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Drug Monitoring and Toxicology

Arsenic Metabolites in Human Urine after Ingestion of an Arsenosugar

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Background: **Arsenic-containing carbohydrates (arsenosugars) are common constituents of marine algae, including those species used as human food. The toxicology of these compounds has not been fully evaluated.**
Methods: **Arsenic metabolites in human urine were monitored over a 4-day period after ingestion of a synthetic specimen of arsenosugar. The metabolites were determined by HPLC–inductively coupled plasma mass spectrometry, and structural assignments were confirmed with liquid chromatography–electrospray ionization mass spectrometry.**
Results: **Approximately 80% of the total ingested arsenic was excreted in the urine during the 4 days of the experiment. There was a lag-period of ~13 h before substantial quantities of arsenic appeared in the urine, and the excretion rate peaked between 22 and 31 h. At least 12 arsenic metabolites were detected, only 3 of which could be positively identified. Dimethylarsinate (DMA) was the major metabolite, constituting 67% of the total arsenicals excreted. A new urinary arsenic metabolite, dimethylarsinoylethanol, represented 5% of the total arsenicals, whereas trimethylarsine oxide was present as a trace (0.5%) constituent. One other significant metabolite cochromatographed with a reduced DMA standard, and hence was possibly dimethylarsinous acid. The second most abundant metabolite in the urine (20% of the total arsenic) remained unidentified, whereas the rest of the excreted arsenic was made up of several trace metabolites and small amounts of unchanged arsenosugar.**
Conclusions: **Arsenosugars are biotransformed by humans to at least 12 arsenic metabolites, the toxicologies of which are currently unknown.**

Although inorganic arsenic has long been recognized as a poison of high acute toxicity, the toxic effects of chronic exposure to low concentrations of arsenic have only recently been well documented. Epidemiologic studies in Taiwan and elsewhere have now clearly linked human cancers and other disorders with long-term exposure to arsenic in drinking water *(1)*. However, in many communities, food (seafood in particular) is a more significant source of arsenic than is water *(2)*.

Fundamental to the toxicologic assessment of arsenic exposure is knowledge of the precise chemical form of the arsenic compound implicated. Arsenic in drinking water is present almost entirely as the two inorganic oxyanions arsenite and arsenate, whereas seafood arsenic comprises several organoarsenic compounds *(3)*. In fish and most shellfish, the predominant arsenical is arsenobetaine, the nontoxic nature of which has been clearly established *(4, 5)*. In edible seaweed (algae), the arsenic is primarily bound to carbohydrate compounds, collectively termed arsenosugars. Arsenosugars can also occur at significant concentrations in marine animals feeding on algae, such as scallops *(6)*. The toxicology of arsenosugars has not been fully assessed, but it is likely to be more complicated than that of arsenobetaine. Whereas arsenobetaine is excreted rapidly and unchanged in human urine *(7)*, arsenosugars appear to be metabolized to several arsenic compounds, only one of which, dimethylarsinate (DMA),[4] has been identified to date *(8, 9)*.

Studies have been performed on arsenic metabolites in

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[4] Nonstandard abbreviations: DMA, dimethylarsinate; LC-electrospray MS, liquid chromatography–electrospray ionization MS; ICPMS, inductively coupled plasma mass spectrometry; MA, methylarsonate; DMAE, dimethylarsinoylethanol; TMAO, trimethylarsine oxide; MA^{3+}, methylarsonous acid; and DMA^{3+}, dimethylarsinous acid.

human urine after ingestion of edible algae, which naturally contain arsenosugars *(8, 9)*. Such investigations are toxicologically sound because they examine what people are actually eating. Interpretation of the results, however, may be complicated by the presence of other arsenicals in the algae, including unknown compounds. These unknown arsenicals may be water insoluble, and thus unidentifiable by current analytical methods, but might be converted to water-soluble metabolites in the gastrointestinal tract. Consequently, the possibility exists that arsenic metabolites in urine after the ingestion of algae (and other seafood as well) might wrongly be ascribed to arsenosugars. The present study aims to clarify this aspect by examining the arsenic metabolites in human urine after ingestion of a pure (synthetically prepared) arsenosugar.

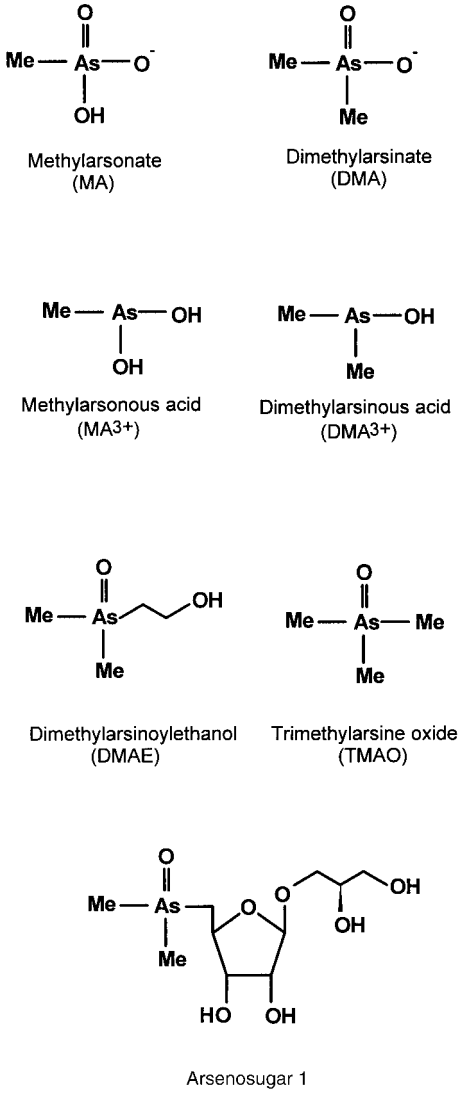

Fig. 1. Structures of arsenic compounds relevant to this study.

The structures of the reduced methylated species MA^{3+} and DMA^{3+}, drawn here as hydroxy species, are uncertain.

Materials and Methods

PREPARATION AND INGESTION OF ARSENOSUGAR

Arsenosugar **1** (Fig. 1), prepared according to the 10-step synthesis of McAdam et al. *(10)*, was obtained as a syrup (214 mg; 12% overall yield). Its purity was verified by liquid chromatography–electrospray ionization mass spectrometry (LC-electrospray MS), and ^{1}H and ^{13}C NMR spectroscopy. Immediately before use in the experiment, the synthetic specimen was tested for "arsenic purity" by HPLC–inductively coupled plasma MS (HPLC-ICPMS); >99.5% of the total arsenic was present as arsenosugar **1**, and there was just a trace amount (<0.5%) of a single arsenic impurity with a retention time matching that of DMA.

The arsenosugar **1** (~70 mg) was dissolved in water (5.00 mL), and the precise arsenic concentration of this stock solution was determined by ICPMS (see below). A portion of this stock solution (450 μL; equivalent to 1220 μg of arsenic) was diluted with water (25 mL) and completely ingested (t = 0) by one male volunteer (47 years of age). The volunteer gave informed consent and was aware of the experimental details and possible effects of ingesting the arsenosugar, and the procedures followed were in accordance with the current revision of the World Medical Association Declaration of Helsinki. This quantity of arsenic is equivalent to that contained in ~25 g of edible seaweed products, such as Nori or Hijiki; the average daily consumption of such products by the Japanese is ~5 g *(11)*. There are no data on the toxicity of arsenosugars to mammals, although it is worth noting that the "seaweed-eating sheep" from the Orkney Islands consume 2–4 kg (wet weight) daily of algae containing 45–90 mg of arsenic, mainly in the form of arsenosugars *(12)*.

COLLECTION OF URINE

All seafood products and other foods in which arsenic can occur at significant concentrations [e.g., mushrooms *(13)*] were avoided for 4 days before the beginning of the experiment. A urine sample was collected on the morning of arsenosugar ingestion and checked for arsenic species by HPLC-ICPMS. The results (~6 μg of total arsenic/L of urine) were within the reference interval for people not consuming seafood *(1)*. The arsenosugar was ingested at 0930 on day 0. All urine samples were collected separately over the subsequent 69 h (and at 94 h); the time of urination and volume of urine (±5 mL) was recorded, and a subsample (~50 mL) was removed and stored in a sealed plastic centrifuge tube at 4 °C in the dark until analysis, which took place within 10 days. The samples were clear and were not filtered before analysis. Arsenic species in urine were stable for up to 2 months when stored under the conditions described above *(14)*.

DRY WEIGHT OF URINE SAMPLES (FOR NORMALIZATION)

A portion of urine (1.00 mL) was transferred to a preweighed plastic test tube and evaporated to dryness

under reduced pressure at 30 °C in a centrifugal lyophilizer (Heto Holton). The test tube plus contents were weighed to determine the weight of the dried residue.

DETERMINATION OF TOTAL ARSENIC

A portion of urine (2.00 mL) or stock solution of arsenosugar **1** (3 × 50 μL) plus water (2.00 mL) was added to a quartz digestion vessel (12-mL capacity) of a ultraCLAVE 2® microwave autoclave (EMLS). Water (1.00 mL) and HNO_3 (2.00 mL) were then added to the quartz vessels. The system was closed, loaded with argon to 4 × 10^6 Pa, and the mixture was heated at 260 °C for 30 min. The samples were then diluted to 25.0 mL with water before analysis for arsenic by ICPMS.

DETERMINATION OF ARSENIC METABOLITES BY LC-MS

STANDARD ARSENIC COMPOUNDS

Arsenate (as Na_2HAsO_4) and arsenite ($NaAsO_2$) were purchased from Merck; DMA, as the sodium salt, was purchased from Aldrich Chemical Company Inc; methylarsonate (MA) was prepared in-house from arsenic trioxide (As_2O_3) and methyl iodide *(15)*; tetramethylarsonium ion was prepared from trimethylarsine *(16)* and methyl iodide; arsenosugar **1** *(10)*, arsenobetaine *(16)*, arsenocholine *(16)*, dimethylarsinoylethanol (DMAE) *(17)*, and trimethylarsine oxide (TMAO) *(18)* were synthesized as reported previously. The reduced forms of MA and DMA [methylarsonous acid (MA^{3+}) and dimethylarsinous acid (DMA^{3+})] were prepared essentially as described in Mandal et al. *(19)* by reducing the corresponding As^{5+} compounds. For this reduction, we added 15.0 mL of 100 mmol/L sodium metabisulfite ($Na_2S_2O_5$) and 2.00 mL of 70 mmol/L sodium thiosulfate ($Na_2S_2O_3$) and acidified this solution by the addition of 0.10 mL of 18 mol/L H_2SO_4. One volume of this reducing solution was added to one volume of a standard solution (1.00 mg/L arsenic) of MA or DMA, and the mixture was allowed to stand for 2 h at room temperature in a tightly capped tube before analysis by HPLC-ICPMS. Reduction of DMA produced a single product peak with retention behavior that was consistent with the literature *(20)*, and a trace of unchanged DMA. In contrast, reduction of MA produced two approximately equal product peaks.

HPLC INSTRUMENTATION AND COLUMNS

Chromatographic separation of arsenic compounds was performed with Hewlett Packard Series 1100 HPLC instruments equipped with solvent degassers, binary pumps, autosamplers, and thermostated column compartments. Separate instruments (in different laboratories) were coupled to ICPMS or to electrospray MS detectors (see below). Chromatographic columns were as follows: PRP-X100 anion-exchange columns (250 or 150 mm × 4.1 mm) from Hamilton Company; Zorbax® 300-SCX cation-exchange column (150 × 4.6 mm) from Hewlett Packard; and a Ionospher-C cation-exchange column (100 × 3 mm) from Chrompack Denmark ApS. Mobile phases and other chromatographic conditions differed slightly depending on the detector used and are detailed separately below.

HPLC-ICPMS

The mobile phases for anion-exchange chromatography (PRP-X100) were aqueous solutions of NH_4HCO_3 (20 mmol/L) at pH 8.0, or adjusted to pH 9.0, 9.5, 10.0, or 10.7 with aqueous ammonium hydroxide. The mobile phases for cation-exchange chromatography were a solution of 20 mmol/L pyridine in water, pH 2.6, adjusted with formic acid (both Zorbax 300-SCX and Ionospher-C columns), or a solution of 10 mmol/L pyridine in water, pH 3.0, adjusted with formic acid (Ionospher-C only). Methanol (3:97 by volume) was added to the mobile phases to enhance the signal response for arsenic *(21)*. The chromatography was carried out at 40 °C with a flow rate of 1.5 mL/min; injection volume was typically 5–20 μL. Polypropylene vials were used for the autosampler because we have found that glass vials can contaminate samples with arsenate.

The outlet of the HPLC column was connected via 800-mm (1/16-inch) polyether-ether-ketone capillary tubing (0.13-mm i.d.) to the Babington-type nebulizer of an ICP mass spectrometer (Hewlett Packard Model 4500). The ion intensities at *m/z* 75 and 77 were monitored. The ICPMS signal was optimized with a solution of the mobile phase containing 20 μg/L arsenic to give maximum response on the arsenic signal (*m/z* 75). Arsenic compounds were quantified by external calibration with standard solutions of MA, DMA, and arsenate (anion exchange) or arsenobetaine, TMAO, arsenocholine, and tetramethylarsonium ion (cation exchange). In general, the type of compound in which the arsenic is bound does not influence the arsenic signal in ICPMS *(22, 23)*.

Chromatographic analysis of the standards and 27-h urine sample was also performed with the HPLC coupled to a continuous-flow, hydride-generation ICP mass spectrometer with an integrated sample introduction system (Agilent) using the conditions described by Frank *(24)*. In this way, only those arsenicals giving volatile arsines were detected.

IDENTIFICATION OF THE ARSENIC METABOLITES BY LC-ELECTROSPRAY MS

The mobile phase for anion-exchange chromatography (PRP-X100; 150 × 4.1 mm) was a mixture of 20 mmol/L NH_4HCO_3 at pH 9.5, adjusted with aqueous ammonium hydroxide, and methanol (7:3 by volume). For cation-exchange chromatography (Ionospher-C), the mobile phase was a mixture of 20 mmol/L pyridine in water, pH 2.6, adjusted with formic acid, and methanol (8:2 by volume). The chromatography was performed at 30 °C and a flow rate of 1.0 mL/min; injection volumes were typically 5 μL.

A single quadrupole mass spectrometer (G1946A MSD; Hewlett Packard) equipped with an atmospheric pressure ionization LC-MS interface was used for characterization of the arsenic compounds. We performed the LC-electrospray MS analyses on urine samples and standard solutions of DMAE, DMA, and TMAO using selected-ion monitoring in the positive-ion mode with variable fragmentor voltages, enabling detection within the one chromatographic run of $[M+H]^+$ species at *m/z* 167 (DMAE), 139 (DMA), or 137 (TMAO) at 70 V; and *m/z* 75 (As^+) at 240 V. The technique has been previously described for other arsenic compounds *(25)*.

PHENOL EXTRACTION OF 27-H URINE SAMPLE

The 27-h urine sample (20.0 mL, containing 21 μg of arsenic in 870 mg of dry weight) was extracted twice (10 mL each time) with a solution of phenol and water (9:1 by volume). The aqueous layer was separated, washed with ether (2 × 20 mL) to remove traces of phenol, and evaporated at 40 °C with a Büchi Rotavapor to give a dry residue (490 mg containing 5.4 μg of arsenic), which was named "phenol-insoluble". The phenol layer was diluted with ether (75 mL), and the mixture was washed with water (3 × 5 mL); these aqueous extracts were combined, washed with ether (2 × 25 mL), and evaporated to give a dry residue (300 mg containing 13 μg of arsenic), which was termed "phenol-soluble". Small emulsion layers that formed at the interface of the two solvents during the various separations were discarded.

Results and Discussion

TIME COURSE OF TOTAL ARSENIC ELIMINATION

The preexperiment total arsenic concentration in the participant's urine was 6 μg/L, which is within the range expected for a person on a nonseafood diet *(1)*. After ingestion of the arsenosugar, the urine arsenic concentrations showed small immediate increases reaching ~30 μg/L after 3 h (Table 1), and these modest concentrations were maintained for at least the next 6 h. After 13 h, arsenic began to appear in the urine at substantial concentrations, peaking at some point between 22 and 30 h (from our sampling times, 27 h gave the highest concentration of 1290 μg/L). The final urine sample collected after 94 h contained 34 μg/L.

Because urine volume is influenced by many factors (e.g., fluid intake and perspiration rate), the quantity of urinary metabolites per unit volume is often normalized in some way, thereby facilitating comparison of samples. Adjustments are usually made against urine creatinine concentrations or specific gravity *(26)*. Accordingly, our data are presented on a dry mass basis (μg of arsenic/g of dry weight of urine; Table 1). This appears to be a convenient way of normalizing the data because the dry weight of 1.00 mL of urine; is an effective index of specific gravity, and we were able to easily obtain dry-mass data with the centrifugal lyophilizer. These normalized data for the synthetic arsenosugar agree well with results reported in two previous studies examining the ingestion of arsenosugars (including compound **1**) naturally

Table 1. Urinary excretion of arsenic after ingestion of arsenosugar 1.

Urine sample, h[a]	Volume, L	Dry weight, g[b]	Arsenic, μg/L	Total arsenic, μg	μg of arsenic/g of dry weight of urine (normalized)	Cumulative arsenic excretion, μg[c]	Cumulative arsenic excretion, % of ingested arsenic[d]
0	0.20	3.56	6.25	1.25	0.35		
1	0.25	2.65	11.8	2.94	1.11	1.4	0.1
3	0.24	3.70	29.5	7.08	1.92	7.0	0.6
6	0.20	2.94	25.3	5.05	1.72	10.8	0.9
9	0.40	7.00	30.5	12.2	1.74	20.5	1.7
13	0.18	5.62	109	19.5	3.48	38.9	3.2
22	0.23	11.7	1160	267	22.9	304	24.9
27	0.15	6.56	1290	194	29.6	498	40.7
31	0.225	6.89	603	136	19.7	632	51.7
35	0.095	4.39	770	73.1	16.7	704	57.6
46	0.28	13.8	526	147	10.6	850	69.5
50	0.26	6.94	162	42.2	6.08	890	72.9
54	0.47	7.14	66.5	31.3	4.38	919	75.2
58	0.46	7.59	54.7	25.2	3.32	941	77.0
60	0.52	4.94	26.7	13.9	2.82	952	77.9
63	0.36	4.25	32.7	11.8	2.78	961	78.7
69	0.25	10.5	103	25.8	2.45	985	80.6
94	0.21	10.6	34.0	7.14	0.67	991	81.1

[a] Values represent how many hours after ingestion of arsenosugar **1**.
[b] Calculated for total sample volume from the dry weight of 1.00 mL of urine.
[c] Small background contribution has been subtracted.
[d] Based on ingestion of 1220 μg of arsenic as arsenosugar **1**.

present in edible algae *(8, 9)*. Both studies showed a delayed absorption of arsenic followed by elimination peaking at ~15–30 h, although the authors noted large individual variability in the quantities of arsenic excreted by the study participants.

The total quantity of arsenic collected in the urine over the first 69 h was 990 μg, representing 81% of the total amount ingested. This value has been corrected for the small contribution of background arsenic, assumed to be constant at the starting concentration. An additional sample collected after 94 h contained a further 7 μg of arsenic, indicating that excretion of ingested arsenic was continuing, but at a low rate. We were surprised by this high-excretion efficiency of arsenosugar **1**; it matched the human excretion reported for arsenobetaine *(27)*, the natural arsenic constituent of fish and other seafood.

The data suggest a biphasic disposition of arsenosugar **1**. There appears to be slow absorption and elimination during the first 13 h, followed by rapid elimination. These data suggest that biotransformation processes are taking place, and the results on the arsenic species present in the samples (see below) support this view.

determination of arsenic metabolites

Arsenic metabolites were identified and quantified with HPLC-ICPMS. The methods have been described in detail elsewhere *(21, 22)*, so we give only a brief description here. A combination of anion-exchange and cation-exchange chromatography (HPLC) provides good separation of the arsenic species, and ICPMS provides sensitive and specific detection of arsenic. Under our conditions, the analytical response is essentially independent of the type of compound, although differences can occur under certain nebulization conditions *(23)*. Compounds are identified by matching retention times with those of standards. Changes in pH can often effect large changes in retention times that are characteristic of some compounds (Table 2). Under one set of chromatographic conditions, a hydride generation system was placed between the column and the ICPMS. This system measures only those arsenicals capable of giving volatile arsines, and thus can provide useful information *(9)*.

Further support for compound identifications made by HPLC-ICPMS was provided by LC-electrospray MS. This technique provides data for the arsenic ion and the protonated molecular species of the compound of interest in the same chromatographic run *(25)*; when combined with retention-time data for standards, structural assignments of sample constituents can be made with some confidence. The method, however, is less sensitive for arsenic and more prone to interferences than is ICPMS *(22)*.

Table 2. Retention times (min) of arsenic standards and four major arsenic metabolites in urine (t = 27 h) on anion-exchange columns at various pH values.[a]

	PRP-X100 (250 × 4.1 mm),	PRP-X100 (150 × 4.1 mm)			
Arsenical	pH 9.0	pH 8.0	pH 9.5	pH 10.0	pH 10.7
TMAO	1.53	NR[b]	NR	NR	NR
DMAE	1.51	NR	NR	NR	NR
As^{3+}	2.1	1.18	1.74	1.8	1.5
DMA	3.05	2.94	2.14	1.8	1.5
MA	7.32	5.92	8.86	6.63	4.25
As^{5+}	22.5	42.5	17.8	10.5	6.27
Peak A1	1.53	1.01	1.01	1.00	0.99
Peak A2	3.04	2.89	2.11	1.77	1.49
Peak A3	7.23	7.55	7.81	7.77	7.31
Peak A4	18.4	24.6	16.9	13.0	9.69

[a] Mobile phase was a mixture of 20 mmol/L NH_4HCO_3 and methanol (97:3 by volume).

[b] NR, not recorded under these conditions.

qualitative assessment of arsenic species in urine

To facilitate the discussion of the time-course data for arsenic species, we provide here an overview of the arsenic species involved, including unknown compounds. The various urine samples showed small differences in their pattern of arsenic species. However, after 13 h, the samples were qualitatively the same; we describe here the arsenic species present at 27 h because this sample most clearly represents the pattern that was common in most samples. Unless stated otherwise, the retention times refer to those obtained on the 250 × 4.1 mm PRP-X100 column at pH 9.0 (Table 2).

The anion-exchange chromatogram for the 27-h sample (Fig. 2) showed the presence of four significant arsenic peaks (A1 to A4) and several trace arsenic constituents (each <1% of total arsenic). When the hydride generation system was used, three of the peaks (A2, A3, and A4) produced volatile arsines. The major peak (A2 at 3.04 min) was assigned as DMA by retention-time matching in several chromatographic systems (Table 2). This assignment was confirmed with LC-electrospray MS by measuring the protonated molecular species [$Me_2As(OH)_2^+$; *m/z* 139] in the urine and in the urine supplemented with the DMA standard (Fig. 3).

The second major metabolite (A4) in the urine was strongly retarded on the anion-exchange column. Its retention time (18.4 min), which did not match any of the standard arsenicals, was greatly influenced by pH, changing from 9.69 min at pH 10.7 to 24.6 min at pH 8.0 (150 mm PRP-X100 column; Table 2). This behavior was similar to that displayed by arsenate (from 6.3 to 42.5 min). Increasing retention times with decreasing pH is not readily explained by simple anion-exchange processes. Possibly, both arsenate and the unknown A4 form carbonate complexes in the mobile phase; arseno-carbonate complexes have been suggested for As^{3+} species *(28)*, although we could see no evidence of this for arsenite under our chromatographic conditions.

A third significant metabolite (A3) had a retention time

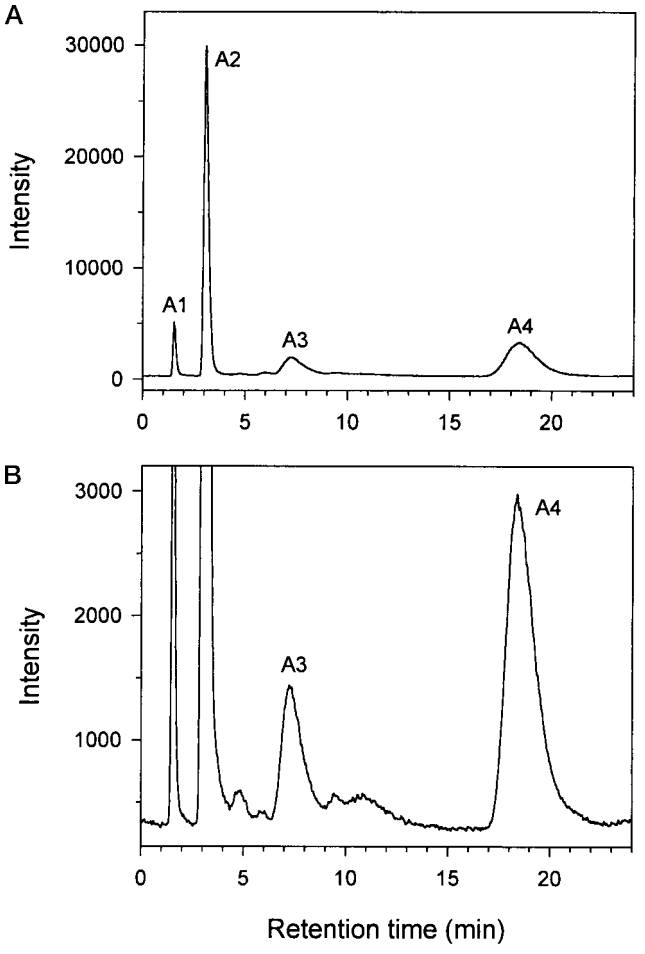

Fig. 2. Anion-exchange chromatograms (HPLC-ICPMS) of arsenic metabolites in urine at 27 h (*A*) and urine at 27 h with an expanded intensity scale (*B*).

Major peaks were at 1.53 min (*A1*), 3.04 min (*A2*), 7.23 min (*A3*), and 18.4 min (*A4*); and minor peaks were at 4.86, 6.0, 9.6, and 10.9 min. Chromatographic conditions were as follows: PRP-X100 anion-exchange column (250 × 4.1 mm) at 40 °C with a mobile phase of 20 mmol/L NH_4HCO_3, pH 9.0, and methanol (97:3 by volume) at a flow rate of 1.5 mL/min.

(7.23 min) at pH 9.0 close to that of MA (Table 2). Further chromatography, however, demonstrated that A3 was not MA because the retention times were markedly different at other pHs (Table 2). Although we could find no evidence for the presence of MA as a metabolite in our study, this compound was present in the urine of sheep that feed on seaweed (and hence consume large quantities of arsenosugars) *(12)*.

Reduced forms of MA and DMA, namely methylarsonous acid (MA^{3+}) and DMA^{3+}, have recently been reported in human urine *(19, 29, 30)*. These compounds may well be of toxicologic significance *(31–33)*. At the time of our experiment, standards for MA^{3+} and DMA^{3+} were not available to us, but we subsequently prepared these arsenic species by the method reported by Mandal et al. *(19)*. We note that these reduced arsenic species have not been well characterized; they are at best "operationally defined" calibrators, and the interpretation of data on the

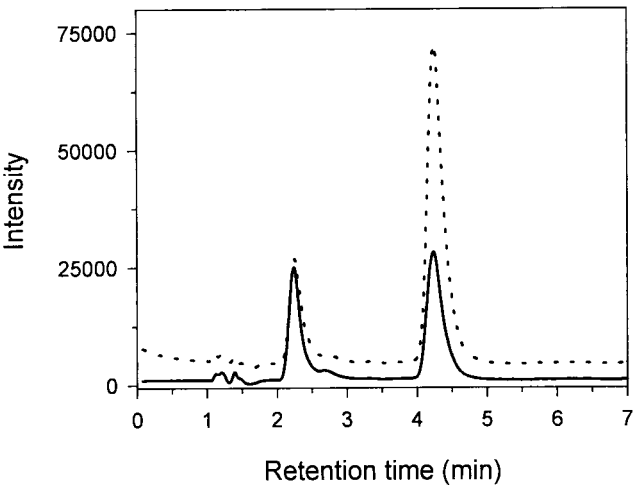

Fig. 3. Anion-exchange chromatograms of urine sampled at 27 h detecting the protonated molecular species (*m/z* 139) by electrospray MS.

Chromatographic conditions were as follows: PRP-X100 anion-exchange column (150 × 4.1 mm) at 30 °C with a mobile phase of 20 mmol/L NH_4HCO_3, pH 9.5, and methanol (7:3 by volume) at a flow rate of 1.0 mL/min. *Solid line*, urine; *dotted line*, urine supplemented with DMA (offset by 3000 intensity units).

basis of comparison among them is tentative. Nevertheless, MA^{3+} and DMA^{3+} were compared with the arsenicals in the 27-h urine sample, which had been stored as a freeze-dried residue at −18 °C for 6 months. One of the urine peaks matched the retention time for DMA^{3+} (Fig. 4). However, the relative quantities of the arsenicals had changed during storage, and consequently, we cannot state that DMA^{3+} was in the original "fresh" urine samples, at least not at the concentrations indicated by Fig. 4. When the urine samples were stored as solutions at 4 °C and −18 °C for 6 months, compounds A3 and A4 virtually

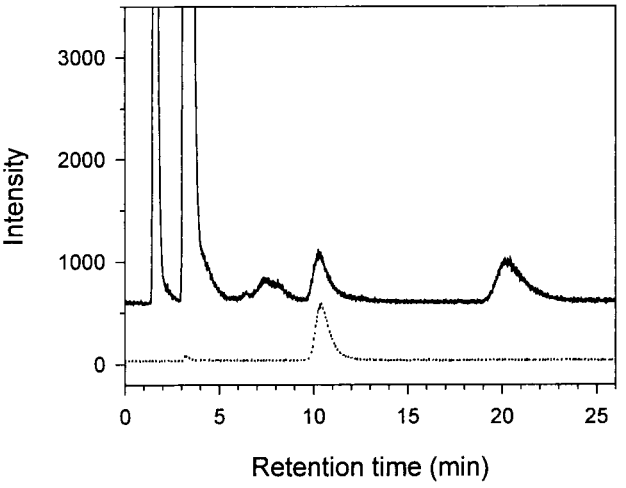

Fig. 4. Anion-exchange chromatograms (HPLC-ICPMS) of arsenic metabolites in urine at 27 h (*solid line*) and DMA^{3+} standard (*dotted line*).

The MA^{3+} standard gave two peaks (2.30 and 13.7 min). Before this analysis, the urine sample had been stored as a freeze-dried residue for 6 months, during which time the ratio of metabolites had changed (compare with Fig. 2B). Chromatographic conditions were as reported in Fig. 2: the column had been recently regenerated, which led to small changes in retention times for standards and metabolites compared with the findings described in Fig. 2.

disappeared and the chromatogram was dominated by DMA. Feldmann et al. *(14)* reported changes in arsenic speciation when urine is stored for >2 months. Possibly, both A3 and A4 were initially present in our samples as dimethylated arsenic species (reduced or complexed with matrix components), and they then reverted to DMA during storage.

The peak A1 at 1.53 min (near the void volume of the anion-exchange column) was thought to comprise cationic arsenic species. This peak was enhanced relative to the other arsenic signals in the phenol-soluble fraction of the 27-h urine sample. The phenol extraction procedure has been used previously with good results to separate naturally occurring organoarsenicals from inorganic salts and highly polar organic compounds present in urine *(7)*. Cation-exchange chromatography of this phenol-soluble fraction gave a complex pattern of arsenicals (Fig. 5, A and B). The two large peaks (C2 and C3) near the void volume corresponded to the two major arsenicals (DMA and the unknown A4) in the anion-exchange chromatogram. In addition, there were 10 arsenicals that were presumably cationic because they were retarded by the column. The major cation (C4) and a minor cation (C5) had retention times that matched those for DMAE and TMAO, respectively (Table 3). Support for the assignment of the major cation as DMAE was provided by cochromatography with an authentic specimen under two sets of chromatographic conditions (Fig. 5C provides one example). Similarly, the C5 peak was assigned as TMAO according to cochromatography with standards. Confirmation of the assignment of C4 as DMAE was provided by LC-electrospray MS (Fig. 6) with the detection of the protonated molecular species (*m/z* 167). The assignment of the minor cationic metabolite C5 as TMAO was established on the basis of HPLC-ICPMS data alone because the concentration was too low to allow detection by LC-electrospray MS.

The presence of TMAO in the human urine samples is of interest. Experiments by Yoshida et al. *(34)* have demonstrated that the rat can readily methylate orally administered DMA and excrete TMAO in the urine. The results from our experiment indicate that humans can also methylate DMA, albeit at a low rate. Consequently, because DMA is a common significant metabolite in human urine, one might expect TMAO also to be present naturally, but only at trace concentrations. Furthermore, it is possible that in our experiment, other dimethylated arsenicals were also transformed to trimethylated metabolites such as arsenocholine (from DMAE) or the trimethylarsonio analog of arsenosugar **1**. However, we could find no evidence for the presence of such compounds in our samples.

DMAE was a significant metabolite in the urine samples. There have been no previous reports of this metabolite occurring in human urine or in the urine of experimental animals. This does not, however, exclude its presence at low concentrations: DMAE is not freely available as a calibrator, so analytical methods cannot easily be developed specifically for this compound. It does not appear to have been present at detectable concentrations in the urine of seaweed-eating sheep in the study by

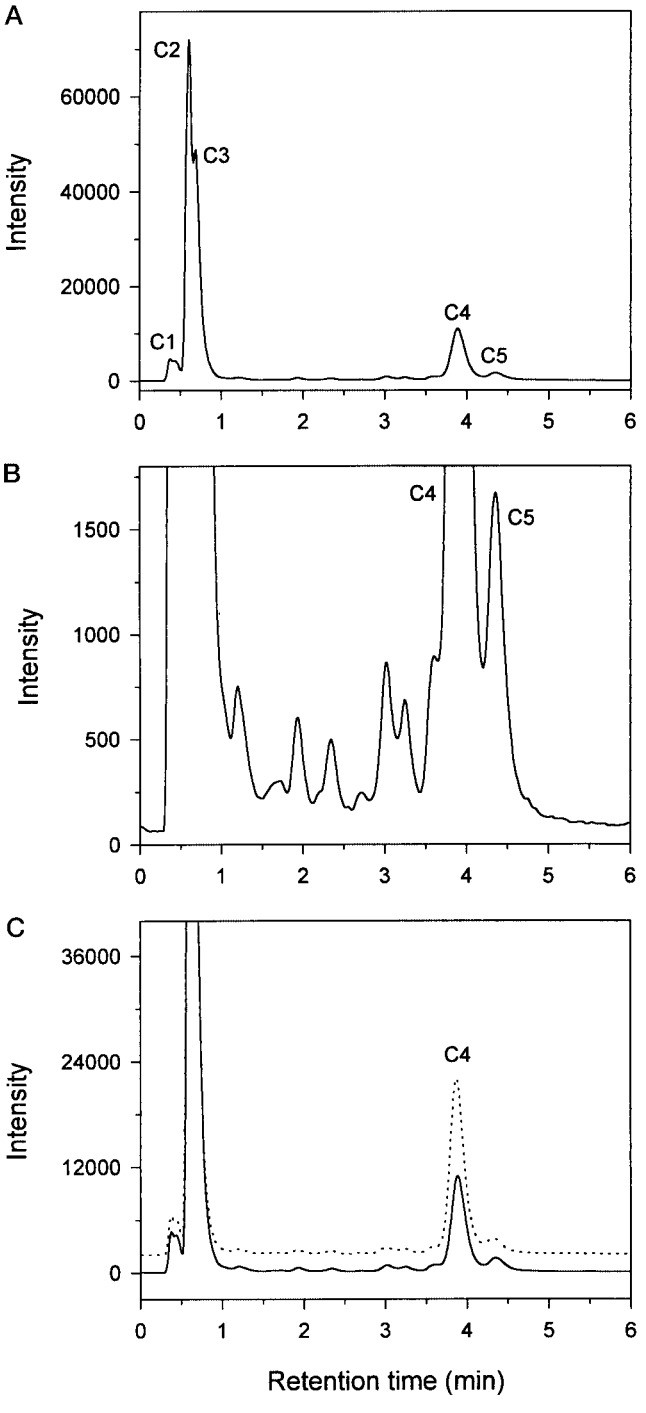

Fig. 5. Cation exchange chromatograms (HPLC-ICPMS) of arsenic metabolites in the phenol-soluble fraction of urine at 27 h.

Chromatographic conditions were as follows: Ionospher-C cation-exchange column (100 × 3 mm) at 40 °C with a mobile phase of 10 mmol/L pyridine, pH 3.0, and methanol (97:3 by volume) at a flow rate of 1.5 mL/min. (*A*), urine at 27 h. (*B*), urine at 27 h with an expanded intensity scale. (*C*), urine at 27 h (*solid line*) and supplemented with DMAE (*dotted line*; offset by 2000 intensity units).

Table 3. Retention times (min) of arsenic standards and the arsenic metabolites in urine (t = 27 h) on cation-exchange columns at various pH values.

Arsenical	Zorbax (150 × 4.6 mm; 20 mmol/L pyridine), pH 2.6	Ionospher-C (100 × 3 mm; 20 mmol/L pyridine), pH 2.6	Ionospher-C (100 × 3 mm; 10 mmol/L pyridine), pH 3.0
Arsenobetaine	1.82	0.97	0.96
Arsenosugar **1**	2.21	1.45	NR[a]
DMAE	NR	2.08	3.90
TMAO	2.60	2.31	4.34
Arsenocholine	3.54	3.32	6.54
Tetra	4.04	4.10	8.07
Peak C1	1.2	0.4	0.4
Peak C2	1.3	0.50	0.60
Peak C3	1.4	Unresolved	Unresolved
Peak C4	2.5	2.08	3.96
Peak C5	Unresolved	2.28	4.35

[a] NR, not recorded under these conditions; Tetra, tetramethylarsonium ion.

Feldmann et al. *(12)*. The formation of DMAE is likely to occur directly from degradation of arsenosugar **1**, possibly carried out by gut microflora, rather than from the addition of the -CH_2CH_2OH moiety to DMA. A previous experiment examined the microbial degradation of arsenosugars in sediments and showed complete conversion to DMAE under anaerobic conditions *(17)*.

TIME COURSE OF ARSENIC METABOLITES IN URINE

The time-course data for the arsenic species are contained in Table 4, but the patterns of excretion can be seen more easily when the data are normalized against dry weight of urine samples and graphically depicted (Fig. 7).

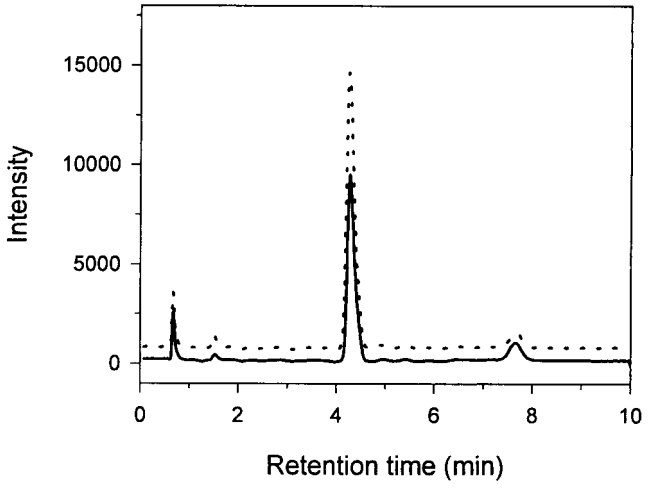

Fig. 6. Cation exchange chromatogram of phenol-soluble fraction of urine sampled at t = 27 h detecting the protonated molecular species (m/z 167) by electrospray MS.

Chromatographic conditions were as follows: Ionospher-C cation-exchange column (100 × 3 mm) at 30 °C with a mobile phase of 20 mmol/L pyridine, pH 2.6, and methanol (8:2 by volume) at a flow rate of 1.0 mL/min. *Solid line*, urine; *dotted line*, urine supplemented with DMAE (offset by 500 intensity units).

Table 4. Urinary excretion of the major arsenic metabolites after ingestion of arsenosugar 1.

Urine sample, h[a]	Volume, L	Arsenic metabolite, μg/L			
		DMA	DMAE	A3	A4
0	0.20	1.5	ND[b]	ND	ND
1	0.25	2.2	ND	ND	ND
3	0.24	4.6	ND	ND	ND
6	0.20	4.6	ND	ND	ND
9	0.40	4.0	ND	ND	ND
13	0.18	16.9	ND	7.3	17.6
22	0.23	455	35.9	88.4	289
27	0.15	674	50.2	58.7	240
31	0.225	346	18.0	19.3	86.2
35	0.095	491	16.2	13.4	102
46	0.28	358	7.5	5.6	51.3
50	0.26	117	3.7	1.0	13.7
54	0.47	48.0	1.6	ND	5.8
58	0.46	37.0	0.64	ND	2.2
60	0.52	20.1	ND	ND	2.2
63	0.36	25.4	ND	ND	3.2
69	0.25	74.9	ND	ND	8.5
94	0.21	25.0	ND	ND	5.4

[a] Values represent how many hours after ingestion of arsenosugar **1**.
[b] ND, not detected.

The background urine sample contained arsenite and DMA, each at ~3 μg/L. After 1 h, small quantities of arsenosugar **1** appeared in the urine, peaking at 15 μg/L

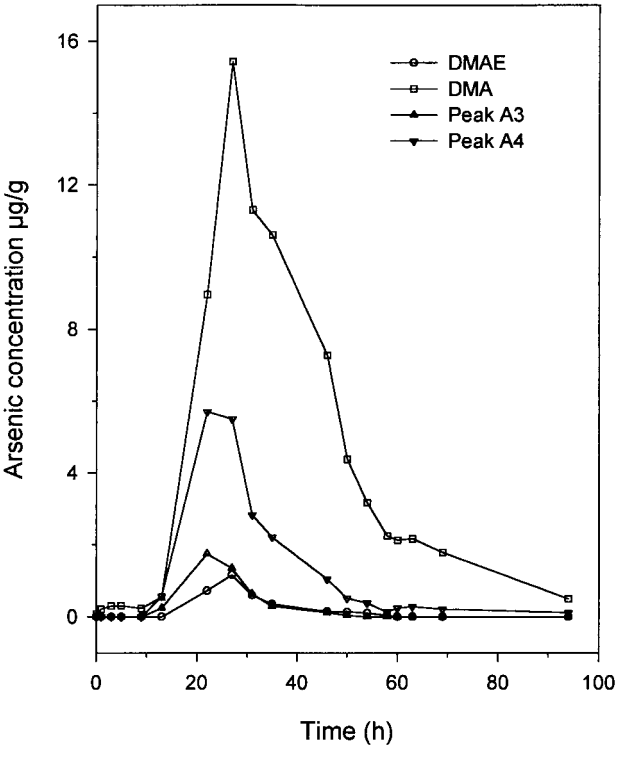

Fig. 7. Urinary excretion of arsenic metabolites normalized to dry weight of urine.

after 6 h before dropping to 7 μg/L at 13 h. Thereafter, the large peak in the cation-exchange chromatograms (e.g., Fig. 5A) associated with DMA (C3), as well as the peak that was attributable to DMAE (C4), precluded the quantification of arsenosugar **1**; however, it was never more than a trace constituent. Small quantities of DMA (slightly above background) also occurred in the early urine samples. Possibly this reflected the trace contamination (<0.5%) of the synthesized arsenosugar **1** with DMA.

From t = 13 h, arsenic metabolites began to appear in the urine at concentrations considerably above background concentrations, peaking between 22 and 31 h. The data suggest that the various metabolites were excreted at slightly different rates. For example, concentrations of compounds A3 and A4 were highest in the sample t = 22 h, whereas both DMA and DMAE concentrations peaked in sample t = 27 h (Fig. 7). This may reflect different rates of biotransformation of arsenosugar **1** to the various metabolites within the body, or it may merely be showing different urinary elimination rates for the metabolites once they are produced. First-order elimination rate constants (K_e) were obtained by fitting the data to a one-compartment toxicokinetic model: the K_e values were 0.03 h^{-1} [equivalent to a half life ($t_{1/2}$) of ~23 h] and 0.05 h^{-1} ($t_{1/2}$= 14 h) for DMA and the unknown A4, respectively. For comparison, urinary excretion of DMA was assigned a K_e value of 0.04 h^{-1} in a pharmacokinetic model for arsenic disposition in humans *(35)*.

DMA was identified as the major urinary metabolite when humans ingested arsenosugars naturally present in edible algae *(8, 9)*. In the study of Ma and Le *(9)*, a maximum DMA concentration of 90 μg /L was reached 26.5 h after ingestion of 10 g of the edible alga Yakinori containing ~200 μg of arsenic in the form of arsenosugars. Those data are closely matched by our results showing a maximum DMA concentration of 674 μg/L in urine 27 h after ingesting 1220 μg of arsenic in the form of the pure arsenosugar **1**.

During the first 69 h (when all urine was collected), the combined amount of arsenic accounted for by the arsenic species measured by HPLC-ICPMS was 820 μg (by anion-exchange) and 1070 μg (by cation-exchange). These amounts represent 83% and 108% of the total arsenic excreted in 69 h (990 μg) as determined by ICPMS. These data might suggest that some arsenic species were retained by the anion-exchange column. However, the values are (just) within the estimated precision of our analytical procedure for these untreated urine samples, so the discrepancy could be accommodated by analytical uncertainty.

When the data from the individual urine samples are considered collectively, the major metabolites were DMA (67% of total urinary arsenicals), unknown A4 (20%), DMAE (5%), unknown A3 (5%), and TMAO (0.5%). Traces of other arsenicals (at least eight compounds) collectively accounted for just 2.5% of the excreted arsenic.

In summary, when arsenosugar **1** was taken orally, it was biotransformed to at least 12 arsenic metabolites that were excreted in the urine. After 3 days, ~80% of the ingested arsenic was accounted for by these urinary metabolites. The toxicologic significance of these metabolites remains to be determined. Finally, we see this work progressing on two fronts: (*a*) to identify the major (and minor) unknown metabolites produced from arsenosugar **1**; and (*b*) to carry out experiments with synthetic compounds and a large study group to obtain data on the individual variability in the way humans metabolize arsenosugars.

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