	DMA^{III}	sum/total
1	44.4	14.9	27.2	0.55	1.8	0.5	89.4
2	46.4	27.2	22.0	1.9	1.2	0.6	99.8
3	58.1	17.2	25.1	4.2	1.0	0.1	105.7
4	26.0	32.0	36.2	4.8	1.1	0.2	100.3
mean	43.7	22.8	27.6	2.8	1.3	0.4	98.8
SD	13.3	8.1	6.1	2.0	0.4	0.2	6.8

Table 3 summarizes percentages of each arsenic species over total arsenic concentration in urine samples collected 24 h after the injection of As^{III}. The mean and standard deviation values from the four patients were 43.7 ± 13.3 (DMA^V), 22.8 ± 8.1 (MMA^V), 27.6 ± 6.1 (As^{III}), and $2.8 \pm 2.0\%$ (As^V). In urine samples collected shorter than 24 h after the injection of As^{III}, lower fractions of methylated arsenic species were observed. Urine samples from the general population that are exposed to lower levels of arsenic typically contain 60−80% DMA^V, 10−20% MMA^V, and 10−20% inorganic arsenic (As^{III} and As^V). The proportion of the methyl arsenic species in the APL patients, especially DMA^V, was substantially lower than those found in people exposed to much lower levels of arsenic. One possible reason for the lower fraction of methyl arsenic metabolites in the APL patients treated with sublethal doses of As^{III} is the short time (<24 h) between the repeat administrations of As^{III}. Another possibility is the inhibition of As^{III} on the methylation of arsenic. Animal studies have shown that As^{III} inhibited the formation of DMA^V (*53*, *54*).

Discussion

In most studies involving treatment of APL with arsenic, including the present study, arsenic trioxide (As_2O_3) is used as the precursor arsenic compound (*1*−*6*). As_2O_3 is typically dissolved in a saline solution for intravenous injection. Dissolving As_2O_3 in an aqueous solution likely leads to the formation of arsenous acid [$As^{III}(OH)_3$] or As^{III}. Therefore, this As^{III} species is the actual arsenic species injected intravenously although most medical literature loosely uses As_2O_3 as the agent for treatment of APL.

The injected As^{III} species can be readily metabolized in the human body through a biomethylation process (Scheme 1) (*54*). As a result of human metabolism of As^{III}, the major metabolites MMA^V and DMA^V have been frequently detected in human urine samples (*17*−*22*). Only recent studies have shown the presence of the intermediate, trivalent methyl arsenic metabolites, $MMA^{I,II}$ and DMA^{III}, in human urine samples collected from people who were exposed to arsenic in drinking water (*24*, *27*, *28*). The present study demonstrates the presence of MMA^{III} and DMA^{III} in urine samples from APL patients treated with high doses of As^{III}. The determination of these trivalent methyl arsenic metabolites is important because of their higher cytotoxicity and geno-

toxicity than the inorganic arsenicals and the pentavalent methyl arsenic metabolites (*30*−*38*).

Cytotoxic effects of MMA^{III} and DMA^{III} have been studied in more than a dozen animal and human cell lines (*55*). In all cases, MMA^{III} and DMA^{III} were found to be more toxic than the inorganic As^{III} species. LC_{50} values for MMA^{III} ranged from 0.4 to 5.5 μM (or 30−412 μg/L as arsenic). In the present study, urine samples from the ALP patients who were treated with arsenic trioxide contained up to 95 μg/L (or 1.3 μM) of MMA^{III} and 171 μg/L (or 2.3 μM) of DMA^{III}. Although MMA^{III} and DMA^{III} each accounted for less than 5% of the total arsenic excreted into the urine, their concentrations are on the same order of magnitude as the LC_{50} values. The actual concentrations of MMA^{III} and DMA^{III} in the bladder could be higher because these trivalent arsenicals are readily oxidized (*45*). Determination of MMA^{III} and DMA^{III} in the urinary bladder is toxicologically relevant because bladder cancer is a major health effect of chronic exposure to arsenic (*51*).

A main analytical challenge for accurate determination of MMA^{III} and DMA^{III} is that these reactive metabolites can be readily oxidized to MMA^{V} and DMA^{V} during sample transport, storage, and processing prior to speciation analysis (*45*). We have found that a chelating agent, DDDC, was able to bind with MMA^{III} and DMA^{III} and improve the stability of these trivalent methyl arsenic species in the samples. Further research is needed to establish a routine method of arsenical preservation using DDDC.

Urinary arsenic measurements in previous studies involving APL patients are inconsistent. Westervelt et al. (*9*) found that "mean urinary excretion of arsenic (from 9 APL patients) during the first 24 h of treatment was 18.7% of the daily dose". However, Shen et al. (*3*) found that urinary arsenic content was slightly increased during administration of arsenic and the total amount of arsenic excreted daily in the urine accounted for only 1−8%. They did not explain how the urine samples were collected and how arsenic was measured. In a study of APL patients administered orally with As_4S_4, Lu et al. (*7*) assumed that the urinary excretion of total arsenic was 70% of the absorbed amount.

Studies involving human volunteers have provided useful information on the urinary excretion of arsenic from the human body (*48*−*50*). Urinary excretion of arsenic depends on the arsenic species administered. Human volunteers who ingested a single dose of 500 μg of As^{III} showed that 46% of the ingested amount was excreted into the urine within 4 days (*49*). For repeated ingestion of As^{III} (125−1000 μg once per day), a steady state in the urinary excretion of arsenic was reached within 5 days. The total amount of arsenic excreted in urine per day was ∼60% of the ingested dose (*48*). In the present study, 32−65% of the arsenic injected intravenously was excreted into the urine.

The concentration of arsenic in the blood of eight APL patients during the course of As_4S_4 treatment (each ingested ∼750 mg of As_4S_4 four times daily for 14 days) was 65 ± 11 μg/L (*7*). This and other previous studies (*51*) suggest that little arsenic is accumulated in the blood. Although analysis of arsenic in the hair and nails indicated elevations of arsenic after repeated administration of arsenic (*3*, *7*), the total amount of arsenic in the hair and nails cannot account for the major excretion of arsenic. Other pathways, in addition to the urinary excretion, may also be responsible for the elimination of arsenic from the body. A likely pathway is the excretion into the bile.

There have been attempts to address whether the arsenic methylation process can be saturated by high levels of arsenic (*48*, *51*, *56*−*58*). Previous studies deal with populations exposed to arsenic from drinking water at the hundreds of micrograms per liter level. They would correspond to daily ingestion of arsenic on the order of micrograms to submilligrams. There was no evidence of saturation of arsenic methylation at these levels of arsenic. However, whether higher arsenic concentration leads to saturation of the methylation process or stimulates the completion of methylation process had not been studied. The APL patients were administered with daily sublethal doses of As^{III} (7.5 mg). This study has shown that a much lower fraction of DMA^{V} (26−58%) was present in the APL patient urine as compared with those from the general population (60−80% DMA^{V}). The reduced levels of DMA^{V} suggest that high levels of As^{III} inhibit the formation of this methyl arsenic metabolite.

In conclusion, we have found that both trivalent and pentavalent methyl arsenic species were excreted into the urine by APL patients who were administered daily doses of 7.5 mg of As^{III}. Daily urinary excretion of arsenic accounted for 32−65% of the daily dose administered. The fraction of DMA^{V} (26−58%) in the patient urine was much lower than the normal range (60−80%) for the general population, suggesting that the high doses of As^{III} administered to the patients interfere with the methylation of arsenic to DMA^{V}. There were large variations among the individual patients with regards to the arsenic metabolic profiles and excretion patterns. Understanding the interpatient differences in metabolism and excretion of arsenic is potentially useful for optimizing treatment of APL with appropriate doses of As^{III} for individual APL patients.

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Supporting Information Available: Chromatograms showing full intensity scales of Figures 1−4. This material is available free of charge via the Internet at http://pubs.acs.org.

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