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Speciation of arsenic metabolites in the urine of occupational workers and experimental rats using an optimised hydride cold-trapping method†

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A hydride cold-trapping technique was developed and optimised for the measurement of urinary arsenic metabolites. The analytical precision of the method was found to be 6.1, 4.0 and 4.8% (*n* = 5) for inorganic arsenic (As_i), monomethylarsonate (MMA) and dimethylarsinate (DMA), respectively, with recoveries close to 100%. The detection limits were 1.0, 1.3 and 3 ng for As_i, MMA and DMA, respectively. The method was then used to analyse urine samples obtained from three groups of workers for occupational exposure in three companies where copper chrome arsenate was used for timber treatment. The results were compared with those for a normal control group of laboratory workers. Arsenic and its metabolites were also measured in experimental rats given 5 mg As kg^{-1} body mass by oral gavage in the form of sodium arsenite, calcium arsenite or sodium arsenate. Occupational workers showed a significantly higher excretion of As_i. Up to two fold increases of urinary As_i excretion in rats compared with control rats were also observed in animals dosed with various forms of arsenicals. The method is suitable for the measurement of arsenic metabolites in urine of both humans and experimental animals.

Keywords: *Arsenic, speciation; monomethylarsonate; dimethylarsinate; arsenic methylation; urinary metabolite; hydride generation atomic absorption spectrometry*

Arsenic is one of the most abundant elements on earth and occurs naturally in many chemical forms. The toxicity of arsenicals varies widely, ranging from relatively harmless organic compounds through the more hazardous inorganic arsenate and arsenite species. In mammals, inorganic arsenic (As_i) is methylated in the body to its less harmful metabolites monomethylarsonate (MMA) and dimethylarsinate (DMA), then excreted in the urine together with the unchanged inorganic species.[1] Organic arsenicals, such as those encountered in seafood, include arsenobetaine and arsenocholine which are relatively non-toxic and pass through the alimentary system unchanged. It is important to speciate chemically arsenic metabolites of inorganic origin and to distinguish them from arsenic of marine origin. The latter is generally regarded as less important to human health risk assessment.

Hydride generation has been used for arsenic speciation since 1973, when Braman and Foreback[2] introduced a cold-trapping step into a basic hydride generation system. Since then, a number of improvements have been made to the system, including the addition of water[3] and CO_2 traps[4] and the use of hydrogen as an atomisation support gas.[5]

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In 1981, Buchet and Lauwerys[6] reported a hydride generation technique in urine analysis to distinguish arsenic levels in occupational exposure to arsenic of marine origin. Total arsenic levels (As_{tot}) were measured by acid digestion of urine followed by hydride generation. The cold trapping speciation technique was used to measure As_i (As^{III} or As^{V}) and its metabolites MMA and DMA in urine without acid digestion where arsenic originating from marine organisms does not undergo hydride formation. The difference, $As_{tot} - (As_i + MMA + DMA)$, was attribute to arsenic of marine origin.

For health risk assessment, the traditional measurement of total urinary arsenic could lead to false positive diagnosis because of the probable presence of arsenic derived from seafood. Therefore, when the total arsenic level is high in the urine, it is essential to separate metabolites of inorganic arsenic origin. In our study, the method reported by Cleuvenbergen *et al.*[5] was used as the basis for the investigation and optimisation of this technique. The optimised method was then used to analyse the arsenic metabolite profile in urine samples obtained from workers for occupational exposure in three companies where copper chrome arsenate was used for timber treatment, and in urine obtained from experimental rats exposed to various arsenicals for comparison. Rats were used as positive controls to demonstrate alterations in the arsenic metabolite profile resulting from exposure to various arsenicals.

Experimental

Arsenic was measured post-hydride generation on a Varian 1475DB atomic absorption spectrometer. The hydride generation system used is illustrated in Fig. 1. The system consisted of a Pyrex glass reaction vessel, two Pyrex glass water traps and a chromatographically packed collection trap. The 100 ml reaction vessel had a B24 ground-glass fitting with 3 mm id carrier

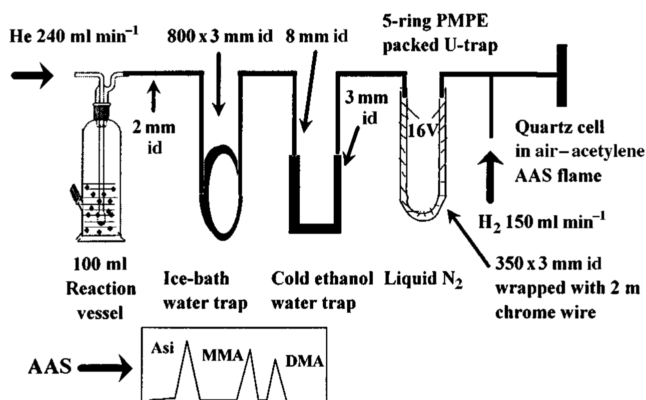

Fig. 1 Schematic diagram for the hydride cold-trap speciation of arsenic metabolites.

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gas inlet and outlet tubes. $NaBH_4$ was injected *via* a side arm near the base of the reaction vessel through a silicone-rubber GC septum (Shimadzu, Kyoto, Japan) secured by silicone-tubing. The two water traps selected consisted of a 150 mm length tapered U-tube (large 8 mm id, small 3 mm id) and a modified GC glass column of 800 × 3 mm id. The hydrides were collected on one of two 350 × 3 mm id collection traps, used alternatively, and packed with 10% polyphenylether [5 rings (PPE)] (Alltech, Baulkham Hills, NSW, Australia) supported on Chromosorb DMCS, 100–120 mesh (Supelco, Bellefonte, PA, USA). Both collection traps contained approximately 1.04 g of this packing, which was secured using dimethyldichlorosilane (DMCS) treated chromatographic grade glass-wool plugs. To aid in drying of the collection-traps after use, approximately 2 m of 1.6 Ω ft^{-1} resistance wire was wound around the outside and connected, on completion of a run, to a variable transformer from which a voltage of 16 V was supplied. The drying was further aided by connecting a high purity nitrogen gas supply to the heated trap with a post-tube flow rate of 300 ml min^{-1}. All connections between glassware were made using 2 mm id silicon tubing.

Helium was used as the carrier gas at a flow rate of 240 ml min^{-1}. The flow rate was referred to the rate measured in the system after passage through the collection trap at room temperature. Hydrogen support gas was brought into the system after the collection-trap by means of a plastic Y-connector. A hydrogen flow rate of 150 ml min^{-1} was used. The flow rates of both gases were controlled using regulators and measured with a soap film flow meter.

Reagents

Water purified with a Milli-Q system (Millipore, Bedford, MA, USA) was used throughout. All reagents were of analytical-reagent grade unless specified otherwise. A stock standard solution of dimethylarsinic acid (1000 mg l^{-1}) was prepared by dissolving the appropriate amount of the solid (Sigma–Aldrich, Castle Hill, NSW, Australia) in 0.06 M HCl. Arsenate and arsenite stock standard solutions were prepared in the same manner using sodium arsenate and sodium arsenite (BDH, Kilsyth, Victoria, Australia), respectively. Monomethylarsenic stock standard solutions were prepared from disodium methylarsenate (Chem Service, West Chester, PA, USA). Working standard solutions (25 µg l^{-1}) of each compound were prepared by serial dilution in 0.06 M HCl. A 10% $NaBH_4$ solution was prepared by sonication of the reagent (Ajax Chemicals, Auburn, NSW, Australia) in 0.02 M NaOH for 10 min followed by filtration through a 0.45 µm filter membrane. All solutions were stored under refrigeration to aid stability. The acidified standard solutions were stable for more than 1 month and the $NaBH_4$ solution for 1 week.

Urine collection

Urine samples were collected from occupational workers in the afternoon of a work shift. The samples were collected into acid washed laboratory beakers and divided into two aliquots in plastic urine specimen jars (Sarstedt, Adelaide, SA, Australia). Wistar rats of about 200 g body mass were dosed by oral gavage at a dose rate of 5 mg As kg^{-1} body mass with a solution of sodium arsenate or sodium arsenite or a slurry of wheat flour spiked with calcium arsenite. Each rat was kept in an individual metabolic cage for 24 h urine collection. All samples were stored frozen until speciation analysis.

Sample preparation

Experimental rat urine samples were mixed thoroughly by vortex mixing and diluted up to 500-fold in 0.06 M HCl before analysis. Human urine samples were analysed without dilution. An appropriate volume of sample or standard (0.1–4.0 ml) was transferred into the reaction vessel containing 4.0 ml of 6 M HCl and one drop of anti-foam reagent. Water (20 ml) was added to bring the level above the septum inlet and the vessel was then closed and sealed. The first column water trap was immersed in an ice-bath and the second (U-tube) in a cold ethanol (−70 °C) bath. The helium carrier gas was started at a flow rate of 240 ml min^{-1} and the system was de-gassed until a constant absorbance was obtained (typically 1 min). The collection trap was then immersed in liquid nitrogen for a further 1 min. The spectrophotometer was zeroed with hydrogen support gas turned on at a flow rate of 150 ml min^{-1}. Five 2 ml portions of 10% $NaBH_4$ solution were then added slowly through the rubber septum using a syringe. The hydrides were collected on the cold trap for 4.5 min after the final addition of $NaBH_4$. The collection-trap was removed from the liquid nitrogen and the signals were recorded on a Model CR4A integrator (Shimadzu).

On completion of the run, the collection trap was removed from the system and the wire was attached to the terminals of the variable transformer. Nitrogen was pushed through this trap at 300 ml min^{-1} and a voltage of 16 V was applied. The next run was carried out using the second collection trap, while the first was being dried, to increase the throughput.

The total arsenic content of urine samples was determined using a standard hydride generation method following an acid digestion process (Australian Standards Method 3502). All urine arsenic measurements were standardised against urinary creatinine concentrations. The analyser for the measurement of creatinine was a Roche Cobas Mira random access system. The creatinine reagent (picric acid method, the Jaffe reaction) was supplied by Trace Scientific P/L (Castle Hill, NSW, Australia). All samples were analysed on the same working day.

Results and discussion

Three wavelengths were investigated, 189.0, 193.7 and 197.3 nm. The most sensitive line was 189.0 nm. However, 197.3 nm (Table 1) was selected as the most suitable wavelength for arsenic determination because it afforded a wider linear working range, reproducibility of peak areas and linearity

Table 1 Analytical characteristics of the optimised system at three wavelengths

Wavelength/nm	Parameter	As_i	MMA	DMA
189.0	Linear working range/ng	0–10 (r = 0.9901)	0–20 (r = 0.9954)	0–25 (r = 0.9905)
	Detection limit/ng	0.3	0.5	0.3
	Reproducibility (%)	> 15 (n = 6)	> 15 (n = 7)	> 15 (n = 7)
193.7	Linear working range/ng	0–15 (r = 0.9902)	0–25 (r = 0.9942)	0–25 (r = 0.9949)
	Detection limit/ng	0.6	0.6	0.6
	Reproducibility (%)	8.5 (n = 6)	9.0 (n = 7)	8.0 (n = 7)
197.3	Linear working range/ng	0–20 (r = 0.9951)	0–50 (r = 0.9995)	0–50 (r = 0.9973)
	Detection limit/ng	1	1.3	3
	Reproducibility (%)	6.1 (n = 5)	4.0 (n = 6)	4.8 (n = 6)

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of calibration lines. Although the sensitivity was lower at this wavelength, it was still ample for the determination of arsenic metabolites in urine. It was essential that peak areas were the basis for analysis, since peak height measurements provided reproducibilities of up to 20%. Typical peak areas for 25 ng of arsenic were 2.5×10^6, 2.2×10^6 and 1.2×10^6 μV s^{-1} for As_i, MMA and DMA respectively.

The analysis of the effect of each individual parameter was optimised in the following manner.

Reaction vessel type

Initial experiments were performed using a Varian (Palo Alto, CA, USA) Model 65 vapour generation accessory. On insertion of the packed collection trap into this system, the back-pressure created in the Model 65 unit allowed flow rates no greater than 200 ml min^{-1}. Modification of the reaction vessel to allow carrier gas inlet and outlet tubes to be fitted using a ground-glass joint allowed flow rates of up to 700 ml min^{-1} to be achieved. Two different sizes of these vessels were investigated. The small 25 ml vessel provided too little headspace for expansion when high flow rates were used. The borohydride reaction resulted in a large increase in pressure, and only the use of a 100 ml reaction vessel prevented gas leaks and blow-outs elsewhere in the hydride generation system.

Reaction stoichiometry and conditions

Oxalic acid and hydrochloric acid were evaluated. Results of experiments following conditions reported by Cleuvenbergen *et al.*[5] (1 ml of 6 M HCl and 2 ml of 4% $NaBH_4$) without using hydrogen support gas showed that these conditions were unsuitable in the current system. The results were unpredictable and not reproducible.

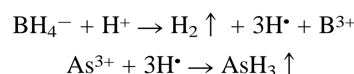
The reaction described by Feldman[7] (20 ml of 1% oxalic acid and 6 ml of 2% $NaBH_4$) improved the performance of the system, but the reproducibility was still unacceptable. Increasing the volume of borohydride solution used to 5 ml of 6% solution and using 8 ml of 2% oxalic acid further improved the performance to RSD 12% for the DMA peak area and the detection limit to 10 ng at a 197.3 nm detection wavelength. These conditions were suitable for the quantitative conversion of MMA, DMA and arsenite into their respective arsines, However, the conversion of arsenate into arsine was only 20% as efficient. As the latter reaction is very pH dependent, being most efficient at pH 1,[5] the volume of 2% oxalic acid used was increased progressively up to 30 ml. This had the undesirable effect of lowering not only the arsenate but also the arsenite and methylated arsenic (species with higher pH optimum[5]) responses to unacceptably poor levels.

The introduction of hydrogen as an atomisation support gas proved to be the most crucial step in the study. When the same acid and borohydride concentrations at which little if any arsine detection occurred previously (*i.e.*, 30 ml of 2% oxalic acid and 5 ml of 6% $NaBH_4$) and a minimum hydrogen flow rate of 150 ml min^{-1} were used, the performance of the system improved markedly. The detection limit of methylated arsines improved to 3 ng with RSD 4.0 and 4.8 for MMA and DMA, respectively, at 197.3 nm (Table 1). In the presence of hydrogen, the arsenate response was as efficient as that of arsenite, with a detection limit of 0.8 ng and RSD 5%.

It was clear that hydrogen played a prominent role in the atomisation of the arsines under these reaction conditions. It was also apparent that different atomisation mechanisms were occurring under different reaction conditions, since acceptable results could be achieved for arsenite, MMA and DMA species without the presence of hydrogen support gas. However, if all arsenic metabolites were to be atomised efficiently and reproducibly, the presence of hydrogen was required.

The role of air in the atomisation process was investigated. Cleuvenbergen *et al.*[5] claimed a 20–60% improvement in sensitivity when a minimum hydrogen to air ratio of 2 : 1 was used. However, in this study, no such improvement was found, and in fact the presence of air in the quartz furnace cell increased the noise significantly and imparted a sloping baseline absorbance to each run.

To avoid excessive use of reagents, *i.e.*, the use of 30 ml of 2% oxalic acid per run, the response of the system was again studied using 6 M HCl. A 2 ml volume of 6 M HCl gave the same response as 30 ml of 2% oxalic acid. However, as shown in Fig. 2, the optimum reaction stoichiometry for the current system proved to be 4 ml 6 M HCl and 10 ml of 10% $NaBH_4$. These concentrations were far in excess of those used by Cleuvenbergen *et al.*[5] and indicated the presence of high concentrations of hydride ions, as suggested by the mechanism



$$BH_4^- + H^+ \rightarrow H_2\uparrow + 3H^\bullet + B^{3+}$$
$$As^{3+} + 3H^\bullet \rightarrow AsH_3\uparrow$$

The main mechanisms of interference in the determination of arsines are proposed to be (i) competitive hydride formation by an interferent,[8] (ii) reduction of a generated hydride to the metal,[9–11] and (iii) adsorption of the arsine on the precipitated metal surfaces. No interference experiments were undertaken in this study, but the final conditions chosen should minimise the potential for interference. The use of high concentrations of $NaBH_4$ must overcome interference from the first mechanism. Experiments run to determine the optimum concentration of $NaBH_4$ showed that the increase in $H^\bullet$ levels did not act to reduce the arsines to arsenic metal as proposed by the second mechanism. The use of 4 ml of 6 M HCl acts to increase the solubility of any transition metal through the formation of chloro complexes,[9–11] thus decreasing their capacity to act through the second and third mechanisms. To confirm that no interference occurred in a typical human urine matrix, a number of spike recovery experiments were carried out and the results are given in Table 2.

Water and CO_2 *traps*

The advent of a wet column packing disrupted the carrier gas flow rate and could cause: (i) the volume of air in the quartz furnace cell to increase, thus causing more noise and a drifting baseline, (ii) the retention time of the arsines to change, (iii) the peak shape and area to change and (iv) the peak resolution to decrease.

Cleuvenbergen *et al.*[5] claimed that their method did not require a drying step; however, the volume of water vapour generated in the current reaction necessitated a rigorous drying procedure (Fig. 1). In the experiments in which oxalic acid was used, an NaOH packed CO_2 trap was added to the system. This served to remove noise and baseline drift associated with CO_2

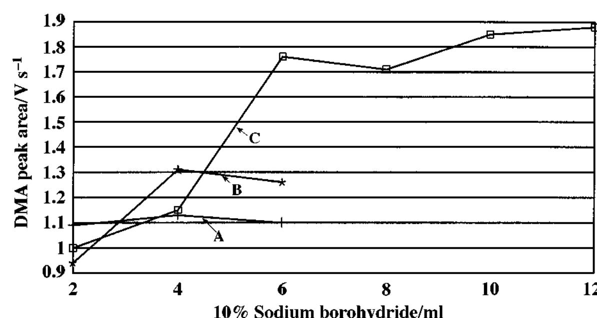


Fig. 2 Dependence of DMA response on 6 M HCl and 10% borohydride volumes. A, 1; B, 2; and C, 4 ml 6 M HCl.

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evolution from oxalic acid. However, the subsequent use of HCl did not require the use of this trap, since CO_2 was not generated and the de-gassing step removed all dissolved gases from the reaction solution prior to the addition of $NaBH_4$.

Collection-trap conditions

Initial experiments were undertaken using silanised glass beads (0.5 mm od) and quartz beads of various diameters as a collection trap stationary phase. The 350 mm length collection trap did not provide sufficient peak resolution with these packings. Modified columns of total length 550 mm provided acceptable results, although this length of packing restricted the carrier gas flow rates that could be comfortably applied to about 200 ml min^{-1}.

Converting the stationary phase to 10% PPE[5] gave better resolution for MMA and DMA and a wider range of carrier gas flow rates. Nonetheless, the total column length needed in the current study (350 mm) was almost double that recommended.[6] Collection times ranging from 4.5 to 15 min were tested, with no discernible difference in response.

Carrier gas

The suitability of nitrogen and helium as carrier gases was evaluated. Dissolved nitrogen in air-saturated water has been shown to act as a positive interference in arsenic determinations,[12] but in this study nitrogen was shown to have a signal suppressing effect, probably interfering in the atomisation process. The areas of the arsine peaks, using nitrogen, were only 20–40% of the areas when helium was used.

Flow rate optimisation experiments showed that provided that the column packing and quartz furnace cell were working efficiently, the response was reproducible with the range 180–300 ml min^{-1}. At the upper end of this range, the MMA–DMA peak resolution begins to deteriorate. A flow rate of 180 ml min^{-1} was generally adopted to minimise the pressure within the system, consequently reducing the possibility of leaks and blow-outs.

Volatilisation

Many complex methods have been described for the volatilisation of arsines after the cold trapping step is complete, ranging from applying a voltage to 11 Ω m^{-1} wire wrapped around the trap[5] to applying a 1400 linear ft min^{-1} velocity of air at room temperature.[6]

In our study, the best resolution was obtained by simply removing the collection trap from the liquid nitrogen and allowing it to warm at room temperature. Attempts to aid the volatilisation process by applying a range of voltages to the wire around the trap failed, resulting in worse resolution and poor peak shapes. The electrical heating was only applied off-line to dry the trap between runs while another trap was in use.

Silanisation

When the system decreased in sensitivity and increased in daily fluctuation in performance, it was necessary to treat all glass parts of the system with a silanising reagent as recommended by Cleuvenbergen *et al.*[5] A 10% DMCS solution in toluene was used for this purpose.

A study by Reamer *et al.*[13] found that up to 23% of ^{75}As was adsorbed on the walls of glass reaction vessels in a hydride generation system. On silanising the glass twice, this was reduced to 2%.

Contrary to this recommendation, the silanisation treatment alone did not return the current system to optimum sensitivity. However, when silanisation was carried out in conjunction with collection-trap repacking every 100 runs, in addition to daily acid washing of the quartz furnace cell, the system performed satisfactorily.

Urine analysis

Confirmation that the urine matrix had no effect on the recovery of the arsenic species was made by a series of spike recovery tests. Table 2 gives the results obtained from the spikes, within the limits of the reproducibility of the method.

Reproducible results were obtained from human samples with at least 30 s mixing on a vortex mixer. The samples could be kept refrigerated for up to1 month, although freezing of the samples was still preferred. The use of one drop of anti-foam reagent did not affect the response of the system.

Table 3 gives the results of the analysis of the human urine samples. Comparison of total metabolites with total arsenic showed that the subjects confirmed as having high seafood intakes had total arsenic levels far in excess of the metabolite total. These results were consistent with the work of Buchet and Lauwerys,[6] who first used this method to distinguish arsenic

Table 2 Spike recoveries from three urine samples using an 1 ml sample volume

Urine sample	As species	Measured in sample/ng	Spike added to sample/ng	Actual total/ng	Found in spiked sample/ng	Recovery (%)
1	As_i	1.9	10.8	12.7	12.9	102
	MMA	3.9	25	28.9	28.2	98
	DMA	10.1	25	35.1	38	109
2	As_i	6.3	0	6.3	7	111
	MMA	4.2	25	29.2	28.3	97
	DMA	4.9	25	29.9	27.1	91
3	As_i	7.6	0	7.6	7.7	101
	MMA	1.5	0	1.5	1.9	127
	DMA	9	25	34	33	97

Table 3 Arsenic metabolite concentrations (As_i + MMA + DMA) in urine of employees of timber treatment companies A, B and C and normal control group of laboratory staff

Group	*n*	Total As/ μg g^{-1} creatinine	Metabolite/ μg g^{-1} creatinine	As_i (%)	MMA (%)	DMA (%)
A	7	76 ± 55	64 ± 39	50 ± 24	21 ± 11	29 ± 18
B	20	33 ± 44	10 ± 8	22 ± 16	18 ± 8	60 ± 18
C	3	25 ± 14	11 ± 7	32 ± 18	30 ± 9	38 ± 17
Control	7	53 ± 101	7 ± 5	7 ± 12	47 ± 37	46 ± 34

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levels resulting from occupational exposure from arsenic levels of marine origin. They found that high total arsenic concentrations did not necessarily imply occupational exposure. Our study substantiates the importance of speciation of arsenic metabolites.

The urinary metabolic profiles of arsenic varies depending on the species of animals, as demonstrated by Vahter.[1] Normally, a large proportion of the arsenic is excreted in the urine by humans as methylated forms and usually less than 27% of the inorganic arsenic is excreted unchanged. However, a recent study[14] found high levels of As_i ($\geqslant$50%) in children with chronic arsenic exposure in northern Argentina.

The average total urinary arsenic metabolites in the urine of the control group was 7 ± 5 μg As g^{-1} creatinine (n = 8 laboratory workers) consisting 7 ± 5, 47 ± 36 and 46 ± 34% of As_i, MMA and DMA, respectively. Factory workers from company A had the highest exposure of arsenic averaging 64 ± 39 μg As g^{-1} creatinine (n = 7) with 50 ± 24, 21 ± 11 and 29 ± 18% of As_i, MMA and DMA, respectively. Company B workers had an average of 10 ± 8 μg As g^{-1} creatinine (n = 20) with 22 ± 16, 18 ± 8 and 60 ± 17% of As_i, MMA and DMA, respectively. Company C workers had an average of 11 ± 7 μg As g^{-1} creatinine (n = 3) with 32 ± 18, 30 ± 9 and 38 ± 17% of As_i, MMA and DMA, respectively. Although the average excretion of arsenic metabolites (As_i + MMA + DMA) from the surveyed industrial workers was below the NHMRC recommended level of 150 μg As g^{-1} creatinine in Australia, it is of particular interest that workers from company A showed significantly higher excretion of As_i. This is similar to the levels found in children in northern Argentina studied by Concha *et al.*[14] The increased As_i excretion was also observed in rats dosed with arsenate and arsenite in this study (Table 4). When rats were given a sub-lethal dose of arsenic, the As_i levels were increased by up to two fold compared with control rats.

Table 4 Arsenic metabolite concentrations (As_i + MMA + DMA) in urine of rats 24 h after a single oral dose of 5 mg kg^{-1} body mass of arsenic in the form of sodium arsenite, calcium arsenite or sodium arsenate

Group	n	Total/ μg l^{-1})	As_i (%)	MMA (%)	DMA (%)
Sodium arsenite	5	5280 ± 150	58 ± 25	16 ± 20	26 ± 24
Calcium arsenite	4	3690 ± 163	66 ± 6	3 ± 4	31 ± 4
Sodium arsenate	4	2590 ± 1118	76 ± 4	4 ± 3	20 ± 3
Control	6	112 ± 27	38 ± 4	8 ± 3	54 ± 2

In conclusion, this method is relatively non-expensive compared with, for example, an HPLC–ICP-MS technique.[15] Although the latter technique is more sensitive, the current method offers sufficient sensitivity, precision and selectivity for the measurement of urinary arsenic metabolites, which is essential for the monitoring of occupational exposure in humans, and is potentially a useful tool for the study of arsenic methylation in sentinel species at arsenic contaminated sites.

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