Glutathione-conjugated Arsenics in the Potential Hepato-enteric Circulation in Rats

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The metabolic pathways for arsenic were precisely studied by determining the metabolic balance and chemical species of arsenic to gain an insight into the mechanisms underlying the animal species difference in the metabolism and preferential accumulation of arsenic in red blood cells (RBCs) in rats. Male Wistar rats were injected intravenously with a single dose of arsenite (iAs^{III}) at 2.0 mg of As/kg of body weight, and then the time-dependent changes in the concentrations of arsenic in organs and body fluids were determined. Furthermore, arsenic in the bile was analyzed on anion and cation exchange columns by high-performance liquid chromatography−inductively coupled argon plasma mass spectrometry (HPLC−ICP MS). The metabolic balance and speciation studies revealed that arsenic is potentially transferred to the hepato-enteric circulation through excretion from the liver in a form conjugated with glutathione (GSH). iAs^{III} is methylated to mono (MMA)- and dimethylated (DMA) arsenics in the liver during circulation in the conjugated form [$iAs^{III}(GS)_3$], and a part of MMA is excreted into the bile in the forms of MMA^{III} and MMA^{V}, the former being mostly in the conjugated form [$CH_3As^{III}(GS)_2$], and the latter being in the nonconjugated free form. DMA^{III} and DMA^{V} were not detected in the bile. In the urine, arsenic was detected in the forms of iAs^{III}, arsenate, MMA^{V}, and DMA^{V}, iAs^{III} being the major arsenic in the first 6-h-urine, and DMA^{V} being increased in the second 6-h-urine. The present metabolic balance and speciation study suggests that iAs^{III} is methylated in the liver during its hepato-enteric circulation through the formation of the GSH-cojugated form [$iAs^{III}(GS)_3$], and MMA^{III} and MMA^{V} are partly excreted into the bile, the former being in the conjugated form [$CH_3As^{III}(GS)_2$]. DMA is not excreted into the bile but into the bloodstream, accumulating in RBCs, and then excreted into the urine mostly in the form of DMA^{V} in rats.

Introduction

The metabolism of arsenic is known to depend on the chemical species of arsenic ingested or injected into animals and also on the animal species, even when the same chemical species is administered (*1*−*5*). Inorganic arsenics such as arsenite (iAs^{III})[1] and arsenate (iAs^{V}) are more toxic to animals than organic forms such as arsenosugars and arsenobetaines of marine origin (*6*−*8*).

Arsenic ingested or injected in the form of arsenite (iAs^{III}) is mostly excreted in the form of methylated metabolites into the urine in diverse animals, dimethylated arsenic (DMA) being the major form (*2*, *9*−*12*). However, the metabolic pathway, such as the distribution among organs/tissues, is quite different among animal species, i.e., arsenic does not accumulate in red blood cells (RBCs) in hamsters (*13*−*15*), but mostly accumulates in RBCs in rats (*16*, *17*).

The animal species difference in the metabolism of arsenic has been stated to depend on the reduction and methylation capacity of the proposed metabolic pathway for arsenic in Scheme 1 (*2*, *18*). Namely, iAs^{III} is oxidatively methylated to monomethylarsonic acid (MMA^{V}), which is reduced to monomethylarsonous acid (MMA^{III}) for further methylation, leading to dimethylarsinic acid (DMA^{V}). Although DMA can be further reduced and methylated to trimethylated arsenic (TMA), the most common and most abundant chemical species of urinary arsenics is DMA (*2*, *9*−*12*, *19*), and DMA has been thought to be the major final urinary metabolite of inorganic arsenics in most animals (*2*, *9*−*12*). It has been generally believed that the methylation of inorganic arsenic comprises a detoxification mechanism (*9*, *20*−*22*). It has been pointed out that urinary DMA consists not only of DMA^{V} but also dimethylarsinous acid (DMA^{III}) in humans drinking arsenic-polluted tube-well water (*23*, *24*).

Rats are known to be one of the most tolerant animal species as to arsenic and show a distinct difference in the metabolism of arsenic from other animals such as hamsters, i.e., arsenic administered to rats preferentially accumulates in RBCs, with a long half-time (*2*, *16*, *17*). It is proposed that the reduction and methylation capacity is the major reason for the animal species difference

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[1] Abbreviations: iAs^{V}, arsenate; iAs^{III}, arsenite; AsB, arsenobetaine; DMA, dimethylated arsenics; DMA^{III}, dimethylarsinous acid; DMA^{V}, dimethylarsinic acid; MMA, monomethylated arsenics; MMA^{III}, monomethylarsonous acid; MMA^{V}, monomethylarsonic acid; TMA, trimethylated arsenic; HPLC, high-performance liquid chromatography; ICP MS, inductively coupled argon plasma mass spectrometry; GSH, glutathione; RBCs, red blood cells.

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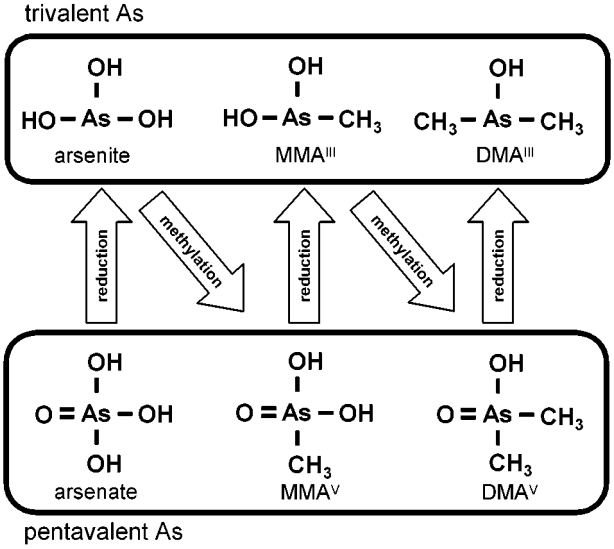
Scheme 1. Reduction and Methylation Reactions in the Metabolic Pathway for Arsenic in Mammals.



in the toxicity and metabolism of arsenic (*2*, *4*). However, additional reasons are required to explain the preferential accumulation of arsenic in RBCs in rats. Our recent results indicated that the uptake of arsenic by RBCs is highly dependent on the animal species in vitro. Namely, DMA^{III} but not DMA^{V} is taken up efficiently by RBCs of rats than of hamsters, mice, and humans (25), suggesting that inorganic aresenics are consecutively reduced and methylated to form DMA in the liver and the DMA^{III} excreted into the bloodstream is taken up by RBCs.

The present study was performed to precisely reveal the metabolic pathway for arsenic administered in the form of arsenite to rats in order to gain an insight into the mechanism underlying the species difference in the metabolism of arsenic. As the first step, the metabolic balance of arsenic injected in the form of iAs^{III} into rats was determined. In addition to determination of the material balance, the chemical species of arsenics in the bile and urine were determined on both anion and cation exchange columns by high-performance liquid chromatography (HPLC)−inductively coupled argon plasma mass spectrometry (ICP MS). The present conditions for the speciation of arsenics in bile demonstrated that iAs^{III}, iAs^{V}, MMA^{III}, MMA^{V}, DMA^{III}, DMA^{V}, and arsenobetaine (AsB) can be detected by means of a single procedure involving two columns with isocratic elution. In addition, it was suggested that iAs^{III} and MMA^{III} can be detected in glutathione (GSH)-conjugated and free forms.

Experimental Procedures

Caution: *Inorganic arsenic compounds have been established to be human carcinogens* (*1*, *19*). *Ingestion of inorganic arsenic may cause cancer of the skin, urinary bladder, kidneys, lungs, and livers, as well as disorders of the circulatory and nervous systems.*

Reagents. All reagents were of analytical grade. Milli-Q SP water (Millipore) was used throughout. Sodium arsenite ($NaAsO_2$) (iAs^{III}), sodium arsenate, dibasic (Na_2HAsO_4 $7H_2O$) (iAs^{V}), dimethylarsinic acid [$(CH_3)_2AsO(OH)$] (DMA^{V}), sulfuric acid, and nitric acid were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Monomethylarsonic acid [$(CH_3)AsO(OH)_2$] (MMA^{V}) was purchased from Tori Chemicals, Ltd. (Yamanashi, Japan). Trizma HCl, Trizma Base and cysteinylglycine were purchased from Sigma (St. Louis, MO). The arsenic standard solution (1000 ppm) for ICP MS was purchased from SPEX CentiPrep (Metuchen, NJ). Arsenobetaine (AsB) was a gift from Professor T. Kaise (Tokyo University of Pharmacy and Life Science, Tokyo, Japan). Sodium disulfite sodium thiosulfate, and other analytical reagents were purchased from Wako Pure Chemical Industries, Ltd. Stock solutions of all arsenic compounds (10 mmol/L) were prepared from the respective standard compounds. All stock solutions were stored in the dark at 0 °C. Dilute standard solutions for analysis were prepared daily prior to use.

Animal Experiments. Male Wistar rats were purchased at 8 weeks of age from a breeder (Clea Japan Co., Tokyo). They were housed in a humidity-controlled room, maintained at 22−25 °C with a 12 h light-dark cycle. The animals were fed a commercial diet (CE−2; Clea Japan Co.) and tap water ad libitum. Following a 1-week acclimation period, the animals, at 9 weeks old and weighing 220−270 g, were used for experiments.

Three rats per group were injected intravenously with sodium arsenite (iAs^{III}), a single dose of 2.0 mg of As/kg of body weight. Immediately before the injection, sodium arsenite (iAs^{III}) was dissolved in saline (Otsuka Pharmaceutical Co., Ltd., Naruto, Japan). The arsenic solutions were adjusted to 1.0 mL/kg of body weight. Control rats were injected with the same volume of saline as that in the experimental group. Rats were sacrificed at 1, 3, 6, and 12 h after the injection.

Bile collection was performed according to Gregus et al. (*26*). The rats were anesthetized with sodium pentobarbital (Dainippon Pharmaceutical Co., Ltd., Osaka) and then the bile duct was cannulated with a polyethylene tube (o.d. 0.61 mm × i.d. 0.28 mm., Natume, Tokyo). The bile collection was started 30 min before the arsenic injection, at the dose of 2.0 mg of As/kg of body weight, and then 30-min-bile was collected up to 300 min after the injection.

Whole Body Perfusion. As arsenic accumulates preferentially in RBCs in rats, the concentrations of arsenic in organs and tissues have to be determined after completely removing blood (RBCs) by whole body perfusion. In the present study, perfusion was performed according to the method developed by Professor H. Yamauchi, St. Marianna University School of Medicine. In brief, rats were injected with 0.2 mL of heparin into the tail vein under sodium pentobarbital anesthesia and then dissected to expose the heart for whole body perfusion. A wing-syringe of 21 guage size was inserted into the left ventricle, and then the heart was pumped full of phosphate-buffered saline (PBS). The right atrium was cut to drain the PBS after confirming string was tight, and then whole body perfusion was performed for 15 min.

Preparation of DMA^{III} from DMA^{V} and MMA^{III} from MMA^{V}. Reduction of DMA^{V} and MMA^{V} was carried out with metabisulfite and thiosulfate as described by Reay and Asher (*27*). Briefly, 0.28 g of sodium metabisulfite was dissolved in 15 mL of 50 mM Tris-buffered saline (TBS, pH 7.4). Then, 2 mL of 1% sodium thiosulfate was added. After the addition of 0.1 mL of concentrated H_2SO_4 to the reaction mixture, 1 vol of the reaction mixture was mixed with 1 vol of a standard DMA^{V} or MMA^{V} solution (100 μmol/L), and then the reaction mixture was incubated at room temperature for 1 h in a tightly capped tube. The reaction mixture was subsequently diluted with each mobile phase for HPLC to a final concentration of 5 μmol/L.

Syntheses of Arsenic Triglutathione [$As(GS)_3$] and Methylarsenic Diglutathione [$CH_3As(GS)_2$]. The synthesis of GSH-conjugated forms of iAs^{III} and MMA^{III} [$As(GS)_3$ and $CH_3As(GS)_2$, respectively] was carried out according to the published procedures (*28*−*31*). Stoichiometric amounts of GSH and the respective arsenic species were mixed, and then the reaction was carried out for 48 h under a nitrogen atmosphere at room temperature. The experimental conditions for each of the arsenic−GSH complexes were as follows: $As(GS)_3$ was prepared by dissolving sodium arsenite (0.129 g; 1 mmol) and GSH (0.921 g; 3 mmol) in 10 mL of degassed water. At the end of the reaction, the $As(GS)_3$ complex was precipitated with 100 mL of

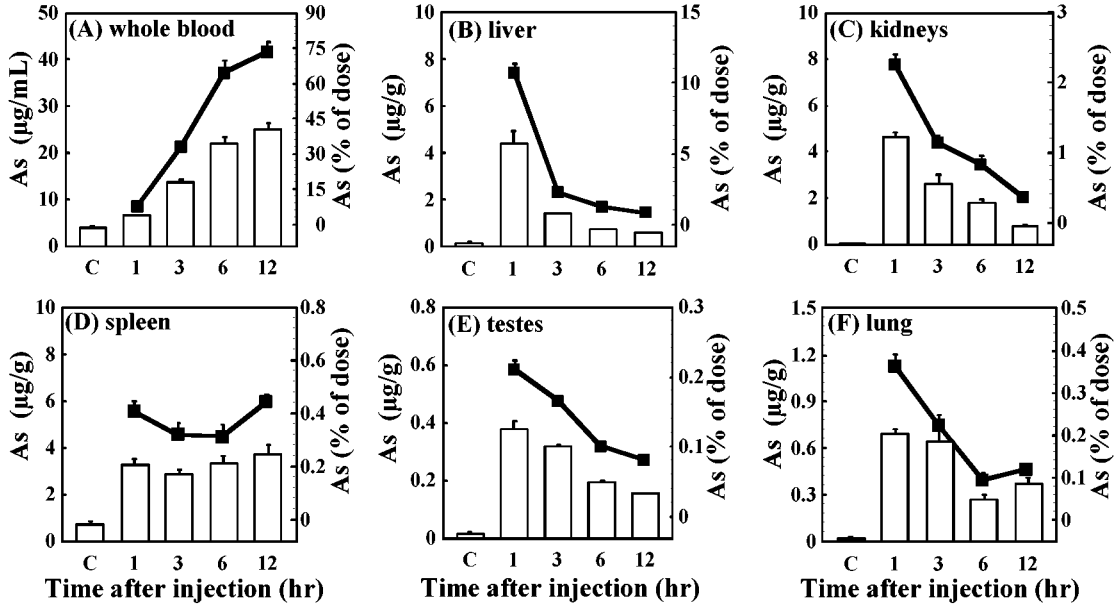


Figure 1. Changes in the concentrations and recoveries of arsenic in the whole blood and five organs of rats after a single intravenous injection of arsenite (iAs^{III}). Male Wistar rats were injected intravenously with iAs^{III} at a single dose of 2.0 mg of As/kg body weight, and sacrificed 1, 3, 6, or 12 h later. The concentrations of arsenic were determined by ICP MS. The concentrations (open column) and recoveries (line graph) of arsenic as to the dose are shown for (A) whole blood, (B) liver, (C) kidneys, (D) spleen, (E) testes, and (F) lungs. In panel A, whole blood is assumed to comprise 7% of the body weight. C denotes control. Data are expressed as means + SE ($n = 3$).

methanol, and the precipitate was filtered and residual water was removed under reduced pressure without heating. Similarly, $CH_3As(GS)_2$ was prepared by dissolving MMA^V (0.056 g, 0.4 mmol) and GSH (0.491 g, 1.6 mmol) in 2 mL of degassed water, the product being precipitated with 40 mL of ethanol, and the precipitate was filtered and residual water was removed under reduced pressure without heating.

Instruments. The HPLC system consisted of a liquid chromatograph solvent delivery pump (PU-610., GL Sciences Co., Tokyo), a DG 660B-2 degasser (GL Sciences Co., Tokyo) and a polymer-based anion exchange column (Shodex Asahipak ES-502N 7C, 100 mm × 7.6 mm i.d., Showa Denko, Tokyo) or a polymer-based cation exchange column (Shodex RSpak NN-614, 150 mm × 6.0 mm i.d., Showa Denko). All the tubing in contact with the mobile phase was made of an inert PEEK material. An ICP MS (HP 4500, Yokogawa Analytical Systems Co., Musashino, Japan) was used as a chromatographic detector. The outlet of the HPLC system was coupled directly (with 300 mm × 0.25 mm i.d. long PEEK tubing) to the inlet of the ICP nebulizer. The signal at *m*/*z* 75 was monitored for arsenic. The signal at *m*/*z* 77 was also monitored to compensate for the isobaric interference by $ArCl^+$. On-line ICP MS data were processed with software developed in house.

Analytical Procedures. The concentrations of arsenic in whole blood, liver, kidneys, spleen, testes, lungs, urine, and bile were determined by ICP MS after wet-ashing with nitric acid and hydrogen peroxide. Aliqouts of 20 μL of the 6-h-urine and 30-min-bile were separately applied to a cation exchange column (NN-614), and the column was eluted with 36 mM formic acid/2 mM ammonium formate buffer (pH 2.81 at 25 °C) at the flow rate of 0.8 mL/min. A 20 μL aliquot of 30-min-bile was also applied to an anion exchange column (ES-502N 7C), and the column was eluted with 15 mM citric acid (pH 2.0 adjusted with nitric acid at 25 °C) at the flow rate of 1.0 mL/min (23).

Results

Changes in the Concentrations of Arsenic in Organs and Body Fluids. The arsenic administered intravenously to rats in the form of iAs^{III} mostly disappeared from the bloodstream within 1 h after the injection, suggesting rapid disappearance of arsenite from the plasma. However, the arsenic concentration in the bloodstream increased with time after 1 h to a high plateau level at 6 h, as shown in Figure 1A, the preferential accumulation of arsenic in RBCs having been reported already by other investigators (*16*, *17*). The arsenic concentration in the bloodstream is high in rats, even in ones fed a normal diet, as shown in Figure 1A, which is attributable to that in RBCs due to the inorganic arsenic contaminating the diet. Likewise, the increased arsenic in the bloodstream after 1 h postinjection was mostly attributable to that in the RBCs, arsenic being marginally detected in the plasma (less than 1 ng/mL plasma) in the present study.

The concentrations of arsenic (μg/g) in the liver (Figure 1B), kidneys (Figure 1C), testes (Figure 1E), and lungs (Figure 1F) were highest at 1 h after the injection, and then decreased with time, showing a different pattern from that in the bloodstream. The arsenic concentration in the spleen (Figure 1D) showed a different pattern from those in other organs and was maintained at a constant level throughout.

The changes in the amounts of arsenic (μg/organ or whole blood) in the organs and whole blood were plotted against time in order to show the recovery relative to the dose in each organ and whole blood. Preferential accumulation of arsenic in RBCs and a rapid decrease of arsenic in organs were observed, as shown in Figure 1, panels A−E.

The amount of arsenic in the 6-h-urine (Figure 2A) indicates that although more than 10% of arsenic was excreted into the urine shortly after the intravenous injection, arsenic was excreted slowly into the urine thereafter. The changes in the urinary excretion of arsenic suggest rapid filtration of iAs^{III} in the glomerulus, followed by its excretion into the urine shortly after the injection. However, arsenic was suggested to be excreted slowly into the urine after arsenic had accumulated in RBCs.

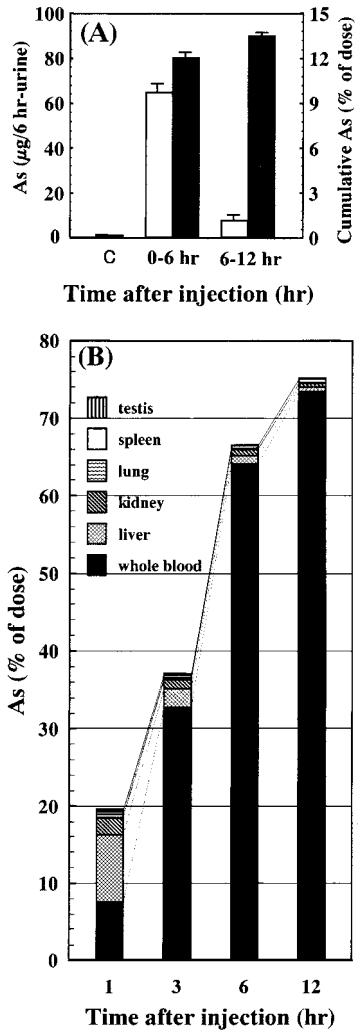


Figure 2. Changes in the summed recovery of arsenic in the five organs and whole blood after a single intravenous injection of arsenite into rats. (A) The amount of arsenic in each 6-h-urine (open column) and cumulative recovery relative to the dose in urine (closed column). (B) The mean amounts of arsenic in the whole blood (assumed to be 7% of the body weight), liver, kidneys, spleen, testes, and lungs shown in Figure 1 were summed up, and the recovery relative to the dose is plotted against time after the injection of arsenite.

The percentile distributions relative to the dose were summed up and plotted against time after the injection, as shown in Figure 2B. Arsenic amounted to only about 20% of the dose in the selected organs and whole blood at 1 h after the injection, indicating the disappearance of arsenic from these five selected organs and whole blood in this period. It is obvious that the amount of arsenic taken up by the liver immediately after the injection decreased with time after the injection, similarly to in other organs. Instead, the amount of arsenic in the whole blood increased rapidly, showing the reappearance of the arsenic that had disappeared from the whole blood (plasma), and the arsenic accumulating in the whole blood at the plateau amounted to more 3-fold of the total arsenic at 1 h.

The rapid disappearance from the plasma and reappearance in the RBCs of arsenic can be explained by the hepato-enteric circulation, and so the biliary excretion of arsenic was then examined together with its chemical speciation.

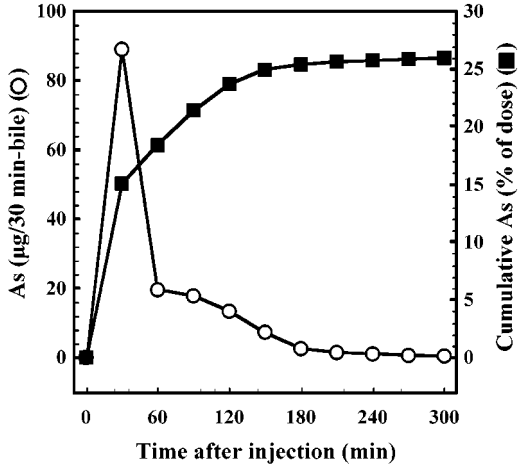


Figure 3. Changes in the biliary amount of arsenic and its recovery as to the dose after a single intravenous injection of arsenite (iAs^{III}) into rats. Male Wistar rats were cannulated for the collection of normal bile for 30 min without injecting iAs^{III}, and then injected with iAs^{III} at a single intravenous dose of 2.0 mg of As/kg body weight, 30-min bile being collected for 300 min. The amounts of arsenic and cumulative recoveries relative to the dose are plotted against time after the injection.

Excretion and Chemical Species of Arsenic in the Bile. Male Wistar rats were cannulated, and iAs^{III} was injected intravenously at the same single dose of iAs^{III} as in Figure 1. The concentration and percentile amount relative to the dose of arsenic were plotted against time after the injection, as shown in Figure 3. A massive amount of arsenic was excreted into the bile within 30 min after the injection, and then the amount decreased gradually with time. The excreted arsenic amounted to approximately 26% of the dose by 5 h after the injection even with continuous collection of bile without feeding of a diet and/or water.

The distribution of arsenic in each 30-min-bile was determined by HPLC−ICP MS on a cation exchange column, as shown in Figure 4A, together with those of authentic samples, as shown in Figure 4B. The six kinds of arsenic species in Scheme 1 were eluted from a cation exchange column in the following order elution: iAs^{V}, MMA^{V}, MMA^{III}, DMA^{III}, iAs^{III}, and DMA^{V}, as shown in Figure 4B, spectra i−iii. Arsenobetaine (AsB) was eluted far more slowly than the six arsenic species at the retention time of 12.5 min. The reduction of DMA^{V} gave one major arsenic peak of DMA^{III} at 4.7 min (Figure 4B, spectrum iii). However, MMA^{V} gave two arsenic peaks for MMA^{III} (Figure 4B, spectrum ii), the major peak at the retention time of 4.3 min being assumed to correspond to MMA^{III} in the bile, as explained below for Figure 4A.

The massive amount of arsenic excreted into the bile within 30 min was shown to be attributable to the major peak at 5.0 min together with the three minor peaks at 3.3, 3.8, and 4.3 min, which correspond to iAs^{III} (major peak), iAs^{V}, MMA^{V} and MMA^{III}, respectively (Figure 4A, 30 min). Although the amount of arsenic in the second and third 30-min-bile samples (Figure 4A, 60 and 90 min) decreased to less than one-fifth of that in the first 30-min-bile, the major arsenic peak was still iAs^{III}, accompanied by the three minor peaks, i.e., iAs^{V} MMA^{V} and MMA^{III}. The two monomethylated arsenic (MMA^{III} and MMA^{V}) peaks increased with time from the beginning to 150 min after the injection (Figure 4A, 30−150 min). At 90 min postinjection, arsenite (iAs^{III}) was no longer

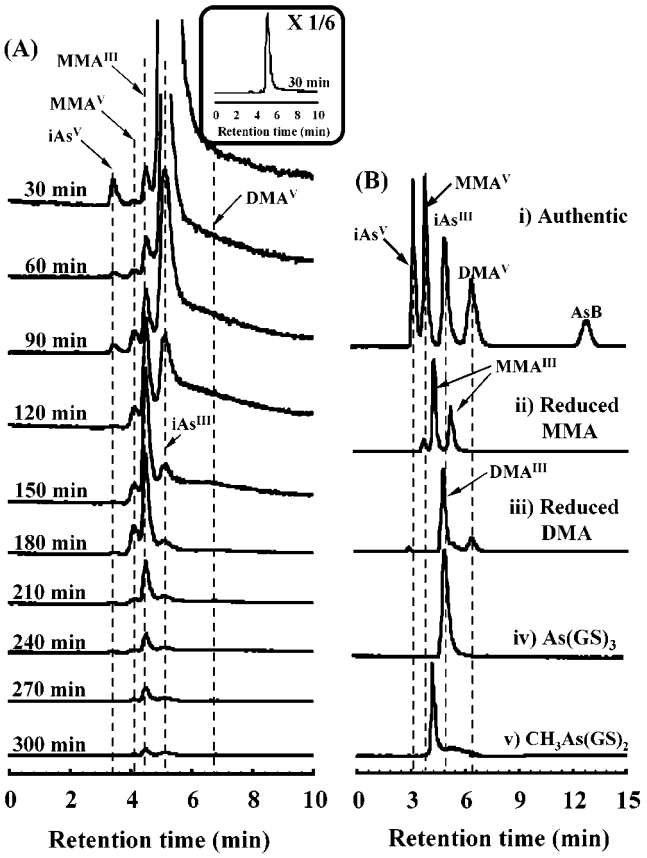


Figure 4. Changes in the distributions of biliary arsenic species with a cation exchange column on HPLC−ICP MS after a single intravenous injection of arsenite (iAs^{III}) into rats. The arsenic species in the bile shown in Figure 3 were assigned based on the retention times of the corresponding authentic samples by HPLC−ICP MS analysis on a cation exchange column (Shodex RSpak NN-614). (A) A 20 μL portion of the 30-min-bile specimens at each time was subjected to HPLC−ICP MS analysis to determine the arsenic distributions. The insert shows the 6-fold reduced distribution of arsenic for the first 30-min-bile. (B) Authentic arsenic samples were subjected to HPLC−ICP MS analysis under exactly the same conditions as those in panel A. (i) Commercially available authentic iAs^V, iAs^{III}, MMA^V, and DMA^V, and known AsB. (ii and iii) Commercially available MMA^V and DMA^V, respectively, were reduced chemically (*27*), and each reaction mixture was diluted 10-fold with 50 mM Tris-HCl buffer, pH 7.4, and then subjected to HPLC−ICP MS analysis. (iv and v) The conjugated forms of iAs^{III} [$iAs^{III}(GS)_3$] and MMA [$CH_3As^{III}(GS)_2$] (*28*−*31*) in the same buffer solution as that in spectra ii and iii were subjected to HPLC−ICP MS analysis.

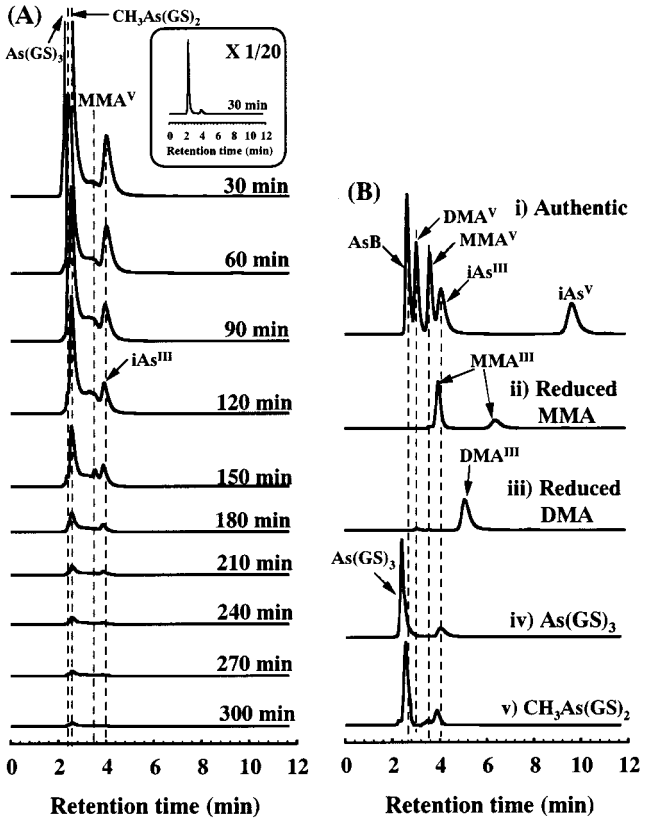


Figure 5. Changes in the distributions of biliary arsenic species with an anion exchange column on HPLC−ICP MS after a single intravenous injection of arsenite (iAs^{III}) into rats. The same 30-min-bile specimens as used in Figure 4 were subjected to HPLC−ICP MS analysis on an anion exchange column (Shodex Asahipak ES-502N 7C). (A) The same bile specimens as used in Figure 4 were subjected to the HPLC−ICP MS analysis to determine the arsenic distributions. The insert shows the 20-fold reduced distribution of arsenic for the first 30-min-bile. (B) Authentic arsenic samples were subjected to HPLC−ICP MS analysis under exactly the same conditions for those in panel A. (i) Commercially available authentic iAs^V, iAs^{III}, MMA^V and DMA^V, and known AsB. (ii and iii), Commercially available MMA^V and DMA^V, respectively, were reduced chemically (*27*), and each reaction mixture was diluted 10-fold with 50 mM Tris-HCl buffer, pH 7.4, and then subjected to HPLC−ICP MS analysis. (iv and v) The conjugated forms of iAs^{III} [$iAs^{III}(GS)_3$] and MMA [$CH_3As^{III}(GS)_2$] (*28*−*31*) in the same buffer solution as that in spectra ii and iii were subjected to HPLC−ICP MS analysis.

the major arsenic peak, MMA^{III} becoming the major one thereafter. DMA^{III} and DMA^V were not detected in the bile at any time point before 5 h after the injection.

The same bile specimens and authentic arsenic species were analyzed on an anion exchange column by HPLC−ICP MS, as shown in Figure 5. The authentic arsenic samples were mostly eluted in the reverse order from an anion exchange column compared with from a cation exchange column, as shown in Figure 5A, spectrum i. AsB was eluted faster than the six arsenics, which were eluted out in the order of DMA^V, MMA^V, MMA^{III} (major peak), iAs^{III}, DMA^{III}, MMA^{III} (minor peak), and iAs^V. The six important arsenic species in the metabolism of arsenic in animals can thus be detected within 10 min on a cation or anion exchange column by HPLC−ICP MS (Figures 4 and 5).

The same bile samples as used for the cation exchange column in Figure 4A were subjected to HPLC−ICP MS analysis on an anion exchange column, as shown in Figure 5B. Apparently two arsenic peaks were detected for the first 30-min-bile. The major one at 2.4 min does not correspond to any of the six authentic arsenics, while the minor one at 4.1 min corresponds to iAs^{III} (Figure 5A, 30 min). The distribution profiles of arsenic in the second to fifth 30-min-bile samples (Figure 5A, 60−150 min) suggest that the major arsenic peak at 2.4 min decreased with time after the injection and another arsenic peak at 2.6 min increased with time, indicating that there are two intense arsenic peaks at 2.4−2.6 min changing in relative intensity. Neither of these arsenic peaks corresponds to any of the six authentic peaks in Figure 5B, spectra i−iii. As the two major arsenic peaks obtained for the bile were assigned as iAs^{III} and MMA^{III} in Figure 4A, the two unknown major arsenic peaks in Figure 5A were assumed to be related to iAs^{III} and MMA^{III}, too. Furthermore, it is assumed that although these two arsenic species were each eluted in the free forms from a cation exchange column, they were eluted

in their original forms without being converted to the free forms under the present analytical conditions. As a result, rational candidates are assumed for the two unknown peaks were the glutathione (GSH)-conjugated forms of iAs^{III} and MMA^{III} because of the bile excretion metabolites.

The GSH-conjugated form of arsenite [$iAs^{III}(GS)_3$] was prepared according to the reported method (*28*−*31*) and was subjected to HPLC−ICP MS analysis on both cation and anion exchange columns, as shown in Figures 4B, spectrum iv, and 5B, spectrum iv, respectively. Although the arsenic prepared as authentic $iAs^{III}(GS)_3$ was eluted at the retention time corresponding to that of the free arsenite (iAs^{III}) peak from a cation exchange column (Figure 4B, spectrum iv), it was eluted as two peaks at 2.4 and 4.1 min from an anion exchange column (Figure 5B, spectrum iv). The former major peak corresponds to the major peak in Figure 5A, while the latter minor peak corresponds to the arsenite (iAs^{III}) peak, suggesting that although $iAs^{III}(GS)_3$ can be eluted as the conjugated form from the present anion exchange column, it is eluted as the free form from the present cation exchange column.

Likewise, the GSH-conjugated form of MMA^{III} [$CH_3As^{III}(GS)_2$] was prepared according to the reported method (*28*−*31*). Although the authentic $CH_3As^{III}(GS)_2$ was eluted at the same retention time as that of MMA^{III} from a cation exchange column, as shown in Figure 4B, spectrum v, it was eluted at a faster retention time of 2.6 min from an anion exchange column, as shown in Figure 5B, spectrum v. The chromatographic behavior of the authentic $CH_3As^{III}(GS)_2$ coincided with that of an unknown peak of arsenic in the bile, suggesting that MMA^{III} is excreted in the GSH-conjugated form and that it is eluted from cation and anion exchange columns in the free and conjugated forms, respectively.

As the conjugated forms of iAs^{III} and MMA^{III} may be present in the bile in the forms conjugated with cysteinylglycine by γ-glutamyl transpeptidase (γ-GTP), the corresponding conjugated forms, $CH_3As^{III}(cysteinylglycine)_2$ and $iAs^{III}(cysteinylglycine)_3$, were also prepared in the similar manner as for $CH_3As^{III}(GS)_2$ and $iAs^{III}(GS)_3$. The conjugated forms with cysteinylglycine were eluted faster than the corresponding forms with GSH, $CH_3As^{III}(cysteinylglycine)_2$ being at 2.5 min as a sharp peak, while $iAs^{III}(cysteinylglycine)_3$ being as a broad peak, indicating the presence of the conjugated forms with GSH in the bile.

Chemical Species of Arsenic in the Urine. The chemical species of arsenic in the two 6-h-urines in Figure 3A were analyzed by HPLC−ICP MS on a cation exchange column, as shown in Figure 6. Four arsenic peaks were detected, the major one being assigned as iAs^{III}, suggesting that approximately 8% of the injected arsenic (arsenite) was excreted into the urine in its intact form immediately after intravenous injection. One of the three minor peaks was assigned as the oxidized form of iAs^{III}, iAs^{V}, and the other two were assigned as MMA^{V} and DMA^{V}. The decreases with time in the amounts of inorganic forms (iAs^{III} and iAs^{V}) and the increases in the methylated forms (MMA^{V} and DMA^{V}) are consistent with the common concept that methylated arsenics, especially DMA^{V}, are the major urinary chemical species (*2*, *9*−*12*).

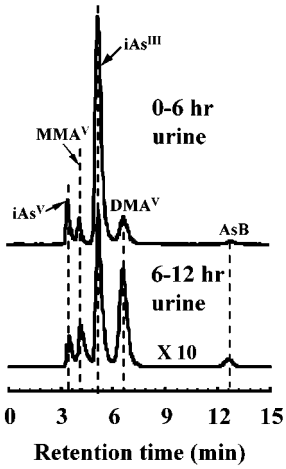


Figure 6. Changes in the distributions of urinary arsenic species with a cation exchange column on HPLC−ICP MS after a single intravenous injection of arsenite (iAs^{III}) into rats. Six-hour-urine collected from the rats shown in Figure 1 was subjected to HPLC−ICP MS analysis on a cation exchange column (Shodex RSpak NN-614) under the same conditions used in Figure 4. The distribution profile of arsenic for the first 6 h-urine is reduced to 10-fold less than that for the second 6 h-urine. The distributions of authentic arsenics are depicted in Figure 4B.

Discussion

The metabolism and toxicity of arsenic are highly dependent on the chemical species ingested and also on the animal species. Although it is well recognized in general that the reduction and methylation capacity is the major contributing factor for the difference in the metabolism (*1*−*5*), the precise mechanisms underlying the animal species difference in the metabolism of arsenic have not been well established. Rats are known to be one of the tolerant animal species as to the toxicity of inorganic arsenic (*2*, *16*, *17*), and the metabolism of arsenic is quite different from in other mammals. Namely, the half-time of arsenic in rats is long, and arsenic preferentially accumulates in RBCs with slow excretion into urine, mostly in the form of DMA^{V} (*9*−*12*). Thus, rats show a distinct difference in the metabolism of arsenic from other animal species, and thus a reasonable explanation in addition to the reduction and methylation capacity for arsenic in rats is required. The present study was carried out to reveal the characteristics of the metabolism of arsenic in rats by applying the speciation method. The present new analytical procedures helped to reveal the chemical species of arsenic in each chemical reaction and the distribution, especially to speciate arsenic in the bile and urine.

As arsenic accumulates rapidly and preferentially in RBCs (Figure 1), the reduction and methylation capacity is considered to be highly important in rats. iAs^{III} is taken up efficiently by the liver, and reduced and methylated there, and then the arsenic is excreted into the bloodstream and preferentially accumulated RBCs (*25*), with some arsenic being excreted slowly into the urine. However, it was revealed that the efficient metabolism of arsenic takes place through the potential hepatoenteric circulation. Furthermore, it was found that a substantial portion of iAs^{III} taken up by the liver is conjugated with GSH, and iAs^{III} is excreted into the bile in the form of GSH-conjugate, $iAs^{III}(GS)_3$. This conjugation and excretion pathway is thought to reduce the acute toxicity of inorganic arsenic by reducing the concentration of reactive iAs^{III} in the liver. As iAs^{III} seems to be retaken up by the liver in the nonconjugated free form, GSH is

assumed to be the vehicle for iAs^{III} in the hepato-enteric circulation.

Although only 26% of the dose was excreted into the bile on cannulation during 300 min, it can be estimated that approximately 70% of the dose was present in the enteric circulation at 1 h after the injection from the material balance shown in Figure 2. Therefore, it is estimated that iAs^{III} gets into the hepato-enteric circulation far more efficiently than expected from the cannulation data in Figure 3. It can be said that more than 70% of the iAs^{III} taken up by the liver initially gets into the circulation. The biological significance for this potential hepato-enteric circulation remains to be explained. However, it was revealed that iAs^{III} is rapidly taken up by the liver and that a major portion of iAs^{III} is conjugated with GSH to form the conjugate $iAs^{III}(GS)_3$, which is then excreted into the bile. The excreted $iAs^{III}(GS)_3$ is transferred to the hepato-enteric circulation, and iAs^{III} is methylated to MMA and DMA in the liver during the circulation. The presence of arsenic in the form of the GSH-conjugate in the bile was also reported recently by other investigators (*31*).

MMA started to be excreted into the bile immediately after the injection in the forms of MMA^{III} and MMA^{V}, the former form being mostly (probably 100%) in the conjugated form and the latter in the free form. The amounts of MMA^{III} and MMA^{V} increased in the bile with time until 150 min after the injection and then started to decrease, reflecting the concentration of MMA in the liver. As the concentration of arsenic in the liver was only about 1% of the dose at 6 h postinjection and 70% of the dose was already present in the RBCs, the change in the biliary excretion of MMA is reasonably explained. However, it should be noted that although MMA^{V} was excreted into the bile in the form of free ions, as in the case of iAs^{V}, MMA^{III} was excreted in the conjugated form, $CH_3As^{III}(GS)_2$, as in the case of inorganic arsenic, iAs^{III}. Alternatively, it may be possible that only conjugated forms of iAs^{III} and MMA^{III} are excreted into the bile and their free forms including their pentavalent forms are artifact during the analytical procedures.

Thus, inorganic and monomethylated arsenics were detected in the bile in the free and conjugated forms of both tri- and pentavalent arsenics. However, DMA was not detected in any form in the bile, suggesting that DMA is not excreted into the bile and hence not into the feces, at least under the conditions on injection of arsenite. It has not been explained why inorganic arsenics and MMA are excreted but DMA is not excreted into the bile. It should also be explained why both the free and conjugated forms of iAs^{III} and MMA are excreted into the bile.

Arsenic is known to be excreted into the urine mostly in the form of DMA (*2*, *9*−*12*). However, it was reported recently that not only inorganic arsenics, pentavalent DMA and MMA, but also trivalent DMA and MMA are detected in the urine of people drinking arsenic tube-well water (*23*, *24*, *32*, *33*). In the present study on rats, urinary arsenic was detected in four forms, iAs^{III}, iAs^{V}, MMA^{V}, and DMA^{V}. iAs^{III} is the major form up to 12 h after the injection, certainly because of the intravenous injection of iAs^{III} and early collection time of urine after the injection. The rapid increase in the DMA peak for the second 6-h-urine and the preferential accumulation of arsenic in RBCs suggest an increase in DMA as the major urinary metabolite after 12 h postinjection. iAs^{III} was mostly present in the nonconjugated form in the urine (data not shown). Although it has to be examined precisely, MMA^{III} and DMA^{III} were not detected at detectable levels in the urine of rats. As arsenic is assumed to be mostly present in the form of DMA in RBCs, excretion of arsenic into the urine in the form of MMA later than 12 h is not possible. In fact, in separate experiments, urinary arsenic in rats was detected in the form of only DMA^{V} in addition to the dietary origin of arsenic, arsenobetaine (AsB). Thus, in rats, arsenic is thought to be excreted into urine in the form of DMA even when arsenic is accumulating heavily in the body, reflecting the accumulation of arsenic in RBCs in the form of DMA.

Summarizing the present observations, arsenic injected with intravenous iAs^{III} is effectively taken up by the liver, and a substantial portion of iAs^{III} is conjugated with GSH in the liver and gets into the hepato-enteric circulation, while the potential reduction and methylation capacity of the liver further metabolizes iAs^{III} to methylated arsenics during the circulation. Among methylated arsenics in the liver, MMA^{III} and MMA^{V} are partly excreted into the bile in completely conjugated and nonconjugated forms, respectively. DMA was not detected in any form in the bile. Arsenic accumulates in the RBCs after being consecutively reduced and methylated to DMA in the liver during the circulation. Although arsenic is excreted into the urine in the forms of nonmethylated iAs^{III} and iAs^{V}, and methylated MMA^{V} and DMA^{V} at an early time after the injection, the major urinary form is DMA^{V} after the acute phase.

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