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Determination of trivalent methylated arsenicals in rat urine by liquid chromatography–inductively coupled plasma mass spectrometry after solvent extraction

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Abstract

A method for the determination of trivalent arsenicals in urine was examined. Trivalent arsenicals, extracted as complexes with diethylammonium diethyldithiocarbamate (DDDC) into carbon tetrachloride, were determined by liquid chromatography–inductively coupled plasma mass spectrometry (LC–ICP-MS). The trivalent methylated arsenicals monomethylarsonous acid (MMA(III)), dimethylarsinous acid (DMA(III)), and trimethylarsine (TMA) were detected in urine of rats that had received dimethylarsinic acid (DMA(V)) or monomethylarsonic acid (MMA(V)) at concentration of 200 μg ml^{-1} in drinking water for 24 weeks. This method is the first to permit quantification of trivalent methylated arsenicals in urine without significant changes in concentration during storage or pretreatment.

Keyword: Trivalent methylated arsenicals

1. Introduction

Arsenic is widely distributed in the environment in a variety of chemical forms. For humans, one of the major sources of arsenic is the ingestion of drinking water contaminated with inorganic arsenic [1]. In most mammals, inorganic arsenic is methylated to the organic pentavalent arsenicals monomethylarsonic acid (MMA(V)), dimethylarsinic acid (DMA(V)), and trimethylarsine oxide (TMAO) and then excreted in the urine [2–5]. In humans, DMA(V) is the endpoint of the metabolism of arsenic [6]. Although arsenic has been shown to be a human carcinogen in numerous epidemiological studies [7], there had been no report on the carcinogenicity of arsenic in animal models. Recently, however, Wei et al. [8] reported that DMA(V) caused cancer in the urinary bladder of rats. Waalkes et al. [9] also reported that inorganic arsenic is a transplacental carcinogen in mice.

The metabolic pathway of arsenic involves two steps, reduction and oxidative methylation [3,10] (Fig. 1). Although methylation has been regarded as a detoxification process, in recent years this interpretation has been questioned. It was reported that the organic trivalent species monomethylarsonous acid (MMA(III)) and dimethylarsinous acid (DMA(III)), which are intermediates of metabolism, are much more cytotoxic than the corresponding pentavalent arsenicals [11–13]. Cohen et al. [13] showed that the LC_{50} (μM) values for human bladder cells of MMA(III), MMA(V), DMA(III), and DMA(V) are 1.0, 1700, 0.8, and 500, respectively. Mass et al. [14] also showed that MMA(III) and DMA(III) exhibit genotoxic effects by directly damaging DNA.

These trivalent methylated arsenicals, MMA(III) and DMA(III), have been recently confirmed in the urine of humans exposed to arsenic in drinking water [15–20]. There has since been increasing interest in the determination of the trivalent methylated arsenicals in biological samples, as this may yield information on the mechanism of arsenic-induced carcinogenicity. However, it is difficult to quantify these trivalent methylated arsenicals in biological

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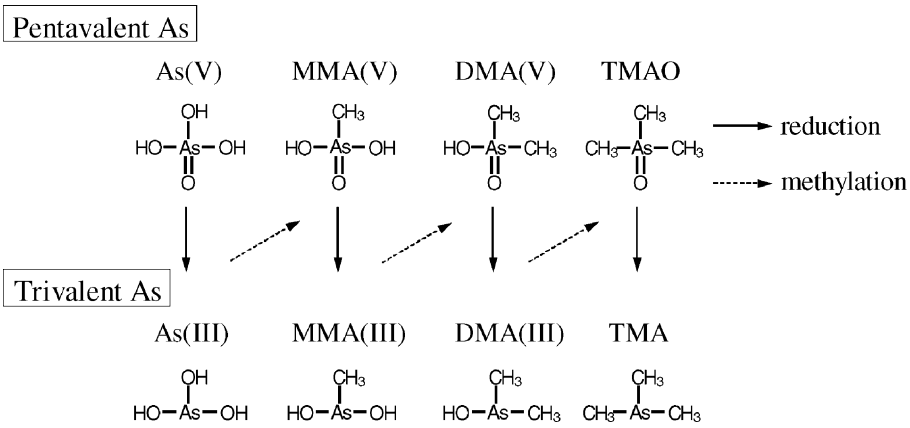

Fig. 1. The biotransformation pathway for arsenic.

samples, since they are unstable and readily oxidized during sample pretreatment or storage [19,21]. Urine samples collected are usually stored for several weeks or more before analysis. Therefore, it is conceivable that the original distribution of species in the sample is lost. It thus is necessary to develop methods for determination of arsenicals in samples without altering their original oxidation state. Hasegawa et al. [22] developed a method which separates trivalent arsenicals from pentavalent species by solvent extraction using diethylammonium diethyldithiocarbamate (DDDC), and determined trivalent arsenicals in natural water by hydride generation atomic absorption spectrometry after chromatographic separation.

To date, several procedures have been developed and documented for speciation of arsenic [15–29]. Of the numerous methods reported, liquid chromatography–inductively coupled plasma mass spectrometry (LC–ICP-MS) with high sensitivity and accuracy is the most effective for determination of urinary arsenic metabolites [20,23–26,28,29].

The purpose of this study was to determine trivalent arsenicals in urine at the native (point of sampling) concentration. We combined separation by solvent extraction using DDDC with determination by LC–ICP-MS for this purpose. We report optimization of a method for determination of trivalent arsenicals and application of this method to urine samples.

2. Experimental

2.1. Reagents

Sodium arsenite, sodium arsenate, MMA(V), DMA(V), TMAO, arsenobetaine (AsBe), and tetramethylarsonium iodide, used for analytical standard solutions, were purchased from Tri Chemical Laboratory (Yamanashi, Japan). Iododimethylarsine (DMA(III)I) was obtained from Dr. Cullen (University of British Columbia, Vancouver, Canada).

Water purified with a Milli-Q system (Millipore, Tokyo, Japan) was used throughout. All reagents were of analytical-reagent grade unless otherwise specified. HNO_3 was of electronics laboratory (EL) grade, and acetic acid and carbon tetrachloride (CCl_4) were of atomic absorption spectrometry (AAS) grade (Kanto Chemical, Tokyo, Japan). DDDC was obtained from Tokyo Chemical Industry (Tokyo, Japan). Sodium acetate was obtained from Kanto Chemical. Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA) was obtained from Dojin (Kumamoto, Japan). The other reagents were obtained from Wako Pure Chemical Industry (Osaka, Japan). The 0.2 mol l^{-1} acetic acid–sodium acetic buffer was adjusted to the desired pH by mixing 0.2 mol l^{-1} acetic acid and 0.2 mol l^{-1} sodium acetic solution. DDDC solution, EDTA solution, and acetic acid–sodium acetic buffer were purified just before use.

2.2. Animals and urine samples

Six-week-old male and female F344/DuCrj rats were purchased from Charles River Japan (Hino, Japan). Male and female rats were separately housed in a box cage with wood-chip bedding at a room temperature of 23 ± 1 °C with 12 h light/dark cycle. The animals were provided with a standard diet (CE2, Clea Japan, Tokyo, Japan) and water ad libitum. After a 1-week acclimatization period, they received DMA(V) or MMA(V) at concentrations of 200 μg ml^{-1} in drinking water. Urine was collected forcibly by pressing the bladder area of a rat at the end of 24 weeks. All urine samples were centrifuged at 6000 rpm for 10 min. On the day of collection, the supernatants were treated as described below. Afterward, specimens were stored at −80 °C and were determined within 1 week.

2.3. Instrumentation

A model HP4500 ICP-MS (Hewlett-Packard, DE, USA) was used for arsenic-specific detection. A model IC 7000 (Yokogawa Analytical Systems, Tokyo, Japan)

was used for separating arsenic species. Chromatographic separations were performed on a Shodex RSpak NN-614 cation-exchange column (6.0 mm × 150 mm i.d., 10 μm, Showadenko, Tokyo, Japan) with a mobile phase (0.8 ml min^{-1}) of 5 mM HNO_3–6 mM NH_4NO_3; or on an Excelpak ICS-A13 anion-exchange column (4.6 mm × 75 mm i.d., 6 μm, Yokogawa Analytical Systems) with a mobile phase (0.8 ml min^{-1}) of 3 mM NaH_2PO_4 at pH 6.0 with 1 mol l^{-1} NaOH. A guard column of the same packing type was used preceding the analytical column. An injection valve with a 50 μl injection loop was used for sample introduction, and separation was carried out at room temperature. The solution eluted from the column was introduced on-line to ICP-MS using an ETFE (ethylenetetrafluoroethylene) tube of 0.3 mm i.d.

2.4. LC–ICP-MS

Measurements using LC–ICP-MS were performed by the method of Inoue et al. [24] with a slight modification. Working standard solutions (1 μg As ml^{-1}) were prepared daily from these stock solutions (100 μg As ml^{-1}) by dilution with water. The samples were injected on the LC column after filtering through 0.45 μm membrane filter. A 1 μg ml^{-1} germanium solution was used as the internal standard for ICP-MS; it was added to the eluate from LC through a mixing joint prior to introduction to the ICP mass spectrometer. Signals at *m/z* 75 ($^{75}As^+$), *m/z* 72 ($^{72}Ge^+$), and *m/z* 77 ($^{40}Ar^{37}Cl^+$) were monitored. The signal at *m/z* 77 was monitored for interference by $^{40}Ar^{35}Cl^+$. Arsenic species in samples were identified by matching the retention time with the arsenic standards.

2.5. Separation and determination of trivalent and pentavalent arsenicals spiked in water for extraction efficiency study

The method for separation of trivalent and pentavalent species was based on that developed by Hasegawa et al. [22]. After 2 μg of an arsenic standard solution was prepared in a 2.0 ml tube, 0.01 mol l^{-1} DDDC in 0.2 mol l^{-1} acetic acid–sodium acetate buffer was added to adjust the final volume to 400 μl. Then, 400 μl of 0.01 mol l^{-1} DDDC in CCl_4 was added and the mixture was vigorously shaken for 5 min. After centrifugation and separation of the organic phase, the aqueous phase was again extracted with 0.01 mol l^{-1} DDDC in CCl_4, and the organic phases were combined. A portion of the aqueous phase was fractioned into another tube and diluted with water. The concentration of pentavalent arsenicals in the solution was determined by LC–ICP-MS. The organic phase containing trivalent arsenicals was back-extracted with 400 μl of NaOH for 5 min. After centrifugation, the organic phase was separated from the aqueous phase and transferred to another tube. This stage was repeated once more and the back-extraction phases were combined. An aliquot of the aqueous phase was fractioned into a tube and adjusted to 3% hydrogen peroxide (H_2O_2) by adding 30% H_2O_2. Subsequently, the solution in the tube was heated at approximately 80 °C for 1 h. After cooling to room temperature, 1.0 mol l^{-1} HNO_3 was added to neutralize the solution. Then, the solution was diluted with water and the concentration of trivalent arsenicals was determined by LC–ICP-MS.

2.6. Analysis of arsenic species in urine

Additional improvements were made in the procedure for extraction of trivalent arsenicals in DMA(V)- or MMA(V)-exposed rat urine. EDTA was added as a masking agent. A 100 μl portion of urine sample, 20 μl of 0.1 mol l^{-1} EDTA, 260 μl of 0.2 mol l^{-1} acetic acid–sodium acetate buffer and 20 μl of 0.2 mol l^{-1} DDDC were placed in a 2.0 ml tube in that order. The solution was then extracted with 400 μl of CCl_4 twice. The subsequent analytical procedures were the same as those described in Section 2.5.

3. Results and discussion

3.1. Optimization of analytical method for trivalent arsenicals

Of the standard reagents of trivalent arsenicals, only that of arsenite (As(III)) has sufficient purity. In addition, As(III) is most difficult to extract; As(III) was quantitatively extracted in a narrower pH range than MMA(III) and DMA(III), and in extraction experiment recovery of As(III) was lower than that of MMA(III) and DMA(III) [22,30]. Therefore, we selected sodium arsenite as a trivalent standard arsenical to use in optimizing the method of analysis for trivalent arsenicals.

The effect of pH was studied on extraction of As(III). Recovery of As(III) was measured over a pH range of 3.0–8.0. A constant and maximum recovery (about 95%) was obtained in the pH range 3.0–5.0 and the recovery decreased as the pH of the buffer increased beyond 5.0. The effect of NaOH concentration on back-extraction was also studied. Recovery of As(III) was measured over a NaOH concentration range of 0.1–1.0 mol l^{-1}. Recovery was about 90% at all concentrations at or above 0.4 mol l^{-1}.

Hasegawa et al. [22] and Sampayo-Reyes et al. [30] observed that trivalent arsenicals were partly oxidized after back-extraction. In our study, As(III) in aqueous phase after back-extraction was partially oxidized to arsenate (As(V)) (Fig. 2A). In addition, an unknown peak appeared with a retention time (872 s) after that of As(V) in the chromatogram (Fig. 2A). This peak appeared to be due to the DDDC–As(III) complexes. On the other hand, when H_2O_2 was used as oxidizing agent, the peak disappeared (Fig. 2B). For these two reasons, trivalent arsenicals in aqueous phase after back-extraction were oxidized to pentavalent arsenicals using H_2O_2. Because an oxidizing agent

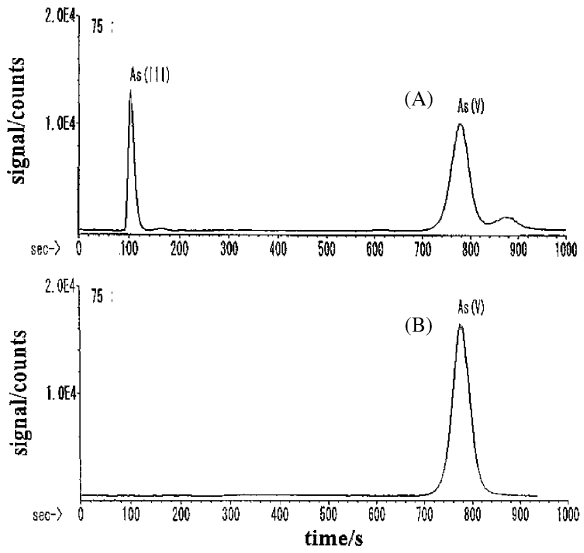

Fig. 2. Effect of H_2O_2 on determination of As(III). Chromatograms obtained from the LC–ICP-MS analyses of As(III) in aqueous phase after back-extraction: (A) without H_2O_2; (B) with H_2O_2. Chromatographic conditions were Excelpak ICS-A13 column at room temperature with a mobile phase of 3 mM NaH_2PO_4 at pH 6.0 with 1.0 mol l^{-1} NaOH; flow rate was 0.8 ml min^{-1}; 50 μl injection.

cannot be injected onto the LC column, the back-extracted aqueous phase was heated to 80 °C to decompose H_2O_2. The effect of heating time on recovery was examined in order to evaluate arsenic loss by heating. Recovery was found to be constant independent of heating time (data not shown).

3.2. Recovery test

To confirm separation and determination of both trivalent and pentavalent species, the recoveries of As(III) and As(V) were assessed by analyzing four replicates of the solutions containing 2 μg of each standard solution of As(III) and As(V). Mean recoveries of As(III) and As(V) were 91.3 and 92.2%, respectively, and relative standard deviations (R.S.D.s) were 3.3 and 6.1%, respectively.

Recovery tests for DMA(III) were also carried out. A standard solution of DMA(III) was prepared by dissolving DMA(III)I in deoxidized water. The amount (as arsenic) added was not precisely fixed, for available DMA(III)I included impurities. We determined recovery by calculating the percentage of the amount of DMA(III) in the aqueous phase after back-extraction to the total amount of that in both the aqueous phase after extraction and the aqueous phase after back-extraction, since arsenic species were negligible in quantity in the organic phase after back-extraction. The effect of pH was studied on extraction of DMA(III). Recovery was measured over a pH range of 3.0–8.0. Recovery nearly leveled off at pH 3.0–8.0, with only a small tendency toward decrease observed. Recovery of DMA(III) was above 91% at every pH tested.

3.3. Analysis of urine samples

The determination of urine was carried out with separation using cation-exchange LC, because anion-exchange LC could not resolve AsBe and tetramethylarsonium (TeMA), which are known to be present in rat urine samples [5], from TMAO.

We first attempted the extraction from urine samples using the procedure described above. However, we could not obtain good recoveries with it. Because urine includes some metal ions (iron, copper, zinc, etc.) [31], it was possible that they interfered with the chelation of trivalent arsenicals and DDDC. EDTA, which is generally used as an effective masking agent, was added to the urine samples to solve this problem. EDTA forms stable chelates with foreign ions, while it does not chelate arsenic. In general, EDTA forms more stable chelates with metal ions at higher pH. Dojozan et al. [32] masked some contaminant metal ions by EDTA at pH 5.5 and obtained high extraction efficiency. We therefore changed the pH of the buffer for extraction to 5.0, and obtained good recoveries of trivalent arsenicals from urine samples with the addition of 20 μl of 0.1 mol l^{-1} EDTA.

We needed to purify the reagents used in the extraction process, especially DDDC solution, since our preliminary experiments showed that DDDC contained a small amount of arsenic as an impurity. Although DDDC aqueous solution could be purified by repeated extraction with CCl_4, we could not purify DDDC dissolved in CCl_4. Therefore, to keep the reagent blank value as low as possible, CCl_4 alone (without the addition of DDDC) had to be used for extraction. We obtained satisfactory recoveries with this extraction (data not shown). We therefore used CCl_4 for extraction of trivalent arsenicals in the urine samples. Because the effects of the reagent blank due to reagents cannot be ignored in trace analysis, we examined contamination of arsenic from reagents in all stages of the extraction procedure and subtracted the reagent blank values from the final results.

A flow chart of the procedure for determination of trivalent and pentavalent arsenicals in urine is shown in Fig. 3. Representative chromatograms are shown in Figs. 4 and 5.

3.4. Linearity

Standard samples between 0.1 and 100 μg As ml^{-1} of As(III) in urine were analyzed in triplicate to determine the linearity of the method. Calibration curve showed good linearity ($r = 0.9989$) over the range of concentrations studied, with an equation of $y = 0.0209$ (±S.E. 0.0003)$x - 0.0015$ (±S.E. 0.0136) (x, concentration of As(III); y, peak area ratio of As(III) to internal standard).

3.5. Reproducibility

The reproducibility of the present method was studied. The intra-day reproducibility was determined by analyzing

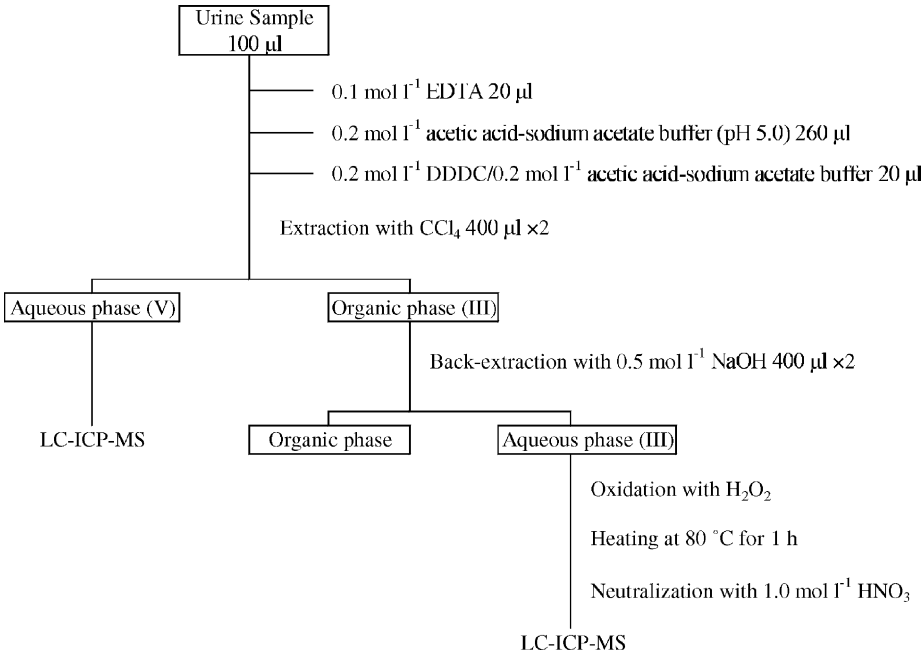

Fig. 3. Flow chart of determination of trivalent and pentavalent arsenic species in urine.

four specimens of As(III) spiked urine sample on the same day. The inter-day reproducibility was determined by analyzing two specimens of As(III) spiked urine sample on four separate days. The intra- and inter-day reproducibility were 5.7 and 6.3%, respectively.

3.6. Sensitivity and recovery

The limits of detection were calculated according to the ratio of the signal peak to the noise peak (S/N). The limits of detection of the method were 0.24 ng As ml^{-1} (As(V)), 0.48 ng As ml^{-1} (MMA(V), DMA(V)), 0.96 ng As ml^{-1} (AsBe), and 1.92 ng As ml^{-1} (TeMA, TMAO) for 100 μl urine samples (S/N = 3).

When 0.02 or 2 μg of As(III) standard solution was added to 100 μl of a urine sample, mean recoveries were 79.1 and 88.0%, and R.S.D.s were 7.7 and 2.1% (four measurements each).

3.7. Arsenic species in urine samples

Three trivalent methylated arsenicals, MMA(III), DMA(III), and trimethylarsine (TMA), were detected in the back-extracted aqueous phase of urine samples from DMA(V)- or MMA(V)-exposed rat (Fig. 4). Concentrations of these trivalent arsenicals are shown in Table 1. MMA(V), DMA(V), TMAO, TeMA, and AsBe were detected in the aqueous phase remaining after extraction of trivalent arsenicals (Fig. 5). In addition, unknown arsenic species (M-1, M-2, and M-3), which were detected in rat urine and feces after long-term oral administration of DMA(V) [5,33], were detected in the same aqueous phase (Fig. 5).

In this study, DMA(III) and MMA(III) were detected in urine of arsenic-exposed rats (Table 1). Cohen et al. [13] detected DMA(III) at concentrations of approximately 1 μmol l^{-1} in fresh urine collected from female rats treated with DMA(V) (100 μg/g of the diet). They reported that this concentration was greater than the LC_{50} for cytotoxicity of DMA(III) for rat and human urothelial cells in vitro. The concentrations of MMA(III) and DMA(III) determined in the present study were also higher than each of the LC_{50}s for cytotoxicity reported by them. We consider it quite likely that DMA(III) and MMA(III) in urine play roles in the toxic or carcinogenic effects on urinary bladder of DMA(V) shown by Wei et al. [8], Cohen et al. [13] and Wanibuchi et al. [34].

Notably, we found TMA in rat urine (Table 1), though there had been no previous report of detection of TMA in urine of arsenic-exposed animals. However, it appears that

Table 1
Trivalent arsenicals found in aqueous phase after back-extraction of DMA(V)- or MMA(V)-exposed rat urine[a]

Treatment	Sex	Arsenic concentration (μg As ml^{-1})		
		MMA(III)	DMA(III)	TMA
DMA(V)	Male	0.016	0.137	0.096
DMA(V)	Male	0.014	0.190	0.218
DMA(V)	Female	0.006	0.259	0.416
DMA(V)	Female	0.004	0.249	0.330
MMA(V)	Male	0.071	0.052	0.030
MMA(V)	Male	0.105	0.042	0.028
MMA(V)	Female	0.236	0.094	0.042
MMA(V)	Female	0.271	0.095	0.053

[a] Single measurement each.

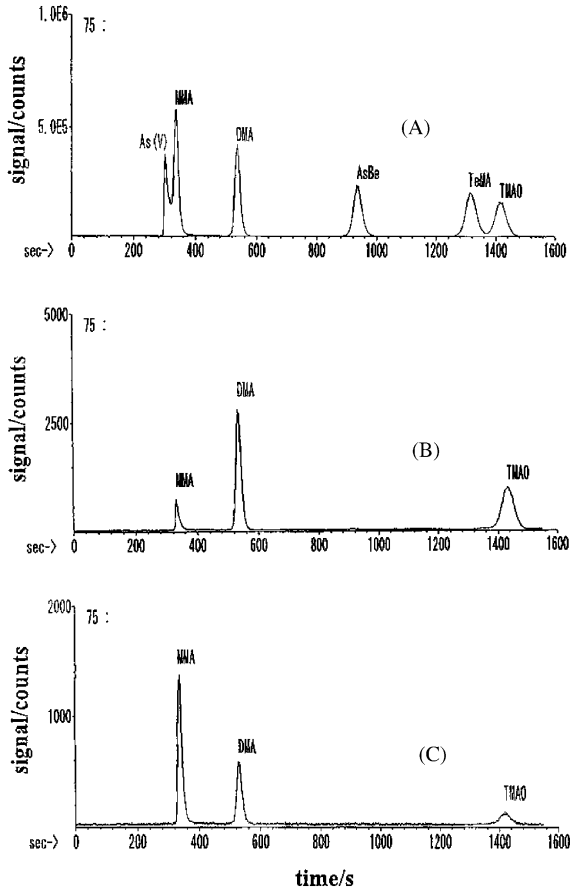

Fig. 4. LC–ICP-MS chromatograms of (A) mixtures (1 μg As ml^{-1} each) of six standard arsenic species, (B) trivalent arsenicals in urine from DMA(V)-exposed rat, and (C) trivalent arsenicals in urine from MMA(V)-exposed rat. Chromatographic conditions were Shodex RSpak NN-614 column at room temperature with a mobile phase of 5 mM HNO_3–6 mM NH_4NO_3; flow rate was 0.8 ml min^{-1}; 50 μl injection.

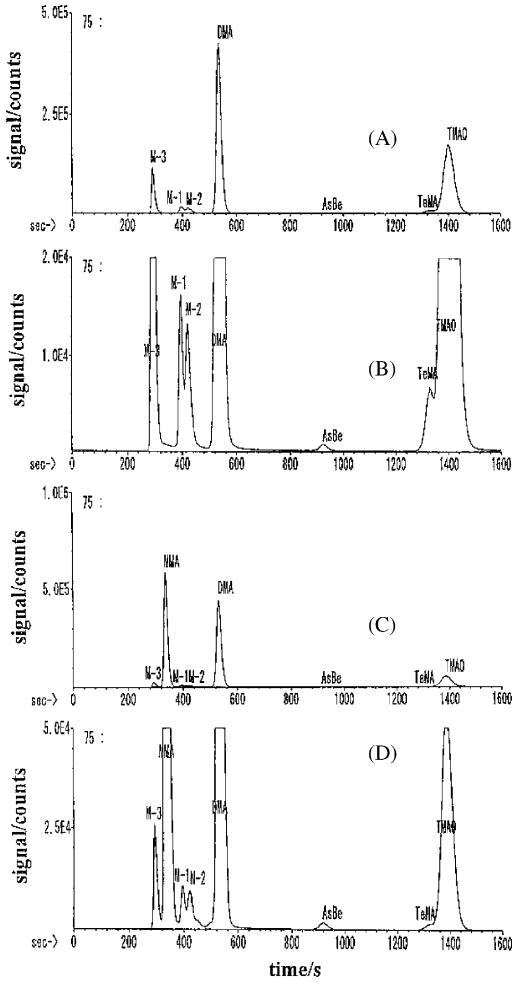

Fig. 5. LC–ICP-MS chromatograms of arsenic species found in aqueous phase after extraction of urine of arsenic-exposed rats: (A) urine from DMA(V)-exposed rat; (B) an expanded scale of chromatogram (A); (C) urine from MMA(V)-exposed rat; (D) an expanded scale of chromatogram (C); M-1, M-2, and M-3 represent unknown arsenic species. Chromatographic conditions were Shodex RSpak NN-614 column at room temperature with a mobile phase of 5 mM HNO_3–6 mM NH_4NO_3; flow rate was 0.8 ml min^{-1}; 50 μl injection.

TMAO is further reduced to TMA in vivo [3,35]. In addition, the present study demonstrated the presence of TeMA, a subsequent metabolite of TMAO, suggesting that TMA is probably present in urine as an intermediate in the process of metabolism. It is thus not surprising that TMA was detected in urine.

The level of the intermediate trivalent species in the methylation pathway was higher in female rats than in male rats (Table 1). van Gemert and Eldan [36] reported that female rats were more susceptible than males to the proliferative effects on bladder following feeding of DMA(V). The difference in the level of trivalent arsenicals between males and females observed in our study might be related to that in the susceptibility reported by them.

3.8. Stability of trivalent arsenicals in urine during pretreatment and storage

No method has previously been available to accurately determine trivalent methylated arsenicals without changes in concentration from the time of sampling, since pretreatment and storage often alter the species present in the original sample [19,21]. Because our method can separate trivalent arsenicals from pentavalent species immediately after sample collection, native concentration of trivalent arsenicals in samples can be preserved. In addition, to study the stability of the trivalent arsenicals extracts during storage, trivalent arsenicals extracts were reanalyzed after storage as back-extracted NaOH solutions at −80 °C for 1 month. There was no difference between the values determined immediately after extraction and those determined after storage in concentration of trivalent arsenicals (data not shown). Our method thus is to be effective for quantification of trivalent arsenicals in urine without significant changes in concentration during pretreatment and storage.

4. Conclusion

A method based on separation by solvent extraction using DDDC was demonstrated to accurately determine trivalent arsenicals in urine samples. With this method, three trivalent methylated arsenicals, MMA(III), DMA(III), and TMA, were detected in rat urine. In conclusion, the method described in this study permits quantification of all arsenicals in urine including trivalent arsenicals without significant changes in concentration.

The present method can separate trivalent arsenicals from pentavalent species immediately after sample collection, and store individually trivalent and pentavalent arsenicals in samples. Therefore, native concentration of trivalent arsenicals in samples can be preserved even if it is a long time before the trivalent arsenicals are determined. This method will be applicable to speciation of arsenic containing trivalent arsenicals in urine from arsenic-exposed individuals.

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