APPLIED ORGANOMETALLIC CHEMISTRY
Appl. Organometal. Chem. 2001; **15**: 271–276
DOI: 10.1002/aoc.138

Metabolites of arsenobetaine in rats: does decomposition of arsenobetaine occur in mammals?†

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Biotransformation following oral administration of arsenobetaine (AsBe), the major arsenic compound in marine animals, was studied in rats. Male F344/DuCrj rats were administered a single dose of AsBe (20 mg As kg^{-1}) orally. Urine was collected at 0, 2, 4, 6, 8, 10, 12, 24, 48, 72, and 96 h after administration by forced urination. Arsenic metabolites in urine were analyzed by ion chromatography with inductively coupled plasma mass spectrometry. Urinary elimination of AsBe, trimethylarsine oxide (TMAO), dimethylarsinic acid (DMA), methylarsonic acid (MMA), tetramethylarsonium (TeMA), an unidentified arsenic metabolite, arsenate, and arsenite was determined at various time points after administration. Unmetabolized AsBe was the most common form. Most elimination of unchanged AsBe occurred within 48 h, with peak elimination between 0 and 2 h. A small portion of administered AsBe was eliminated as TMAO and TeMA, with peak elimination between 0 and 2 h. Elimination of the unidentified metabolite, MMA, DMA, and inorganic arsenic occurred later and to a slight extent. A delay in elimination of the unidentified compound, MMA, DMA, and inorganic arsenic compared with that of TMAO suggests that the former compounds may be formed from TMAO. Degradation of AsBe by an intestinal bacterium, *Escherichia coli*, did not occur in rats. These results suggest that TMAO may be formed from AsBe, and that TMAO may subsequently be converted to the unidentified compound and demethylated arsenic compounds.

Keywords: arsenobetaine; arsenic; metabolism; urine; rats; degradation; demethylation; microorganism; trimethylarsine oxide

Received 9 December 1999; accepted 7 August 2000

INTRODUCTION

Several different organic compounds have been reported to be present in seafood, e.g. arsenosugar,[1–3] arsenocholine,[4,5] and arsenobetaine (AsBe).[4–7] Repeated ingestion of seafood over a lifetime can result in significant intake of arsenic, since most seafood contains high levels of arsenic.[8,9] Studies of the metabolism and toxicity of seafood arsenic are therefore needed. Since Edmonds *et al.*[6] identified AsBe in western rock lobster tail, AsBe has been shown to be the major arsenic compound in lobsters, shrimps, crabs, flounders, sharks, and octopus.[5,7,10] Although relatively abundant literature is available on the toxicity or metabolism of inorganic arsenic, findings on the toxicity and metabolism of AsBe are quite limited. The toxicity of arsenic compounds is known to depend on their chemical form. Inorganic arsenic, monomethylarsonic acid (MMA), and dimethylarsinic acid (DMA) are generally considered to be toxic forms of arsenic, but the organic arsenic compounds in seafood have less acute toxic

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† Based on work presented at the Ninth Symposium of the Japanese Arsenic Scientists' Society (JASS-9), held 21–22 November 1999 at Hiroshima, Japan.

effects than inorganic arsenic and methylated arsenic compounds. The LD_{50} value of AsBe in mice is more than 10 g kg^{-1}.[10,11] Orally administered AsBe is almost completely absorbed from the gastrointestinal tract and excreted unchanged via the urine in rats, mice, rabbits and hamsters.[12,13] There was no evidence that decomposition of AsBe to trimethylarsine oxide (TMAO) occurs in mammals,[12,13] whereas decomposition of AsBe to TMAO, DMA, and inorganic arsenic by microorganisms has been reported.[14–16] However, these studies of the metabolism of AsBe in mammals were performed after a single exposure. Little is known concerning the metabolism of AsBe after long-term exposure. Information on metabolism after long-term exposure to AsBe is vital for evaluation of the toxicological implications and health risks of seafood consumption. The metabolism of arsenic compounds is influenced by the duration of administration.[17,18] In our recent study,[18] it was shown that urinary metabolites of various arsenic compounds administered for 7 months in rats were different from those for compounds administered for 1 week, and that AsBe was partly metabolized to tetramethylarsonium (TeMA) and TMAO after 7 months of administration, although it was mostly eliminated in urine without transformation. The purpose of the present study was to determine the chemical forms of arsenic excreted in the urine following oral administration of AsBe to rats and *in vivo* biotransformation of AsBe.

Urinary excretion is the major pathway of elimination of arsenic from the body.[12,13,19,20] Vahter *et al.*[12] have reported that urinary excretion for 3 days following oral administration of AsBe was above 98.5%. Chemical analysis of urine samples is thus considered a convenient and reliable approach to the study of metabolism of AsBe. In efforts to elucidate the biotransformation of arsenic, special attention should be paid to the analytical methods used to characterize arsenic compounds in urine. Ion chromatography with inductively coupled plasma mass spectrometry (IC–ICP-MS)[21,22] permits examination of the pattern of elimination and concentrations of several urinary arsenic metabolites simultaneously and with a high degree of sensitivity. The results obtained by IC–ICP-MS methodology eliminate analytical artifacts. In this study, temporal changes in the metabolism of AsBe were determined by measuring urinary metabolites following forced urination at various time points after orally administration of AsBe.

MATERIALS AND METHODS

Reagents

Sodium arsenite, sodium arsenate, MMA, DMA, TMAO, tetramethylarsonium iodide, and AsBe, used for analytical standard solutions and with purities of at least 99.99%, were obtained from Tri Chemical Lab. (Yamanashi, Japan). AsBe for administration, with a purity of at least 99.99%, was also obtained from Tri Chemical Lab. The purity of these compounds was confirmed by IC–ICP-MS. AsBe for administration contained no other arsenic compounds as impurities. GAM was purchased from Nissui (Tokyo, Japan). Other chemicals (analytical grade) were also from Wako Pure Chemical Industry (Osaka, Japan).

Animals and treatments

Six-week-old male F344/DuCrj rats weighing 109–122 g were obtained from Charles River Japan (Hino, Japan) and allowed to acclimatize for 1 week. Five rats were housed in a box cage with wood-chip bedding and provided with a standard diet (CE2, Clea Japan, Tokyo, Japan) and water *ad libitum*. The room was kept on a 12/12 h light/dark cycle at a temperature of 23 ± 1 °C.

Five rats were given AsBe orally at a single dose of 20 mg As kg^{-1} body weight. Urine was collected by forced urination at 0, 2, 4, 6, 8, 10, 12, 24, 48, 72 and 96 h after administration. The urine samples were centrifuged to remove particulate materials and stored at −20 °C until analysis.

Cultivation

Escherichia coli (A3-6) was isolated from cecae contents of rats exposed to arsenic. The culture medium, GAM, which had been used for the intestinal microbial degradation experiments, was used in this study. A3-6 and AsBe were added to the autoclaved growth medium. The initial concentration of AsBe in the growth medium was 100 mg l^{-1}. Control experiments without inoculation of bacteria were also run simultaneously. After 16 h of incubation under aerobic conditions at 37 °C, the culture media were centrifuged and the supernatants were ultrafiltered using Urtrafree-MC (Millipore, MA, USA) with a cut-off value of 10 000. The filtrate was stored at −20 °C until analysis.

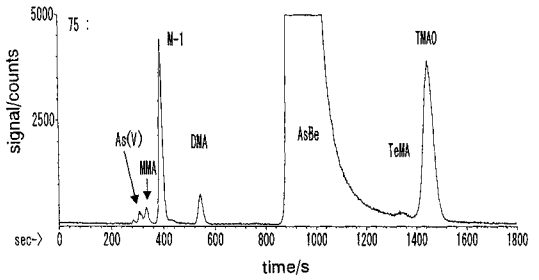

Figure 1 IC–ICP-MS chromatogram of urinary elimination of AsBe, TMAO, DMA, MMA, TeMA, an unidentified arsenic metabolite (M-1), and arsenate (As(V)) 6 h after administration after a single oral dose of 20 mg As kg^{-1} body weight of AsBe. Column, NN-614 (150 mm × 4.6 mm i.d.); mobile phase, 5 mM HNO_3–6 mM NH_4NO_3; flow rate, 0.8 ml min^{-1}.

Instrumentation

A Model HP4500 inductively coupled plasma mass spectrometer (Hewlett-Packard, DE, USA) was used for arsenic-specific detection. The operating conditions for ICP-MS were established in accordance with those reported by Inoue *et al.*[21] The ion chromatograph was a Model IC7000 from Yokogawa Analytical Systems (Tokyo, Japan). The analytical column was a Showdex NN-614 column (150 mm × 4.6 mm i.d.) packed with cation-exchange resin (Showadenko, Tokyo, Japan). A guard column of the same packing type was used for analysis of urine. Ion chromatography was performed under the following conditions: mobile phase 5 mM HNO_3–6 mM NH_4NO_3, flow rate 0.8 ml min^{-1}, and injection volume 50 µl. An outlet from the separation column was connected directly to the nebulizer of the spectrometer using an ethylenetetrafluoroethylene tube of 0.3 mm i.d.

IC–ICP-MS analysis

Measurements using IC–ICP-MS were performed by the method established by Inoue *et al.*[21] with modifications. Stock standard solutions of sodium arsenite, sodium arsenate, MMA, DMA, TMAO, tetramethylarsonium iodide, and AsBe were prepared by dissolving each compound in pure water at an arsenic concentration of 100 mg l^{-1}. The final diluted aqueous standard mixtures were prepared from each stock standard just before use. The urine samples were thawed and diluted 50-fold with distilled water just before measurement by IC–ICP-MS. The samples and the standards were injected into the ion chromatograph using a 50 µl loop. In order to obtain precise measurements, 1 mg l^{-1} of germanium solution was used as the internal standard for ICP-MS; the internal standard was added to the eluate from IC through a mixing joint prior to introduction to the spectrometer.[18] The ICP-MS detection mass was set to *m/z* 75 ($^{75}As^{+}$), *m/z* 72 ($^{72}Ge^{+}$), and *m/z* 77 ($^{40}Ar^{37}Cl$). The ion intensity at *m/z* 77 was of diagnostic value only in the examination for the possible occurrence of $^{40}Ar^{35}Cl^{+}$ interference on *m/z* 75. This method was linear in the arsenic range 0.001–10 mg l^{-1}, and the reproducibility (RSD) for 0.01 mg As l^{-1} of standard arsenic compound was about 2%.

RESULTS

Arsenic species confirmed by IC–ICP-MS

The urinary metabolites of arsenic were measured by IC–ICP-MS. Using ICP-MS as a detector, trace amounts of arsenic compounds can be measured accurately and with a high degree of sensitivity. The detection limits of IC–ICP-MS for arsenic species using a Showdex NN-614 column were estimated to be 0.05 µg As l^{-1}, 0.05 µg As l^{-1}, 0.07 µg As l^{-1}, 0.05 µg As l^{-1}, 0.11 µg As l^{-1}, 0.24 µg As l^{-1}, and 0.21 µg As l^{-1} respectively for arsenate (As(V)), MMA, arsenite (As(III)), DMA, AsBe, TeMA, and TMAO taking the limit as $S/N = 2$. In addition, this method, in which urine samples diluted with pure water are injected into the ion chromatograph and detected directly by ICP-MS, enables efficient measurement of AsBe and its urinary metabolites, since they can be analyzed in unchanged forms. Urine often causes analytical problems because of its high salt content. The urine matrix can also cause column overload or broadening of the analyte signals. Furthermore, interference resulting from the elution of chloride and subsequent formation and detection of $^{40}Ar^{35}Cl^{+}$ ion at *m/z* 75 can be substantial. Therefore, a high degree of dilution of urine samples might be necessary.[23] In this study, the urine was diluted 50-fold with distilled water. Elimination of eight arsenic compounds was found in urine after oral administration of AsBe, including AsBe, As(V), As(III), MMA, DMA, TMAO, TeMA, and one unidentified arsenic peak, metabolite 1 (M-1), which eluted just after MMA. M-1 was the same metabolite as that detected after long-term exposure to MMA, DMA, and TMAO in our previous

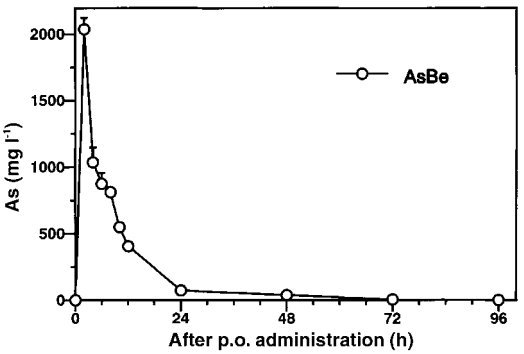

Figure 2 Excretion of AsBe in urine after a single oral administration of 20 mg As kg^{-1} body weight of AsBe.

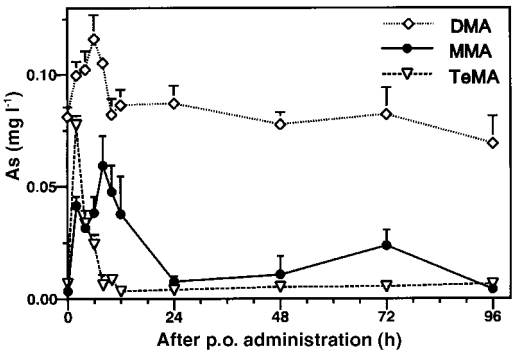

Figure 4 Excretion of DMA, MMA and TeMA in urine after a single oral administration of 20 mg As kg^{-1} body weight of AsBe.

study.[18] The cation-exchange IC–ICP-MS chromatogram of arsenic species in urine at 6 h after administration of AsBe is shown in Fig. 1.

Arsenic metabolites in urine after ingestion of AsBe

Basal excretion of arsenic in urine before administration of AsBe was 0.353 ± 0.033 mg As l^{-1}, 0.081 ± 0.004 mg As l^{-1}, 0.015 ± 0.002 mg As l^{-1}, 0.007 ± 0.001 mg As l^{-1}, and 0.009 ± 0.004 mg As l^{-1} respectively for AsBe, DMA, TMAO, TeMA and As(V). These trace amounts of arsenic species may have been due to the presence of arsenic in feed.

The time course of urinary AsBe elimination following oral administration of AsBe is shown in Fig. 2. Most elimination of unchanged AsBe occurred within 48 h, with peak elimination between 0 and 2 h. The maximum concentration of the excreted AsBe was 2039 ± 86.8 mg As l^{-1} at 2 h. Thereafter, the concentration of excreted AsBe decreased rapidly. At 24 h post-administration, the amount of AsBe in urine was about one-thirtieth that in 2 h urine. The concentrations of TMAO in urine after administration of AsBe are shown in Fig. 3. Elimination of TMAO appeared within 2 h, with peak elimination between 0 and 2 h. The maximum concentration of excreted TMAO was 2.53 ± 0.56 mg As l^{-1} at 2 h. As shown in Fig. 3, urinary elimination of the unidentified metabolite M-1 occurred after 2 h, with peak excretion between 6 and 8 h. The maximum concentration of excreted M-1 was 0.721 ± 0.168 mg As l^{-1} at 6 h. Urinary elimination of MMA occurred over a long period of time, and the time to peak elimination occurred later (8–10 h). The maximum concentration of

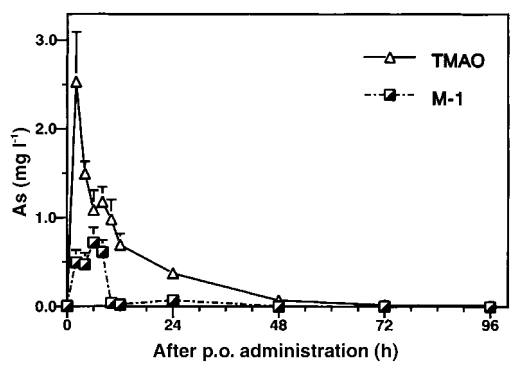

Figure 3 Excretion of TMAO and an unidentified arsenic metabolite (M-1) in urine after a single oral administration of 20 mg As kg^{-1} body weight of AsBe.

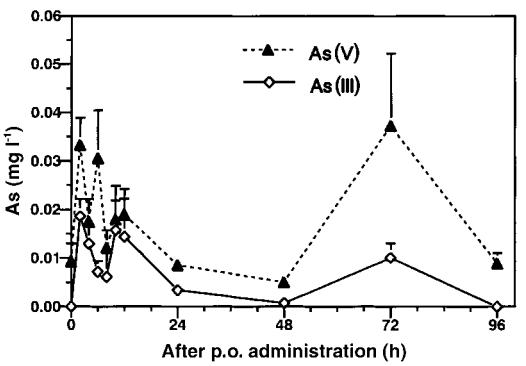

Figure 5 Excretion of and arsenate (As(V)) and arsenite (As(III)) in urine after a single oral administration of 20 mg As kg^{-1} body weight of AsBe.

excreted MMA was 0.060 ± 0.013 mg As l^{-1} at 8 h (Fig. 4). Elimination of TeMA in urine was detected within the first 6 h. The highest concentration of TeMA was found 2 h after administration, and was 0.078 ± 0.004 mg As l^{-1} (Fig. 4). Peak elimination of DMA occurred at 6 h (Fig. 4). After administration of AsBe, the As(V) concentration in urine was usually near normal, although urinary elimination at 2, 6, and 72 h after administration was slightly higher than basal excretion at 0 h (Fig. 5). The As(III) concentration in urine increased within the first 12 h and at 72 h following ingestion (Fig. 5).

Conversion of AsBe by intestinal bacteria

No arsenic compound other than AsBe was found in the growth medium after 16 h of incubation, as determined by IC–ICP-MS.

DISCUSSION

The studies performed with a single exposure of rats, mice, rabbits, and hamsters to AsBe suggested that AsBe is not biotransformed,[12,13] whereas our recent study performed with long-term exposure of rats to AsBe showed that AsBe was partly metabolized to TeMA and TMAO.[18] This is the reason why we investigated AsBe metabolism in rats. In the present study, urinary metabolites were measured following forced urination at various time points after oral administration of AsBe. This method of collecting urine can detect temporal changes in the metabolism of AsBe. We found that orally administered AsBe was mainly eliminated in urine in the form of unchanged AsBe, but that a small part of administered AsBe was eliminated as TMAO with peak elimination between 0 and 2 h (Figs 2 and 3). Welch and Landau[24] found production of a strong garlic odor from AsBe *in vivo* as well as *in vitro* in rats, suggesting cleavage of AsBe to trimethylarsine. The degradation of AsBe to TMAO, DMA, and inorganic arsenic by microorganisms in sediments,[14] particles,[15] and mollusk intestine[16] has also been reported. In this study, we examined the possibility of degradation of AsBe by an intestinal bacterium, *E. coli*, in rats. It is not evident from the present findings that the microflora present in the cecum are responsible for the decomposition of AsBe. The degradation of AsBe to TMAO might take place in the liver.

In addition to TMAO, an unidentified metabolite, M-1, was detected in urine (Figs 1 and 3). In unpublished research we found that the M-1 was present in the GAM medium added A3-6 and TMAO after 16 h of incubation. Microflora present in the cecum are probably responsible for the transformation from TMAO to M-1. The delay in M-1 elimination compared with that of TMAO found in this study also suggests that M-1 is formed from TMAO. In this study, demethylated metabolites, MMA, DMA, and inorganic arsenic were detected to a slight extent (Figs 4 and 5). The occurrence of demethylation of methylated arsenics in mammals is supported by the finding of Cullen *et al.*[25] that homogenates of mouse cecum demethylate the methylarsine oxide to arsenate and that ceca from mice administered methylarsine oxide contain arsenate. It is possible that the microflora present in the cecum are responsible for demethylation of TMAO. The finding that MMA, DMA, and inorganic arsenic were also excreted later than AsBe, TMAO, and TeMA supports the hypothesis that intestinal bacteria participate in demethylation in rats. The variability of elimination of MMA, DMA and inorganic arsenic may be ascribed to demethylation by microorganisms. The variability of elimination of inorganic arsenic may be also ascribed to oxidation of As(III) to As(V) and/or reduction of As(V) to As(III). Taken together, these results indicate that TMAO is formed from AsBe, and that a portion of TMAO is subsequently converted to M-1 and demethylated arsenic compounds by intestinal microorganisms.

Marked species differences in the biliary excretion of arsenic were reported by Klaassen.[26] The rate of biliary excretion of arsenic in rats was much greater than that in rabbits and dogs.[26] Arsenic was rapidly excreted into bile.[26] A comparison of biliary and fecal excretion rates in rats revealed that arsenic undergoes intestinal reabsorption.[27] The biliary excretion and enterohepatic circulation in rats may, in part, explain how arsenic becomes available to bacteria in the gut and why arsenic excretion in rats is biphasic.

In conclusion, our findings revealed that a small portion of administered AsBe was converted to arsenic species such as TMAO, M-1, MMA, TeMA, DMA, and inorganic arsenic after oral administration in rats. The metabolic pathways for AsBe described in the present study may be of toxicologic importance, since AsBe is ingested by man via seafood and toxic arsenic compounds may subsequently be formed.

Acknowledgements The authors thank Mieko Yoshimura, Department of Preventive Medicine and Environmental Health, Osaka City University Medical School, for her excellent technical assistance.

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