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Journal of Chromatography B, 807 (2004) 169–176
JOURNAL OF CHROMATOGRAPHY B
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Method for the determination of five toxicologically relevant arsenic species in human urine by liquid chromatography–hydride generation atomic absorption spectrometry

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Received 17 July 2003; received in revised form 19 March 2004; accepted 25 March 2004

Abstract

An analytical method for the simultaneous quantitation of arseneous acid (As(III)), arsenic acid (As(V)), monomethylarsonic acid (MMA), dimethylarsinic acid (DMA) and trimethylarsine oxide (TMAO) in human urine by coupling of high-performance liquid chromatography with hydride generation atomic absorption spectrometry (HPLC/HG-AAS) via a flow-injection interface is presented. After arsenic species separation by anion-exchange displacement chromatography the compounds are on-line reduced to their corresponding hydrides and detected by atomic absorption spectrometry. Detection limits range from 1.1 (TMAO) to 2.6 μg/L (As(V)). The method has been applied to determine arsenic species in the urine of a volunteer before and after consumption of seafood as well as to analyse certified reference urine samples for their arsenic species content.

Keywords: Arseneous acid; Arsenic acid; Monomethylarsonic acid; Dimethylarsonic acid; Trimethylarsine oxide

1. Introduction

Arsenic speciation in samples of biological origin, especially human urine, has been the topic of many papers. The most important species of toxicological concern, arsenic acid (As(V)), arseneous acid (As(III)), monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) are usually determined coupling HPLC on-line with atomic absorption spectrometry (AAS) [1], inductively-coupled plasma mass spectrometry (ICP-MS) [2] or atomic fluorescence spectrometry (AFS) [3].

Arsenobetaine and arsenocholine are regarded as non-toxic and therefore are not topic of this paper [4]. But little attention has yet been paid to the determination of the toxic arsenic metabolite trimethylarsine oxide (TMAO) in human urine and attempts to determine all five species simultaneously in urine samples under practical conditions have been failed so far. Larsen et al. [5] described a cation-exchange HPLC/ICP-MS method for the quantitation of TMAO in urine, but all other anionic arsenic species eluted in the void volume. Londesborough et al. [6] used an ion-chromatographic method to quantify all five species in pure aqueous solutions. However, in the presence of matrix constituents of biological specimens coelution of MMA and As(III) as well as peak splitting occurred. Tsalev et al. [7] report a coelution of TMAO and DMA in their high-performance liquid chromatography with hydride generation atomic absorption spectrometry (HPLC/HG-AAS) method and with the HPLC/HG-ICP-MS coupling of Nakazato et al. [8]. TMAO does not elute till 63 min after sample injection. Approaches applying gas chromatography for determination of these species in urine cannot discriminate between As(III) and As(V) and suffer from poor detection limits when using small sample volumes [9].

Therefore, the objective of this study was to develop and validate a rugged and *reliable* method in order to determine As(III), As(V), MMA, DMA and TMAO in human urine within a single chromatographic run.

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doi:10.1016/j.jchromb.2004.03.051

2. Experimental

2.1. Chemicals

Arsenic(III)-oxide (>99%), Arsenic(V)-oxide (>99%), disodium methylarsonate hexahydrate (>95%), sodium dimethylarsinate trihydrate (>99%) and trimethylarsine oxide (>95%) were supplied by Baker (Phillipsburg, USA), Merck (Darmstadt, Germany), Argus Chemicals (Prato, Italy), Fluka (Neu-Ulm, Germany) and Argus Chemicals, respectively. Sodium hydroxide, sodium borohydride, sodium chloride, hydrochloric acid 30%, sodium dihydrogenphosphate monohydrate, buffer solution pH 4.00 and 7.00 were purchased from Merck. Sodium tetraborate decahydrate and disodium hydrogenphosphate dihydrate were from Fluka (Buchs, Switzerland). Ortho phosphoric acid and ethanol abs. were supplied by Baker and argon 5.0 by Messer Griesheim (Krefeld, Germany). In addition, Seronorm® Trace Elements Urine from Nycomed (Majorstua, Norway) and ClinRep®-Control Level I, control urine lyophilised, from Recipe (Munich, Germany) as well as silicon anti-foaming agent Dow Corning DB 110 A from Perkin-Elmer (Ueberlingen, Germany) were applied. Generally, all chemicals were of the highest purity available. Doubly-deionized water was prepared by an apparatus from Millipore (Eschborn, Germany).

2.2. Equipment

All species investigated have in common that they can be reduced to their volatile arsine derivatives. By this procedure they are separated from matrix constituents and are amenable to very sensitive AAS detection The used HPLC/HG-AAS apparatus is depicted in Fig. 1. A 250 LC binary pump (Perkin-Elmer) equipped with a Rheodyne 7125 six-port injection valve out of titanium and a 100-μL sample loop manufactured out of PEEK (Alltech, Unterhaching, Germany) was connected to a pre and analytical column (Hamilton, Martinsried, Germany). A model FIAS 400 (controlled with the "FIAS&DMS" software) with a MHS-FIAS quartz cell (Perkin-Elmer) was used to form the volatile arsines. Detection was carried out with an atomic absorption spectrometer model 3030 and an arsenic electrodeless discharge lamp (EDL) with an EDL 2 power supply (Perkin-Elmer). The wavelength of the monochromator was set to 193.7 nm with a spectral slid-width of 0.7 nm. The analogous voltage output signal of the spectrometer was acquired with a sampling rate of 1 Hz by a Nelson 950 A interface (Perkin-Elmer). Data processing was performed using Turbochrom 3.0 software (Perkin-Elmer).

2.3. Sample preparation

Urine specimens were sampled in cleaned 100-mL polyethylene bottles and stored at −18 °C or below. Before analysis samples were heated to room temperature in a water bath and were diluted 1 + 1 with mobile phase A (cf. 2.4). Afterwards, they were filtered through a syringe filter (0.45 μm pore diameter). Fortified urine samples served as matrix-matched standards for quantitation. They had the same matrix concentration as the urine samples to be analysed.

2.4. Column liquid chromatography

HPLC separation was carried out on a PRP-X100 anion-exchange column with a polystyrene divinylbenzene stationary phase and trimethylammonium as functional group (250 mm × 4.1 mm stainless steel, 10 μm particle diameter, Hamilton). A PRP-X100 pre-column containing the same stationary phase was attached to the analytical column (25 mm × 2.3 mm stainless steel, Hamilton). A 100-μL volume of the sample was injected at a flow rate of 1.4 mL/min. A step gradient was applied for elution of the analytes (0–4 min, 100% mobile phase A: 10 mM $Na_2B_4O_7 \cdot 10H_2O$/20 mM NaCl, pH 9; 4–11 min, 100% mobile phase B: 50 mM $Na_2B_4O_7 \cdot 10H_2O$/30 mM NaCl, pH 9).

2.5. Post-column derivatization

The peristaltic pump tubings consisted of ethylene-vinylacetate polymers (Tygon®) and were part of the

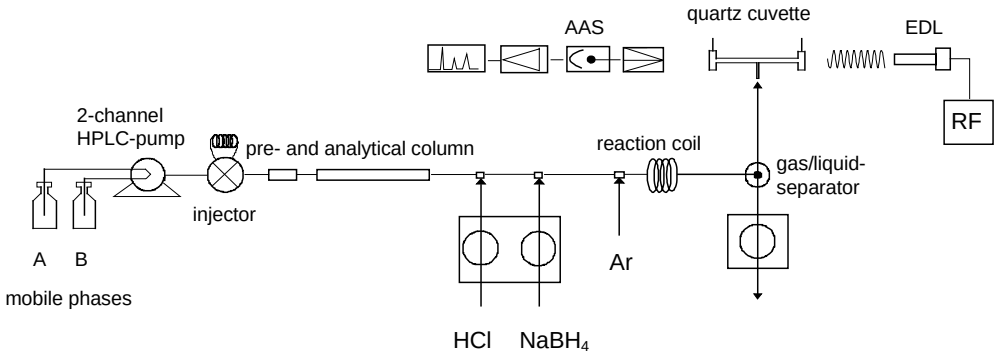

Fig. 1. Coupling of HPLC and AAS with a flow-injection interface (HPLC/HG-AAS).

commercially available "FIAS 400" system described above. The effluent of the HPLC column was acidified with a 5% HCl (v/v) solution at 40 rpm of the peristaltic pump (using red/red tubing of 1.14 mm i.d.). Sodium borohydride solution for reduction of the arsenic species to arsines (0.5% in 0.05% aqueous sodium hydroxide (w/v)) was pumped into the system at 40 rpm (blue/yellow tubing). A violet/violet coloured drainage tubing (2.06 mm i.d.) was applied at 60 rpm. The argon flow to transfer the volatile compounds into the quartz cell was set to 45 mL/min.

3. Results and discussion

3.1. Optimization of the mobile phase

In order to improve the separation of the arsenic species the retention behaviour as a function of pH was investigated. According to the eluotropic series an aqueous buffer solution as strongest eluent for anion-exchange supports was chosen. Buffer salts based on phosphoric acid are convenient, since phosphoric acid is a three-protic acid resulting in a good buffer capacity over a wide pH range. In Fig. 2 retention times of all five species are depicted as a function of pH. Conditions between pH 8.5 and 9 result in a complete resolution of all five species. Below pH 8.0, As(III) and TMAO coelute, above 9.5 so do As(III) and DMA.

The retention behaviour of the arsenic species in this experiment can be explained considering the effective or apparent charge $Q_{app} = \sum n \times \delta$ (δ = the concentration of the ion with charge n/total concentration of the analyte) [13]. For As(V) this expression amounts to:

$$Q_{app} = \frac{3[AsO_4^{3-}] + 2[HAsO_4^{2-}] + [H_2AsO_4^{-}]}{[AsO_4^{3-}] + [HAsO_4^{2-}] + [H_2AsO_4^{-}] + [H_3AsO_4]}$$

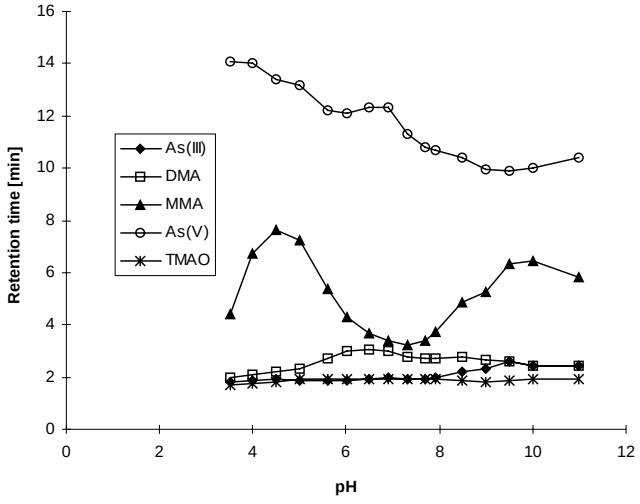

Fig. 2. Retention times of As(III), As(V), MMA, DMA and TMAO as a function of pH of the mobile phase (10 mmol phosphate/L, column flow: 1.4 mL/min).

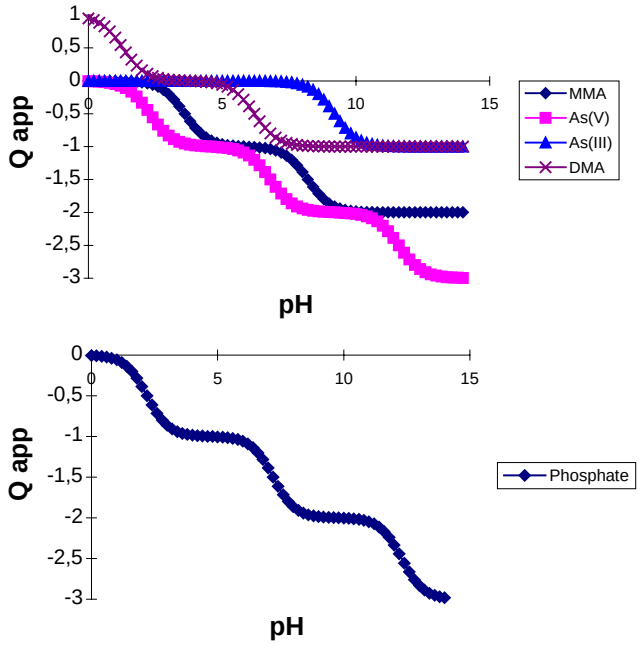

Fig. 3. Effective charge Q_{app} of As(III), As(V), MMA and DMA as a function of pH (calculated values).

Considering a chemical equilibrium $HMe + H_2O = Me^- + H_3O^+$ with a dissociation constant K_a, δ is calculated according to:

$$\delta = \frac{1}{1 + [H_3O^+]/10^{-pK_a}}$$

When calculating δ for As(V) at a given pH only two equilibrium species are regarded (acid and corresponding base) and the third one is neglected due to the great difference in concentrations.

Fig. 3 presents calculated values for Q_{app} of As(III), As(V), MMA and DMA and phosphate buffer. The acid constants presented in Table 1 were used for calculation.

Q_{app} for TMAO was not calculated, as the chemical properties of TMAO in aqueous solution are not well understood. Gailer and Irgolic [14] suppose a protonation of the As = O group due to the known hygroscopic properties of TMAO leading to a cationic species:

$$(CH_3)_3AsO + H_2O = (CH_3)_3AsO \cdots H_2O$$
$$= (CH_3)_3As(OH)_2 = [(CH_3)_3As\text{–}OH]^+OH^-$$

Table 1
pK_a values for arsenic species and ortho phosphoric acid

Analyte	pK_a [3,10–13]
As(III)	9.2
As(V)	2.3, 6.8, 11.6
MMA	3.6, 8.2
DMA	1.3, 6.2
TMAO	No data
H_3PO_4	2.2, 7.2, 12.3

According to this assumption, TMAO may have an effective charge of $0 \leq Q_{app} \leq 1$ and would elute in the void volume of the column provided that no hydrophobic interactions with the stationary phase take place.

Between pH 0 and 8.5, As(III) has a Q_{app} of 0 and therefore elutes in the void volume. Exceeding this value, deprotonation of the molecule decreases Q_{app} and increases the retention time (t_R) due to ion-exchange retention. At the beginning of the examination at pH 3.5, Q_{app} of DMA and As(III) amounts to 0; hence, both species coelute. At pH 5, Q_{app} (DMA) becomes negative and separates from TMAO and As(III). The retention time decreases slightly with increasing pH due to competition with phosphate ions for ion exchange sites. This also causes a decrease in t_R of As(V), although its Q_{app} decreases from −0.93 (pH 3.5) to −2.2 (pH 11). Between pH 3.5 and 5.2, Q_{app} of MMA decreases stronger than Q_{app} of phosphate (−0.39 to –0.98 versus −0.9 to −1.0) resulting in an increase of t_R of MMA. Between pH 5.2 and 7.4, Q_{app} (MMA) remains nearly constant (−0.98 to −1.14), but Q_{app} of phosphate further declines (−1.0 to −1.6). This leads to a more effective displacement of MMA from its binding sites and in turn to a decreases of t_R. Around pH 7.4 the opposite effect occurs: Q_{app} (MMA) decreases more rapidly than phosphate (−1.14 to −1.98 versus −1.61 to −2.0) causing t_R to increase again.

3.2. Optimization of the column flow

In Figs. 4 and 5 the influence of the column flow on t_R of the arsenic species is shown for pH 8.75 and 9.00, respectively. At pH 8.75 the retention times of As(III) and TMAO differed only slightly between 0.8 and 2.0 mL/min, so that no baseline resolution could be obtained for both species. However, the variation of the flow velocity at pH 9 resulted in a baseline resolution for all five species. The investigation was not extended beyond 1.4 mL/min due to a gradually decreasing resolution.

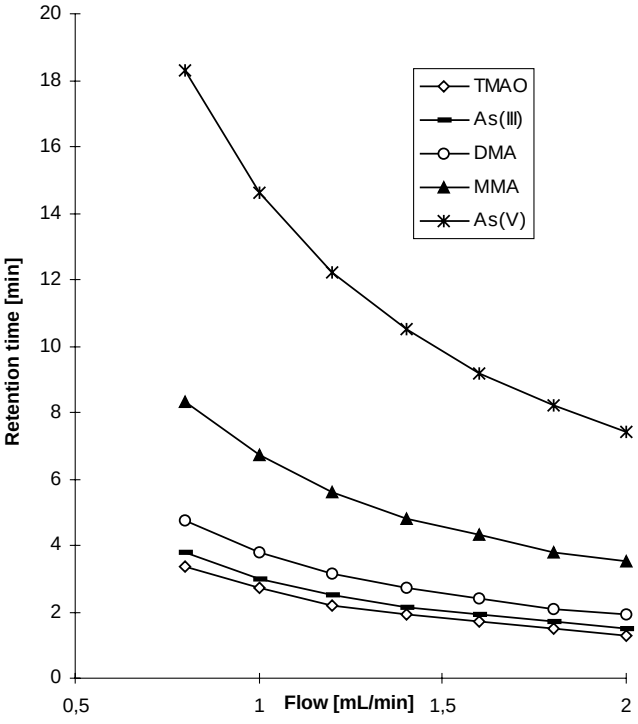

Fig. 4. Retention times of As(III), As(V), MMA, DMA and TMAO as a function of column flow (phosphate buffer 10 mmol/L, pH 8.75).

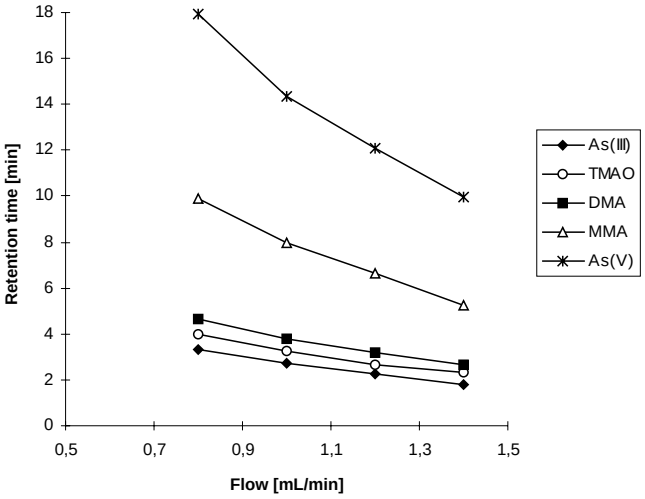

Fig. 5. Retention times of As(III), As(V), MMA, DMA and TMAO as a function of column flow (phosphate buffer 10 mmol/L, pH 9.0).

3.3. Optimization of the boric acid buffer

The next step was to change the buffer substance, because the buffer capacity of phosphate at pH ≈ 9 is not sufficient when analysing urine samples (pK_a $[H_2PO_4^-/HPO_4^{2-}] = 7.21$). An aqueous solution of 10 mM sodium tetraborate–decahydrate (pK_a $[H_3BO_3/H_4BO_4]$ = 9.25) at pH 9.0 (adjusted with HCl) was chosen for further investigations. Neither MMA nor As(V) eluted even after 40 min, as the elution strength of the tetraborate ion was too low. Therefore NaCl was added to the mobile phase and the resulting retention times are shown in Table 2. After addition of 20 mmol NaCl/L all species eluted within 18 min from the column. A further increase of the elution strength was not desired to prevent peak overlapping of TMAO, As(III) and DMA.

In order to shorten the retention times of MMA and As(V) without influencing those of the three other species a step gradient was applied: Following the elution of TMAO,

Table 2
Retention times (in min) of arsenic species as a function of NaCl concentration (in mmol/L) (mobile phase: 10 mmol tetraborate/L, pH 9.0, isocratic elution)

[NaCl]	TMAO	As(III)	DMA	MMA	As(V)
0	2	3.4	5.7	>40	>40
10	2	2.7	4	17.3	>40
20	2	2.5	3.4	9.9	18

Table 3
Retention times (in min) of arsenic species as a function of NaCl concentration (in mmol/L) of mobile phase B (50 mmol tetraborate/L, pH 9.0), mobile phase A: 10 mmol tetraborate/L, 20 mmol NaCl/L, pH 9.0 (0–4 min: 100% mobile phase A, >4–11 min: 100% mobile phase B)

[NaCl]	TMAO	As(III)	DMA	MMA	As(V)
30	1.9	2.4	3.3	8.5	10.3
60	1.9	2.5	3.4	8.2	9.3

As(III) and DMA with the original solution (10 mmol tetraborate/L and 20 mmol NaCl/L), the mobile phase was changed to a borate solution containing 50 mmol tetraborate/L and 30 mmol NaCl/L. A further increase of the NaCl concentration to 60 mmol/L caused only a slight decrease in the retention times for MMA and As(V) (Table 3).

Decreasing the pH of mobile phase B to pH 8.0 did not have an effect on t_R of MMA, in the case of As(V) a decrease of 0.2 min could be observed.

Finally, the separation was performed with 30 mmol/L NaCl at pH 9.0 in order to have two similar mobile phases leading to shorter equilibration times after each chromatographic run. In Fig. 6 a chromatogram of all five baseline separated arsenic species is depicted (standard solution with 10-μg/L each).

3.4. Optimization of the acid concentration

The acid concentration of the flow-injection interface had a great influence on the efficiency of hydride generation and therefore on the sensitivity of the analytical method. Fig. 7 compiles the signal intensities for the five species as a function of HCl concentration. DMA and TMAO show a decrease in peak heights with increasing concentration of HCl. Using 20% HCl (v/v) the intensities are only 40 and 20% of those with 5% HCl, respectively. As the intensities of MMA and As(III) are almost constant over the examined range, the As(V) peak height increases gradually with the acid concentration. As a compromise a 5% HCl solution was used in order to achieve best sensitivity for TMAO, which represents the species with the lowest concentration in human body fluids [22].

3.5. Retention factors, separation factors and resolutions for TMAO, As(III), DMA, MMA and As(V)

Retention factors (α), separation factors (k) and resolutions (R_s) for TMAO, As(III), DMA, MMA and As(V) are compiled in Table 4. Baseline resolution was achieved for all species. The resolutions are comparable to analytical methods using HPLC/ICP-MS coupling [6].

3.6. Calibration curves

In Table 5 calibration data for aqueous as well as spiked urine samples are shown for As(III), As(V), MMA, DMA and TMAO. For fortification a pooled urine sample of three individuals was used. The instrumental detector response increases in the order As(V), TMA, As(III), MMA and DMA

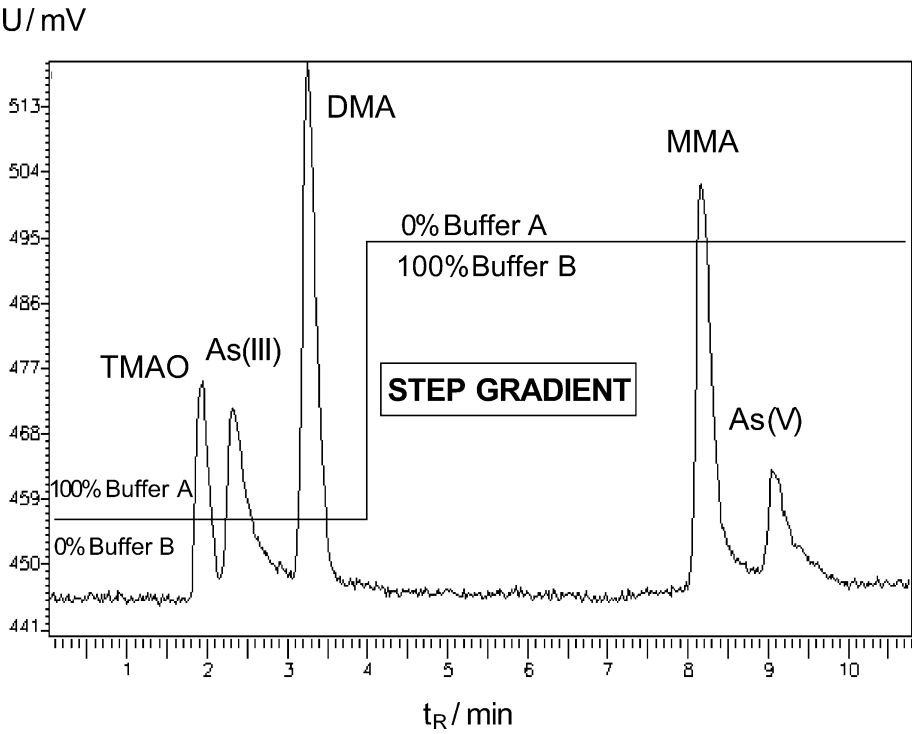

Fig. 6. Chromatogram of a standard solution containing all five arsenic species (mobile phases A: 10 mmol tetraborate/L, 20 mmol NaCl/L, pH 9.0; B: 50 mmol tetraborate/L, 30 mmol NaCl/L, pH 9.0; column flow: 1.4 mL/min). Concentrations of arsenic species are generally expressed as μg arsenic/L.

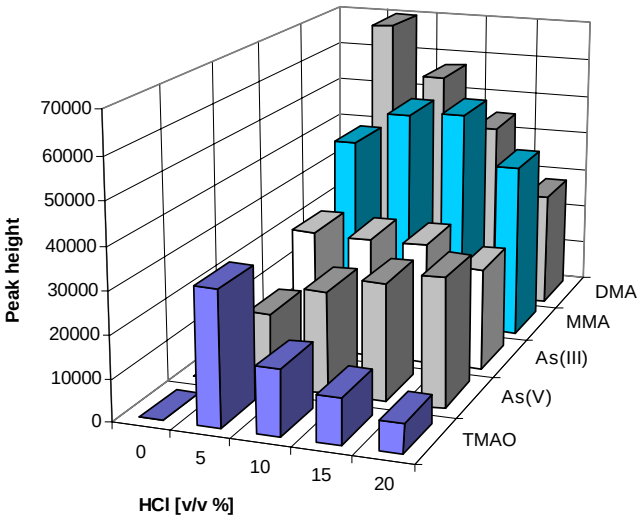

Fig. 7. Peak intensity of each 10 μg/L As(III), As(V), MMA, DMA and TMAO as a function of HCl concentration added with the flow-injection interface (experimental conditions: cf. Fig. 6).

by a factor of about 4. The high *y*-axis intercepts of the matrix standard curves are caused by blank concentrations of these species in the unfortified urine (As(III): 2.4 μg/L, As(V): 6.6 μg/L, MMA: 4.5 μg/L, DMA: 5.1 μg/L, TMA: <1.1 μg/L). All concentrations are expressed as μg arsenic/L.

3.7. Limit of detection, precision and matrix effects

Limit of detection (LOD), precision and matrix effects as quality parameters of the presented method are summarized in Table 6. Precision has been evaluated with urine samples, which were spiked with a species concentration of 10 μg/L, each. The detection limit has been calculated as 3σ of the background signal. Matrix effects were determined comparing the slopes of calibration curves using matrix-matched standards with those applying pure solvent standards. Matrix effects >0% may be encountered in flow-injection systems in the presence of salts coeluting with arsenic species and hence reducing the solubility of the corresponding arsine [15]. The LODs of this method for As(III), As(V), MMA and DMA are comparable to those of most other studies or are even lower [1,5,7,16–18]. Lintschinger et al. [2] report LODs of an HPLC/ICP-MS method for As(V), MMA and DMA being lower by a factor between 50 and 100, the LOD for As(V) amounts to 5 μg/L. But the method only works with aqueous standard solutions and not with urine samples. Le and Ma [19] developed an HPLC/HG-AFS method with LODs ranging from 0.4 to 0.8 μg/L for the four species. But all of these methods have in common

Table 4
Retention factors (k), separation factors (α) and resolutions (R_s)

	TMAO	As(III)	DMA	MMA	As(V)
k	0	0.2	0.7	3.2	3.7
α	nd	3.5	4.6	1.2	–
R_s	0.9	2.2	14.4	2.1	–

Separation factors (α) and resolutions (R_s) are determined for adjacent peaks for which $t_{R2} > t_{R1}$. $k = (t_R - t_M)/t_M$, where t_R is the retention time of the analyte, t_M is the elution time of the unretained species, here defined as the retention time of TMAO; $\alpha = k_2/k_1$. $R_s = 1.177(t_{R2} - t_{R1})/(W_{H1} + W_{H2})$, where W_H is the peak height at half height; nd (not defined), because $k_1 = 0$.

Table 5
Calibration data for pure solvent and matrix-matched standard solutions

Spiked concentration (μg/L)	As(III): peak area (arbitrary units)	As(V): peak area (arbitrary units)	MMA: peak area (arbitrary units)	DMA: peak area (arbitrary units)	TMAO: peak area (arbitrary units)
Matrix standards					
0	27236	62014	42812	172506.5	0
5	No data	117581[a]	No data	No data	No data
10	346817	180927	464788	632069	199133
20	610193	290200	931936	961635	369224
100	2656358	1122621	3374890	4086193	1762287
Linear regression					
Slope	25974	10550	32559	38880	17507
Intercept	65989	69822	145444	199513	13672
r^2	0.9994	0.9997	0.9956	0.9997	0.9998
Aqueous standards					
0	0	0	0	0	0
20	365289	243688	776874	848379	389945
40	860300	448973	1373783	1656221	736589
Linear regression					
Slope	20859	11224	35244	41406	18631
Intercept	0	6401	0	6756	0
r^2	0.9910	0.9976	0.9932	0.9998	0.9986

[a] Additional data point due to low detector sensitivity for As(V).

Table 6
Precision (intra-laboratory repeatability R.S.D.), detection limit and matrix effect for As(III), As(V), MMA, DMA and TMAO

	TMAO	As(III)	DMA	MMA	As(V)
R.S.D. (%)	3.2	5.9	5.7	5.6	6.4
LOD (μg/L)	1.1	2.4	2.3	2.4	2.6
Matrix effect (%)	−5	25	−8	12	−2

Matrix effect = (slope (matrix standard curve) − slope (solvent standard curve))/slope (solvent standard curve).

Table 7
Results of speciation analysis of two certified reference urine samples

	ClinRep®	Seronorm®
As(V) (μg/L)	29.8	105.2
DMA (μg/L)	43.9	5.4
Certified range (μg/L)	53.1–83.9	90–110

that detection and quantitation of TMAO beside the other species is not possible. Larsen et al. [5] report an LOD of 2.8 μg/L for TMAO in urine with an HPLC/ICP-MS-based method; but As(III), As(V), MMA and DMA elute in the void volume and cannot be determined. Odanaka et al. [9] applied Purge-and-trap-GC/MS for the determination of As(III)/As(V), MMA, DMA and TMAO in urine. This method cannot discriminate between As(III) and As(V) but yields LODs between 0.2 and 0.4 μg/L. The main drawback is, that a sample volume of 50 mL is needed for analysis and that due to very laborious sample preparation including liquid–liquid extraction and distillation of the arsines from a cryogenic trap in the GC this method is not applicable for routine analysis.

3.8. Application to urine samples

The method was applied to determine arsenic species in the morning urine of a test person before and 12 h after the consumption of 200 g shrimps and 100 g hering, as ingestion of seafood is known to elevate levels of arsenic in urine. The DMA concentration was found to increase by a factor of about 6 from 4.2 ± 0.9 to 24.0 ± 2.2 μg/L. The concentrations of the other species were below their respective LOD. An increase of urinary DMA excretion after seafood consumption has been reported, whereas the excretion of the other toxic species hardly increased [20]. Arbouine and Wilson [21] examined 20 persons before and after the consumption of different seafood meals and found in none of the samples As(III) and As(V) and in only three traces of MMA. The LODs of all three species were 2 μg/L.

3.9. Quality assurance

Analysis of certified reference materials is an important means to determine the trueness of an analytical method. However, a commercially available reference urine for arsenic species is not available. Only the sum of hydride-forming species is certified. Therefore, the sum of arsenic species of two reference urine samples was determined with the present method and compared with the total certified amount (Table 7). The species As(III), MMA and TMAO were <LOD. As(V) and DMA were detectable in both urine samples as indicated by Fig. 8. The sum of these species was within the certified range of "ClinRep®" and only slightly out of range in the case of "Seronorm®". However, the certified total amounts of arsenic have been

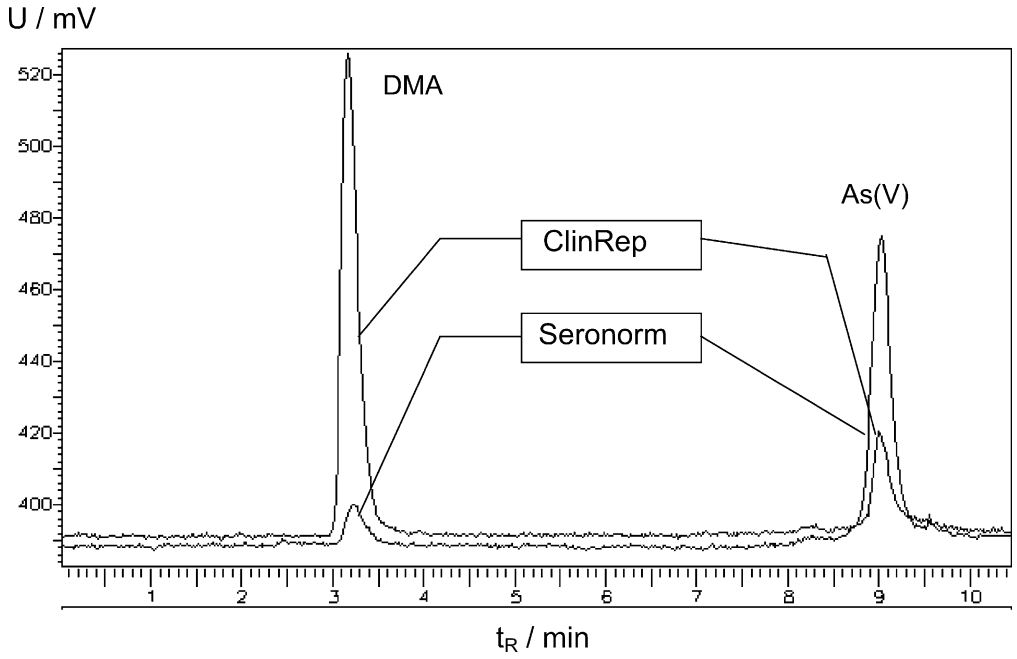

Fig. 8. Chromatograms of two certified reference urine samples for the determination of the trueness of the analytical method.

determined using a calibration with As(V) not regarding the response of the individual species. A species-specific calibration as applied in the present method would have been more accurate.

4. Conclusions

With the presented HPLC/HG-AAS method the toxicologically relevant arsenic species As(III), As(V), MMA, DMA and TMAO can be determined in urine samples within a single chromatographic run. To the best of our knowledge this method is the first one that can simultaneously determine TMAO and the other four species in urine. Baseline resolution of all species is achieved and HPLC separation is completed within 11 min. The LODs for As(III), As(V), MMA and DMA are comparable to those of other methods. In addition, the metabolite TMAO can be determined down to 1.1 μg/L. As normal concentrations of TMAO in humans are supposed to range between <0.1 and 2.7 μg/L [5,22] future work has to focus on the lowering of the LOD of TMAO.

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