Speciation of Key Arsenic Metabolic Intermediates in Human Urine

ARTICLE *in* ANALYTICAL CHEMISTRY · DECEMBER 2000

Impact Factor: 5.64 · DOI: 10.1021/ac000527u · Source: PubMed

6 AUTHORS, INCLUDING:

X. Chris Le

University of Alberta

248 PUBLICATIONS **7,753** CITATIONS

SEE PROFILE

Xiufen Lu

University of Alberta

45 PUBLICATIONS **2,034** CITATIONS

SEE PROFILE

Mingsheng Ma

University of Alberta

22 PUBLICATIONS **1,769** CITATIONS

SEE PROFILE

Baoshan Zheng

Chinese Academy of Sciences

104 PUBLICATIONS **2,152** CITATIONS

SEE PROFILE

Anal. Chem. **2000,** *72,* 5172−5177

Speciation of Key Arsenic Metabolic Intermediates in Human Urine

X. Chris Le,*,† Xiufen Lu,† Mingsheng Ma,† William R. Cullen,‡ H. Vasken Aposhian,§ and Baoshan Zheng||

Department of Public Health Sciences, University of Alberta, Edmonton, Alberta, T6G 2G3, Canada, Department of Chemistry, University of British Columbia, Vancouver, BC, Canada, Department of Molecular and Cellular Biology, University of Arizona, Tucson, Arizona 85721-0106, and Institute of Geochemistry, Academia Sinica, Guiyang, Guizhou, China

Biomethylation is the major human metabolic pathway for inorganic arsenic, and the speciation of arsenic metabolites is essential to a better understanding of arsenic metabolism and health effects. Here we describe a technique for the speciation of arsenic in human urine and demonstrate its application to the discovery of key arsenic metabolic intermediates, monomethylarsonous acid (MMA^{III}) and dimethylarsinous acid (DMA^{III}), in human urine. The study provides a direct evidence in support of the proposed arsenic methylation pathway in the human. The finding of MMA^{III} and DMA^{III} in human urine, along with recent studies showing the high toxicity of these arsenicals, suggests that the usual belief of arsenic detoxification by methylation needs to be reconsidered. The arsenic speciation technique is based on ion pair chromatographic separation of arsenic species on a 3-μm particle size column at 50 °C followed by hydride generation atomic fluorescence detection. Speciation of MMA^{III}, DMA^{III}, arsenite (As^{III}), arsenate (As^{V}), monomethylarsonic acid (MMA^{V}), and dimethylarsinic acid (DMA^{V}) in urine samples is complete in 6 min with detection limits of 0.5−2 μg/L. There is no need for any sample pretreatment. The capability of rapid analysis of trace levels of arsenic species, which resulted in the findings of the key metabolic intermediates, makes the technique useful for routine arsenic speciation analysis required for toxicological and epidemiological studies.

Chronic exposure to arsenic in drinking water at levels of several hundred micrograms per liter has contributed to cancers of skin, bladder, and lung, as well as noncancer effects, which have been seen in many parts of the world.[1−9] Most of the chronic arsenic poisoning cases are caused by the ingestion of inorganic arsenic in well water.[1−3] The inorganic arsenic species undergo methylation in the human body and the metabolites monomethylarsonic acid (MMA^{V}) and dimethylarsinic acid (DMA^{V}), which are less acutely toxic, are more readily excreted into the urine than the inorganic arsenic species. Thus, methylation of arsenic is usually considered to be a process of detoxifying arsenic.[10,11]

Methylation of arsenic involves a two-electron reduction of the pentavalent arsenic species to the trivalent arsenic species followed by oxidative addition of a methyl group to arsenic (Scheme 1).[12,13] Glutathione can act as a reducing agent, and *S*-adenosylmethionine (SAM) is the methyl donor. The metabolites MMA^{V} and DMA^{V} have been commonly observed in human urine,[14−19] providing supporting evidence for the proposed mechanism of arsenic methylation (Scheme 1). However, until recently,[20−25] little was

* Corresponding author: (telephone) 780 492-6416; (fax) 780 492-0364; (e-mail) xc.le@ualberta.ca.

† University of Alberta.

‡ University of British Columbia.

§ University of Arizona.

|| Academia Sinica.

(1) National Research Council. *Arsenic in Drinking Water;* National Academy Press: Washington, DC, 1999.

(2) Bagla, P.; Kaiser, J. *Science* **1996**, *274*, 174−175.

(3) Lepkowski, W. *Chem. Eng. News* **1998**, (Nov 16), 27−29.

(4) Tseng, W.-P. *Environ. Health Perspect.* **1977**, *19*, 109−119.

(5) Chen, C.-J.; Chuang, Y.-C.; Lin, T.-M.; Wu, H.-Y. *Cancer Res.* **1985**, *45*, 5895−5899.

(6) Cebrian, M. E.; Albores, A.; Aguilar, M.; Blakely, E. *Human Toxicol.* **1983**, *2*, 121−133.

(7) Bates, M. N.; Smith, A. H.; Hopenhayn-Rich, C. *Am. J. Epidemiol.* **1992**, *135*, 462−476.

(8) Chatterjee, A.; Das, D.; Mandal, B. K.; Chowdhury, R. R.; Samanta, G.; Chakraborti, D. *Analyst* **1995**, *120*, 643−650.

(9) Hopenhayn-Rich, C.; Biggs, M. L.; Fuchs, A.; Bergoglio, R.; Tello, E. E.; Nicolli, H.; Smith, A. H. *Epidemiology* **1996**, *7*, 117−124.

(10) Goyer, R. A. Toxic effects of metals. In *Casarett and Doull's Toxicology: The Basic Science of Poisons*, 5th ed.; Klaassen, C. D., Ed.; McGraw-Hill: New York, 1996; pp 696−698.

(11) Yamauchi, H.; Fowler, B. A. In *Arsenic in the Environment. Part II: Human Health and Ecosystem Effects*; Nriagu, J. O., Ed.; Wiley: New York. 1994; pp 35−43.

(12) Cullen, W. R.; Reimer, K. J. *Chem. Rev.* **1989**, *89*, 713−764.

(13) Thompson, D. J. *Chem.-Biol. Interact.* **1993**, *88*, 89−114.

(14) Smith, T. J.; Crecelius, E. A.; Reading, J. C. *Environ. Health Perspect.* **1977**, *19*, 89−93.

(15) Buchet, J. P.; Lauwerys, R.; Roels, H. *Int. Arch. Occup. Environ. Health* **1981**, *48*, 111−118.

(16) Foà, V.; Colombi, A.; Maroni, M.; Burrati, M.; Calzaferri, G. *Sci. Total Environ.* **1984,** *34*, 241−259.

(17) Vahter, M. *Acta Pharmacol. Toxicol.* **1986**, *59*, 31−34.

(18) Le, X. C.; Cullen, W. R.; Reimer, K. J. *Clin. Chem.* **1994**, *40*, 617−624.

(19) Hopenhayn-Rich, C.; Biggs, M. L.; Smith, A. H.; Kalman, D. A.; Moore, L. E. *Environ. Health Perspect.* **1996**, *104*, 620−628.

(20) Styblo, M.; Delnomdedieu, M.; Thomas, D. J. Biological mechanisms and toxicological consequences of the methylation of arsenic. In *Toxicology of metals−biochemical aspects, Handbook of experimental pharmacology*; Goyer, R. A., Cherian, G., Eds.; Springer-Verlag: Berlin, 1995; pp 407−433.

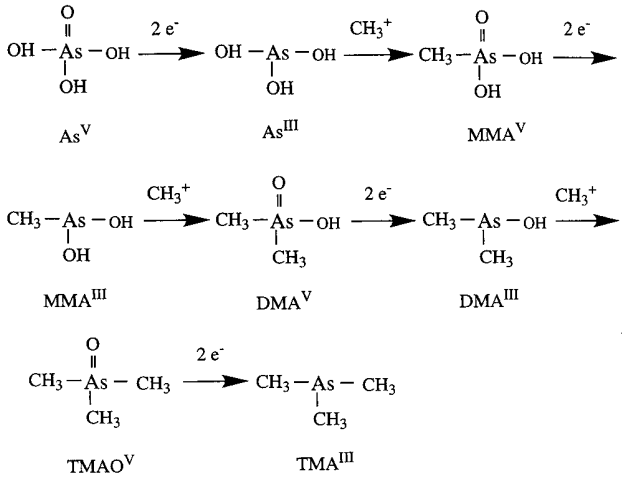
(21) Zakharyan, R. A.; Ayala-Fierro, F.; Cullen, W. R.; Carter, D. M.; Aposhian, H. V. *Toxicol. Appl. Pharmacol.* **1999**, *158*, 9−15.

(22) Styblo, M.; Serves, S. V.; Cullen, W. R.; Thomas, D. J. *Chem. Res. Toxicol.* **1997**, *10*, 27−33.

(23) Lin, S.; Cullen, W. R.; Thomas, D. J. *Chem. Res. Toxicol.* **1999**, *12*, 924−930.

10.1021/ac000527u CCC: $19.00
Published on Web 09/21/2000

Scheme 1. Pathway for Biotransformation of Inorganic Arsenic



known about the proposed essential intermediates, monomethylarsonous acid (MMA^{III}) and dimethylarsinous acid (DMA^{III}), in the human system, a result of the lack of techniques for the determination of these key arsenic species. To this end, we have developed a technique that allows for the speciation analysis of MMA^{III} and DMA^{III}, as well as MMA^{V}, DMA^{V}, and inorganic arsenite (As^{III}) and arsenate (As^{V}).

Here we report on the finding of the metabolic intermediates MMA^{III} and DMA^{III}, in human urine, providing direct evidence in support of the proposed pathway for arsenic biomethylation. Recent studies indicate that these arsenic methylation intermediates are more toxic than the inorganic arsenic species.[22−24,26] Our finding of MMA^{III} and DMA^{III} in human urine confirms that the reactive trivalent arsenic species are formed in intact organisms in the course of metabolism. This complements other published results indicating that trivalent arsenicals are especially toxic in in vitro systems and in cultured cells. The capability of the speciation technique makes this study possible.

EXPERIMENTAL SECTION

Instrumentation. A HPLC system consisted of a Gilson (Middleton, WI) HPLC pump (model 307), a Rheodyne six-port sample injector (model 7725i) with a 20-μL sample loop, and an appropriate column. The column was mounted inside a column heater (model CH-30, Eppendorf), which was controlled by a temperature controller (model TC-50, Eppendorf). Column temperature was maintained at 50 °C, and the mobile phase was preheated by using a stainless steel capillary tubing of 50 cm long, which was also placed inside the column heater. Isocratic HPLC operation was performed under a flow rate of 1.2−1.5 mL/min. A reversed-phase C18 column (ODS-3, 150 × 4.6 mm) with 3-μm particle size packing materials (Phenomenex, Torrance, CA) was used for most of the study. A C8 column (250 × 4.6 mm, Phenomenex) was used to separate trimethylarsine oxide from the other arsenic species.

A hydride generation atomic fluorescence detector (HGAFD) (model Excalibur 10.003, P.S. Analytical, Kent, U.K.) was used for the detection of arsenic. The combination of HPLC and HGAFD has been described previously.[27,28] Briefly, a 20-μL sample was injected onto the HPLC column for separation. Effluent from the HPLC column directly meets at two T-joints, with continuous flows of hydrochloric acid and sodium borohydride that were introduced by using a peristaltic pump. Upon mixing of the HPLC effluent, acid, and borohydride solutions, hydride generation takes place. Gaseous hydride generated from the reaction is separated from liquid waste in a gas/liquid separator apparatus and carried by a continuous flow of argon to the atomic fluorescence detector. 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 an IgorPro software (WaveMetrics, Lake Oswego, OR).

Reagents. Solutions of standard arsenic compounds, As^{III}, As^{V}, MMA^{V}, and DMA^{V}, were prepared by appropriate dilutions with deionized water from 1000 mg/L stock solutions, as described previously.[27−29] The source of MMA^{III} was the solid oxide (CH_3-AsO) and the source of DMA^{III} was the iodide [$(CH_3)_2$AsI], which were prepared following literature procedures.[30,31] Trimethylarsine oxide [$(CH_3)_3$AsO, TMAO] was synthesized following the procedures of Merijanian and Zingaro.[32]

Tetrabutylammonium hydroxide, malonic acid, and Na_2HPO_4 were obtained from Aldrich (Milwaukee, WI). HPLC grade methanol was from Fisher (Pittsburgh, PA). The HPLC mobile-phase solutions were prepared in deionized water and filtered through a 0.45-μm membrane prior to use. Sodium borohydride (Aldrich) solutions (1.3%) in 0.1 M sodium hydroxide (Fisher) were prepared fresh daily. All reagents used were of analytical grade or better.

A Standard Reference Material (SRM), Toxic Metals in Freeze-Dried Urine SRM 2670, was obtained from National Institute of Standards and Technology (NIST, Gaithersburg, MD). The freeze-dried urine was reconstituted by the addition of 20.0 mL of deionized water as recommended by the supplier.

Urine Samples. A total of 164 urine samples were collected from 41 people in Inner Mongolia, China. Arsenic levels in their well water that was used for consumption were 510−660 μg/L.[25] They were asked to exclude seafood consumption for 3 days prior to and during the urine sample collection period. They stopped drinking well water and were provided with distilled water to drink during the course of this study. They were fasted overnight and then orally administered 300 mg of sodium 2,3-dimercapto-1-propanesulfonate (DMPS).[25] This chelating agent has been previously used as an antidote to treat acute arsenic and mercury poisoning. A urine sample was collected from each participant before the administration of DMPS (between 11 h prior to and the time of DMPS administration). Three urine samples were

(24) Petrick, J. S.; Ayala-Fierro, F.; Cullen, W. R.; Carter, D. E.; Aposhian, H. V.. *Toxicol. Appl. Pharmacol.* **2000**, *163*, 203−207.
(25) Aposhian, H. V.; Zheng, B.; Aposhian, M. M.; Le, X. C.; Cebrian, M. E.; Cullen, W.; et al. *Toxicol. Appl. Pharmacol.* **2000**, *165*, 74−83.
(26) Cullen, W. R.; McBride, B. C.; Manji, H.; Pickett, A. W.; Rglinski, J. *Appl. Organomet. Chem.* **1989**, *3*, 71−79.
(27) Le, X. C.; Ma, M. *Anal. Chem.* **1998**, *70*, 1926−1933.
(28) Le, X. C.; Ma, M.; Wong, N. A. *Anal. Chem.* **1996**, *68*, 4501−4506.
(29) Feldmann, J.; Lai, V. W−M.; Cullen, W. R.; Ma, M.; Lu, X.; Le, X. C. *Clin. Chem.* **1999**, *45*, 1988−1997.
(30) Cullen, W. R.; McBride, B. C.; Manji, H.; Pickett, A. W.; Reglinski, J. *Appl. Organomet. Chem.* **1989**, *3*, 71−78.
(31) Burrows, G. J.; Turner, E. E. *J. Chem. Soc. Trans.* **1920**, *117*, 1373−1383.
(32) Merijanian, A.; Zingaro, R. *Inorg. Chem.* **1966**, *5*, 187.

collected 0−2, 2−4, and 4−6 h after the administration of DMPS. Informed consents were obtained from the participants prior to the study.

Urine samples were collected in 3-L polyethylene containers (Baxter Laboratories, Inc., Morton, Grove, IL), and the containers were then placed in portable ice boxes containing dry ice. The samples were kept frozen during transportation and were stored at −20 °C until before the analysis when the samples were thawed at room temperature and an aliquot (20 μL) was analyzed for arsenic species.

HPLC−HGAFD Analysis. Analyses of arsenic speciation in urine samples were carried out by using ion pair chromatographic separation with hydride generation atomic fluorescence detection (HPLC−HGAFD). An aliquot of a sample was filtered through a 0.45-μm membrane prior to be subjected to HPLC−HGAFD analysis. A mobile-phase solution (pH 5.9) contained 5 mM tetrabutylammonium hydroxide, 3 mM malonic acid, and 5% methanol, and its flow rate was 1.2 mL/min. The concentrations of hydrochloric acid (1.2 M) and sodium borohydride (1.3%) for hydride generation were optimized for maximum sensitivity of less abundant arsenic species in urine, such as MMA^{III}, DMA^{III}, and MMA^{V}. Arsines generated were separated from liquid waste and carried by a continuous flow of argon to the atomic fluorescence detector for quantitation.

To probe trimethylarsine oxide in urine, a reversed-phase C8 column (250 × 4.6 mm, Phenomenex) was used with a mobile phase containing 20 mM phosphate (pH 7.4) and 10% methanol. The flow rate was 1.0 mL/min, and the column temperature was maintained at 50 °C.

RESULTS AND DISCUSSION

Our primary objective was to determine new arsenic species at trace levels in human urine in which several common arsenic species may coexist. High sensitivity and the capability of differentiating individual arsenic species are our main considerations for the development of a speciation technique. Ion pair chromatography was chosen to separate a mixture of neutral and anionic arsenic species. We have previously demonstrated that the combination of elevated column temperature (50−70 °C) and short HPLC column (3−15 cm) with small particle size (3 μm) resulted in improved separation efficiency and rapid analysis, consistent with the benefits observed by others who used high-temperature ultrafast HPLC.[33−36] The combination allowed for rapid separation of As^{III}, As^{V}, MMA^{V}, and DMA^{V} within 3 min.[27] We now take the advantage of the improved separation and further enhance it for the speciation of the two key metabolic intermediates of arsenic methylation, MMA^{III} and DMA^{III}. However, our initial studies showed that MMA^{III} coeluted with As^{III} and DMA^{V} and that DMA^{III} coeluted with MMA^{V} and As^{V} within a 3-min period of retention time. Therefore, a wider separation window is needed, especially between As^{III} and DMA^{V} and between MMA^{V} and As^{V}. This is achieved primarily by adjusting the concentration of malonic acid and methanol in the mobile phase. Under the optimized conditions, we are able to achieve the separation of the six target arsenic compounds within 6 min (Figure 1).

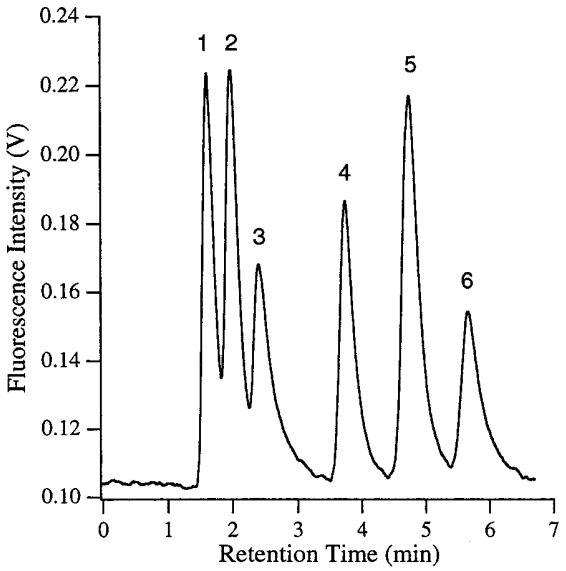


Figure 1. Chromatogram showing speciation analyses of As^{III}, As^{V}, MMA^{V}, DMA^{V}, MMA^{III}, and DMA^{III} in deionized water. Separation was carried out on an ODS-3 column with a mobile phase containing 5 mM tetrabutylammonium hydroxide, 3 mM malonic acid, and 5% methanol. The HPLC column (15 cm × 4.6 mm, 3-μm particle size) was maitained at 50 °C. Peaks labeled 1−6 correspond to As^{III}, MMA^{III}, DMA^{V}, MMA^{V}, DMA^{III}, and As^{V}, respectively. The concentration of As^{III}, DMA^{V}, MMA^{V}, and As^{V} is 50 μg/L for each species. The concentrations of MMA^{III} and DMA^{III} are approximately 100 and 150 μg/L, respectively.

High-sensitivity detection was achieved by a combination of hydride generation with atomic fluorescence detection. A limiting factor in atomic fluorescence detection has been light scattering and interference due to sample matrix. Hydride generation, as an efficient derivatization process, converts arsenic species in the solution to gaseous hydrides. Only the hydrides are introduced to the atomic fluorescence detector, and sample matrix is left in the liquid waste. Thus, spectral and chemical interferences encountered in the conventional atomic fluorescence detection system are essentially eliminated. Without scattering and background interference from sample matrix, the detection limit by using atomic fluorescence is dramatically improved. We have obtained detection limits of 0.5−2 μg/L (for 20 μL of sample) for the six target arsenic species. This detection capability allows for direct speciation of arsenic in human urine samples from the general population without any pretreatment. Examples of the reported concentrations of arsenic in human urine from the general population are (mean ± standard deviation, μg/L) 9 ± 7 from a U.S. population,[37] 17 ± 11 and 11 ± 6 from European studies,[16,38] 21 ± 7 from Taiwan,[39] and 121 ± 101 from Japan.[40] People exposed to higher levels of arsenic from drinking water

(33) Antia, F. D.; Horvath, C. *J. Chromatogr.* **1988**, *435*, 1−15.
(34) Chen, H.; Horvath, C. *J. Chromatogr., A* **1995**, *705*, 3−20.
(35) Hancock, W. S.; Chloupek, R. C.; Kirkland, J.; Snyder, L. R. *J. Chromatogr.* **1994**, *686*, 31−43.
(36) Yan, B.; Zhao, J.; Brown, J. S.; Blackwell, J.; Carr, P. W. *Anal. Chem.* **2000**, *72*, 1253−1262.
(37) Kalman, D. A.; Hughes, J.; van Belle, G.; Burbacher, T.; Bolgiano, D.; Coble, K.; Mottet, N. K.; Polissar, L. *Environ. Health Perspect.* **1990**, *89*, 145−151.
(38) Buchet, J. P.; Lison, D.; Ruggeri, M.; Foa, V.; Elia, G. *Arch. Toxicol.* **1996**, *70*, 773−778.
(39) Lin, T. H.; Huang, Y. L. *Biol. Trace Elem. Res.* **1995**, *48*, 251−261.
(40) Yamauchi, H.; Takahashi, K.; Mashiko, M.; Yamamura, Y. *Am. Ind. Hyg. Assoc. J.* **1989**, *50*, 606−612.

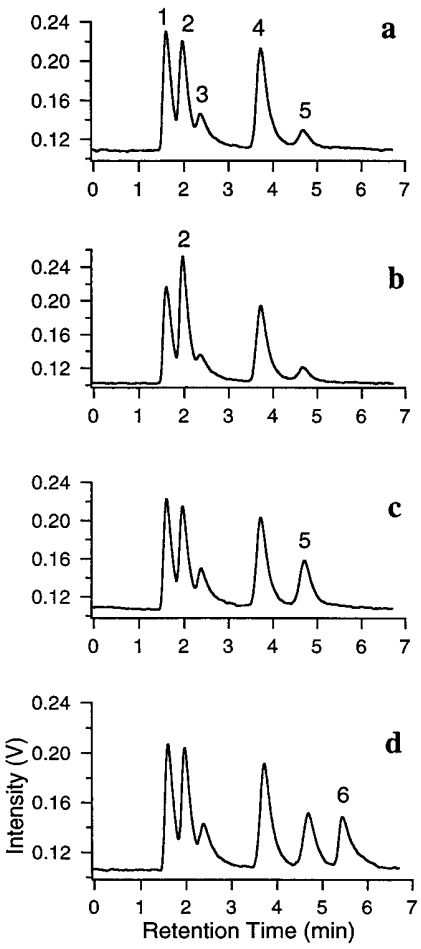


Figure 2. Chromatograms showing speciation analyses of arsenic in a urine sample (a) and the urine sample with addition of MMA^{III} (b), DMA^{III} (c), and As^{V} (d). Same conditions as shown in Figure 1 were used.

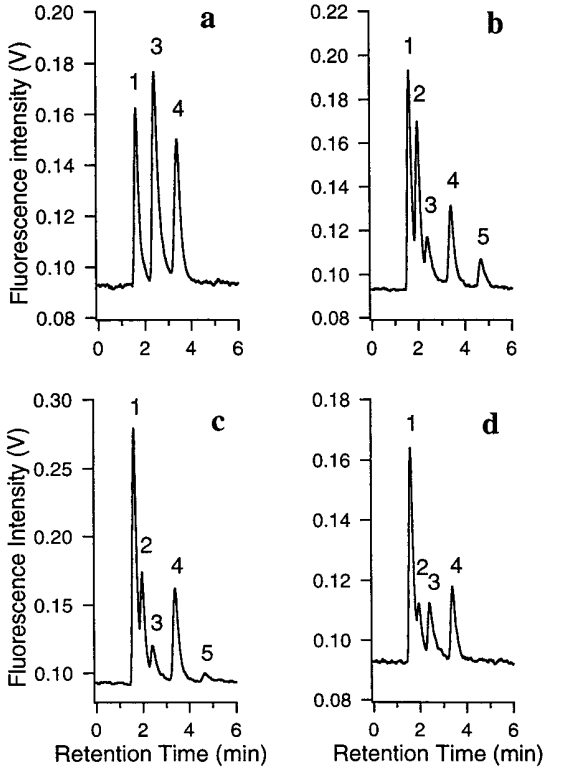


Figure 3. Chromatograms obtained from speciation analyses of a series of urine samples collected from a participant before and after a single oral dose of 300 mg of DMPS: (a) between 11 h before and the time of the DMPS administration and (b) 0−2, (c) 2−4, and (d) 4−6 h after the administration of DMPS. Same conditions as shown in Figure 1 were used.

and food have corresponding higher levels of urinary arsenic, e.g., 56 ± 13 from black food disease patients,[39] 274 ± 98 from a highly exposed Argentina population,[41] and 450−700 from a highly exposed Mexican group.[42]

A Standard Reference Material, SRM2670 (from NIST), was used for method validation purpose. The certified value for total arsenic concentration is 480 ± 100 μg/L in the urine containing elevated levels of toxic metals. Results for the speciation of arsenic in the SRM2670 using the HPLC−HGAFD method are 46 ± 5 for DMA^{V}, 11 ± 3 for MMA^{V}, and 460 ± 25 for As^{V}. These results are in good agreement with the certified and literature values.[43]

Figure 2 shows chromatograms obtained from the analyses of a urine sample collected 2 h after a person ingested 300 mg of DMPS (Figure 2a). Co-injection of the urine sample with authentic MMA^{III} standard (Figure 2b) demonstrates the coelution of the suspected MMA^{III} in the sample with that of the standard MMA^{III}, confirming the identity of MMA^{III} in the urine sample. Similarly, co-injection of the urine sample with standard DMA^{III} (Figure 2c) and As^{V} (Figure 2d) confirms the presence of DMA^{III} in the sample. This is the first report demonstrating the presence of both DMA^{III} and MMA^{III} in human urine.

From a total of 164 Inner Mongolia samples from 41 people, we found detectable (>2 μg/L) MMA^{III} in 51 samples and DMA^{III} in 2 samples. The highest MMA^{III} concentration in the urine samples was 240 μg/L.

Figure 3 shows arsenic species in urine samples collected from a participant over a 6-h period. No MMA^{III} or DMA^{III} is detected in the urine sample collected prior to the DMPS ingestion; and inorganic As^{III}, DMA^{V}, and MMA^{V} are the major arsenic species (Figure 3a). Following the DMPS treatment, MMA^{III} is observed in all the three samples collected 2, 4, and 6 h after the treatment. DMA^{III} is present only in the 2- and 4-h urine samples after DMPS treatment. Concentrations of arsenic species in these samples are summarized in Table 1.

To confirm that MMA^{III} and DMA^{III} species are formed in vivo, we first tested whether DMPS is involved with the in vitro reduction of MMA^{V} and DMA^{V} species to MMA^{III} and DMA^{III}. Figure 4 shows representative chromatograms from periodic analyses of urine samples containing added DMPS (438 μM), MMA^{V} (0.3 μM), and DMA^{V} (0.7 μM) for up to 44 h. No MMA^{III} or DMA^{III} is detectable in the mixture, although the molar concentration of DMPS is approximately 1460-fold that of MMA^{V} and 626-fold that of DMA^{V}. The concentration of DMPS in human urine after a single oral administration of DMPS is below this

(41) Vahter, M.; Concha, G.; Nermell, B.; Nilsson, R.; Dulout, F.; Natarajan, A. T. *Eur. J. Pharmacol. Environ. Toxicol. Pharmacol.* **1995**, *293*, 455−462.
(42) Del Razo, L. M.; Garcia-Vargas, G. G.; Vargas, H.; Albores, A.; Gonsebatt, M. E.; Montero, R.; Ostrosky-Wegman, P.; Kelsh, M.; Cebrian, M. E. *Arch. Toxicol.* **1997**, *71*, 211−217.
(43) Crecelius, E.; Yager, J. *Environ. Health Perspect.* **1997**, *105*, 650−653.

Table 1. Concentrations of Arsenic Species (μg/L) in Urine Samples Shown in Figure 3[a]

sample	sampling time	As^{III}	MMA^{III}	DMA^{V}	MMA^{V}	DMA^{III}	As^{V}	sum
A	−11 to 0 h	46 ± 2	nd[b]	161 ± 6	56 ± 2	nd	nd	263 ± 6
B	0−2 h	59 ± 2	127 ± 4	57 ± 6	36 ± 2	38 ± 3	nd	317 ± 6
C	2−4 h	111 ± 3	137 ± 4	60 ± 2	64 ± 2	21 ± 2	nd	393 ± 4
D	4−6 h	43 ± 2	32 ± 2	37 ± 2	21 ± 1	nd	nd	133 ± 2

[a] Results are mean ± 1 standard deviation from triplicate analyses of each sample. [b]nd, below detection limit (1 μg/L for As^{V} and 2 μg/L for MMA^{III} and DMA^{III}).

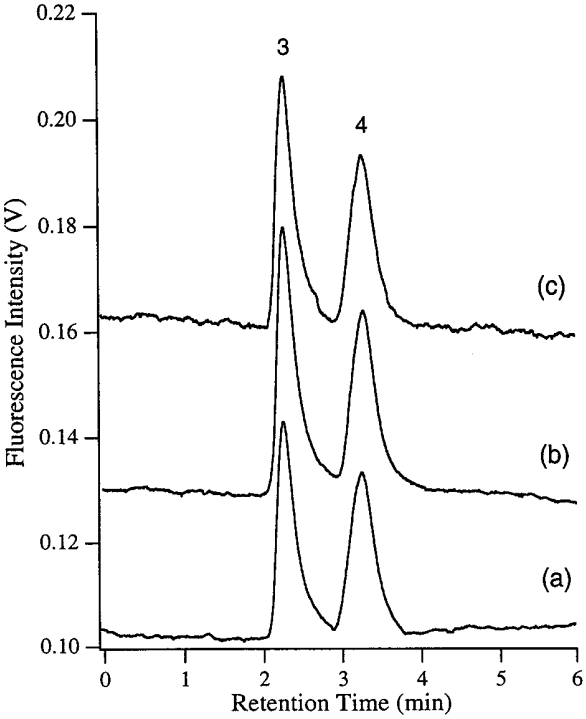


Figure 4. Representative chromatograms from the HPLC−HGAFS analyses of a mixture containing a background urine sample with added DMPS, MMA^{V}, and DMA^{V}: (a) 20 min, (b) 23 h, and (c) 44 h after the mixing of DMPS (438 μM), MMA^{V} (0.3 μM), and DMA^{V} (0.7 μM) in urine. Same conditions as shown in Figure 1 were used.

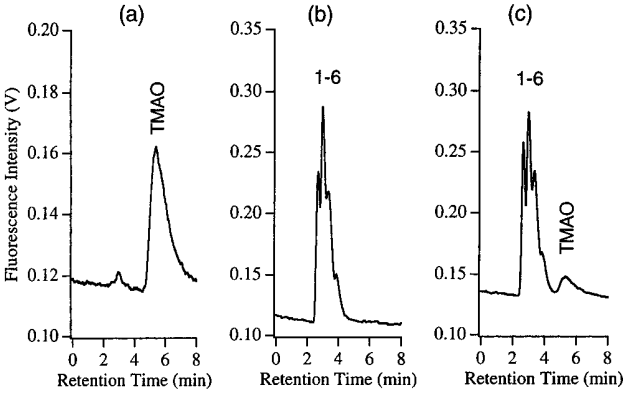


Figure 5. Chromatograms showing HPLC−HGAFD analyses of (a) trimethylarsine oxide (TMAO) in deionized water; (b) the urine sample as shown in Figure 2a, containing As^{III}, MMA^{III}, DMA^{V}, MMA^{V}, and DMA^{III}; and (c) the urine sample spiked with TMAO. A C8 column was used for separation, with 20 mM phosphate (pH 7.4) and 10% methanol as the mobile phase. Peaks labeled 1−6 correspond to As^{III}, MMA^{III}, DMA^{V} MMA^{V}, DMA^{III}, and As^{V}, respectively.

level.[44] The results confirm that the MMA^{III} and DMA^{III} detected in urine samples of DMPS-treated subjects is not the result of the reduction of MMA^{V} and DMA^{V} by DMPS in vitro. In addition, recent studies[45,46] have also found MMA^{III} and DMA^{III} in urine samples from people without DMPS treatment. Thus, the finding of MMA^{III} and DMA^{III} in human urine samples suggests that these species are present in vivo as a result of arsenic biomethylation. They are normally bound to proteins and glutathione in the body because the trivalent arsenic species have a high affinity for thiol groups in these molecules.[1,12,13,20,21] When DMPS is administered, these MMA^{III} or DMA^{III} species presumably become chelated to the excess DMPS and excreted into the urine. The finding of MMA^{III} and DMA^{III} provides a key evidence in support of the arsenic methylation pathway (Scheme 1).

We further probed the presence of TMAO because this arsenical is an expected product of the methylation of DMA^{III}. By using two different chromatographic separation systems, we found no detectable TMAO in any of the urine samples. Under the ion pair chromatographic conditions described above, TMAO coeulted with As^{III} and, thus, could not be differentiated. Thus, we used a different separation mode, reversed-phase separation on a C8 column (Figure 5). Under this setting, TMAO is well resolved from the other arsenicals. We analyzed all the urine samples that contained DMA^{III}. No TMAO was detected in any of these urine samples.

TMAO has been identified as a metabolite in bacterial systems. Little is known about TMAO in humans. Failure to observe TMAO suggests that either its concentration in urine is below the detection limit of the method or it is further metabolized to trimethylarsine and subsequently exhaled into the breath as it is a volatile species.

In vitro experiments using cultured cells have shown that MMA^{III} and DMA^{III} are more toxic than the inorganic arsenic species.[22−24,26] The observation of MMA^{III} and DMA^{III} species in human urine together with recent studies on arsenic toxic effects indicates that methylation of arsenic may not be entirely a detoxification process for humans, as previously believed. Toxicological consequences of MMA^{III} and DMA^{III} in humans need to be examined. Determination of these arsenic methylation intermediates in different tissues may provide useful information for a better understanding of arsenic metabolism and health

(44) Maiorino, R. M.; Dart, R. C.; Carter, D. E.; Aposhian, H. V. *J. Pharmacol. Exp. Therap.* **1991**, *259*, 808−814.
(45) Aposhian, V. H.; Gurzau, E. S.; Le, X. C.; Gurzau, A.; Healy, S. M.; Lu, X.; Ma, M.; Yip, L.; Zakharyan, R. A.; Maiorino, R. M.; Dart, R. C.; Tirus, M. G.; Gonzalez-Ramirez, D.; Morgan, D. L.; Avram, D.; Aposhian, M. M. *Chem. Res. Toxicol.* **2000**, *13*, 693−697.
(46) Del Razo, L. M.; Styblo, M.; Thomas, D. J. Presented at the 4th International Conference on Arsenic Exposure and Health Effects, San Diego, CA, June 18−22, 2000, Abstract p 75.

effects. The technique we have developed for speciation analysis of arsenic metabolites, which resulted in the discovery of the key metabolic intermediates in human urine, should contribute to a better understanding of arsenic metabolism and health effects. The capability of rapid analysis of trace levels of arsenic species makes the technique useful to survey human populations at risk and to study individual variability in arsenic metabolic behavior. The urinary MMA^{III} and DMA^{III} concentration may also be an indicator of the body burden of these species, which may be a potential biomarker for the assessment of human exposure to arsenic.

While the technique is suitable for the speciation of arsenicals in human urine, an important issue to be addressed in the future is the stability of MMA^{III} and DMA^{III} species. These trivalent species are readily oxidized to MMA^{V} and DMA^{V} in solution. Our preliminary results showed that approximately 60% of MMA^{III} and 95% of DMA^{III} were oxidized to MMA^{V} and DMA^{V}, respectively, after 1 μM MMA^{III} and DMA^{III} was separately spiked into a urine sample and the sample was stored at 4 °C for 2 weeks. Thus, it is possible that many more freshly collected urine samples from the 41 people could have contained MMA^{III} and DMA^{III}. Some of the MMA^{III} and DMA^{III} could have been oxidized during the storage prior to speciation analysis. Further detailed studies of the stability of these arsenic species need to be conducted and possible method for stabilizing these species be developed.

ACKNOWLEDGMENT

This work was supported in part by the Natural Sciences and Engineering Research Council of Canada (NSERC).

Received for review May 11, 2000. Accepted August 3, 2000.

AC000527U