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Automated, continuous, and dynamic speciation of urinary arsenic in the bladder of living organisms using microdialysis sampling coupled on-line with high performance liquid chromatography and hydride generation atomic absorption spectrometry

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An on-line and fully automated method was developed for the continuous and dynamic *in vivo* monitoring of four arsenic species [arsenite (As^{III}), arsenate (As^{V}), monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA)] in urine of living organisms. In this method a microdialysis sampling technique was employed to couple on-line with high performance liquid chromatography (HPLC) and hydride generation atomic absorption spectrometry (HGAAS). Dialysates perfused through implanted microdialysis probes were collected with a sample loop of an on-line injector for direct and automated injection into HPLC system hyphenated with HGAAS. The saline (0.9% NaCl) solution was perfused at the rate of 1 μl min^{-1} through the microdialysis probe and the dialysate was loaded into 50 μl of sample loop. The separation conditions were optimally selected to be in phosphate buffer solution at a pH 5.2 with a flow rate of 1.2 ml min^{-1}. The effluent from the HPLC was first mixed on-line at the exit of the column with HCl (1 M) solution and then mixed with a $NaBH_4$ (0.2% m/v) solution. Based on the optimal conditions obtained, linear ranges of 2.5–50 ng ml^{-1} for As^{III} and 6.75–100 ng ml^{-1} for the other three arsenic species were obtained. Detection limits of 1.00, 2.18, 1.03 and 2.17 ng ml^{-1} were obtained for As^{III}, DMA, MMA and As^{V}, respectively. Typical precision values of 3.4% (As^{III}), 5.4% (DMA), 3.6% (MMA) and 7.5% (As^{V}) were obtained, respectively, at a 25 ng ml^{-1} level. Recoveries close to 100%, relative to an aqueous standard, were observed for each species. The average *in vivo* recoveries of As^{III}, DMA, MMA and As^{V} in rat bladder urine were 56 ± 5%, 60 ± 9%, 49 ± 3% and 55 ± 7%, respectively. The use of an on-line microdialysis-HPLC-HGAAS system permitted the determination of four urinary arsenic species in the bladder of an anesthetized rat with a temporal resolution of 50 min sampling.

Introduction

As is well known, inorganic arsenic, when present as arsenite (As^{III}) and arsenate (As^{V}), is highly toxic. Some methylated arsenic species such as monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) are only moderately toxic, and are considered part of some detoxification mechanisms in living organisms. Other quaternary organic compounds such as arsenobetaine (AsB) and arsenocholine (AsC) are nontoxic.[1] Methylation of inorganic arsenic is considered a detoxification mechanism in humans because DMA is rapidly excreted in urine. In toxicological analysis, the urine medium is most often used as a proof of intoxication, because urinary excretion is the principle route of arsenic elimination.[2] It is generally accepted that a species-selective determination of arsenic is needed for a meaningful risk assessment and better understanding of its biological and metabolic pathways and its analytical behavior. Arsenic speciation has been addressed in more than 500 original publications.[3] However, few studies have been carried out on the real-time biotransformation of arsenic in living organisms. The reason for this is the absence of an *in vivo*, real-time and continuous sampling technique. Over the past decade, microdialysis sampling has become a standard *in vivo* sampling technique in the extracellular fluid (ECF) of virtually any tissue, organ or biological fluid. Microdialysis sampling makes it possible to continuously monitor the biotransformation of arsenic in living organisms.

Microdialysis is a powerful sampling technique wherein regional chemical (or biochemical) information is obtained by implanting a probe consisting of a hollow fiber semi-permeable membrane into the organ or biological fluid of interest. Interest in microdialysis has increased significantly over the past decade as discussed in several reviews.[4–9] Microdialysis sampling is a diffusion controlled method used to obtain macromolecule-free samples from the ECF of tissues or from biological fluids. The main advantage of using microdialysis sampling is that a single animal can be used and the physiological and anatomical features of its tissue remain intact during sampling with the microdialysis probe. A second advantage of microdialysis sampling coupled with appropriate analytical chemical technology is the ability to continuously monitor chemical changes within the ECF with respect to time at one implantation site.[9]

The analytical approach in the majority of arsenic speciation studies involves coupling the high separatory power of liquid

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chromatography with the inherent selectivity and sensitivity of element-specific spectrometric detections. In particular, high performance liquid chromatography (HPLC) for the separation of arsenic species has been coupled to atomic absorption spectrometry (AAS),[3,10–12] atomic fluorescence spectrometry (AFS),[13,14] inductively coupled plasma atomic emission spectrometry (ICPAES),[1,15] inductively coupled plasma mass spectrometry (ICPMS),[16,17] and electrospray mass spectrometry (ESMS).[18,19] Although the ICPAES, ICPMS and ESMS detectors have unique analytical capabilities for speciation, their instrumental and running costs make them unlikely to be adopted widely as common chromatographic detectors. In general, hydride generation atomic absorption spectrometry (HGAAS) is one of the most common techniques for the speciation of arsenic.

Microdialysis coupled on-line with HPLC has been widely applied for continuous *in vivo* monitoring of unbound drugs and neurotransmitters by various detectors, such as ultraviolet,[20] fluorescence[21] or electrochemical.[22] On-line HPLC with microdialysis perfusion provides many advantages, such as simplified sample preparation and automated analyses. Furthermore, urinary arsenic species are not stable when exposed to ambient air. To obtain real time, *in situ* urinary arsenic species in the bladders of living organisms, on-line microdialysis coupled with HPLC would be a suitable method. However, to our knowledge, the technique of microdialysis coupled on-line with HPLC has not been used for arsenic speciation with element-specific spectrometric detection. Thus, a fully automated method for continuously monitoring two inorganic (As^{III} and As^{V}) and two organic (MMA and DMA) species by an on-line microdialysis-HPLC-HGAAS system was developed in this study. This on-line system has been evaluated for use in the determination of the real-time concentration of urinary arsenic species in the bladder of an anesthetized rat after the administration of sodium arsenite.

Experimental

Instrumentation

A schematic diagram of the fully automated on-line microdialysis-HPLC-HGAAS system for the analysis of four arsenic species is shown in Fig. 1.

The microdialysis system was purchased from the Carnegie Medicine Associate (CMA, Stockholm Sweden). The sampling system used consists of a microinjection pump (CMA/100), a microdialysis probe (CMA/20) with a 10 mm length and a 0.5 mm diameter polycarbonate membrane, which is metal free and has a cut-off at 20 kDa, and an on-line injector (CMA/160) with a 50 μl sample loop.

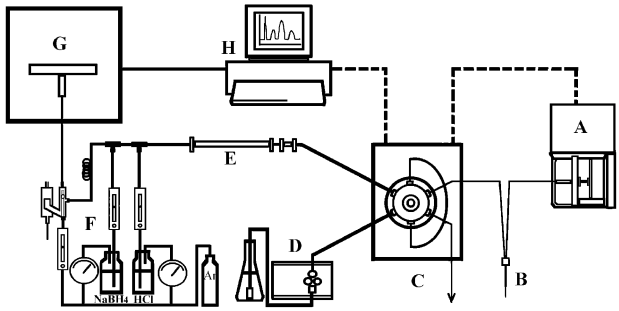

Fig. 1 Schematic diagram of the fully automated on-line microdialysis-HPLC-HGAAS system for the analysis of four arsenic species. A, Microinjection pump; B, microdialysis probe; C, on-line injector with a 50 μl sample loop; D, HPLC delivery pump; E, separation column; F, continuous hydride generation system; G, atomic absorption spectrophotometer; H, computer integrated system.

The HPLC system used consists of a Waters 501 solvent delivery pump, a silica-based anion-exchange column (Nucleosil 10 SB, 250 × 4.6 mm, Phenomenex, CA, USA) with a guard column packed with the same material.

A home-made continuous hydride generation (CHG) system[23] was used in this study. As shown in Fig. 1, the system comprised two sets of gas displacement pumps, two Teflon liquid flow meters (Cole-Parmer, IL, USA), two T-connectors (model 1010, Omnifit, Cambridge, UK), a reaction coil (180 cm × 0.8 mm id, PTFE, Omnifit), a gas liquid separator and an argon gas supply system. The gas displacement pump comprised a pressure regulator (model 3120, Omnifit), a 3-way connector (model 3220, Omnifit) and a 1000 ml glass reagent bottle (model 3200, Omnifit). In order to reduce the sample dispersion in the axial direction in the reaction coil, the PTFE tubing was knitted coiled. The U-shaped gas liquid separator was made from glass and the interior dead volume was 10 cm^3. To supply a stable argon flow for carrying the hydride, a precise flow meter (Kofloc, model RK1600R, Kojima, Japan), a bellows valve (Kofloc, model 5330) and a mass flow controller were employed.

A PerkinElmer model 4000 atomic absorption spectrometer, an electrodeless discharge lamp (EDL) power supply (System 2, PerkinElmer), and an arsenic EDL lamp (PerkinElmer) operating at 400 mA were used for the determination of arsenic. For the atomization of the arsenic hydrides (detected at 193.7 nm), a heating controller, a heating mantle (parts of MHS-20 system, PerkinElmer) and a quartz cell (160 × 7.6 mm, PerkinElmer), heated to 900 °C, were used. A personal computer was used to operate the integrated software (Chem-Lab, Taipei, Taiwan) for acquiring the arsenic absorption signal.

Reagents

All reagents used were of analytical reagent grade. High purity water (18.3 MΩ cm) was used throughout. The perfusion solution was prepared by 0.9% m/v NaCl (suprapure, E. Merck, Darmstadt, Germany). Stock solutions (1000 mg l^{-1}) of arsenite, arsenate, MMA and DMA were prepared in 0.2% v/v sulfuric acid from sodium arsenite (E. Merck), disodium hydrogen arsenate (E. Merck), sodium dimethylarsenate trihydrate (E. Merck) and disodium methylarsenate (Chem Service), respectively, and stored at 4 °C until use. Working solutions diluted by saline (0.9% m/v, NaCl) solution for each arsenic species were prepared fresh daily.

The buffered mobile phase (20 mM phosphate solution) was prepared by mixing $NaH_2PO_4{\cdot}2H_2O$ (Riedel-de Haën, Seelze, Germany) and Na_2HPO_4 (Riedel-de Haën) and adjusting the pH with HNO_3 (E. Merck) and NaOH (Riedel-de Haën). For the hydride generation reaction, the $NaBH_4$ solution was prepared by dissolving tetrahydroborate powder (Riedel-de Haën) in water stabilized by NaOH. The HCl solution was prepared by diluting concentrated HCl (Riedel-de Haën) in water.

Animal preparation

Adult male Sprague–Dawley rats (350–450 g) were obtained from the Laboratory Animal Center at the National Science Council of Republic of China (Taipei, Taiwan). These animals were specifically pathogen-free and were allowed to acclimate to their environmentally controlled quarters (25 °C and 12 : 12 h light–dark cycle) for at least 5 days before experimentation. The rats were initially anesthetized with urethane (ethyl carbamate) (1200 mg kg^{-1} body weight, i.p.), and remained anesthetized throughout the experimental period. Following a midline incision of the abdomen, the bladder was exposed and one needle was drilled on the bladder for insertion of a dialysis probe. Dialysate samples collected over the first 2 h were

discarded to prevent any interference from the acute effects of the surgical procedures. Sodium arsenite (400 μg kg^{-1} body weight) was injected into the subcutaneous tissue of the back of the neck. Arsenic species were monitored every 50 min after the subcutaneous injection.

In vivo recovery

A retrodialysis technique was used for the assessment of the *in vivo* recovery. The microdialysis probe was inserted into the rat bladder under anesthesia with urethane. A perfusion solution containing the four arsenic species (25 ng ml^{-1} of As^{III} and 50 ng ml^{-1} of the other species) was perfused through the probe at a constant flow-rate (1 μl min^{-1}) using the perfusion pump (CMA/100). After a 2 h stabilization period following the surgical procedure, the perfusate (C_{perf}) and dialysate (C_{dial}) concentrations of the four arsenic species were determined by using the on-line HPLC-HGAAS system. The relative loss of the four urinary arsenic species during retrodialysis (L_{retro}) or *in vivo* recovery ($R_{in\ vivo}$) by dialysis, was then calculated as follows:[24] $L_{retro} = R_{in\ vivo} = [(C_{perf} - C_{dial})/C_{perf}]$.

Results and discussion

Microdialysis sampling

Sheppard *et al.*[16] demonstrated that urine contains about 0.15 mol dm^{-3} NaCl (or about 9 mg NaCl ml^{-1}). The perfusate selected in the present study should be saline solution (0.9% NaCl). The concentration of sample in the dialysate is a fraction of that in the surrounding matrix. The transport properties of the probe are described by the extraction fraction.[9,25] The extraction fraction values of the microdialysis probe for the four arsenic species are evaluated by a commercial probe (CMA/20) at various flow rates of 1–5 μl min^{-1}. As is well known, if the perfusion flow rate is increased, the extraction fraction will be decreased. For the performance of the on-line system it is important to balance the perfusion flow rate, sample volume and concentration detection limits. In our experiments, the saline solution was perfused at a flow rate of 1 μl min^{-1} through the microdialysis probe and the dialysate was loaded into 50 μl of sample loop present on the on-line injector.

Optimization of the anion-exchange chromatographic separation

The arsenic species that have been reported in urine are As^{III}, As^{V}, DMA, MMA, AsB, AsC, trimethylarsine oxide (TMAO) and tetramethylarsonium (TMAs) ion.[26] Among these, four are presented in an anionic form (As^{III}, As^{V}, DMA and MMA), and the others in a cationic form (AsB, AsC, TMAO and TMAs).[26] In the present study four arsenic species (As^{III}, As^{V}, DMA and MMA) were monitored. For separation, anion-exchange chromatography was chosen because of its inherent long-term stability, operational simplicity and low cost of analysis. A strong anionic column packed with Nagel-$N(CH_3)_2$ was used to separate the arsenic species in the dialysate. Phosphate buffers of different pH were used to investigate the separation efficiency of the four arsenic species. As^{III} is eluted first whereas As^{V} is eluted last. The retention time for both As^{III} and As^{V} was not significantly affected by a change of pH. However, the influence of pH on the retention time of DMA and MMA was noticeable. The retention time for DMA increased markedly with pH from 5 to 6, and decreased at a higher pH while the retention time for MMA decreased with an increase of pH from 5 to 7.

The reason for the wide variation of the effect of pH on the retention time for these four arsenic species can be explained on the basis of their respective ionic dissociation constant (pK_a). Due to the different pK_a values of the arsenicals, various ionizing species distributions are expected, depending upon the pH of the medium.[1,27–29] In the range of pH 4–7, As^{III} is not dissociated in the mobile phase and exists in neutral form because of its high pK_a (9.3). Whereas As^{V} would exist in the form $H_2AsO_4^-/HAsO_4^{2-}$ from pH 4 to 7 because of its pK_{a1} (2.3) and pK_{a2} (6.9). DMA has a slightly high pK_a value (6.2) and can be considered to basically exist in a neutral form at pH lower than 5. However, as the pH of the mobile phase increases from 5 to 6, DMA begins to dissociate into a negative species to be more attractive to the resin, and this consequently results in an increased retention time. By further increasing to pH > 6, the apparent charge of DMA will remain constant because the only hydroxide group has already dissociated. A decreased retention time for MMA is observed as the pH increases from 5 to 7. This phenomenon can also be considered as a competition between MMA ion and phosphate ion on the resin surface.

Based on the above discussion, the separation conditions were optimally selected to be phosphate buffer solution at pH 5.2 with a flow rate of 1.2 ml min^{-1}. 20 mM phosphate buffer solution was chosen to be the analytical eluent in this work, despite the fact that a higher concentration of phosphate buffer solution would give a higher capacity, because a low eluent concentration was expected to increase the lifetime of the silica-based column and can be used for a aged column[1]

Design of the CHG system

For continuous determination of arsenic species separated by HPLC, HGAAS is the most commonly used detection method. The use of hydrides in AAS has been extensively reviewed.[30] With the continuous system, sample solution and reduction reagent are continuously delivered for the production of the hydrides. The hydrides of the analytes are instantly generated and are separated from the liquid waste with a gas/liquid separator. The gases generated from the reaction are subsequently introduced into the detector for signal measurement.In the CHG system (see Fig. 1), the flows of reagent (HCl and $NaBH_4$) are delivered by gas displacement pumps and are controlled by the flow meter. The arsenic species eluted from the HPLC are continuously mixed with these two reagents *via* two T-connectors. After flowing through a 180 cm long reaction coil, the gaseous products evolved from the reaction mixture are separated with a home-made gas/liquid separator. By coupling the CHG system with an AAS detector, the gaseous products can be carried by a stable argon stream, provided by a bellows and mass-flow controller, to the detection system.

In the present study the generation efficiencies for each species were investigated in terms of concentration of $NaBH_4$ (0.25, 0.5, 1 and 2% m/v) and HCl (0.25, 0.5, 1 and 2 M), and the results are shown in Fig. 2. For As^{III}, As^{V} and MMA, a relatively high concentration of HCl (higher than 1 M) is favorable for their generation; while for MDA, a lower concentration (0.5 M) would be more favorable. In general, the hydride generation efficiency of each species increases with an increase of concentration of $NaBH_4$. However, as the concentration of $NaBH_4$ increases to higher than 2% m/v, abrupt formation of large amount of hydrogen may cause water droplets to be entrained into the atomization tube and result in a noisy baseline.[31] The maximum usable concentration of $NaBH_4$ solution is about 2% m/v. According to the above results, the hydride generation conditions for the use of the CHG system were optimally set at 2% m/v $NaBH_4$ and 1 M HCl.

The effect of the argon flow rate on the signals for the arsenic species was also investigated. The sensitivity increased proportionally as the flow rate decreased. This can be explained in that

the number of atoms in the quartz tube will increase at low gas flow rate, and consequently result in increasing signal. An argon flow rate of 50 ml min^{-1} was used throughout this study.

Method validation

The optimized operating conditions for the on-line microdialysis-HPLC-HGAAS system are summarized in Table 1. Based on the optimized operating conditions, the chromatograms obtained for the determination of a standard solution and a urine sample by the on-line system developed in this study are shown in Fig. 3. Fig. 3a shows a typical chromatogram of the four arsenic species at concentrations of 50 ng ml^{-1} for As^{III} and 100 ng ml^{-1} for the other arsenic species. Fig. 3b shows a chromatogram of a urine sample containing 30 ng ml^{-1} of As^{III} and 80 ng ml^{-1} of the other arsenic species.

The analytical performance of the HPLC-HGAAS system for continuous arsenic speciation was evaluated in terms of calibration curves, detection limits (DLs), precision and recoveries. These results are shown in Table 2. The concentration–response relationship of the present method indicated linearity (correlation coefficient value >0.995) over the concentration range 2.5–50 ng ml^{-1} for As^{III} and 6.75–100 ng ml^{-1} for DMA, MMA and As^{V}. The DL was estimated from seven replicate peak area measurements as a concentration equivalent to three times the standard deviation of the background signal corresponding to each peak. Precision was estimated from six successive replicate urine dialysate samples containing a mixture of the four species at an arsenic concentration level of 25 ng ml^{-1}. Precision for the four arsenic species was within 10% relative standard deviation (RSD) under normal operation conditions. By spiking different amounts of arsenic species into the urine dialysates, recovery efficiencies were assessed. As shown in Table 2, a recovery of close to 100% relative to aqueous standard for each species is observed.

In vivo experiment

Bungay *et al.*[32] indicated the salient features of mass transport resistance in microdialysis. In this study, using a retrodialysis technique, the average *in vivo* recoveries of As^{III}, DMA, MMA

Table 1 Optimized operating conditions for the on-line microdialysis-HPLC-HGAAS system

Microdialysis sampling—	
Perfusate	Saline (0.9% NaCl) solution
Perfusion flow rate	1 μl min^{-1}
Probe (CMA/20)	10 × 0.5 mm membrane, metal free and cut-off at 20 kDa
Sample loop	50 μl
HPLC separation—	
Eluent	20 mM of phosphate buffer at the pH 5.2
Eluent flow rate	1.2 ml min^{-1}.
Column	Anion-exchange column (Nucleosil 10 SB, 250 × 4.6 mm)
HGAAS detection—	
Mixed solution	1 M HCl at 3.0 ml min^{-1}
Reduction solution	2% m/v $NaBH_4$ (in 0.5% m/v NaOH) at 3.0 ml min^{-1}
Argon purge flow rate	50 ml min^{-1}

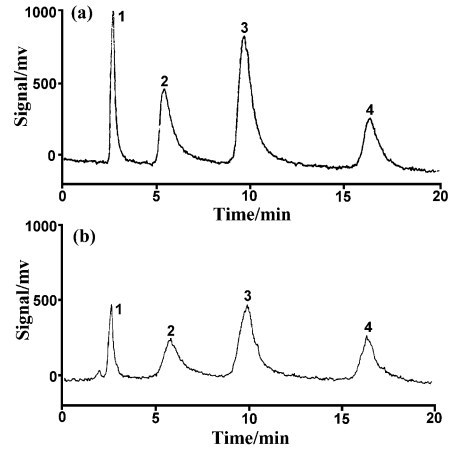

Fig. 3 Chromatograms obtained by the on-line microdialysis-HPLC-HGAAS system for solutions containing: 1, As^{III}, 2, DMA, 3, MMA and 4, As^{V}. (a) Standard solution containing 50 ng ml^{-1} As^{III} and 100 ng ml^{-1} of the other arsenic species, (b) urine sample containing 30 ng ml^{-1} As^{III} and 80 ng ml^{-1} of the other arsenic species. Injection volume, 50 μl.

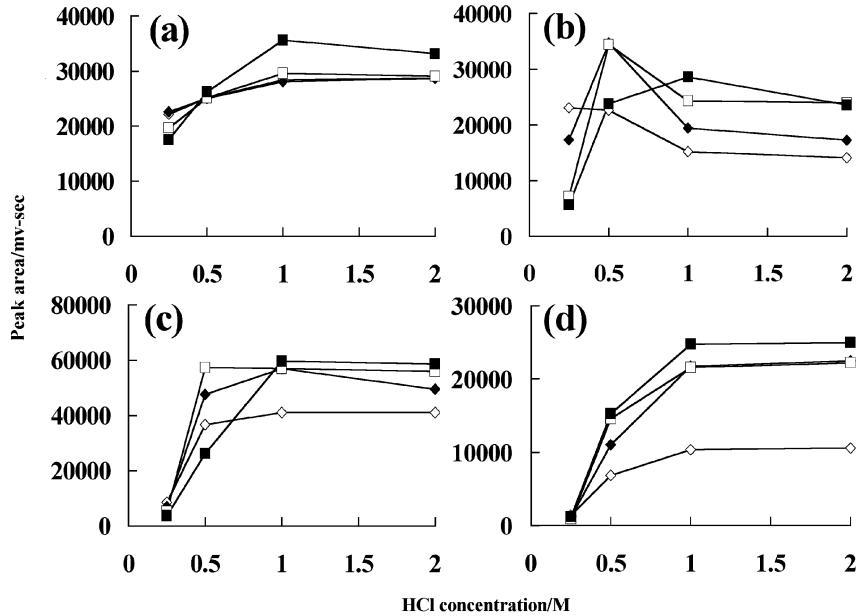

Fig. 2 Effect of HCl and $NaBH_4$ concentration (◇, 0.25%; ◆, 0.5%; □, 1%; ■, 2%) on the hydride generation efficiency of As^{III} (a), DMA (b), MMA (c) and As^{V} (d).

Table 2 Analytical performance of the proposed HPLC-HGAAS system

	As^{III}	DMA	MMA	As^{V}
Calibration curve—				
Linear range/ng ml^{-1}	2.5–50	6.75–100	6.75–100	6.75–100
Slope	285.8	90.5	441.3	100.9
Correlation coefficient (r)	0.999	0.999	0.999	0.999
Detection limit/ng ml^{-1} (n = 7)	1.00	2.18	1.03	2.17
Precision (RSD%) (n = 6)	3.4	5.4	3.6	7.5
Recovery[a] (%) (n = 3)	98± 5	103 ± 7	101 ± 9	105 ± 10

[a] Spiking amounts are 1.0, 2.5, 1.5 and 2.0 ng for As^{III}, DMA, MMA and As^{V}, respectively.

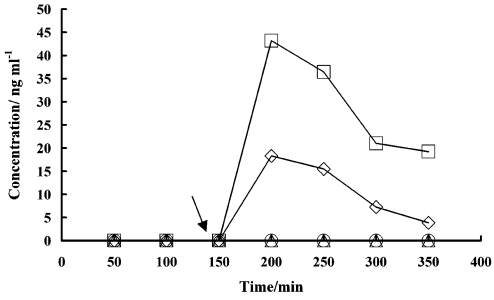

Fig. 4 The time course of the concentration of four urinary arsenic species in the bladder of a rat following a subcutaneous injection of arsenite. ↓, Subcutaneous injection of 400 μg kg^{-1} body weight arsenite. The arsenic species in the collected dialysate were measured using the method developed in the present study. ◇, As^{III}; □, DMA; △, MMA; ○, As^{V}.

and As^{V} in rat bladder urine were 56 ± 5%, 60 ± 9%, 49 ± 3% and 55 ± 7%, respectively. A quiescent solution does provide resistance to mass transport and therefore cannot be ignored.

In order to demonstrate *in vivo* monitoring of arsenic species by the on-line microdialysis-HPLC-HGAAS system, urinary arsenic species were measured in the bladder of anaesthetized rats after administration of sodium arsenite. Subcutaneous injection of 400 μg kg^{-1} body weight sodium arsenite caused increases in As^{III} and DMA levels as illustrated in Fig. 4. The urinary As^{III} and DMA concentration reached a maximum value at 50 min post-injection, and were still higher than the base line values at 150 min after injection. A time-course study, as shown in Fig. 4, demonstrates the reliability of the on-line microdialysis-HPLC-HGAAS system, which allows continuous monitoring of baseline and stimulation.

In conclusion, a method involving microdialysis sampling, fully automated on-line injection and HPLC analysis for the continuous monitoring of the concentrations of arsenic species in living organisms has been developed. This analytical technique may be employed advantageously in a mechanistic study of the biotransformation of arsenic in any tissue, organ or biological fluid (such as blood, cerebrospinal fluid and bile).

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