Fast Determination of Arsenic Species and Total Arsenic in Urine by HPLC–ICP-MS: Concentration Ranges for Unexposed German Inhabitants and Clinical Case Studies

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Abstract

A fast and reliable high-pressure liquid chromatography (HPLC)–inductively coupled plasma-mass spectrometry (ICP-MS) routine method was developed for the determination of inorganic arsenic [As(III) and As(V)], organic monomethylarsonate [MMA(V)], dimethylarsinate [DMA(V)], and arsenobetaine (As-B) in human urine. The complete method validation is described, including internal and external quality assurance. Limits of quantification for the As species are 0.1 µg/L, which is sufficient to determine background concentrations of the arsenic species in human urine. Additionally, total As in all urine samples was determined by conventional ICP-MS. Mean concentrations for 82 non-exposed inhabitants from northern Germany are 12.7, 5.9, 4.0, 0.23, 0.52, and 0.17 µg/L for total As, As-B, DMA(V), As(III), MMA(V), and As(V), respectively. Approximately 15% of the total As was not identified by the anion exchange HPLC–ICP-MS method, and could be other As metabolites in urine. Two case studies underline the need for As speciation, especially when total urinary arsenic concentrations are elevated. In the first case, we investigated the effect of seafood consumption on the concentration of different arsenic species in urine for different persons. A maximum enhancement of total As from 1 up to 2200 µg/L (2000 µg/L for As-B) was observed after a normal fish meal. The second case describes the exposure of a 7-year-old child to As(III) by inhalation of calcium arsenite powder. Five hours after exposure, the concentrations in the child's urine for As-B, DMA(V), As(III), MMA(V), and As(V) were < 0.1, 189, 304, 229, and 27 µg/L, respectively, and these concentrations were reduced to normal background values after 4 days.

Introduction

Inorganic arsenic (As) is a human carcinogen, and many people in different parts of the world are exposed to As mainly via drinking water or food. In some regions, the As contamination in drinking water is hazardous to health; for example, in the Ganga plain (1) and West Bengal in India (2), or in Bangladesh (3). In other regions, environmental exposure to As was described via arsenate-treated wood (4) or via freshwater fish from arsenic-contaminated waters (5). Examples of occupational exposure to As have been published for art glass manufacture (6), the timber treatment and semiconductor industries (7,8), and also for miners (9) and smelter workers (10).

In the past, human biomonitoring of exposure to arsenic was mainly performed using atomic absorption spectrometry (AAS). In most cases, hydride generation atomic absorption spectrometry (HG-AAS) was applied, due to the assumption that only toxicologically relevant As is forming the volatile hydride. Because arsenobetaine (As-B), arsenocholine, and some other important arsenicals are not forming the hydride, it was assumed that HG-AAS determines the sum of the toxicologically relevant As species, which are inorganic As(III) and As(V), organic monomethylarsonate [MMA(V)], and dimethylarsinate [DMA(V)]. However, recently, it was pointed out that arsenosugars are also forming volatile As compounds (11) and can increase the urinary As concentration determined by HG-AAS. Originating from food, the urine may also contain organic As species such as arsenosugars and arsenolipids (12), which are less toxic than inorganic As. Another analytical problem of HG-AAS for As determination is that the inorganic and methylated species have different sensitivities of determination, which makes the calibration difficult when commercially available flow injection AAS instruments are used. Because of these problems, we developed and validated a method not only for the speciation of As, but also to obtain better analytical accuracy and practicability. In this method, high-pressure liquid chromatography (HPLC) was coupled to inductively coupled plasma-mass spectrometry (ICP-MS). This combination of chromatography and atomic spectrometry was also recommended by other authors (13–15), and HPLC–ICP-

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MS methods for As speciation have been developed with focus on sample preparation and storage (16), fast chromatographic separation of the species (17), or the determination of thio-arsenic compounds in urine (18). In our case, the goal was to develop a very fast, accurate, sensitive, and reliable routine method for high sample throughput in a medical laboratory.

Despite the large number of publications describing analytical As speciation, there are few data available on actual urinary concentrations of the As species. For some countries, data for the non-exposed populations are described, such as Hungary, Romania, and Slovakia (19), the United Kingdom (7), Italy (6), Germany (20), Japan (21), and the U.S. (22,27). As mentioned previously, urine also may contain various organic As species (23), and the number of As species found in urine is increasing. In a recent paper, Schmeisser et al. (12) describe the excretion of arsenic-containing fatty acids after ingestion of cod liver. Hansen et al. (24) have reported about different thio-arsenic compounds in the urine of a particular breed of sheep. Ma and Le (25) reported some unknown As species in human urine after arsenosugar ingestion.

This study provides valuable information about the concentration of five arsenic species in human urine of unexposed persons in northern Germany. Additionally, total As was determined by conventional ICP-MS in all urine samples in order to calculate the concentration of other As species, which are not determined by the HPLC–ICP-MS method. This calculation of the recovery is mostly not performed, though there are many published papers for the determination of arsenic species in urine.

Finally, we demonstrate the applicability of our method and the requirement of As speciation in practical case studies. In the first case, we investigated the effect of fish or seafood consumption on the urinary arsenic concentration of all species. In the second case, we describe the urinary As species concentration of a child after exposure to As(III). These case studies are important practical examples, because inorganic As compounds have a significantly higher toxicity than most of the organic As compounds. Today, many medical laboratories only determine total arsenic in urine, and some physicians are not familiar with the fact that high total arsenic concentrations in urine require further specification of the individual As compounds, which was also underlined in a recent paper by Kales et al. (26).

Materials and Methods

Study population

The urine samples of 82 occupationally non-exposed human subjects were collected in April 2007. The subjects live in the greater area of Bremen in northern Germany. This area has more than 500 inhabitants/km^2 and is close to a few industrial regions such as car, steel, or food industries. Information on exposure conditions was collected by questionnaire-based interviews, and the following data are available: age, gender, place of residence, occupation, smoking habits, and fish consumption prior to sample collection. The group ranged in age from 18 to 65 years. Fifty subjects of this group were female, and 32 subjects were male. Fourteen subjects were smokers, and 68 subjects were non-smokers. None of the participants had consumed fish or seafood 72 h prior to the sample collection. All volunteers gave their agreement for the use of their urine samples for this biomonitoring survey.

Three volunteers, consisting of a 40-year old male and 2 females whose ages were 23 and 50 years, participated in the study about the urinary arsenic excretion after one-time ingestion of fish. All persons had strictly not eaten fish four days prior to this fish meal. Urine samples of these persons were collected during a period ranging from 60 h before to 50 h after the meal. Each volunteer consumed 150 g of a special type of fish or seafood: crabs, wolffish (*Anarhichas lupus*), and limandes (*Microstomus kitt*).

Instrumentation

An Agilent 1100 series HPLC with an isocratic pump, autosampler, vacuum degasser, and anion exchange column was coupled to an Agilent 7500ce ICP-MS with an octopole collision/reaction cell (Agilent Technologies, Waldbronn, Germany). The Agilent main chromatographic column (65001, 4.6 mm × 150-mm i.d.) and the guard column (65002, 4.6 mm × 10-mm i.d.) had a chemical bonded hydrophilic anion exchange resin. A Babington nebulizer with a Scott spray chamber (Agilent Technologies) was applied for on-line sample introduction from the column into the ICP. The spray chamber was Peltier cooled at 5°C to ensure temperature stability and to reduce the water vapour present in the nebulizer gas flow. The ICP operating conditions were 1500 W generator power, 15 L/min outer gas flow, 1 L/min intermediate gas flow, 1.1 L/min nebulizer gas flow, and 1.2 mL/min sample uptake rate. The ion lenses of the ICP-MS were optimized to achieve the highest signal/background ratio for the isotope ^{75}As. The data evaluation was based on the determination of peak areas of the As species and was performed with the Agilent chromatographic software.

Urinary creatinine concentrations were determined by the Jaffè method on the Hitachi 911 Autoanalyzer (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's procedure. For quality assurance, the control materials Liquichek 1 and 2 (Lot 0504, Bio-Rad, Irvine, CA) were used in the beginning and after every 20 samples.

Chromatographic conditions

Various salts for matrix matching and a wide range of pH (5–12) were evaluated. Optimized conditions were found for a mobile phase, which consists of a solution containing 1.6 mM NaH_2PO_4, 0.16 mM Na_2-EDTA, 8 mM CH_3COONa, 2.4 mM $NaNO_3$, and 1% (v/v) ethanol. This solution was always prepared freshly and was adjusted with 1 M NaOH to pH 11. CH_3COONa and $NaNO_3$ were used for matrix matching, and ethanol increases the signal-to-background ratio for ^{75}As. All chemicals were supplied by Merck (Darmstadt, Germany) and are used in suprapure quality.

Sample collection

Morning middle stream urine samples were collected in polyethylene containers (Sarstedt, Nümbrecht, Germany) and

transported to the laboratory at room temperature. Prior to collection the containers were cleaned with 5% (v/v) HNO_3 (Merck) in ultrapure quality. The samples were stored in a refrigerator at 4°C. Two hours before sample preparation, the urine samples were brought to room temperature.

Sample preparation and calibration

Five-hundred microliters of the urine sample were diluted 1:10 (v/v) with 4.5 mL deionized water in a polypropylene tube, and 500 µL of this solution was added to a 1-mL polypropylene autosampler vial with a polyethylene cap and PTFE septum (Agilent Technologies). These tubes are recommended because it was found that different glass vials for the autosampler were contaminated with As and are not useful for this application.

Four calibration stock solutions with 1 mg/L were prepared for all the As species in 1% (v/v) HNO_3, except for the As(III) solution, which was prepared in 0.1% (v/v) HCl. The As(III) stock solution was prepared from an As_2O_3 solution in 2% (v/v) HCl (Spex, Grasbrunn, Germany), and the As(V) stock solution from an As_2O_5 solution in 3% (v/v) HNO_3 (Merck). The DMA(V) stock solution was prepared by dissolving 98% (m/m) sodium cacodylate (Sigma, Taufenkirchen, Germany), and the MMA(V) stock solution was made by dissolving 99% (m/m) monosodium acid methane arsonate (Chem Service, Chester, PA). The As-B stock solution was prepared by dilution of the reference material BCR 626 (Promochem, Wesel, Germany). One-hundred microliters and 1 mL of each stock solution were diluted with deionized water up to 10 mL in polypropylene tubes to obtain a mixed calibration solution with 10 and 100 µg/L of each As compound, respectively. These mixed solutions were used to prepare the final calibration solutions with concentrations of 0.1, 0.25, 0.5, 2, and 10 µg/L, respectively. This solution was always prepared freshly. The concentration of the arsenic species in this mixed calibration solution was verified by comparison with the concentration in solutions of single species. Transformation of the species (for example, As(III) to As(V)] was not observed during the sample preparation and calibration. Further details about sample preparation and storage for As speciation have also been discussed by Feldmann et al. (16), who reported that arsenic species are stable in urine when stored at low temperatures (–20°C to 4°C for up to 2 months). Morton and Mason (7) also recommended low temperatures for storage of the urine samples prior to analysis. In our case, we transported the urine samples to the laboratory within a few hours, and the samples and all stock solutions were stored in the refrigerator at 4°C. All samples were analyzed within 2 days.

Results and Discussion

Analytical characteristics and quality assurance

It was the goal to develop a sensitive, rapid, robust, and reliable routine method for the determination of background concentrations of As species in urine by HPLC–ICP-MS. The optimized conditions for good resolution, low detection limits, and low retention times are achieved using a mixture of reagents for the mobile phase, as described previously. At pH 11, we found the best resolution of As(III), DMA(V), and As-B, as well as good resolution of the arsenic peaks from chloride, which is necessary to avoid the $^{40}Ar^{35}Cl$ interference on ^{75}As. Because of this resolution, it is not necessary to use the collision cell modus of the ICP-MS. The duration of a chromatographic run is only 10 min, which is a significant improvement compared with other routine HPLC–ICP-MS methods described previously (27), where the time for a full chromatogram was more than 20 min. The retention times for As-B, DMA, As(III), MMA, and As(V) are 1.8, 2.1, 2.4, 5.5, and 8.8 min, respectively. The linearity of the method was investigated by calibration using solutions containing 0.1, 0.5, 1, 5, 10, 20, and 50 µg/L of the As species. No deviation from linearity of calibration curves was observed for solutions up to 50 µg/L, which corresponds to 500 µg/L of the As species calculated to the undiluted urine. Real samples with extremely high concentration were diluted 1:2 or 1:5 (v/v) with deionized water. Limits of quantification (LOQs) calculated for the undiluted urine are 0.1 µg/L for each of the five arsenic species determined by HPLC–ICP-MS, and 0.02 µg/L for total As, which was determined by conventional ICP-MS with pneumatic nebulisation for sample introduction. The LOQs were defined as the analyte concentration corresponding to 10 times the standard deviation of 5 measurements of a spiked sample. This

Table I. Internal and External Quality Assurance

	Concentration (µg/L)							
	CRM NIES 18		Sample 38A (A)*		Sample 38B (A)		Sample 07U-2 (B)†	
Analyte	Result	Target	Result	Target	Result	Target	Result	Target
As-B	64 ± 4	69 ± 12						
DMA	35 ± 3	36 ± 9	34 ± 3	32.6 ± 11.5	52 ± 5	50.6 ± 12.8		
As(III)			9.4 ± 1.6	11 ± 3.6	16.5 ± 2.1	19.5 ± 5.2		
MMA			3.5 ± 0.3	3.7 ± 1.6	14.7 ± 0.3	15.1 ± 4.4		
As(V)			8.2 ± 0.7	7.7 ± 2.6	20.2 ± 0.7	20.6 ± 6.6		
Total As	146 ± 7	137 ± 11	123 ± 8	120 ± 21	272 ± 19	269 ± 42	79 ± 4	82 ± 5

* A: Out-Patient Clinic for Occupational, Social and Environmental Medicine of the Friedrich-Alexander-University Erlangen-Nuremberg.
† B: Canadian Institut National De Sante Publique Du Quebec.

sample was urine with the lowest arsenic concentration we could get, because a real blank is not available. The power of detection of this HPLC–ICP-MS method is sufficient to determine background concentrations of all As species for environmental health. This is an improvement compared with an earlier study (20) about persons from northern Germany, where limits of detection of 1, 10, 2, and 2 µg/L were reported for As(III), As(V), DMA(V), and MMA(V), respectively.

For internal quality assurance for the determination of total As, we analyzed the quality control materials ClinChek 1 and ClinChek 2 (Lot 607, Recipe, Munich, Germany), Seronorm Urin (Lot 2525, Sero, Billingstad, Norway), and Lyphochek 1 (Lot 69091, Bio-Rad, Irvine, CA) with target values of 41, 82, 184, and 71 µg/L, respectively. Our average concentrations from day-to-day (n = 20) for these control materials were 40, 83, 191, and 69 µg/L, which is in very good agreement with the target values for total As. The RSDs of the average concentrations of total arsenic from day-to-day (n = 20) for these control samples are in the range 6.3–8.7%. For the species DMA and As-B, a certified reference material is available, which was described in the literature (28). The target results for this CRM NIES 18 are in good agreement with our measured results for As-B and DMA(V) (Table I). For total As, we found slightly higher concentrations (Table I) within the accepted range of the target values. Another group has also found slightly higher concentrations for total As (18), and they reported about the presence of thio-arsenicals in this urine reference material, which are approximately 10% of the total As.

For external quality assurance, we participated in the 38th German External Quality Assessment Scheme from the Institute and Out-Patient Clinic for Occupational, Social, and Environmental Medicine of the Friedrich-Alexander-University Erlangen-Nuremberg in November 2006, and in the Quebec Multielement External Quality Assessment Scheme from the Canadian Institut National De Sante Publique Du Quebec in March 2007. In the German program, four different As species and total As were determined. The results are summarized in Table I. For all species in both samples (38A and 38B), the measured concentrations are within the acceptable range defined by the organization of the program. The highest deviation from the target value is 14 % for As(III) in sample 38A. In the Canadian program, where only total As was determined (sample 07U-2), there is also a good agreement of measured results and target concentrations (Table I). The RSDs of the average concentrations of the arsenic species in sample 38A from day-to-day (n = 20) are 4.2, 6.4, 9.4, 9.9, and 8.7% for As concentrations of 50.5 (As-B), 32.6 (DMA), 7.7 [As(III)], 3.7 (MMA), and 8.6 µg/L [As(V)], respectively.

Study results

The analysis results for 82 occupationally unexposed adults living in northern Germany are summarized in Tables II and III, which describe mean concentrations, concentration ranges, geometric mean concentrations, selected percentiles, and the number of values below the LOQ. The concentrations are expressed in micrograms per liter urine (Table II) and also in micrograms per gram creatinine (Table III) to adjust for effects of urinary dilution. These tables make an important contribution to the evaluation of reference values for As species in urine. The mean concentration for total As is 13.7 µg/L, which is similar to values reported for the unexposed Italian (6) and German population (29). As-B and DMA(V) have mean concentrations of 5.9 and 4 µg/L, respectively, and these two compounds are approximately 72% of the total As (mean: 13.7 µg/L). Because of the relatively high mean concentration for As-B, we assume that some participants in this study have eaten fish or seafood unknowingly. In 12% of all urine samples, As-B was below the LOQ. Inorganic As(III) and As(V) and organic MMA(V) were determined in the majority of samples, but their mean concentrations are below 1 µg/L (Table II). The relative distribution of the four toxicologically relevant As species is 5% As(III), 4% As(V), 11% MMA, and 80% DMA. This is in agreement with an earlier paper (30), in which the average distribution of arsenic metabolites in urine was 10–30% inorganic arsenic, 10–20% MMA(V), and 60–70% DMA(V). In our study, the mean values of all determined As species sum up to only 85% of the mean value of the total As, which indicates that approximately 15% of As in the real urine samples could be other species. This possible presence of additional As species in urine has been mentioned before by different authors, such as for thio-arsenic compounds (31), arsenic containing fatty acids (12), trimethylarsine oxide (18), dimethylarsinoylethanol (32), and other unidentified As compounds (25). The occurrence of MMA(III) and DMA(III) in human urine was also discussed (33,34). To

Table II. Statistical Data about the Concentration of As Species in Urine of the Unexposed Population (n = 82) in Micrograms per Liter

Analyte	Mean*	Range	Geometric Mean*	% < LOQ	Percentiles* 5%	Percentiles* 95%
As-B	5.9	< 0.1–23	1.4	12	0.1	22.7
DMA	4.0	< 0.1–18	2.6	1	0.6	9.1
As(III)	0.23	< 0.1–2.3	0.15	27	< 0.1	0.49
MMA	0.52	< 0.1–2.7	0.33	11	< 0.1	1.6
As(V)	0.17	< 0.1–0.6	0.11	69	< 0.1	0.53
Total As	12.7	0.5–103	7.9	0	2.0	42

* Concentrations below the LOQ were calculated as LOQ/2.

Table III. Statistical Data about the Concentration of As Species in Urine of the Unexposed Population (n = 82) in Micrograms per Gram Creatinine

Analyte	Mean*	Range	Geometric Mean*	% < LOQ	Percentiles* 5%	Percentiles* 95%
As-B	6.3	< 0.1–88	1.6	12	0.1	23.3
DMA	4.9	< 0.1–43	3.0	1	1.3	15.8
As(III)	0.31	< 0.1–5.9	0.17	27	< 0.1	0.65
MMA	0.76	< 0.1–6.9	0.38	11	< 0.1	2.3
As(V)	0.3	< 0.1–4.2	0.11	69	< 0.1	1.1
Total As	15.3	1.9–158	8.8	0	2.9	51.6

* Concentrations below the LOQ were calculated as LOQ/2.

our knowledge, biomonitoring surveys with a statistically relevant number of samples were not performed for these species, and further studies are required.

Case studies

The consumption of seafood within 2–3 days of sample collection might increase the toxicologically less relevant As-B concentration and can result in unnecessary action, when only the total As concentration of the patients' urine is determined. Not all clinicians and environmental scientists are familiar with this species-specific toxicological aspect, and sometimes high total As concentration are misinterpreted. In order to investigate whether fish consumption is increasing the concentration of other species than As-B in urine, we performed studies with three persons. All persons had strictly not eaten fish or seafood three days before and three days after a fish meal. In this meal, each test person consumed 150 g of fish or seafood. We measured the concentrations of the 5 As species and total As in urine during a period of 60 h before and 50 h after the meal. Person 1 consumed crabs from the North Sea, and the total urinary As concentration was increased from 1.3 µg/L before the meal up to a maximum of 480 µg/L in urine 14 h after the meal (Figure 1). Person 2 consumed a wolffish (*Anarhichas lupus*), which is a seawater fish living in the Northern Atlantic Ocean. For person 2, we found the highest increase of the total urinary As concentration from 4 µg/L up to a maximum concentration of 2200 µg/L 14 h after the meal. After this peak maximum, the As concentration has decreased rapidly within the next two days. Person 3 showed a similar behavior of As excretion in their urine samples after consumption of a limandes (*Microstomus kitt*), which is a bottom-living seawater fish from the North Sea and Atlantic Ocean. For all fish or seafood species, As-B is the major compound in urine with more than 90 % of the total As (Figure 1). Compared with total As and As-B, the DMA(V) only increases slightly (Figure 1) in the range of 4–10 µg/L, and for the other species As(III), As(V), and MMA(V), we did not find a significant increase of the concentrations after fish consumption.

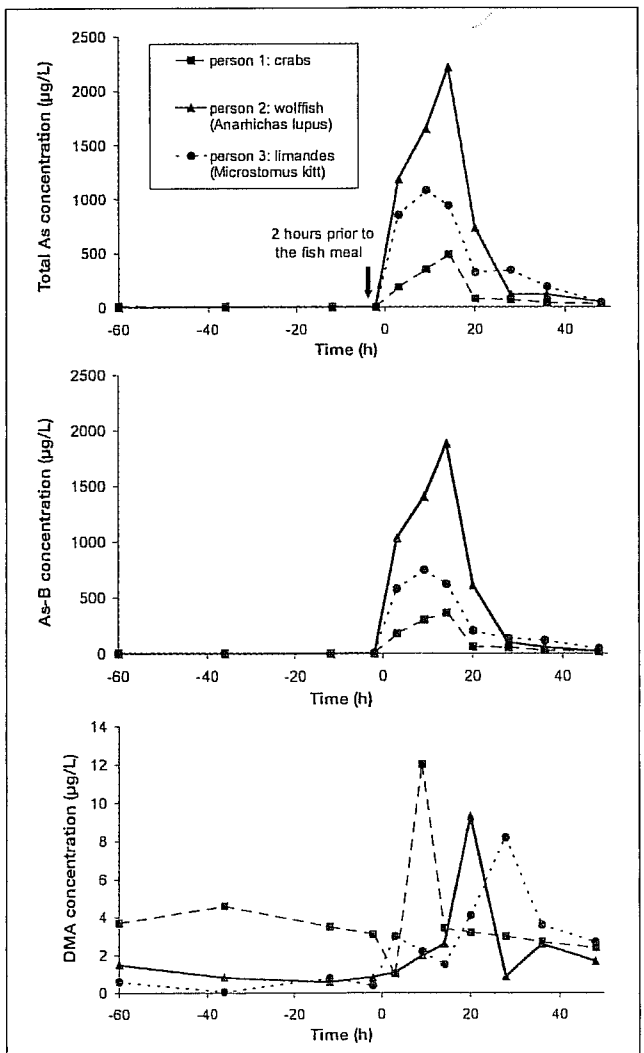

Figure 1. Variation of the chromatogram of total As, As-B, and DMA in the urine of three subjects with respect to the time of fish consumption.

The chromatogram (Figure 2) of the As species 5 h after fish consumption is completely different from a chromatogram 5 h after exposure to As(III). Person 1 in Figure 2 is the 25-year-old woman who also consumed the limandes. The excreted urine 2 h prior to the meal contains 1.6, 0.1, and 1.1 µg/L for total As, As-B, DMA(V), respectively, compared with 1118, 1059 and 1.1 µg/L, respectively, measured in the urine collected 5 h after the meal. Though the As-B increased up to the mg/L-range, no significant enhancement of the As(III), As(V), DMA(V), and MMA(V) concentrations was observed after fish consumption.

Person 2 in Figure 2 is a 7-year-old child who was playing with a 50-year old bag of calcium arsenite in a derelict barn. Originally, the As(III) compound in the bag was used as a plant protectant decades ago. The child inhaled the powder during the time he was playing with this bag, and 5 h after exposure, the concentrations in the child's urine for As-B, DMA(V), As(III), MMA(V), and As(V) were < 0.1, 189, 304, 229, and 27 µg/L, respectively. After four days, the concentrations in the morning urine sample were reduced to significantly lower values of < 0.1, 4.6, 1.5, 1.4, and 0.5 µg/L, respectively. This case confirms earlier descriptions (35) that arsenic is rapidly eliminated through the kidneys, and the methylated species were excreted together with the inorganic species after exposure to inorganic As. Our results are also in agreement with Buchet et al. (36,37) who describe that the urinary excretion

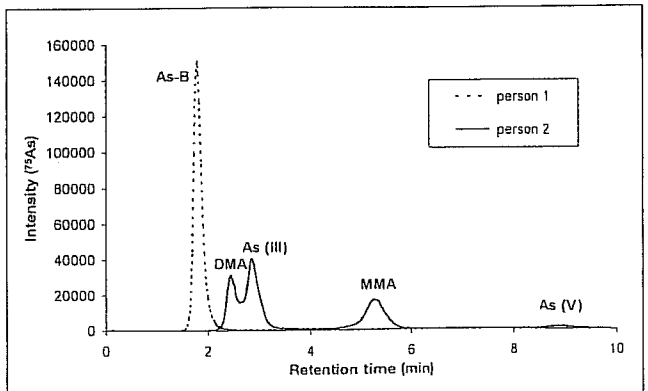

Figure 2. Chromatogram of the arsenic species of two subjects, both of whom have an extremely high value of approximately 1000 µg/L total urinary As (person 1: an adult after fish consumption; person 2: a child after exposure to calcium arsenite).

occurs in the form of the inorganic species during the first hours after the exposure, and then the methylation process is rapidly triggered and leads to a predominant excretion of the methylated species (mainly DMA) after 8 h.

Conclusions

After more than 20 years of research and method development, the speciation of several As compounds in urine has become a routine application. For medical laboratories with high sample throughput, HPLC–ICP-MS is the method of choice due to low detection limits, good precision and accuracy, and the high number of As species which can be determined. However, since today, it is not completely clear how many organic As compounds occur in human urine, and more information is needed about concentration ranges of organic As species, as well as of their metabolism and toxicity. Our study contains a biomonitoring of the total As concentration and the concentration of five different As species in urine samples from inhabitants of northern Germany. This is a valuable contribution to the definition of a reference value; however, it is necessary to do more research in the identification of additional urinary As species and to carry out more biomonitoring surveys with a higher number of As species and for different populations.

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