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The Separation of Dimethylarsinic Acid, Methylarsonous Acid, Methylarsonic Acid, Arsenate and Dimethylarsinous Acid on the Hamilton PRP-X100 Anion-Exchange Column

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In order to separate the potential arsenite metabolites methylarsonous acid and dimethylarsinous acid from arsenite, arsenate, methylarsonic acid and dimethylarsinic acid, the pH-dependent retention behaviour of all six arsenic compounds was studied on a Hamilton PRP-X100 anion-exchange column with 30 mM phosphate buffers (pH 5, 6, 7, 8 and 9) containing 20% (v/v) methanol as mobile phase and employing an inductively coupled plasma atomic emission spectrometer (ICP–AES) as the arsenic-specific detector. Baseline separation of dimethylarsinic acid, methylarsonous acid, methylarsonic acid, arsenate and dimethylarsinous acid was achieved with a 30 mmol dm^{-3} phosphate buffer (pH 5)–methanol mixture (80:20, v/v) in 25 min. Arsenite is not baseline-separated from dimethylarsinic acid under these conditions. Copyright © 1999 John Wiley & Sons, Ltd.

Keywords: arsenite; arsenate; methylarsonic acid; dimethylarsinic acid; methylarsonous acid, dimethylarsinous acid; speciation; HPLC–ICP–AES

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INTRODUCTION

After the ingestion of inorganic arsenic [As(III) and As(V)] by humans, methylarsonic acid and dimethylarsinic acid together with unchanged inorganic arsenic have been detected in human urine.[1–4] Recently, however, chronic exposure of rats to drinking water containing arsenite, methylarsonic acid or dimethylarsinic acid for seven months and subsequent analysis of urine by HPLC coupled online to an inductively coupled plasma mass spectrometer (ICP–MS) revealed two unidentified, probably novel, arsenic metabolites.[5] The molecular identification of these peaks brings about the need to develop HPLC methods that are capable of separating potential new metabolites from those that are already known.

Although methylarsenicals are commonly found in the environment, these are usually reported to be As(V) derivatives, largely because the most commonly used analytical methodologies, such as hydride generation under acid conditions, are poorly capable of making the distinction between As(III) and As(V). The proposed pathway for the enzymatic methylation of arsenite in mammals involves its oxidative methylation to methylarsonate,[6] with *S*-adenosylmethionine being the likely methyl donor.[4,7,8] After subsequent reduction of methylarsonate [As(V)] to methylarsonous acid [As(III)]—which may be accomplished chemically by the endogenous thiol glutathione (GSH)[9,10]—methylarsonous acid is then oxidatively methylated

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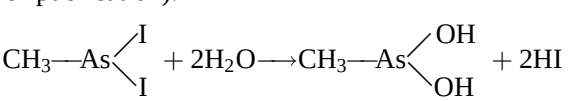
to dimethylarsinate.[6] Dimethylarsinate could subsequently be reduced chemically to dimethylarsinous acid by intracellular GSH.[9,11] Since this methylation of arsenite involves the stepwise production of arsenicals in both oxidation states, methylarsenic(III) species are expected to be found in some abiotic environments.[6,12] In fact, the hydride-generation analysis of sediment pore water showed the presence of methylarsenic(III) species as judged by the evolution of the hydrides CH_3AsH_2 and $(CH_3)_2AsH$ when the analysis was performed at pH 6.[13] The As(V) species do not form hydrides under these conditions, a fact that has long been used to separate As(III) from As(V) species. Diethylammonium diethyldithiocarbamate extraction into carbon tetrachloride has been used to separate As(III) species from As(V).[14] Subsequent hydride-generation analysis of the separated fractions revealed the presence of methylarsenic(III) species in the waters of Lake Biwa, Japan, and in some coastal waters.[15–17]

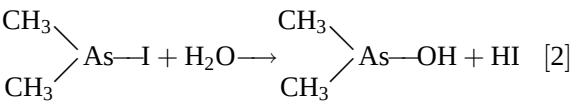
Because methylarsonous acid and dimethylarsinous acid are key intermediates in the proposed pathway for the methylation of arsenite in mammals, both compounds could also be excreted in the urine of mammals after chronic exposure to arsenite in drinking water. So far, however, methylarsonous acid and dimethylarsinous acid have never been detected either *in vivo* or *in vitro*.[18] In addition, both compounds are not well characterized chemically, their pK_a values have not been determined, and consequently their aqueous solution chemistry is poorly understood.[19] Altough the organoarsenic species $RAs(OH)_2$ and $R_2As(OH)$ are essentially unknown, the esters $RAs(ER')_2$ and $R_2As(ER')$ (E = As or S) are known. These species are usually isolated as the polymers $(RAsE)_n$ and the dimers $(R_2As)_2$. In particular, the mass spectrum of one form of $(CH_3AsO)_n$ indicates that it is a predominantly cyclic trimer or tetramer; a cyclic trimer is found for the solid-state structure of (CH_3AsS) (C. Wang, W. R. Cullen and S. J. Rettig, unpublished results).

Methylarsonous acid can be formed after hydrolysis of methyldi-iodoarsine (CH_3AsI_2) in water according to Eqn 1 (R. A. Zakharyan, F. Ayala-Fierro, W. R. Cullen, D. M. Carter and H. V. Aposhian, Toxicol. Appl. Pharmacol., submitted for publication).



[1]

Similarly, dimethylarsinous acid can be formed after the chemical reaction of dimethyliodoarsine $[(CH_3)_2AsI]$ with water according to Eqn. 2.



[2]

To investigate the aqueous solution chemistry of methylarsonous acid and dimethylarsinous acid, the retention behaviour of aqueous solutions of methyldi-iodoarsine and dimethyliodoarsine (essentially containing methylarsonous acid and dimethylarsinous acid) was investigated on a Hamilton PRP-X100 anion-exchange column with phosphate buffer solutions (pH 5–9) containing 20% methanol and using an inductively coupled atomic emission spectrometer (ICP–AES) as the arsenic-specific detector. A comparison of the retention behaviour of methylarsonous acid and dimethylarsinous acid with that of arsenic compounds with well-understood solution chemistries will provide information about the 'molecular status' of these compounds in aqueous solution, which is important for an understanding of the molecular mechanisms involved in the enzymatic methylation of arsenite.

EXPERIMENTAL

Chemicals

$NaAsO_2$ (>99%) was purchased from GFS Chemicals (Columbus, OH, USA), Na_2HPO_4 (>99%) from Mallinckrodt Chemicals (Paris, KY, USA), $NaH_2PO_4 \cdot H_2O$ was obtained from Curtin Matheson Scientific Inc. (Houston, TX, USA), and $Na_2HAsO_4 \cdot 7H_2O$ and sodium dimethylarsinate $\cdot 2.5H_2O$ were purchased from Sigma (St Louis, MO, USA). Disodium methylarsonate monohydrate was provided by Dr H. B. F. Dixon (Department of Biochemistry, University of Cambridge, UK). Methanol (HPLC grade) was purchased from Baxter Healthcare Corporation (Muskegon, MI, USA). Methyldi-iodoarsine and dimethyliodoarsine were synthesized according to published procedures.[20,21] The purity was checked by ^1H NMR and mass spectrometry. Arsenobetaine bromide was synthesized by the method described by McShane.[22] Purification by recrystallization from ethanol yielded white crystals with a melting

point of 225 °C (lit.[22] 227 °C). Water for preparing stock solutions and mobile phases was doubly distilled before use.

Solutions

Stock solutions of the individual arsenic compounds containing 50 mg As dm^{-3} were prepared by dissolving 86 mg $NaAsO_2$, 136 mg sodium dimethylarsinate·$2.5H_2O$, 134 mg disodium methylarsonate·H_2O, 208 mg $Na_2HAsO_4 \cdot 7H_2O$, 172 mg arsenobetaine bromide, 154 mg $(CH_3)_2AsI$ and 229 mg CH_3AsI_2 in distilled water and, filling to 1.0 dm^3. To complete solubilization/hydrolysis of $(CH_3)_2AsI$ (yellow oil), the weighed amount was stirred with distilled water for 30 min. After dissolution the pH had decreased to 3.3 and was brought back to 7.0 by dropwise addition of 0.2 mol dm^{-3} NaOH. Similarly, the pH of the solution obtained after dissolution of methyldiiodoarsine in distilled water was adjusted to 7.0 by dropwise addition of 0.2 mol dm^{-3} aqueous NaOH. A solution containing 50 mg As dm^{-3} of arsenite, arsenate, methylarsonic acid, dimethylarsinic acid, methylarsonous acid and dimethylarsinous acid was prepared by dissolving the appropriate amounts of each arsenic compound (or the precursor) in 1.0 dm^3 distilled water and stirring for 30 min. Then the pH was brought to 7.5 by dropwise addition of 0.2 mol dm^{-3} NaOH.

Solutions of 30 mmol dm^{-3} NaH_2PO_4 and 30 mmol dm^{-3} Na_2HPO_4 were mixed in appropriate ratios to obtain mobile phases with pH values from 5.0 to 9.0 (PHM 220, Radiometer, Copenhagen, Denmark). The 30 mmol dm^{-3} phosphate buffer of the desired pH (5, 6, 7, 8 and 9) was subsequently mixed with methanol (8:2, v/v).

Instrumentation

The HPLC system consisted of a Beckman 110 B Solvent Delivery Module, an Altex Vent 210 injector with a 100 μl loop and a PRP-X100 anion-exchange column (Hamilton, Reno, NV, USA; 25 mm × 4.1 mm i.d., spherical 10 μm particles of a styrene–divinylbenzene copolymer with trimethylammonium exchange sites; stable between pH 1 and 13; exchange capacity 0.19 meq g^{-1}). A guard cartridge filled with the same stationary phase (PRP-X100; Hamilton) protected the analytical column.

ICP–AES

Arsenic-specific detection was achieved with a Thermo Jarrel Ash (Franklin, MA, USA) IRIS HR radial-view ICP–AES. A ThermoSPEC/CID (version 2.10.04) provided the necessary time-scan functions and the multitasking controller allowed the processing of one atomic emission line every 0.02 s. The nebulization gas-flow was maintained at 2 dm^3 min^{-1}, the plasma forward power at 1150 W, and the CID temperature at −85 °C.

HPLC–ICP–AES

The HPLC column exit was coupled to the Meinhard TR-30-K2 concentric glass tube nebulizer (J. E. Meinhard Assoc. Inc., Santa Ana, CA, USA) with a polyethylene tube (length 4 cm, i.d. 0.2 mm). Accumulating charge on the detector from both the 228.812 nm (order 147) and 234.984 nm (order 143) As lines was observed simultaneously throughout chromatographic runs. Subarrays of 15 × 3 pixels were interrogated to represent each analytical line, with the center 3 × 3 pixel section as the peak and the extreme 1 × 3's as the background.

Chromatography

The column was equilibrated by passing at least 100 cm^3 of each mobile phase through the column before the arsenic compounds were injected. Aliquots (100 μl) of each of the 50 mg As dm^{-3} aqueous solutions of arsenite, arsenate, methylarsonate, dimethylarsinate, methylarsonous acid, dimethylarsinous acid and arsenobetaine containing 5 μg arsenic were injected separately at 24 °C with all the mobile phases at a flow rate of 1.5 cm^3 min^{-1}. Each retention time was determined three times (relative standard deviation <1%). Because in aqueous solution and above pH 4.5 arsenobetaine is present as a zwitterion and remains zwitterionic throughout the investigated pH range (pH 5–9),[23] the dead volume of the column was determined by injection (100 μl) of an aqueous solution of arsenobetaine (50 mg As dm^{-3}) to be 2.17 cm^3 (over the entire pH range) corresponding to a dead time of 87 s at a flow rate of 1.5 cm^3 min^{-1}. For the determination of the dead volume, mixtures of aqueous 30 mmol dm^{-3} phosphate buffers of pH 5, 6, 7, 8, and 9 with methanol (80:20, v/v) were used as mobile phases and arsenic was monitored on-line by ICP–AES. Since the retention time of arsenobetaine was 87 s over the

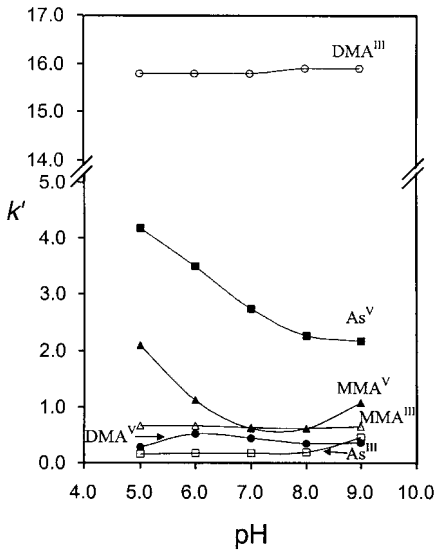


Figure 1 Dependence of the k' values of arsenite (As^{III}), arsenate (As^{V}), methylarsonic acid (MMA^{V}), methylarsonous acid (MMA^{III}), dimethylarsinic acid (DMA^{V}) and dimethylarsinous acid (DMA^{III}) on the pH of the mobile phases in the pH range 5–9. Mobile phase: 30 mmol dm^{-3} phosphate buffer (pH 5, 6, 7, 8 or 9)/methanol (8:2, v/v); Column: Hamilton PRP-X100, 250 mm × 4.1 mm i.d.; flow rate: 1.5 cm^3 min^{-1}; Detector: ICP–AES at 228.812 nm; Injection volume: 100 μl; 5 μg of each arsenic compound injected separately.

entire pH range investigated and smaller than the retention time of arsenite (101 s between pH 5 and 8), the retention time of arsenobetaine was used to calculate the k' values (retention factor) of the arsenic compounds.

RESULTS AND DISCUSSION

Arsenic compounds (arsenite, arsenate, methylarsonic acid, dimethylarsinic acid, methylarsonous acid, dimethylarsinous acid and arsenobetaine) were chromatographed on a Hamilton PRP-X100 anion-exchange column with mixtures of 30 mmol dm^{-3} phosphate buffer solutions (pH range 5 to 9) and methanol (8:2, v/v). The pH-dependent retention behaviour of all six arsenic compounds is shown in Fig. 1. Preliminary experiments showed that dimethylarsinous acid does not elute from the column with aqueous 30 mmol dm^{-3} phosphate buffer in a reasonable time. Hence, methanol was added to the mobile phase in 5% increments to elute dimethylarsinous acid from the column. A concentration of 20% methanol in the 30 mmol dm^{-3} phosphate buffer proved to be sufficient to elute dimethylarsinous acid from the column in a reasonable time. The methanol content of the mobile phase was not increased any further since it tended to extinguish the plasma before the end of the run.

Arsenite

The k' of arsenite is 0.17 and independent of the pH in the range 5–8 (Fig. 1). This behaviour can be rationalized in terms of the pK_1 of arsenite of 9.2 which implies the presence of neutral $As(OH)_3$ in the pH range 5–8.[24] At pH 9 the k' of arsenite is 0.46, thus indicating slight retention by the stationary phase. This can be explained by the presence of a substantial fraction (approximately 50%) of the singly charged anion $As(OH)_2O^-$, which is retained on the anion-exchange column under these conditions.

Dimethylarsinic acid

The k' for dimethylarsinic acid is 0.28 at pH 5, it increases to 0.53 at pH 6 and subsequently decreases gradually to 0.37 at pH 9 (Fig. 1). This retention behaviour in the pH range 5–8 is essentially the same as that previously reported for dimethylarsinic acid on the same anion-exchange column with a 30 mmol dm^{-3} aqueous phosphate buffer (without methanol).[24] The k' value does not change upon increase of the pH from 8 to 9 because the apparent charge (for definition see J. Gailer[24]) on the dimethylarsinic acid anion remains at -1.[24]

Methylarsonic acid

At pH 5 the k' for methylarsonic acid is 2.09; it decreases to 1.13 at pH 6, is 0.61 at pH 7 and remains at 0.61 upon an increase in the mobile phase pH to 8. At pH 9 k' is 1.07 (Fig. 1). In the pH range between pH 5 and 8, the retention behaviour is essentially identical to that reported previously for this compound on the same anion-exchange column with a 30 mmol dm^{-3} aqueous phosphate buffer (without methanol).[24] The observed increase in k' upon raising the pH of the mobile phase from 8 to 9 can be explained by the pK_2 of methylarsonic acid, which is 9.1. At pH 8 the apparent charge on the methylarsonic acid molecule is -1, whereas at pH 9 it is approximately -1.5. Thus the increase in negative charge on the methylarsonic acid molecule

results in increased interaction with the ammonium groups on the stationary phase with a concomitant increase in k' (Fig. 1).

Arsenate

The k' for arsenate decreases gradually from 4.18 at pH 5 to 2.17 at pH 9. This pH-dependent retention behaviour is similar to the retention behaviour of arsenate reported previously for arsenate with a 30 mmol dm^{-3} aqueous phosphate buffer (without methanol) in the pH range between 5 and 8 on the same anion-exchange column.[24] No increased retention is seen upon an increase in the pH from 8 to 9 because the apparent charge on the arsenate anion remains at -2.[24]

Methylarsonous acid

The k' of methylarsonous acid is 0.67 and invariant with pH in the range between 5 and 9 (Fig. 1). Because all the other arsenic compounds investigated (arsenite, arsenate, methylarsonic acid, dimethylarsinic acid) have pK_a values in the investigated pH range corresponding to substantial retention shifts with increasing pH, the pH-independent retention behaviour of methylarsonous acid could be caused by the presence of either neutral $CH_3As(OH)_2$ or by completely deprotonated $CH_3As(O_2)^{2-}$. Because other doubly charged arsenic compounds, such as arsenate (between pH 8 and 9), have k' values between 2.17 and 2.27 while methylarsonous acid has a k' value of 0.67, the doubly charged anion $CH_3As(O_2)^{2-}$, however, is unlikely to be present over the pH range between 5 and 9. Hence, methylarsonous acid is likely to be present as a neutral molecule in the pH range investigated. This is in accord with the fact that methylarsonous acid is the methyl derivative of arsenite, which has a pK_1 of 9.2. Consequently, the pK_1 of methylarsonous acid (which has not yet been determined)[19] is likely to be around 9 and hence the molecule should exist mainly in the un-ionized state at physiological pH.[25]

In the pH range 5–8, methylarsonous acid has k' values that are approximately 0.47 k' units larger than those obtained for arsenite. This comparatively stronger retention of methylarsonous acid is probably caused by the hydrophobic interaction of the methyl group of methylarsonous acid with the organic backbone (styrene/divinylbenzene) of the stationary phase, thus causing stronger retention than arsenite.The pH-dependent retention behaviour of methylarsonous acid and methylarsonic acid (Fig. 1) clearly demonstrate that the right mobile phase pH is absolutely essential to separate these methylarsonicals and hence to avoid speciation errors.

The chromatogram of the 50 mg As dm^{-3} stock solution of methylarsonous acid showed a single peak over a period of three weeks after preparation of the stock solution. Thereafter a small peak corresponding to the retention time of methylarsonic acid was detected in the chromatogram, suggesting that oxidation had occurred.

Dimethylarsinous acid

The k' of dimethylarsinous acid is independent of the pH in the range 5 to 9 and is 15.8 which is indicative of a comparatively hydrophobic compound (Fig 1). This retention behaviour suggests the presence of either neutral $(CH_3)_2As\text{-}OH$ or a singly negatively charged $(CH_3)_2As\text{-}O^-$ species. However, since other singly charged arsenic compounds, such as methylarsonic acid (between pH 5 and 8) or arsenate (between pH 5 and 6)[24] generally have k'-values between 0.61 and 4.18 (Fig. 1), the unusually strong retention of dimethylarsinous acid is probably caused by the two methyl-groups of the dimethylarsinous acid molecule which hydrophobically interact strongly with the organic backbone (styrene/divinylbenzene) of the stationary phase. However, since the pK_1 of dimethylarsinous acid has never been measured,[19] the presence of $(CH_3)_2As\text{-}O^-$ over the investigated pH range cannot be excluded.

The entirely different retention behaviour of dimethylarsinous acid and dimethylarsinic acid (Fig. 1) warrants further explanation. Dimethylarsinous acid has been reported to form dimers of the type $(CH_3)_2As\text{-}O\text{-}As(CH_3)_2$ in aqueous solution.[26] It is therefore possible that these dimers are also present under our chromatographic conditions and thus explain the more than 30-fold increased k' values of dimethylarsinous acid as compared to dimethylarsinic acid. These dimers have four methyl groups per molecule and thus should be much more strongly retained on the hydrophobic PRP-X100 stationary phase then dimethylarsinic acid.

Because of the "apparent" hydrophobic character and its strong retention on hydrophobic stationary phases (e.g. PRP-X100 stationary phase), dimethylarsinous acid may thus have so far escaped detection in environmental samples by HPLC-ICP-MS.

The chromatogram of the 50 mg As dm^{-3} stock

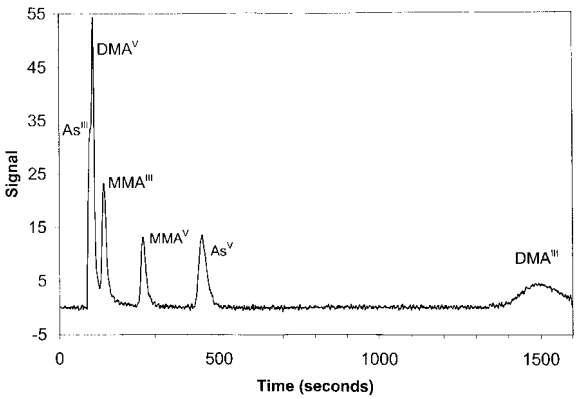


Figure 2 Separation of arsenite (As^{III}), dimethylarsinic acid (DMA^{V}), methylarsonous acid (MMA^{III}), methylarsonic acid (MMA^{V}), arsenate (As^{V}) and dimethylarsinous acid (DMA^{III}) with 30 mmol dm^{-3} phosphate buffer (pH 5)/methanol (8:2, v/v). Column: Hamilton PRP-X100, 250 mm × 4.1 mm i.d.; flow rate: 1.5 cm^3 min^{-1}; Detector: ICP–AES; Injection volume: 100 μl; 5 μg As of each compound; the chromatogram is constructed as 1600 1s time slices of the background-subtracted 228.812 nm signal (order 147), with five-point moving-average smoothing before the derivative was taken.

solution of dimethylarsinous acid showed a single peak over a period of one week.

Optimal conditions for the separation of arsenite, arsenate, methylarsonous acid, methylarsonic acid, dimethylarsinous acid and dimethylarsinic acid

An optimal chromatographic separation of the six arsenic compounds requires baseline separation at short retention times. Among the mobile phases investigated, the mixture of 30 mmol dm^{-3} phosphate buffers (pH 6, 7, 8 and 9) with methanol (8:2, v/v) are least suited to separate all the arsenic compounds from each other because the k' values of arsenite, methylarsonic acid, dimethylarsinic acid and methylarsonous acid are very similar in this pH range (Fig. 1). If these mobile phases were employed, overlapping chromatographic peaks could not be avoided, and baseline separation would be impossible. The mobile phase containing 30 mmol dm^{-3} phosphate buffer of pH 5 with 20% methanol (80:20, v/v) gave the best results for a separation of the arsenic compounds (Fig. 2). Under these conditions dimethylarsinic acid, methylarsonous acid, methylarsonic acid, arsenate and dimethylarsinous acid can be baseline-separated from each other within 25 min at a flow rate of 1.5 cm^3 min^{-1}. The retention times for the arsenic compounds are: arsenite, 101s; dimethylarsinic acid, 111s; methylarsonous acid, 145s; methylarsonic acid, 269s; arsenate, 451s; and dimethylarsinous acid, 1462s. The arsenite peak, which elutes close to the solvent front, partially overlaps with the peak of dimethylarsinic acid under these conditions (Fig. 2).

CONCLUSION

An investigation of the retention behaviour of arsenite, arsenate, methylarsonous acid, methylarsonic acid, dimethylarsinous acid and dimethylarsinic acid on the PRP-X100 anion-exchange column in the pH range 5–9 showed that optimal separation of these arsenic compounds (ICP–AES detection; flow rate 1.5 cm^3min^{-1}) is possible with 30 mmol dm^{-3} phosphate buffer (pH 5)/methanol, 8:2 (v/v). The retention behaviour of methylarsonous acid over the pH range 5–9 suggests that this compound exists as a neutral molecular entity at physiological pH. The unusually strong retention of dimethylarsinous acid (k' 15.7) compared with the other arsenic compounds on this lipophilic styrene–divinylbenzene copolymer column suggests a very hydrophobic character of this compound. The hydrophobicity of dimethylarsinous acid and the chemistry of methylarsonous acid, however, may be changed significantly *in vivo* where a chemical reaction of the OH group(s) with endogenous glutathione (GSH) is likely to occur.[27]

Chromatograms in this work represented 50 mg As dm^{-3} per compound with emission data being taken in 1 s increments. Lower concentrations can clearly be detected, and a variety of optimizations and trade-offs can improve the detection sensitivity of the HPLC–ICP–AES system significantly. However, the expected total arsenic concentrations in the urine of arsenic-exposed humans are in the range 0.06–1.35 mg As dm^{-3},[28] and thus may require detectors with lower detection limits, such as ICP–MS.[29] The results presented in this paper serve as a basis for the development of a chromatographic separation of these arsenic compounds in complex matrices, such as urine.

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