ELSEVIER

Journal of Chromatography B, 740 (2000) 179–186

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

On-line reversed-phase liquid chromatography hydride generation emission spectrometry: speciation of arsenic in urine of patients intravenously treated with As_2O_3

B. Do[a], P. Alet[a], D. Pradeau[a], J. Poupon[b], M. Guilley–Gaillot[b], F. Guyon[a,*]

[a]*Laboratoire Central d'Analyses, Pharmacie Centrale des Hôpitaux de Paris, 7 Rue du Fer à Moulin, 75221 Paris Cedex 03, France*
[b]*Laboratoire de Biochimie Toxicologie, Hôpital Fernand Widal, 200 rue du Faubourg Saint-Denis, 75010 Paris, Cedex, France*

Received 5 October 1999; received in revised form 20 December 1999; accepted 25 January 2000

Abstract

Hydride generation inductively coupled plasma–atomic emission spectrometry (HG ICP–AES) was used as a continuous detection system for the determination of arsenic in the eluate from a high-performance liquid chromatographic (HPLC) system. Four arsenic species [arsenite As(III), arsenate As(V), monomethylarsonate (MMA), and dimethylarsinate (DMA)] present in the urine samples of patients treated intravenously with arsenite, were analyzed separately by HPLC–HG-ICP–AES using a non-polar C_{18} column. This analytical method allowed the sensitive determination of the arsenic species in the submicrogram per liter range. Urine samples collected on different days after arsenite administration were found to contain arsenite predominantly – monomethylarsonate and dimethylarsinate were also detected.

Keywords: Urine; HPLC; Hydride generation; ICP-AES; Arsenic

1. Introduction

Arsenic administration in myelogenia leukaemia treatment was first described by Lissauer in 1865. Recently, a chinese team has shown the efficiency of arsenite in promyelogenia leukaemia [1]. Another team has confirmed arsenite can be used as an alternative to *trans*-retinoic acid or stump cell transplant resistance towards this pathology [2]. At present, a clinical study, conducted by St. Louis Hospital (Paris, France) one of our collaborators, evaluates the efficiency of and the tolerance to intravenous arsenite in voluntary patients suffering from chronic myelogenia leukaemia.

After administration, arsenite was metabolized and distributed in the human body and both the parent compound and its metabolites were mainly excreted into the urine [3]. The toxic actions of arsenical compounds are highly dependent on their valence states and chemical forms. Indeed, the median lethal dose (LD_{50}) indicates that inorganic forms are more toxic than metabolism products in mammals [4]. Therefore, it is essential to identify and quantify individual chemical forms of arsenic so as to assess health risks and more particularly in our case, the tolerance of the treatment associated with intravenous arsenic.

*Corresponding author.

0378-4347/00/$ – see front matter
PII: S0378-4347(00)00047-5

Several analytical methods were employed in arsenic speciation either in urine or in other matrixes [5–11]. These methods are based on coupling liquid chromatographic (LC) separation with atomic spectrometry [12] or mass detection [13–19]. Gas chromatography coupled with atomic absorption spectrometry (GC–AAS) or with inductively coupled plasma atomic spectrometry (GC–ICP–AES) have been reported [20], but previous derivatization of arsenic compounds was necessary due to their low volatility.

Furthermore, arsenic species separation were successfully improved by LC without any previous treatment. The most suitable methods are anion-exchange or ion-pair reversed-phase LC coupled with elemental detection modes [12–19]. Other detection techniques utilised in arsenic analysis were UV and amperometrics [21–23].

In the present study, in order to measure and follow arsenical species concentrations according to time in urine of patients suffering from myelogenia leukaemia, a sensitive and specific analytical technique permitting the simultaneous quantification of As(III), As(V), MMA and DMA was developed. The method consisted of the coupling of ion-pair reversed-phase LC, with tetrabutylammonium (TBA) phosphate as an ion-pair reagent, with HG ICP–AES. Concerning the sensitivity, the detection mode used is not competitive with ICP–MS [19], but offers very sufficient performance to carry out arsenic analysis in urine samples. Therefore, new and dedicated equipment was not required for our laboratory. Moreover, ICP–MS detection requires low salt concentrations levels, which could yield analysis of critical urinary matrices without dilution.

The sensitivity of the graphite furnace atomic absorption detection (GFAAS) is higher than the technique described previously [24] and has been employed as a detector for arsenic analysis, but its use in continuous monitoring is not possible.

2. Experimental

2.1. Instrumentation

The speciation of arsenic described here was performed with the combination shown in Fig. 1.

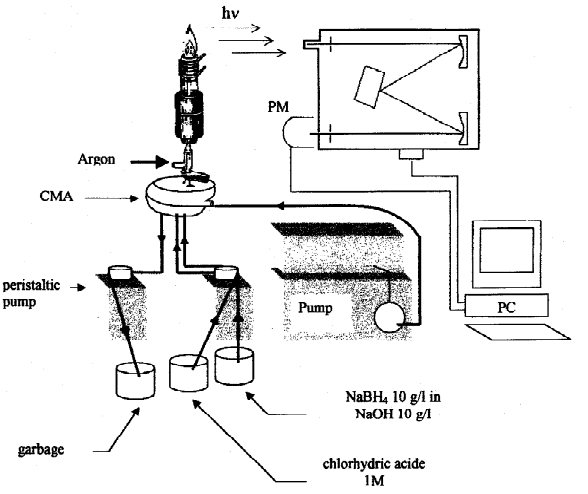

Fig. 1. Principe of the combination HPLC–HG ICP–AES.

HPLC separation was followed by hydride generation and ICP–AES detection. Effluent from the HPLC column entered a HG system (CMA: concomitant mercury analysis), two continuous flows of hydrochloric acid and sodium borohydride introduced by using a peristaltic pump.

A commercial JY 38S sequential analyser for the detection of arsenic was used. It was equipped with an inductived plasma torch, a model nebulizer Meinhard and a JYS professional computer (all from Jobin Yvon, Longjumeau, France). Detailed descriptions of the instrument system and conditions are given in Table 1.

The HPLC system consisted of a Gilson HPLC pump (model 302) with a 5 ml min^{-1} stainless steel pump head, a module sample processor and a rheodyne 6-port sample injector with a 100 μl sample loop, and a HPLC column. A reversed-phase C_{18} column (250×4.6 mm, 5 μm particles, Macheray-Nagel, Hoerdt, France), linked to a C_{18} guard column (15 mm×4.6 mm I.D., Interchim, Montluçon, France) was used for the separation. The connection between the column cartridge and the nebulizer was made by a capillary tube 0.125 μm I.D. This system did not contribute much to post-column dispersion and the loss of numbers of theoretical plates. Isocratic HPLC operation was performed at 1 ml min^{-1} flow-rate.

A HG system (CMA), a prototype from Jobin Yvon, was connected directly to the plasma torch

Table 1
Experimental conditions for ICP–AES[a]

Argon flow-rates/l min^{-1}	
Plasma gas[b]	13
Auxilliary gas[b]	0
Sheathing gas[b]	0.25
Nebulizer gas[b]	0.4
Sample flow-rate	1 ml min^{-1}
Forward power	1200 W
Studied wavelength	188.9 nm
Chromatic splits	
Entry	20 μm
Exit	100 μm

[a] The data acquisition was realized with a specific software TSP (Jobin Yvon). The peaks integration were achieved by area measurement.

[b] These parameters were adjusted daily to optimize the ion signal.

(Fig. 2). This is a cyclonic chamber without gas/liquid separator. The device lets both hydrides and non-hydrides reach the inductived plasma.

2.2. Reagents

Sodium arsenite and sodium arsenate were purchased from Fluka Chemica (Steinheim, Sweden). Sodium dimethylarsinate, sodium borohydride and phosphate tetrabutylammonium were obtained from Sigma Aldrich (St Louis, MO, USA). Sodium monomethylarsonate was synthesized by Seratec (Epinay, France). Sodium hydroxide was from Prolabo (Fontenay sous Bois, France) and hydrochloric acid from Labosi (Elancourt, France). Disodium hydrogen phosphate was purchased from Merck (Nogent sur Marne, France) and acetonitrile (HPLC grade) from Carlo Erba (Val de Reuil, France).

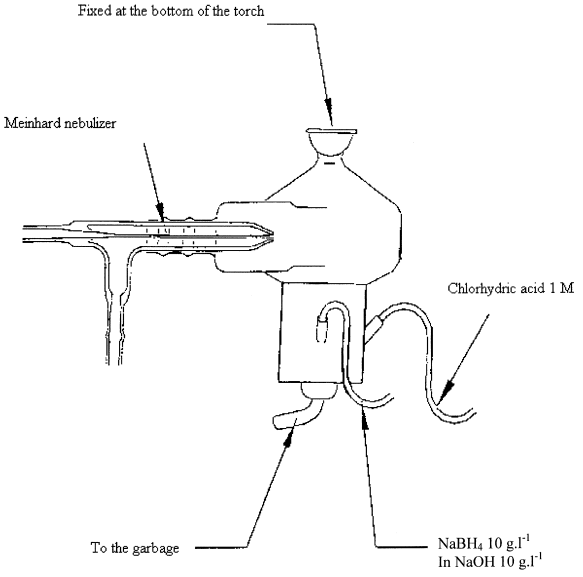

Fig. 2. Concomitant Mercury Analyzer system (CMA), a prototype from Jobin Yvon (France).

Deionized water from an ultrapure water system Milli Q (Molsheim, France) was used for the preparation and the dilutions of all reagents, samples and calibrators.

Stock solutions of arsenic compounds (1000 μg l^{-1}) were prepared by dissolving appropriate amounts and were stored in polypropylene flasks. The standard solutions were freshly prepared by dilution of the stock solutions before use.

Buffer solution resulted from a mixture of TBA (4 mmol l^{-1}) and Na_2HPO_4 (2 mmol l^{-1}). The pH of the solution was 7.1. The mobile phase was a binary mixture containing 1:100 acetonitrile–buffer (v/v). It was filtered through a 0.45 μm membrane filter Millipore (Molsheim, France), degassed and sonicated before use.

HCl for HG was diluted to a concentration of 1 mol l^{-1} and 1% $NaBH_4$ solution was prepared by dissolving $NaBH_4$ in 1% NaOH solution.

2.3. Samples preparation

Urine samples from patients were directly injected into the chromatographic system without previous dilution. Nonetheless, they were first centrifuged within 15 min at 1200 *g* and drawn through the 0.45 μm filter Millipore in order to exclude calcium oxalate crystals and other waste precipitates. The filtered samples were collected in polypropylene bottles and stored at 4°C [25].

3. Results and discussion

3.1. Performance of the HPLC system

Several experiments at different pH values, demonstrated that the chromatographic resolution of the four compounds were dependent on their apparent

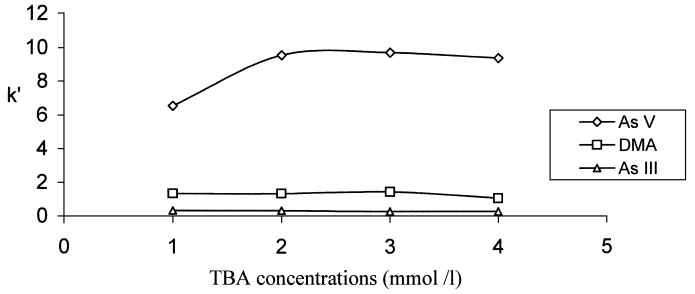

Fig. 3. Influence of TBA concentrations on the k' of the arsenical compounds (pH 7; Na_2HPO_4 2 mmol l^{-1}).

charge (AC) resulting from their pK_a. In acidic environment (pH 4), both As(III) and DMA (AC: 0 and 0, respectively) can not form ion-pairs with TBA and are co-eluted in the dead volume of the column cartridge. While As(V) and part of MMA (AC: −1 and −0.5, respectively) are converted into neutral complexes, they are eluted according to the hydrophobic interactions between the complexes and the stationary phase. Under neutral conditions (pH 7), all of the above arsenic compounds are anions except As(III). The ionized molecules As(V), MMA and DMA (AC: −1.5 and −0.7 and −1, respectively) can combine with TBA. Their resulting degrees of hydrophobia account for their difference in retention times on this packing material. However, in spite of a good separation of the four arsenic forms at pH 7, a 1:100 acetonitrile ratio had to be added to the mobile phase to enhance its elution power towards As(V) as that retention time exceeded 25 min. A gradient elution mode with increasing acetonitrile concentration was not undertaken due to the baseline drift and the delay necessary to obtain an equilibrium state between two injections. Moreover, the detection mode used can not tolerate much more organic solvent rate without disturbing the plasma stability. In basic environment (pH>8), the AC (−2) of As(V) is increased and its retention time is situated beyond 25 min so that was unapplicable for routine analysis.

Furthermore, the capacity factors (k') of the arsenic were dependent on TBA concentration levels and mobile phase ionic force (Figs. 3 and 4). The ion-pair reagent effect on k' was mainly established for the methylated metabolites and especially for As(V). Indeed, the ion-pair formation stability was dependent on the AC of the compounds. In this case, the non-charged As(III), because of the neutral environment, seemed to be unaffected by TBA rate variations. At pH 7, the eluent solution ionic-force could be modified by Na_2HPO_4 and H_3PO_4 addition, increasing salts level. Fig. 4 shows a relative slight influence of the solution ionic-force on the k' values of As(III) and methyled metabolites. As for the k' value of As(V), its significant decrease according to

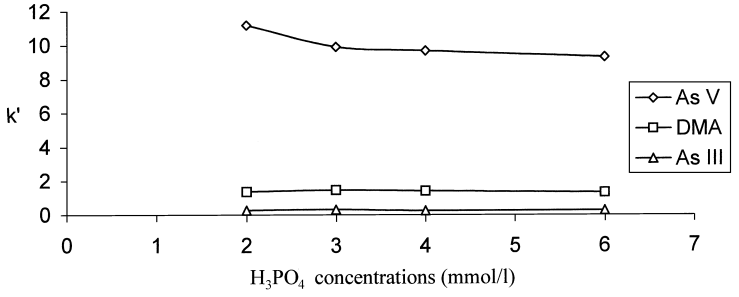

Fig. 4. Influence of the mobile phase ionic force on the k' of the arsenic compounds (pH 7; TBA 4 mol l^{-1}).

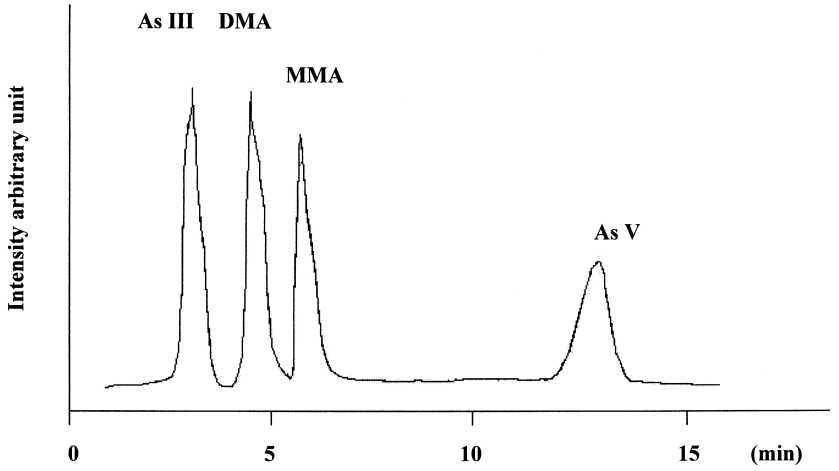

Fig. 5. Chromatogram obtained with 450 ng ml^{-1} of As III, DMA, MMA and As V in spiked urine.

this parameter was interesting in order to accelerate the run time analysis. Also, the most effective separation (Fig. 5) of the four analytes taking time analysis into account, was obtained with a compromise between these different parameters. The work was then undertaken with a mobile phase containing 4 mmol l^{-1} TBA, 2 mmol l^{-1} Na_2HPO_4 and a 1% acetonitrile concentration. Consequently, the observed retention times were at 3.10±0.05 min, 4.70±0.05 min, 5.3±0.05 min and 14.80±0.10 min, for As(III), DMA, MMA and As(V) respectively.

3.2. Detection parameters optimization

3.2.1. Hydride generation

As shown in Fig. 2, hydride generation reactions occurred inside the CMA chamber. It is a cyclonic reaction cell permitting efficient mobilization of arsenic, because it also allows volatile species as well as the less volatiles to enter the plasma. However, without a gas–liquid separator, matrix elements could not be completely eliminated. This could be implicated in matrix interferences. In spite of this drawback, Fig. 6 shows that the signal responses of arsenic with HG were at least ten times more intense compared to the ones obtained without HG for the four compounds, taking the matrix effect into account. Moreover, spectral interferences were minimized by analyzing at a sensitive and specific emission wavelength 188.9 nm. In this way no memory effect was noted with analysis of 100 ng arsenic, which corresponds to our highest working level.

The most efficient recovery of HG for the arsenic compounds was obtained with a pH situated between 1 and 2, as was demonstrated by Braman [26], and with $NaBH_4$ rate between 1% and 4%. However, hydride generation produces systematically and quantitatively hydrogen molecules, which can be a disruptive element for plasma stability. This phenomena was observed with a $NaBH_4$ rate over 1%. After several experiments, we noted that 1% $NaBH_4$ rate and analysis at pH 1 allowed the highest signal-to-noise ratio.

Under these conditions, the limits of quantification which were evaluated as the minimum concentration that can be measured routinely with acceptable precision (C.V. less than 15%), were found to be 36±6 ng ml^{-1} for As(III), 92±16 ng ml^{-1} for DMA, 57±9 ng ml^{-1} for MMA and 101±14 ng ml^{-1} for As(V). These correspond to 3.6, 0.9, 0.6 and 10.0 ng of arsenic, respectively, for a 100 μl sample injected for analysis. These quantification limits (LQ) are 50 times better than those obtained by using HPLC–ICP–MS techniques [13–19] and are sufficient to achieve analysis of the arsenic compounds in urine of our patients.

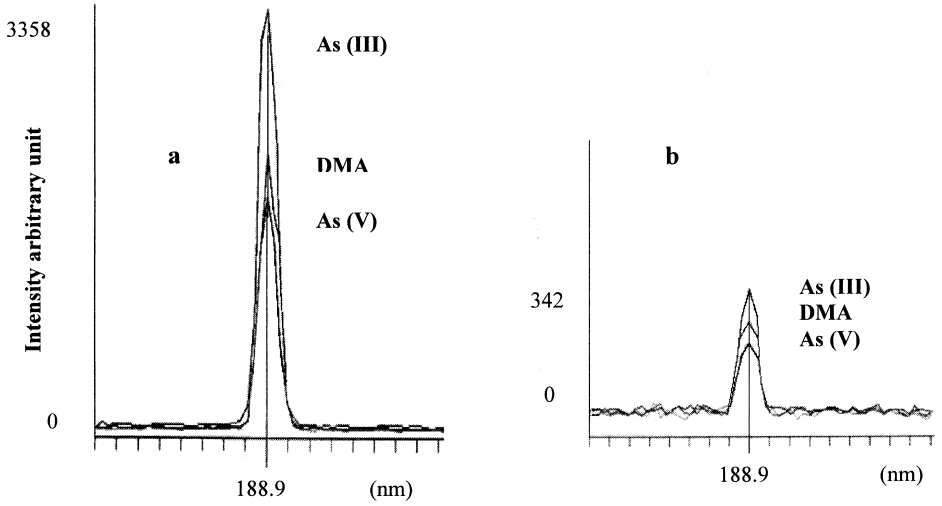

Fig. 6. Emission signal records at 188.9 nm of As(III), DMA and As(V) (50 ng ml^{-1}) (a) with HG, (b) without HG.

3.2.2. As(V) signal enhancement by L-cysteine additional

The As(V) detection signals were less sensitive than the others at the same concentration levels. It was established that the valence state could influence the speed of reduction to the hydride [27]. Indeed, the reduction speed to hydride for a higher oxidation state such as As(V) is slower than for As(III). Because it has a lower oxidation state, As(III) can react easier and faster to generate arsine with $NaBH_4$, it was thus relevant to pre-reduce As(V) to As(III). L-cysteine can act as a pre-reduction reagent for As(V) to As(III), by generating a more potent reducing agent than tetrahydroborate, resulting from the reaction between the tetrahydroborate and the sulfhydryl group of L-cysteine [28].

3.3. Calibration curves and precision

Linearity was studied in the range 5–100 ng (50–1000 ng ml^{-1}) urine corresponding to the ranges observed in clinical samples. Calibration curves were

Table 2
Calibration and precision of the HPLC assay using urine samples ($n=6$)

Spiked concentration (ng ml^{-1})	As III		MMA		DMA		As V	
	Measured concentration (mean±SD)	C.V. (%)	Measured concentration (mean±SD)	C.V. (%)	Measured concentration (mean±SD)	C.V. (%)	Measured concentration (mean±SD)	C.V. (%)
50	53.96±3.44	6.4	48.19±5.47	11.4	47.11±7.33	16.0	44.37±8.32	19.1
100	105.81±7.81	7.4	97.08±5.93	6.1	109.25±9.89	9.1	100.52±6.83	6.7
200	217.12±10.52	4.8	199.26±6.33	3.2	197.40±10.40	5.3	192.29±15.82	8.2
500	515.13±22.46	4.4	492.03±30.42	6.2	469.86±13.38	2.8	479.57±20.34	5.9
1000	990.43±17.54	0.2	997.99±18.44	0.2	992.07±22.43	2.3	1009.09±57.62	5.7
Batch standards ($n=10$)								
200	215.17±9.83	4.5	198.86±6.21	3.1	198.96±10.39	5.2	194.25±11.04	5.6
500	511.29±19.01	3.7	495.74±16.45	3.3	473.11±12.02	2.5	489.12±20.91	4.2
Calibration graphs	$y=7.022x-2.743$		$y=7.016x-1.577$		$y=6.855x-1.201$		$y=7.152x-2.269$	
Correlation coefficients r^2	0.9995		0.9997		0.9989		0.9993	

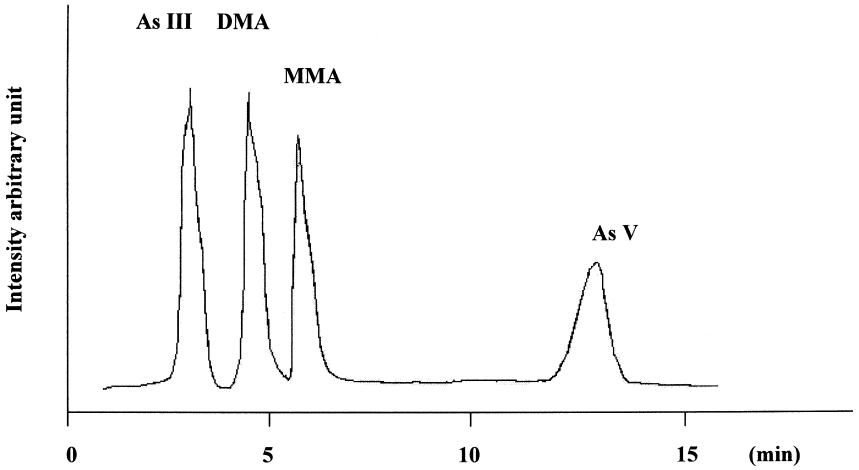

Fig. 7. Chromatogram obtained with 450 ng ml^{-1} of As III, DMA, MMA and As V in spiked urine.

obtained by weighted least-squares linear regression analysis of the peak-area of the four arsenic species versus their concentrations. A statistical test of linearity was performed for each curve separately using an unweighted analysis of variance (ANOVA).

Analysis of all calibration series showed an excellent linearity. The mean correlation coefficients were given in Table 2.

The precision (expressed by the coefficient of variation of replicate analysis) of the method was evaluated over a concentration range of 50–1000 ng ml^{-1} arsenic species in urine. The intra-assay reproducibility was determined to analyze ten specimens of spiked urine on the same day. The inter-assay reproducibility was obtained by analyzing one specimen of a spiked urine sample on various days using a separate calibration each day. The data shown in Table 2 demonstrate the good precision of the method over the concentration range investigated.

3.4. Clinical application

The analytical method was applied to a clinical study with arsenic trioxide. Fig. 7 shows a repre-

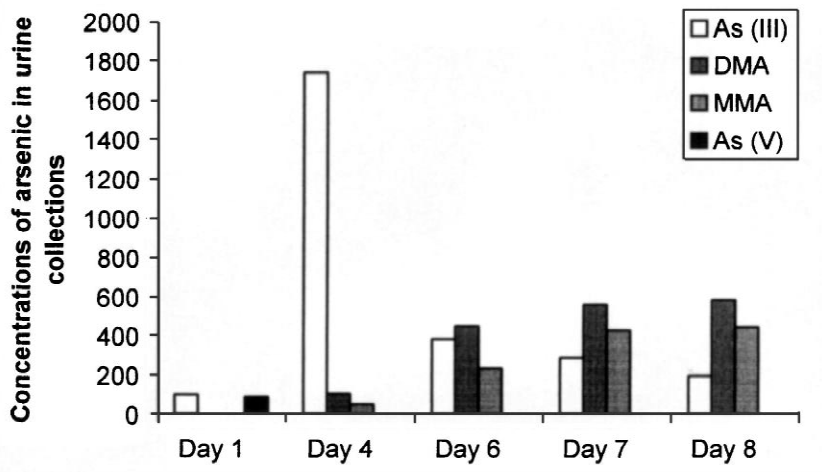

Fig. 8. Concentrations of various forms of arsenic in human urine following intravenous administration of 1 mg kg^{-1} As_2O_3.

sentative chromatogram from this study. It was obtained after 6 days after 1 mg kg^{-1} (body) intravenous As_2O_3 administration to patients. The measured concentrations obtained were 385 ng ml^{-1}, 447 ng ml^{-1} and 233 ng ml^{-1} for As(III), MMA and DMA respectively. The following concentrations of the four species in urine according to time for the same patient, are represented in the histogram of Fig. 8. In the present case, over the fourth day after arsenic intravenous administration, the As(V) species was no longer detectable, due either to the lack of this metabolite in urine or to the limit of sensitivity of our method.

4. Conclusion

ICP–AES hydride generation is a detection mode with its sensitivity placed between ICP–MS and AAS flame. We have demonstrated the reliability of a rapid and direct method to speciate individual arsenic species. Although the chosen detection mode in this study can not compete with ICP–MS, its performance was widely sufficient to bring our urinary analysis to a successful conclusion without further investments for our laboratory.

Further studies are required in other matrixes as serum and whole blood order to give more clues to determine both efficacy and tolerance of the treatment, according to the chemical forms, and to explain any possible correlations of pharmacokinetics–pharmacodynamics.

References

[1] Z.Q. Shen, G.Q. Chen, J.H. Ni, Blood 89 (1997) 3354–3360.
[2] S.L. Soignet, P. Maslak, G.Z. Gang, S. Jhanwar, N. Engl. J. Med. 339 (1998) 1341–1348.
[3] S. Mann, P.O. Dros, Toxicol. Appl. Pharmacol. 140 (1996) 471–486.
[4] H. Yamauchi, B.A. Fowler, Part 2: Human health and ecosystem effects, in: J.O. Nriager (Ed.), Arsenic in the environment, Vol. 3, John Wiley and sons, New York, 1991, pp. 35–53.
[5] M.A. Lovel, J.G. Farmer, Human Toxicol. 4 (1985) 203–214.
[6] J.P. Buchet, R. Loweris, H. Roels, Int. Arch. Occup. Environ. Health 48 (1981) 111–118.
[7] J.P. Buchet, R. Loweris, H. Roels, Int. Arch. Occup. Environ. Health 48 (1981) 71–79.
[8] E.A. Crecelius, J. Yager, Environ. Health. Perspec. 105 (1997) 650–653.
[9] V. Foa, A. Colombi, Sci. Tot. Env. 34 (1984) 241–259.
[10] M.O. Andreae, Anal. Chem. 49 (1977) 820–823.
[11] Z. Zhang, R. Cornelis, L. Mees, R. Vanholder, N. Lameire, Analyst 123 (1998) 13–17.
[12] I. Martin, M.A. Lopez-Gonzalvez, M. Gomez, M. Camara, M.A. Palacios, J. Chromatogr. B. 666 (1995) 101–109.
[13] X.C. Le, M. Ma, J. Chromatogr. A. 764 (1997) 55–64.
[14] K.E. Lafreniere, V. A Fassel, D.E. Eckels, Anal. Chem. 59 (1987) 879–887.
[15] C. Casiot, O.F.X. Donard, M. Potin-Gautier, Spectra Analyse 206 (1999) 17–27.
[16] J. Mattusch, R. Weinnrich, Anal. Chem. 70 (1998) 3649–3655.
[17] J. Lintschinger, P. Schramel, A. Hatalak-Rauscher, J. Anal. Chem. 362 (1998) 313–318.
[18] S.C. Shum, R. Neddersen, R.S. Hook, Analyst. 117 (1992) 577–582.
[19] Y. Shibala, L. Morita, Anal. Sci. 5 (1989) 107–109.
[20] K. Dix, C. Cappon, T. Toribara, J. Chrom. Sci. 25 (1987) 164–169.
[21] J. Zheng, W. Goessler, W. Kosmus, Chromatographia 47 (1998) 257–263.
[22] P. Morin, M.B. Amran, M.D. Lakkis, M.J.F. Leroy, Chromatographia 33 (1992) 581–585.
[23] L. Benramdane, M. Accominotti, J.J. Vallon, Toxicorama numéro spécial (1997) 346–350.
[24] H. Heng-bin, L. Yan-Bing, M. Shi-Fen, N. Zhe-Ming, J. Anal. Atom. Spectrom. 8 (1993) 1085–1090.
[25] J. Feldmann, V.W.M. Lai, W.R. Cullen, M. Ma, X. Lu, X.C. Le, Clin. Chem. 45 (1999) 1988–1997.
[26] R.S. Braman, D.L. Johnson, C.C. Foreback, J.M. Ammons, J.L. Bricker, Anal. Chem. 49 (1977) 621–625.
[27] H.W. Sinemus, At. Spectrosc. 3 (1981) 81–86.
[28] H. Chen, I.D. Brindle, X. Le, Anal. Chem. 64 (1992) 667–672.