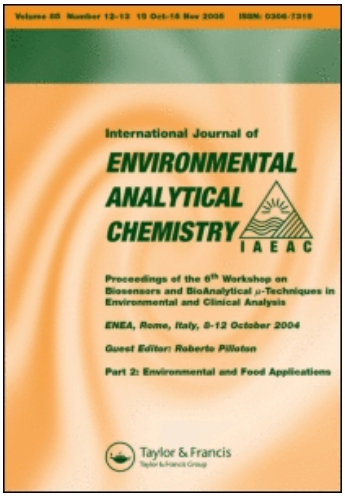

International Journal of Environmental Analytical Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713640455

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To cite this Article Georgieva, Rossitsa B. , Petrov, Panayot K. , Dimitrov, Plamen S. and Tsalev, Dimiter L.(2007) 'Observations on toxicologically relevant arsenic in urine in adult offspring of families with Balkan Endemic Nephropathy and controls by batch hydride generation atomic absorption spectrometry', International Journal of Environmental Analytical Chemistry, 87: 9, 673 — 685
To link to this Article: DOI: 10.1080/03067310701218981
URL: http://dx.doi.org/10.1080/03067310701218981

Intern. J. Environ. Anal. Chem.
Vol. 87, No. 9, 10 August 2007, 673–685

Observations on toxicologically relevant arsenic in urine in adult offspring of families with Balkan Endemic Nephropathy and controls by batch hydride generation atomic absorption spectrometry

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(*Received 14 September 2006; in final form 15 January 2007*)

The aim of this study was to develop a method for the characterization of internal exposure to arsenic, which is thought to play a role in the development of a kidney disease, known as Balkan Endemic Nephropathy, typical for a district in Bulgaria, and to investigate whether the As body burden differs in the offspring *versus* control individuals. For this case study, an analytical procedure for the determination of toxicologically relevant arsenic (the sum of arsenite, arsenate, monomethylarsonate, and dimethylarsinate) in urine by batch-type hydride generation atomic absorption spectrometry was developed. Optimization experiments for levelling off the sensitivity of inorganic arsenic and its mono- and dimethylated species in dilute HCl–L-cysteine medium were performed. The limit of detection for hydride forming arsenic fraction was 0.5 ng As, i.e. 0.25 $\mu g\,L^{-1}$ in 10 mL of 1 + 4 v/v diluted urine. The relative standard deviation was typically 1.5–1.8% for aqueous solution and 2–6% for urine samples at 1.0 $\mu g\,L^{-1}$ As. The sample throughput rate was 15 h^{-1}. No statistical correlation and cross-correlation between individuals case-control and sex at 95% confidence were found: controls ($n = 99$), mean 3.5 ± 2.1 (SD), range 0.9–10.4, median 3.0 $\mu g\,L^{-1}$ As and cases ($n = 102$), mean 3.6 ± 2.2 (SD), range 0.5–11.0, median 3.2 $\mu g\,L^{-1}$ As. On the basis of this study, arsenic can be excluded as a factor involved in BEN development.

Keywords: Arsenic; Urine; Balkan Endemic Nephropathy; Monomethylarsonate; Dimethylarsinate; Hydride generation atomic absorption spectrometry

1. Introduction

Arsenic is a nonessential toxic trace element for humans, and its determination in biological samples, and particularly in urine, represents an invaluable analytical task in environmental, nutritional, occupational, forensic, and clinical fields [1–4]. The chemistry of arsenic involves a number of organic and inorganic compounds.

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ISSN 0306-7319 print/ISSN 1029-0397 online © 2007 Taylor & Francis
DOI: 10.1080/03067310701218981

Methylated arsenicals naturally occur as a result of biological activity. Numerous As compounds have been employed as pesticides, herbicides, and fungicides in agriculture and livestock breeding and forestry. Arsenic migrates mainly by waters, but its immobilization on Al and Fe hydroxides could be substantial, forming polluted sites slowly liberating arsenic. Therefore, humans could be exposed for a long time in certain areas to abnormal As levels. Humans have natural metabolite-detoxicating mechanisms, including glutathione, albeit with limited capacity [5]. Pinto *et al.* [6] found a direct relation between arsenic in air and As in human urine. Their subsequent investigations [7] showed a linear relation between death caused by respiratory cancer and urinary arsenic. The need for continuous monitoring of As and its relevant toxic species became a necessity in the early 1980s, and the World Health Organization (WHO) issued a document [1] outlining the urgent need for additional investigations for toxicologically relevant As in the environmental objects. As the most appropriate biomarker for human internal exposure, the WHO recommends the measurement of As in urine. It is known that some organoarsenicals like arsenobetaine from seafood pass through the human body and are excreted by urine almost unchanged. As far as the most toxic species rather than the total arsenic represent a major health concern, in the early 1980s, arsenic assays in urine were given along with a record on recent diet [1]. Some sampling protocols recommend abstention from seafood consumption for 3–10 days before urine sampling [4, 8–10].

Balkan Endemic Nephropathy (BEN) was first characterized in the Vratza District, Bulgaria [11] and has remained a geographically constant and persistant endemic for over 50 years [12, 13]. BEN is a tubulo-interstitial kidney disease and progresses slowly over many years. The final disease stage is characterized by renal failure and shrinkage of both kidneys to the size of a walnut [14]. The disease shows familial clustering and develops only in particular areas and villages. Usually, BEN patients are diagnosed in the late stages of the disease. Causes of BEN are still unclear.

One of the proposed hypotheses for explanation of BEN etiology indicates that the geochemistry of the environment may cause or play a role in distribution of BEN. A recent pilot study on soils in Vratza region [15] has shown that the BEN soils have significantly higher concentrations for some toxic elements (As, Cd, Pb) *versus* soils from non-BEN villages. A possible correlation between toxic element exposure and developing of the kidney disease was postulated, and a vast investigatory project was started. Around 200 study subjects, carefully selected on the basis of parental health status and sex, were involved in the research. Statistical data treatment was aimed at revealing possible relations between As contents in urine and health problems in the district of Vratza.

Among several techniques for arsenic determination and speciation analysis as reviewed recently [16–18], hydride-generation atomic absorption spectrometry (HGAAS) has been selected on the basis of its capabilities for direct measurements without decomposition of organic matter and persistent organoarsenicals, high sample throughput, and possibilities for differentiation between toxicologically relevant As fractions [19–21]. A statistically planned, experimentally optimised and validated analytical procedure was developed for determination of the hydride-forming and toxicologically relevant arsenic, viz. the sum of inorganic (i) arsenic (III) (i-As(III), arsenite), i-As(V) (arsenate), monomethylarsonate (MMA, $CH_3As(OH)_2O^-$), and dimethylarsinate (DMA, $(CH_3)_2As(OH)O^-$) in urine as a biomarker for dietary and respiratory exposure, thus excluding contribution from dietary 'fish' arsenic

(arsenobetaine, arsenocholine, arsenosugars, etc.). The main endeavour for the analytical procedure was to determine suitable conditions for hydride generation in order to level off analytical signals yielded by different hydride-forming arsenic species: i-As(III), i-As(V), MMA, and DMA (AsH_3, AsH_3, CH_3AsH_2 and $(CH_3)_2AsH$, respectively) using HCl and L-cysteine with batch-type HGAAS. The advantages of L-cysteine as a reagent for pre-reduction of As(V), MMA and DMA in HGAAS are well documented in the literature [19, 22–24] and applications to environmental waters [24–26], urine [20, 21, 23, 27], and other samples [19] are reported. Its main limitation is the strong effect of sample acidity on responses of inorganic and methylated species, which depend on the type of hydride-generation system and cannot be overcome by standard addition calibration. This effect called for multivariate optimization of the batch HGAAS in order to find robust compromise conditions for the determination of this arsenic fraction.

2. Experimental

2.1 *Selection of groups of studied individuals, control groups, and sample collection*

During October 2003 to April 2004 and October 2004 to April 2005, a group of individuals (102 study subjects) whose father or mother were included in the Vratza hospital registry of BEN patients and an almost equally sized control group (99 study subjects) were enrolled in the study; details on both groups are given in table 1. Individuals were instructed to abstain from seafood consumption for 10 days before sampling so as to exclude any possible contributions of seafood-derived As species and metabolites to urinary arsenic. Forty-millilitre urine samples were collected in 50-mL polypropylene containers, acidified with 2 mL of 1 mol L^{-1} HCl, and immediately frozen.

Table 1. Group parameters and individual selection.

		Case, $n = 102$		Control, $n = 99$	
Characteristics		*n*	%	*n*	%
Gender	Men	51	50.0	47	47.5
	Women	51	50.0	52	52.5
Age	30–39	17	16.7	23	23.2
	40–54	55	53.9	48	48.5
	≥55	30	29.4	28	28.3
Place of birth	BEN village	96	95.0	72	72.7
Smoking status	Current smoker	40	39.2	34	34.4
	Ex-smoker	23	22.6	12	12.1
	Non-smoker	39	38.2	53	53.5
Parental history of BEN	Mother	39	38.2	0	0
	Father	26	25.5	0	0
	Both	37	36.3	0	0
Parental history of other kidney diseases	Mother	10	9.8	2	2.0
	Father	10	9.8	0	0

2.2 *Instrumentation*

A Perkin-Elmer model 3030 atomic absorption spectrometer equipped with an electrodeless discharge lamp system 2 power supply (operated at 380 mA) and a batch-type mercury hydride system MHS-20 with externally heated quartz tube atomizer (T_{cell} 900°C) was used. Background correction has been proven to be unnecessary, so the deuterium corrector was switched off in all further work. The As line at a wavelength of 193.7 nm was isolated with a bandpass setting of 2 nm. Integrated absorbance measurements (peak-area, A_{int}) with a delay time of 1 s and signal integration time of 45 s were used throughout. Argon purge gas was applied at 0.25 MPa. The optimized MHS-20 program was: purge time I 25 s, reaction time 15 s (ca 7 mL of 0.65 mol L^{-1} $NaBH_4$ introduced) and purge time II 40 s.

2.3 *Standards and reagents*

Reagents of analytical grade or higher quality were used. Stock standard solutions used for arsenic measurements (1 g L^{-1} As) were: 1000 μg mL^{-1} As(III) atomic absorption spectroscopy standard solution no. 11082 (Fluka, Buchs); 1000 μg mL^{-1} As(V), As standard solution traceable to SRM from NIST, and H_3AsO_4 in 0.5 mol L^{-1} HNO_3 (CertiPUR®, Merck, Darmstadt); organoarsenicals were prepared by dissolving sodium methylarsonate (MMA), $CH_3AsO(ONa)_2 \cdot 6H_2O$ (Cod. 371205, Carlo Erba, Milan), 0.3896 g in 100 mL aqueous solution; and sodium cacodylate (DMA), $C_2H_6O_2AsNa \cdot 3H_2O$ (Cod. 367455, Carlo Erba), 0.28568 g in 100 mL aqueous solution. Working standard solutions were prepared before use. A stock solution of sodium tetrahydroborate(III) reductant was prepared daily in a 1-L glass flask by dissolving 4.00 g of NaOH in 100 mL of H_2O, adding 25.00 g of $NaBH_4$ (Fluka) and filling up with water. The final solution containing 0.65 mol L^{-1} of $NaBH_4$ in 0.1 mol L^{-1} of NaOH was filtered and left for 15 min prior to use. A stock aqueous solution of L-cysteine, 0.5 mol L^{-1}, was prepared fresh daily from solid reagent (>99.5%, Fluka) and diluted as required. A silicon antifoaming agent (Rhodia Silicolapse A 430) from Rhodia Silicones Europe (Saint Fons, Lyon, France) was applied (5–20 μL per 10 mL of diluted sample). Double-distilled water (all quartz still) was used in all operations.

2.4 *Certified reference materials*

The Certified Reference Materials (CRM) 'Lyphochek® Urine Metals Control', Level 1, Lot No. 69031, Level 2, Lot 69032 and Level 1, Lot No. 69071 were obtained from Bio-Rad Laboratories (Munich, Germany).

2.5 *Sample preparation and measurement procedure*

Defrosted urine in polypropylene containers was treated for 45 min in an ultrasonic bath. A 10-mL urine aliquot and 5 mL of 0.5 mol L^{-1} L-cysteine and 2.5 mL of 1 mol L^{-1} HCl were diluted to 50 mL with double-distilled water. The blank, 2.0 μg L^{-1} As(V), and 5.0 μg L^{-1} As(V) standard solutions were prepared in the same way, except 3.00 mL of 1 mol L^{-1} HCl was added in order to exactly match the final HCl concentration in calibration standards with acid level in urine samples (already acidified with

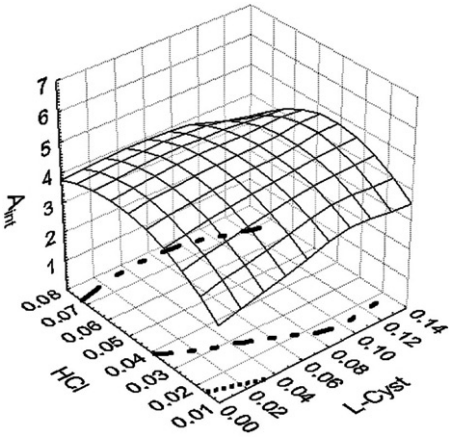

Figure 1. Integrated absorbance for 4.0 μg L^{-1} As(V) *vs.* concentrations of L-cysteine and HCl (mol L^{-1}).

HCl after sampling). Samples and standard solutions were left for 1 h at room temperature for complete pre-reduction. Ten millilitres of diluted urine sample or standard was pipetted into a polypropylene reaction vessel, followed by ca 10 μL of antifoam. The blank was measured several times until a steady signal, indicating cleanup of the sample tubing line, was obtained. Calibration was based on several-fold measurements: eightfold for blank and fourfold for calibration standards; the mean values were plotted and further used for estimation of metrological characteristics. One or two CRMs were measured randomly (twice) with every batch of samples for quality control, and the mean CRM value was plotted on control charts. Each sample was typically measured twice, supplemented by two more replicates at the end of series in case of unusually high or low signals. A 2.0 μg L^{-1} As standard ('sensitivity check') was randomly analysed between samples for correction of drift. Data were processed using MS Excel^{TM}: blank correction, concentration, SD, RSD%, slope, intercept, and r^2 for the current calibration. A calibration graph was automatically plotted and visually examined.

2.6 *Optimization procedures*

Initial simplex-type maximum searching routines were conducted for three As species (As(V), MMA and DMA) as a function of added HCl and L-cysteine. In the concentration range where As(V), MMA, and DMA gave relatively high signals, a central composition plan was conducted. Further, it was extended with several additional points for acid (0.02–0.07 mol L^{-1} HCl) and L-cysteine (0.01–0.14 mol L^{-1}) for better circumscription of the maximum (figures 1–3).

2.7 *Data treatment*

A large number of measurements ($n = 936$) were grouped in a database and statistically analysed with the help of STATISTICA 6.0 software. A distance weighted least-squares smoothed graph for the three species signals and their rate of coincidence (RC) is shown in figure 4.

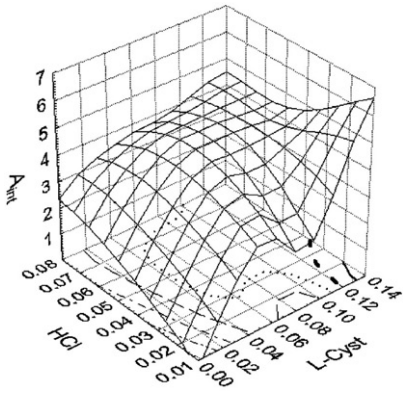

Figure 2. Integrated absorbance for $4.0\,\mu g\,L^{-1}$ MMA *vs.* concentrations of L-cysteine and HCl ($mol\,L^{-1}$).

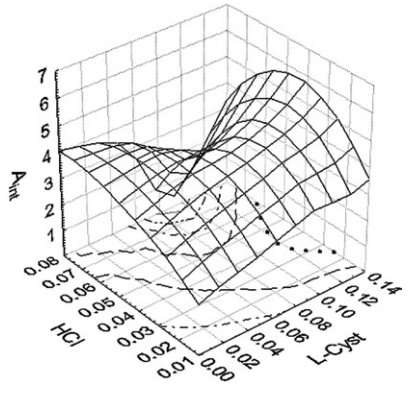

Figure 3. Integrated absorbance for $4.0\,\mu g\,L^{-1}$ DMA as function of L-cysteine and HCl concentrations ($mol\,L^{-1}$).

The RC parameter is calculated in the following steps:

(1) calculating the average value for the A_{int} for each of the three species for the current conditions, i.e. L-cysteine and HCl concentration;
(2) calculating the sum of squares of deviation of the forms from their mean at the same chemical conditions ($DEVSQ_{ij}$), where i and j are the indexing L-cysteine and HCl concentrations, respectively;
(3) finding the highest deviation ($DEVSQ_{max}$);
(4) dividing the $DEVSQ_{ij}$ values by $DEVSQ_{max}$ for normalization;
(5) expressing the $RC_{i,j}$ value as a percentage for the current chemical conditions (expressed by i,j subscripts):

$$RC_{ij} = \left(1 - \frac{DEVSQ_{ij}}{DEVSQ_{max}}\right) \times 100.$$

Results are presented in figure 4, and selected representative figures are given in table 2.

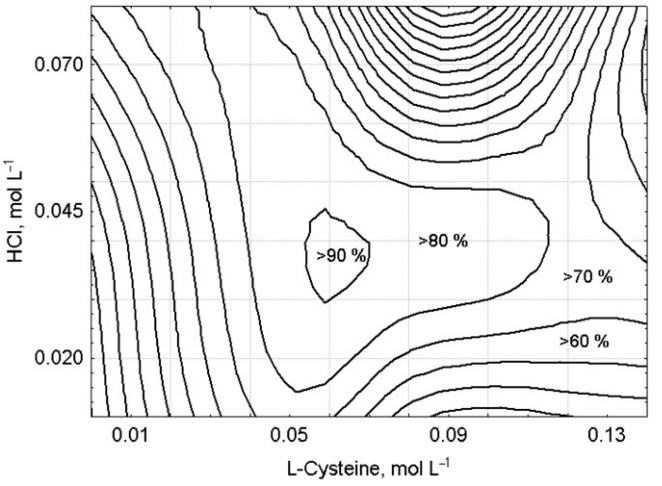

Figure 4. Contour plot of the rate of coincidence (RC%) for hydride-forming As species As(V), MMA and DMA at 4.0 μg L^{-1} As levels *vs.* concentrations of L-cysteine and HCl (mol L^{-1}).

Table 2. Rate of coincidence in % for As(V), MMA, and DMA at 4.0 μg L^{-1} levels as a function of L-cysteine and HCl concentrations.

L-Cysteine (*i*) (mol L^{-1})	HCl (*j*) (mol L^{-1})	RC_{ij}
0.01	0.045	43
0.01	0.070	72
0.01	0.020	36
0.05	0.045	99
0.05	0.045	100
0.05	0.020	97
0.05	0.070	99
0.05	0.045	98
0.09	0.020	72
0.09	0.070	0
0.09	0.045	100
0.13	0.020	72
0.13	0.045	88
0.13	0.070	96

At the optimum experimental conditions, i.e. 0.045 mol L^{-1} HCl and 0.050 mol L^{-1} L-cysteine, the experiment was repeated five more times for all species for estimation of experimental error. Data were compared by *F* and *t*-tests (see table 3). Although the *t*-test gave differences at $\alpha = 0.05$, the rate of coincidence of signals is sufficiently high (>90%). The response plot graph for As(V) resembles a quadratic function; hence, a quadratic data fit was applied.

2.8 *Quality control*

The accuracy and the absence of long-term drift in measurements were tested by randomly analysing three urine CRMs: Bio-Rad® 1, Lot 69031, Bio-Rad® 2-Lot 69032 and Bio-Rad® 2, Lot 69071 with As levels 50 (40–60), 51 (41–62),

Table 3. *t*-test and *F*-test applied to As(V), MMA, and DMA at 4.0 μg L^{-1} levels at the optimum conditions.

	SD (1)	SD (2)	*F*-ratio	*P*	Mean A_{int} (1)	Mean A_{int} (2)	*t*-value	df	*p*
As(V) *vs.* MMA	0.15	0.09	2.7	0.30	4.47	4.27	2.9	10	0.02
As(V) *vs.* DMA	0.15	0.13	1.2	0.82	4.47	4.20	3.3	10	0.01
MMA *vs.* DMA	0.09	0.13	2.2	0.41	4.27	4.20	1.0	10	0.33

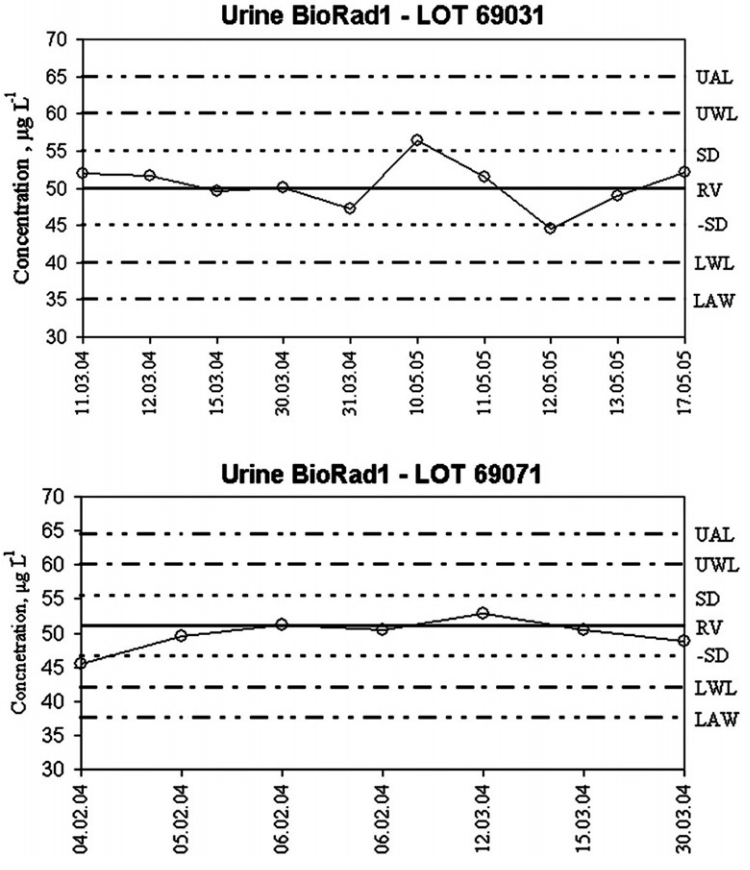

Figure 5. Examples of control charts demonstrating accuracy and long-term stability of measurements. RV, reference value; SD, standard deviation of the certified value; LWL and UWL, lower and upper warning level, respectively; LAL and UAL, lower and upper action level, respectively.

and 154 (123–185) μg L^{-1} As, respectively. These CRMs have been shown previously to contain predominantly hydride-forming arsenic [21], so the certified and experimental values should agree well. The arsenic levels found in within-day analyses of Lots 69031 and 69032 (mean ± confidence interval) were 52.1 ± 10.3 μg L^{-1} As ($n = 5$, RSD 8.1%, passing *t*-test, $t_{exp} < t_{tab}$ $1.0 < 2.45$ and 152.9 ± 18.1 μg L^{-1} As ($n = 5$, RSD 4.1%, $t_{exp} < t_{tab}$ $0.30 < 2.92$), respectively. Examples of control charts plotted within 2 months of measurements for CRMs Lots 69031 and 69071 are shown in figure 5 with SDs taken from CRM certificates, demonstrating good statistical control.

3. Results and discussion

3.1 *Analytical aspects of assays*

Optimization of chemical parameters for HG resulted in the best sensitivity levelling for all four examined species in 0.050 mol L^{-1} L-cysteine and 0.045–0.06 mol L^{-1} HCl reaction medium. With undigested urines, the method of standard addition calibration cannot be applied because the exact composition of various As species in different urine samples is unknown. Individual spike experiments on three different urines (two men and one woman) with three different arsenic species, MMA, DMA, and As(V), at a 2.0 μg L^{-1} As level resulted in recoveries between 2.1 and 2.4 μg L^{-1} for all species against the As(V) standard. Considering biological variability and within-day individual As variations, this bias could be neglected. Peaks were asymmetric with a longer right tail towards the end of integration time. Completeness of pre-reduction with L-cysteine was tested with As(V), MMA, and DMA for 0, 60, and 180 min under the optimal conditions, and no statistical difference was observed between the 1-h and 3-h duration of pre-reduction. No visible drift (above 1 SD) could be revealed over a 6-h measurement run.

Sample stability studies performed with 10 urines before starting the campaign of actual sample collection had indicated that defrosted urines gave lower results for supernatant *versus* mixed sample by −6.8% (median) (range −1.5 to −17.5%), so all defrosted urines were gently mixed before taking a 10-mL aliquot for dilution and subsequent analysis (without vigorous shaking to avoid foaming).

The use of a silicon antifoaming agent is essential for reliable foam control. The reagent used, Rhodia Silicolapse A 430, has proven to be very efficient when applied between 5 and 20 μL per 10 mL of fivefold-diluted urine, so 10 μL was used in all further work. The remarkable efficiency of foam suppression could be explained by the puncture effect of the hydrophobized SiO_2 microparticles suspended in poly(dimethylsiloxane) resulting in rupture of thin foam films and subsidence of foam.

The LOD for toxicologically relevant As was between 0.2 and 1.2 μg L^{-1} during optimization experiments for aqueous solutions and around 0.25 μg L^{-1} in 10 mL of (1 + 4) diluted urine samples. Blank values were between 0.1 and 0.3 μg L^{-1}. The RSD at 1.0 μg L^{-1} As was typically 1.5–1.8% for aqueous solutions and between 2 and 6% for urines.

3.2 *Statistical considerations of results*

Tests for variations between years 2004 and 2005 were initially conducted by means of Friedman ANOVA and Levene statistics (table 4). These non-parametric tests rely on fewer assumptions and do not require normal distributions of variables or homogeneity of variances, while an ANOVA test could be affected if the variances (SD^2) of the groups to be compared are substantially different. The Levene test checks this assumption. The results of these statistical tests (table 4) allow further application of ANOVA and *t*-tests, as presented in table 5, despite the non-compliance with normality assumption [28].

The results for As concentration in urine and statistical characteristics for different groups are compiled in table 6. There is no statistical difference in mean values between case, control, men and women individuals. Other statistical parameters are also comparable, except for distribution characteristics. An average SD of 0.4 for the set of paired measurements is obtained. This value is negligible compared to the average SD for

Table 4. Non-parametric statistics comparing multiple dependent samples.

Friedman ANOVA	Year	Average rank	Sum of ranks	Mean ($\mu g\,L^{-1}$)	SD ($\mu g\,L^{-1}$)	n	df	χ^2	p
	2004	1.5	287	3.6	2.9	187	1	0.91	0.34
	2005	1.5	274	3.5	2.7				
Levene statistics							386	F	p
								0.005	0.941

Table 5. Comparison of multiple dependent variables for urine samples.

n 2004	n 2005	SD 2004	SD 2005	F	p	Mean 2004 ($\mu g\,L^{-1}$)	Mean 2005 ($\mu g\,L^{-1}$)	t-value	df	p
200	188	2.84	2.74	1.07	0.62	3.6	3.5	0.35	386	0.73

Table 6. As concentration in urine ($\mu g\,L^{-1}$) with statistical characteristics of groups.

Group	n	Min.	Max.	Mean	SD	Median	25th	75th	Skeweness	Kurtosis
2004										
Total	200	0.5	18.2	3.6	2.8	3.0	1.7	4.6	2.1	6.3
Men	97	0.5	15.8	3.5	2.6	3.1	1.7	4.0	2.0	5.4
Women	103	0.5	18.2	3.7	3.1	3.0	1.7	4.8	2.2	6.5
Women controls	52	0.5	14.2	3.4	2.7	2.5	1.8	4.8	2.0	5.1
Men controls	46	0.5	15.8	3.5	2.7	3.0	1.8	4.0	2.5	9.0
All controls	98	0.5	15.8	3.5	2.7	2.7	1.8	4.4	2.2	6.6
All cases	102	0.5	18.2	3.7	3.0	3.2	1.6	4.8	2.1	6.2
Women cases	51	0.5	18.2	3.9	3.4	3.6	1.6	4.8	2.3	6.8
Men cases	51	0.7	11.6	3.5	2.5	3.1	1.6	4.9	1.3	1.4
2005										
Total	188	0.5	15.8	3.5	2.7	2.8	1.6	4.6	1.8	4.2
Men	90	0.6	14.5	3.5	2.7	2.8	1.7	4.3	1.9	4.4
Women	98	0.5	15.8	3.5	2.8	2.9	1.6	4.7	1.8	4.2
Women controls	51	0.5	15.8	3.6	3.2	2.4	1.6	4.8	1.9	4.3
Men controls	45	0.8	9.3	3.3	2.0	2.9	1.9	4.1	1.2	1.1
All controls	96	0.5	15.8	3.5	2.7	2.9	1.7	4.7	1.9	5.2
All cases	92	0.5	14.5	3.5	2.8	2.8	1.5	4.5	1.8	3.6
Women cases	47	0.5	11.7	3.3	2.4	3.1	1.5	4.6	1.4	2.5
Men cases	45	0.6	14.5	3.7	3.2	2.6	1.4	4.4	1.8	3.2
Average 2004–2005										
Total	201	0.5	11.0	3.5	2.1	3.0	2.0	4.5	1.2	1.3
Men	98	1.0	11.0	3.5	2.0	3.0	2.1	4.2	1.6	2.8
Women	103	0.5	10.5	3.6	2.2	3.4	1.8	4.6	0.9	0.4
Women controls	52	0.9	9.5	3.6	2.3	2.5	1.8	5.0	0.9	0
Men controls	47	1.0	10.4	3.5	1.9	3.0	2.3	4.0	1.9	4.3
All controls	99	0.9	10.4	3.5	2.1	3.0	2.0	4.4	1.3	1.3
All cases	102	0.5	11.0	3.6	2.2	3.2	2.1	4.5	1.2	1.4
Women cases	51	0.5	10.5	3.6	2.2	3.5	2.1	4.5	0.9	1.1
Men cases	51	1.3	11.0	3.6	2.2	3.0	2.1	4.8	1.5	2.1

the mean of all samples within groups and year of sampling, thus indicating that differences between individuals stem from their individual As concentration levels, rather than from measurement uncertainty: cf. $F_{tab}\,(1.2) < F_{exp}\,(7.0)$ for duplicate measurements ($n = 393$, SD = 0.4) *versus* individuals variation (2004–2005) ($n = 200$, SD = 2.8).

The scatter plot (not shown) and further robust statistics plot (figure 6) does not reveal a significant correlation (within <3% chance of error) between As concentrations in urine

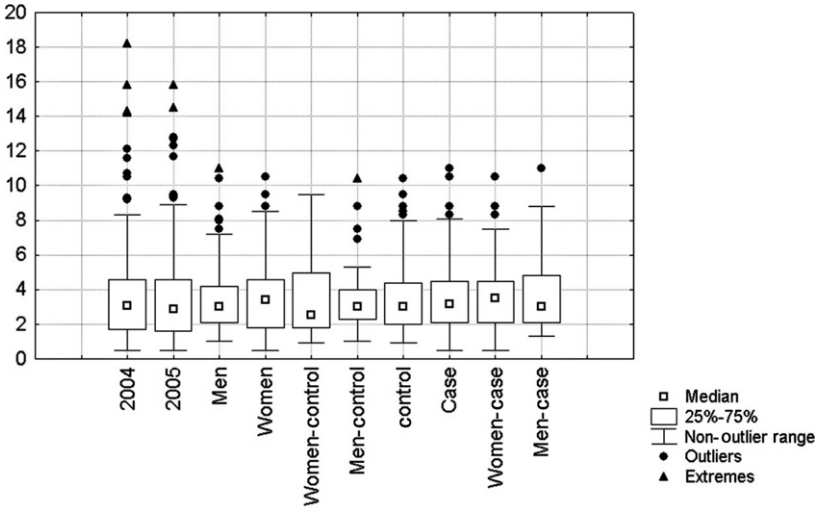

Figure 6. Robust statistics: arsenic concentration ($\mu g\,L^{-1}$) vs. affiliation to different groups (boxes are interquartile ranges, 25–75% of results).

samples from the same individual in two successive years 2005 *versus* 2004 ($n = 201$, $r^2 = 0.03$, $p = 0.03$). Only in a few pairs of results (three cases and three controls) could a correlation be seen, viz. Man Case 155; Man Case 137; Woman Case 194; Woman Control 9, Woman Control 71; Woman Control 163. Since sex is not a factor for As concentration (see ANOVA results), it could be accepted that these six correlations between years are occasional and cannot be attributed to the parental health status.

The results for the As concentration from the factorial ANOVA with two factors on two levels and their mixed effect are summarized in table 7. The experimental p-values suggest a rejection of the hypothesis for a correlation between As contents and sex or health status.

On the basis of smoking habits, all examined individuals ($n = 201$) could be split into two groups: non-smokers ($n = 127$), mean 3.3 ± 2.1 (SD), range 0.6–11.0, median 2.8, interquartile range (25–75%) 1.8–4.3 $\mu g\,L^{-1}$ As and smokers ($n = 74$), mean 3.9 ± 2.1 (SD), range 0.5–8.8, median 3.6, interquartile range (25–75%) 2.4–5.0 $\mu g\,L^{-1}$ As. No statistical difference could be revealed between these two groups: F-test ($p = 0.75$) and t-test ($p = 0.07$).

Median values for the sum of inorganic As (III + V), MMA and DMA in urine from controls (3.0 $\mu g\,L^{-1}$) and cases (3.2 $\mu g\,L^{-1}$) could be compared with recent data for non-exposed populations from the Czech Republic, 3.5 µg (per gram of creatinine) [27]; four regions from Hungary with 12, 16, 32, and 11 $\mu g\,L^{-1}$ As, respectively; two regions from Romania with 2.1 and 2.1 $\mu g\,L^{-1}$; and two regions from Slovak Republic with 4.2 and 5.0 $\mu g\,L^{-1}$ [29].

4. Conclusions

No excessive internal exposure to arsenic could be revealed in the offspring of BEN-affected parents, known as a population at high inheritance risk, *versus* control

Table 7. Results from factorial ANOVA (two factors at two levels).

Factor	Factor MS[a]	Error MS	F	p
2004				
Case-control	2.89	8.17	0.35	0.55
Sex	1.45	8.17	0.18	0.67
Cross-combination	2.61	8.17	0.32	0.57
2005				
Case-control	0.19	7.61	0.02	0.87
Sex	0.00	7.61	0.00	0.99
Cross-combination	6.49	7.61	0.85	0.36
Average				
Case-control	0.32	4.58	0.07	0.79
Sex	0.13	4.58	0.03	0.87
Cross-combination	0.05	4.58	0.01	0.92

[a]MS: mean square, within-group SD.

group in a two-year study of urinary arsenic. The levels of hydride forming arsenic fraction with known toxicological relevance (inorganic As(III + V) + MMA + DMA) are in the low $\mu g\,L^{-1}$ concentration range and are comparable to the levels found in other neighbouring countries. There is no statistical correlation, nor a cross-correlation between two successive years of sampling, sex, and case-control studies. Additional instruction of volunteers to exclude seafood and related products of aquatic origin from their diet for 10 days before sampling insured a better reliability of results, since a possible contribution of DMA, MMA, and other hydride-forming metabolites was greatly minimized. The analytical methodology for batch-type hydride generation atomic absorption spectrometry has been refined and thoroughly validated by elaborating the chemical vapour generation conditions, low dilution factors, efficient foam control owing to dispersed microparticles of silanized SiO_2 in poly(dimethylsiloxane) antifoaming agent, prolonged reaction and integration times, and integrated absorbance measurements.

Acknowledgements

This research was supported by NIH Research Grant R01 TW006192 funded by the Fogarty International Center and the National Institute of Environmental Health Sciences, National Institutes of Health, USA, awarded to the National Centre for Public Health Protection, Sofia, Bulgaria.

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