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Identification of methylated metabolites of inorganic arsenic by thin-layer chromatography

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Abstract

TLC on cellulose plates was used to identify methylated products of inorganic arsenic metabolism (monomethylarsonate and dimethylarsinate) in biological samples. Two solvent systems were tested: methanol–ammonium hydroxide (8:2) and isopropanol–acetic acid–water (10:1:2.5). The latter solvent system produced the most satisfactory separation of radiolabelled methylated arsenic compounds in aqueous solution, in rat liver cytosol incubated with carrier-free or 1 μM [^{73}As]arsenite and in urine of mice given carrier-free [^{73}As]arsenate or 5 mg of [^{73}As]arsenate/kg per os. Oxidation of samples by hydrogen peroxide improved the separation and quantitation of monomethylarsonate in both biological matrices.

1. Introduction

The metabolism of arsenic involves the cycling between trivalent and pentavalent states and its conversion to mono-, di- and trimethylated metabolites [1]. In urine and feces of animals and humans exposed to inorganic arsenic, dimethylarsinate (DMAs) is the predominant metabolite. Smaller amounts of methylarsonate (MAs), arsenate (iAs^{V}) and arsenite (iAs^{III}) are excreted in urine [1]. The methylation in vitro of inorganic arsenic is catalyzed by cytosolic enzyme(s) which require S-adenosylmethionine (AdoMet), glutathione (GSH), methylcobalamin and Mg^{2+} for activity [2–4]. A variety of analytical methods have been developed to separate and quantify inorganic arsenic species and their methylated metabolites in biological matrices [5–8]. A recent critical review of these methods has noted the inherent difficulties of separating arsenic species in biological matrices [9].

In the work reported here, a TLC separation on cellulose plates developed with isopropanol–acetic acid–water (10:1:2.5) solvent system was

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examined for its ability to separate inorganic arsenic species (iAs^V, iAs^{III}) from their methylated metabolites (MAs, DMAs) in aqueous solution, in liver cytosol incubated with iAs^{III} and in urine of mice exposed to iAs^V. This novel TLC separation was compared with the method of Tam et al. [6] for the separation of these arsenic species. Oxidation of samples by treatment with hydrogen peroxide before TLC separation was found to improve quantitation of MAs separated with either solvent system.

2. Experimental

2.1. Radioactively labelled compounds

Carrier-free [^{73}As]arsenic acid was purchased from Los Alamos Meson Production Facility (Los Alamos, NM, USA). Sodium [^{73}As]arsenite was prepared from [^{73}As]arsenic acid by the method of Reay and Asher [10]. Disodium [^{14}C]methylarsonate (10 mCi/mmol) and sodium [^{14}C]dimethylarsinate (10 mCi/mmol) were obtained from ICN Radiochemicals (Irvine, CA, USA). Sodium arsenate (Sigma, St. Louis, MO, USA), sodium arsenite (Sigma), disodium methylarsonate (Chem Service, West Chester, PA, USA), dimethylarsinic acid (Strem, Newburyport, MA, USA) were added as carriers. All radioactive compounds were stored at −20°C.

2.2. Incubation of rat liver cytosol with [^{73}As]iAs^{III}

Adult (120 days old) Fischer 344 rats (Charles River Breeding Laboratories, Raleigh, NC, USA) were used in the experiment. Liver cytosol was prepared from 20% (w/v) tissue homogenate in 0.02 *M* Tris, 0.25 *M* sucrose buffer (pH 7.6) by centrifugation for 30 min at 145 000 *g* (4°C). Rat liver cytosol (25 μl) was incubated in 60 m*M* Tris (pH 7.6) with [^{73}As]iAs^{III} (1–2 μCi) at 37°C in the presence of 10 m*M* GSH (Sigma), 1 m*M* AdoMet (Sigma), 1.2 m*M* $MgCl_2$ (Sigma), and 100 μg of methylcobalamin/ml (Sigma), following the procedure of Buchet and Lauwerys [3]. Final volume of the incubation mixture was 51 μl (52 μl when iAs^{III} carrier was added). After incubation, the incubation mixture was transfered to an ice bath for several minutes and then ultrafiltered using Microcon microconcentrators (Amicon, Beverly, MA, USA) with 10 kDa cut-off by centrifugation at 12 000 *g* for 10 min at 4°C. Retentate on the filter was washed with 25 μl of ice-cold 0.1 *M* Tris buffer (pH 7.6) and centrifuged at 12 000 *g* for 10 min at 4°C. Ultrafiltrate and wash were combined. An aliquot of combined ultrafiltrate and wash was oxidized with 30% H_2O_2 (Sigma) for 30 min. The final concentration of H_2O_2 in a treated sample was 10%. Fresh unoxidized and oxidized samples were analyzed by TLC. Repeated TLC analysis of oxidized samples showed that composition of arsenic metabolites did not change during storage at 4°C for three months.

2.3. Collection of urine from mice given [^{73}As]iAs^V

Six female B6C3F1 (163–170 days old) mice (Charles River Breeding Laboratories) were placed in two metabolic cages (Nagle Co., Rochester, NY, USA), 3 mice per cage, 2 days before dosing and allowed free access to tap water and Dustless Precision Pellets (BioServe, Frenchtown, NJ, USA). The mice received an oral dose of either carrier-free [^{73}As]iAs^V (11 μCi/mouse) or 5 mg of [^{73}As]iAs^V/kg (13.5 μCi/mouse) in water. Urine was collected at 4, 8 and 24 h post-administration. The collected urine was cooled during the two earlier collection periods (0–4 h and 4–8 h) with U-Tek Refrigerant packs (Polyfoam Packers Corp., Wheeling, IL, USA) and with dry ice during the overnight collection (8–24 h). Collected urines were stored (maximum 20 h) at −70°C prior to chromatographic analysis.

2.4. Thin-layer chromatography

Baker-flex PEI-F cellulose TLC plates (20 × 20 cm) with 254 nm fluorescent indicator (J.T. Baker, Phillisburg, NJ, USA) were used for separation of aqueous solutions of radiolabelled

arsenic compounds and biological samples. The following solvent systems were tested: (i) methanol–1 *M* ammonium hydroxide (8:2) as described by Tam et al. [6] for separation of iAs^{V}, iAs^{III}, MAs and DMAs and (ii) isopropanol–acetic acid–water (10:1:2.5).

The aqueous solutions of radiolabelled arsenic compounds (0.25–1.0 nCi) were separated with or without addition of carrier. The amount of carrier ranged from 25 to 50 nmol. Ultrafiltrates of rat liver cytosol and urine samples (0.5–1.3 μl) were separated along with arsenic standards that were prepared by mixing aqueous solution of radiolabelled arsenic compounds with liver cytosol ultrafiltrate or urine obtained from untreated mice. The front of the mobile phase was allowed to migrate 13 to 15 cm. This required about 90 min in the methanol–ammonium hydroxide solvent system and about 160 min in the isopropanol–acetic acid–water solvent system.

Radiolabelled arsenic compounds were detected on the dried plates using an AMBIS 4000 imaging detector (Ambis, San Diego, CA, USA). The AMBIS 4000 software package was used to create plate images, to calculate R_F values (based on the peak of radioactivity), to evaluate the distribution of radioactivity among chromatographic fractions and to generate histograms. To evaluate radionuclide recoveries after separation, developed plates were cut and the radioactivity of plate segments was measured with a Minaxi γ 5000 counter or a Tri-Carb 2200CA liquid scintillation analyzer (Packard Instruments Co., Downers Grove, IL, USA) after mixing with UltimaGold scintillant (Packard Instruments, Meriden, CT, USA).

3. Results and discussion

3.1. Separation of arsenic compounds from aqueous solution

The methanol–1 *M* ammonium hydroxide solvent system of Tam et al. [6] was developed for separation of iAs^{V}, iAs^{III}, MAs and DMAs. It has been widely used for arsenic speciation in biological samples [6,11,12]. In the present study, the R_F values obtained for iAs^{V}, iAs^{III}, MAs and DMAs (Table 1) were similar to those previously reported [6]. However, the methanol–ammonium hydroxide solvent system (pH 11) oxidized more than 70% of iAs^{III} to iAs^{V}, resulting in the tailing of the [^{73}As]iAs^{III} spot. Addition of iAs^{III} carrier (25 nmol) only partly stabilized iAs^{III} during separation. Tailing of the [^{73}As]iAs^{III} spot made it impossible to separate MAs from iAs^{III} and iAs^{V} when a mixture of the arsenic compounds was analyzed. Recoveries of radioactivity for all compounds separated in the methanol–ammonium hydroxide solvent system were higher than 92%.

Table 1
R_F values for the standard arsenic compounds separated in aqueous solutions

	Methanol–1 *M* NH_4OH (8:2)		Isopropanol–acetic acid–water (10:1:2.5)	
	R_F	Recovery[a]	R_F	Recovery[a]
iAs^{V}	0.00	>98%	0.00	>99%
iAs^{III}	0.35	<30%[b]	0.35	>95%
MAs	0.19	>99%	0.23	>99%
DMAs	0.60	>99%	0.66	>99%

[a] Percentage of radioactivity detected in the position of separated compound as compared with total radioactivity in corresponding lane after separation.
[b] Oxidation of iAs^{III} during separation caused tailing of the iAs^{III} spot.

To improve the TLC separation of the arsenic compounds, several mobile phases were tested in the present study, using different types of cellulose and silica plates. TLC on Baker-flex PEI-F cellulose plates developed with an isopropanol–acetic acid–water (10:1:2.5) solvent system provided a better separation of all four arsenic compounds (Table 1). In repeated separations with or without addition of carrier arsenite, oxidation of iAs^{III} did not exceed 5%. Addition of carrier had no significant effect on the chromatographic behavior or recovery of either radiolabelled arsenic compound. Recoveries of radioactivity after separation were greater than 95%.

3.2. Separation of methylated ^{73}As-metabolites from rat liver cytosol

TLC was used to characterize the pattern of inorganic arsenic methylation in an in vitro system for enzymatic synthesis of methylated arsenic compounds [3]. The formation of methylated arsenic metabolites was examined in rat liver cytosol incubated with carrier-free or 1 μM [^{73}As]iAs^{III}. Ultrafiltrates from this incubation mixture were separated on the Baker-flex PEI-F cellulose plates in the methanol–1 M ammonium hydroxide (8:2) solvent system (Fig. 1) or in the isopropanol–acetic acid–water (10:1:2.5) solvent systems (Fig. 2). A mixture of arsenic standards prepared in ultrafiltrate from untreated liver cytosol was chromatographed in parallel with ultrafiltrates of the incubation mixture.

Because of oxidation (tailing) of iAs^{III} which interfered with separation MAs, the mixture of arsenic standards prepared in cytosol ultrafiltrate was not adequately separated by the methanol–ammonium hydroxide solvent system (Fig. 1, I). To allow separation and quantitation of MAs, iAs^{III} in the standard mixture and in ultrafiltrates from liver cytosol samples was oxidized to iAs^{V} by 10% H_2O_2 (Fig. 1, II–VIII). However, even after oxidation, partial overlay of the MAs and iAs^{V} fractions occurred in samples in which a relatively large fraction of the arsenic was present as inorganic arsenic (Fig. 1, III,VI,VII). Oxidation of samples did not significantly affect R_F values for either MAs or DMAs. Recovery of radioactivity after separation in the methanol–ammonium hydroxide solvent system ranged from 99% to 110%.

Almost complete separation of all four arsenic standards in cytosol ultrafiltrate was accomplished on Baker-flex TLC plates developed with the isopropanol–acetic acid–water (10:1:2.5) solvent system (Fig. 2, I). However, a small interference between MAs and iAs^{III} fractions still persisted. To minimize this interference, the standard mixture and ultrafiltrates from incubated cytosol were oxidized with 10% H_2O_2. The R_F value for MAs increased slightly in oxidized samples. Oxidation with H_2O_2 permitted complete separation of iAs^{V}, MAs and DMAs fractions (Fig. 2, II–VIII). Recovery of radioactivity from standards and samples separated in the isopropanol–acetic acid–water (10:1:2.5) solvent system ranged from 97% to 108%.

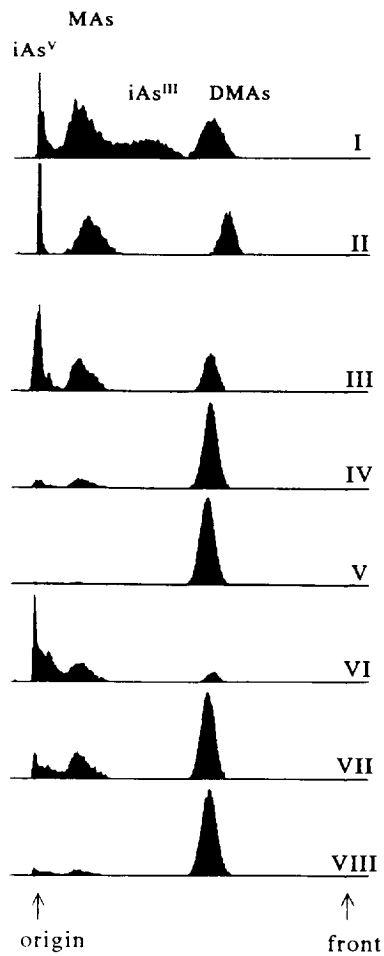

Fig. 1. Separation of arsenic compounds from rat liver cytosol ultrafiltrate by a methanol–1 M NH_4OH (8:2) solvent system: unoxidized arsenic standards in cytosol ultrafiltrate (I); oxidized arsenic standards in cytosol ultrafiltrate (II); oxidized ultrafiltrate from cytosol incubated with carrier-free [^{73}As]iAs^{III} for 5 (III), 15 (IV) and 30 min (V); oxidized ultrafiltrate from cytosol incubated with 1 μM [^{73}As]iAs^{III} for 5 (VI), 15 (VII) and 30 min (VIII).

Table 2 summarizes the patterns of arsenic metabolites in oxidized cytosol ultrafiltrates that

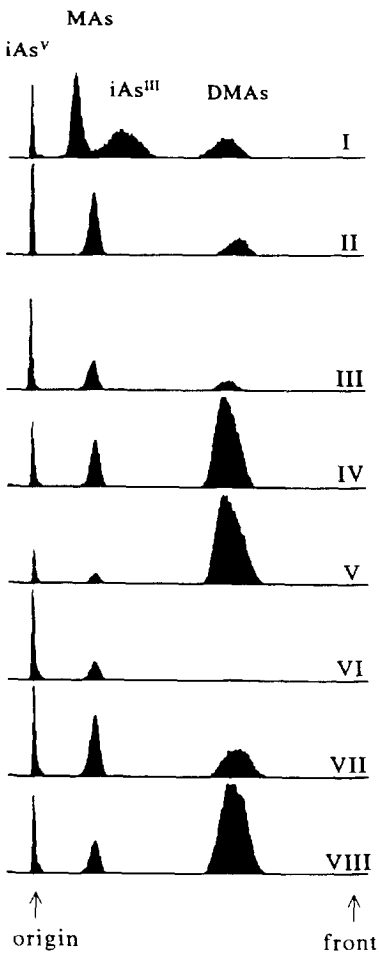

Fig. 2. Separation of arsenic compounds from rat liver cytosol ultrafiltrate by an isopropanol–acetic acid–water (10:1:2.5) (for lane labels legends see Fig. 1).

were separated by either a methanol–ammonium hydroxide (8:2) or an isopropanol–acetic acid–water (10:1:2.5) solvent system. In addition to iAs^{V}, both MAs and DMAs were identified in cytosol ultrafiltrates. The percentage of the total ^{73}As present as MAs was highest at 5 min in cytosol incubated with carrier-free [^{73}As]iAs^{III} and at 15 min in cytosol incubated with 1 μM [^{73}As]iAs^{III}. The amount of DMAs increased continuously with incubation time. At 30 min, DMAs accounted for 89.9–91.2% of the total ^{73}As in samples with carrier-free [^{73}As]iAs^{III} and for about 81.0–83.7% in samples with 1 μM [^{73}As]iAs^{III}. In samples containing 1 μM iAs^{III} carrier, the percentage of the radionuclide present as iAs^{V} was higher throughout the incubation period. This suggested that the rate of methylation at a higher concentration of substrate was relatively slower than that observed under carrier-free conditions. This finding is consistent with previously published data on arsenic metabolism [1]. In all samples, there was good agreement between the amounts of DMAs and MAs determined by the methanol–ammonium hydroxide (8:2) solvent system and the isopropanol–acetic acid–water (10:1:2.5) solvent system.

Regardless of iAs concentration in the assay mixture and the time of incubation, about 55–65% of the ^{73}As was bound to cytosolic protein (i.e. it was retained on a microconcentrator filter with a 10 kDa cut-off).

3.3. Separation of methylated ^{73}As-metabolites from mouse urine

TLC on Baker-flex PEI-F cellulose plates with methanol–1 *M* ammonium hydroxide (8:2) and isopropanol–acetic acid–water (10:1:2.5) solvent systems was used to separate and quantify the methylated arsenic metabolites in urine obtained from mice which received carrier-free [^{73}As]iAs^{V} or 5 mg of [^{73}As]iAs^{V}/kg.

Only DMAs could be unambiguously identified in a mixture of arsenic standards prepared in urine from untreated mice that was separated with the methanol–ammonium hydroxide solvent system (Fig. 3, I). The other arsenic standards were not resolved. Oxidation of the arsenic standard mixture in urine with 10% H_2O_2 did not improve their separation (Fig. 3, II). Chromatography of the individual arsenic compounds in mouse urine showed that radioactivity from [^{73}As]iAs^{V} was eluted in three fractions with R_F values of 0, 0.07 and 0.12. Similarly, separation of radiolabelled iAs^{III} in urine yielded several fractions with R_F values ranging from 0 to 0.2. Two radioactive fractions (R_F 0.12 and 0.16) were detected after chromatography of [^{14}C]MAs. [^{14}C]DMAs migrated as a single

Table 2
Arsenic metabolites in oxidized liver cytosol ultrafiltrates as determined by TLC in methanol–ammonium hydroxide (8:2) or isopropanol–acetic acid–water (10:1:2.5) solvent systems

Incubation time (min)	As species	Cytosol incubated with carrier-free [^{73}As]iAs^{III}		Cytosol incubated with 1 μM [^{73}As]iAs^{III}	
		Methanol–1 M NH_4OH (8:2)	Isopropanol–acetic acid–water (10:1:2.5)	Methanol–1 M NH_4OH (8:2)	Isopropanol–acetic acid–water (10:1:2.5)
5	iAs^{V}	35.1	39.7	53.7	59.3
	MAs^{V}	33.3	35.4	31.2	30.1
	DMAs^{V}	29.7	25.6	14.2	10.9
15	iAs^{V}	6.5	8.2	15.3	18.6
	MAs^{V}	12.7	15.8	27.3	32.5
	DMAs^{V}	79.6	76.1	55.6	48.5
30	iAs^{V}	3.6	5.2	6.7	8.4
	MAs^{V}	4.0	4.7	8.1	10.2
	DMAs^{V}	91.2	89.9	83.7	81.0

Percentage of ^{73}As recovered after TLC separation; average of duplicates.

radioactive peak with an R_F value of 0.58. This chromatographic pattern indicates that iAs^{V}, iAs^{III} and possibly MAs react with unknown substance(s) present in mouse urine to yield stable compounds which cannot be resolved with the methanol–ammonium hydroxide (8:2) solvent system. Consequently, only DMAs could be identified and quantified in mouse urine using this solvent system (Fig. 3, III–VIII). Recovery of radioactivity from urine separated in the methanol–ammonium hydroxide solvent system ranged from 85% to 100%.

All four standard arsenic compounds were separated in mouse urine when the isopropanol–acetic acid–water solvent system (10:1:2.5) was used for TLC separation (Fig. 4, I). Oxidation with 10% H_2O_2 of the arsenic standard mixture before application to the TLC plate improved the separation of MAs from inorganic arsenic (Fig. 4, II). The chromatographic pattern for methylated arsenic metabolites in urine of mice treated with [^{73}As]iAs^{V} matched that for the arsenic standards (Fig. 4, III–VIII). Recovery of radioactivity from the standards and samples separated in urine by the isopropanol–acetic acid–water solvent system ranged from 97% to 110%.

Table 3 shows the content of arsenic metabolites in urine of mice given carrier-free [^{73}As]iAs^{V} and 5 mg of [^{73}As]iAs^{V}/kg. DMAs was the major urinary metabolite in mice treated with carrier-free [^{73}As]iAs^{V}. It represented more than 99% of the total radionuclide excreted in urine at all collection intervals. Inorganic arsenic and MAs as determined by TLC with isopropanol–acetic acid–water solvent system accounted for less than 0.5% and 0.1% of the total radioactivity, respectively. Higher relative amounts of these compounds were found in urine of mice given 5 mg of [^{73}As]iAs^{V}/kg especially during the first 4 h after administration. Over the 24-h period, inorganic arsenic accounted for more than 13% of total ^{73}As excreted in urine. Analysis of unoxidized samples showed that about half of the inorganic arsenic in cumulative 24-h urine was iAs^{III} (data not shown). MAs was a minor urinary metabolite, accounting for 0.73% of excreted radioactivity. DMAs was the major

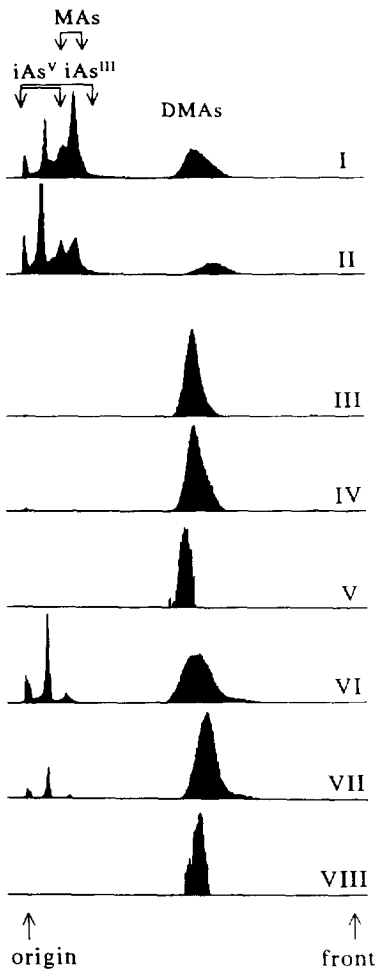

Fig. 3. Separation of arsenic compounds from urine by a methanol–NH_4OH (8:2) solvent system: unoxidized arsenic standards in urine (I); oxidized arsenic standards in urine (II); oxidized urine from mice given carrier-free [^{73}As]iAs^{V} collected in 0–4 (III), 4–8 (IV) and 8–24 h (V) time intervals after administration; oxidized urine from mice given 5 mg of [^{73}As]iAs^{V}/kg collected in 0–4 (VI), 4–8 (VII) and 8–24 h (VIII) time intervals after administration.

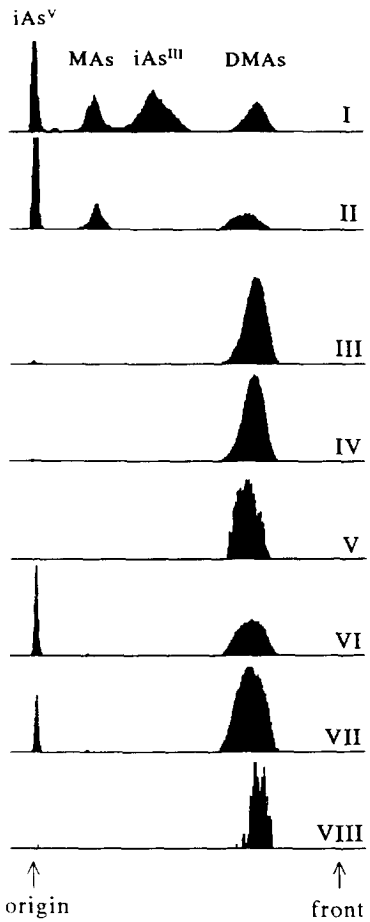

Fig. 4. Separation of arsenic compounds from urine by an isopropanol–acetic acid–water (10:1:2.5) solvent system (for lane labels legends see Fig. 3).

urinary metabolite and accounted for 86% of total excreted radioactivity. Analysis by ion-exchange and reversed-phase HPLC of urine collected from mice after oral administration of 5 mg of [^{73}As]iAs^{V}/kg found a similar pattern of arsenic metabolism [8].

4. Conclusions

The present study showed that the methanol–ammonium hydroxide (8:2) solvent system widely used for TLC speciation of arsenic in biological samples cannot properly separate MAs from both iAs^{V} and iAs^{III} especially when used for analysis of methylated arsenic metabolites in urine. A newly developed isopropanol–acetic acid–water (10:1:2.5) solvent system can unmistakably identify methylated arsenic metabolites

Table 3
Arsenic metabolites in oxidized mouse urine as determined by TLC in methanol–ammonium hydroxide (8:2) or isopropanol–acetic acid–water (10:1:2.5) solvent systems

Collection time (h)	As species	Mice administered with carrier-free [^{73}As]iAs^{V}		Mice administered with 5 mg of [^{73}As]iAs^{V}/kg	
		Methanol–1 *M* NH_4OH (8:2)	Isopropanol–acetic acid–water (10:1:2.5)	Methanol–1 *M* NH_4OH (8:2)	Isopropanol–acetic acid–water (10:1:2.5)
0–4	iAs^{V}	–	0.3	–	27.2
	MAs	–	0.1	–	1.3
	DMAs	99.8	99.7	73.3	72.5
4–8	iAs^{V}	–	0.4	–	9.6
	MAs	–	0.1	–	1.0
	DMAs	99.7	99.5	89.9	90.7
8–24	iAs^{V}	–	0.3	–	0.2
	MAs	–	ND[a]	–	ND[a]
	DMAs	99.9	99.6	87.1	86.0

Percentage of ^{73}As recovered after TLC separation; average of duplicates.
[a] Not detected.

in aqueous solution, in urine and in liver cytosol ultrafiltrate when separated on Baker-flex PEI-F cellulose TLC plates. Oxidation of samples with H_2O_2 as performed in the present study may help to improve quantitation of the MAs fraction.

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