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Urinary antimony speciation by HPLC-ICP-MS

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This is the first study to report on the determination of Sb species in urine. To this end, HPLC was coupled online to an ICP-MS instrument using ultrasonic nebulization (USN) or hydride generation (HG) for sample introduction into the ICP-MS. The high chloride concentration in urine seriously hampered the chromatographic separation of Sb(v) and Sb(iii) on the Dionex AS14 anion exchange column. Distinct signal suppression, shifting of retention times and severe peak broadening did not allow the application to urine samples. Progress to avoid these problems in HPLC-USN-ICP-MS could be made by employing a Hamilton PRP-X100 anion-exchange column. However, Na eluting in the void volume of the column gave rise to a Na-induced peak overlapping with the Sb(v) signal when USN was used to aspirate the HPLC eluents into the plasma. Therefore, a HG system was placed between the HPLC and ICP-MS instrumentation to overcome this dilemma. Thus, Sb(v) and Sb(iii) were separated in urine with the PRP-X100 column using 20 mM EDTA at pH 4.7 as the mobile phase. Similarly, an ION-120 anion-exchange column was employed to separate trimethylantimony dichloride ($TMSbCl_2$) and Sb(v) with a mobile phase containing 2 mM NH_4HCO_3 and 1 mM tartaric acid at pH 8.5. Detection limits of 20 ng l^{-1}, 12 ng l^{-1} and 8 ng l^{-1} for Sb(v), $TMSbCl_2$ and Sb(iii), respectively, could be established in a 1+2 diluted urine matrix. The developed HPLC-HG-ICP-MS method was applied to the speciation of Sb in the urine of occupationally exposed and non-exposed subjects. Additionally, two lyophilised urine reference materials were investigated. Sb(v) was by far the predominant Sb species, followed by $TMSbCl_2$. Only ultratraces of Sb(iii), if any detectable, could be found. The sum of the concentrations of Sb(v), Sb(iii) and $TMSbCl_2$ in urine samples ranged between 51 and 78% of their total Sb concentrations.

Introduction

Speciation analysis of antimony in environmentally relevant areas increasingly attracts the scientific community. Although distinct drawbacks hamper the application of speciation procedures for Sb, tremendous improvements have been accomplished during recent years.[1] The growing interest in Sb speciation is documented in particular by a growing number of publications, including three reviews published on the issue during the last five years.[1–3]

Speciation analysis of Sb in aqueous systems is conventionally performed by high-performance liquid chromatography (HPLC) coupled online with adequate element-specific detection, such as hydride generation-atomic absorption spectrometry (HG-AAS), inductively coupled plasma atomic emission spectrometry (ICP-AES) or inductively coupled plasma mass spectrometry (ICP-MS).[1–3] The lowest detection limits reported for the determination of Sb species by HPLC-HG-AAS are in the range of 1 μg l^{-1}, namely 0.4 μg l^{-1} for trimethylantimony dichloride ($TMSbCl_2$), 0.7 μg l^{-1} for Sb(iii) and 1.0 μg l^{-1} for Sb(v).[4] Employing HPLC-ICP-MS, these values can be lowered about 100 times, thus detection limits in the low ng l^{-1} can be realised.[5,6]

A major problem encountered with the speciation of Sb in real samples consists of the generally low extraction yield for Sb. Quite frequently, only a few per cent. of the total Sb could be released from the matrix.[6] Thus most of the Sb species information is retained within the samples and Sb speciation procedures are not that useful in gaining further knowledge about the fate of Sb in environmental specimens.

The direct analysis of liquids such as urine, without requiring any extraction of Sb, might be advantageous in studying the occurrence and metabolism of Sb species in certain cases. Moreover, it would allow regular human biomonitoring with respect to these compounds of concern. However, high matrix loads in urine, which are normally not present in extracts of solid samples, may hamper this endeavour. Moreover, total Sb concentrations in urine of non-exposed persons are below 1 μg l^{-1}.[7] Therefore, only analytical procedures with detection limits in the low ng l^{-1} range can be utilised for the identification of Sb species in urine.

Based on our previously reported set-up utilising HPLC-HG-AAS[4] and HPLC-ICP-MS[6] for the speciation of Sb(v), Sb(iii) and $TMSbCl_2$ in aqueous standard solutions, this study was intended to develop reliable speciation procedures for Sb compounds in the urine of occupationally non-exposed and exposed persons and to provide urinary Sb speciation data for the first time.

Experimental

Instrumentation

The high-performance liquid chromatographic (HPLC) system consisted of a metal-free pump (L-6220 Intelligent Pump, Merck, Darmstadt, Germany), a Rheodyne metal-free six-port injector valve with a 100 μl sample loop made from polyether ether ketone (PEEK), and one of the anion-exchange columns listed in Table 1. All tubings of the chromatographic device coming into contact with the mobile phase were made from inert PEEK material. Eluents of the chromatographic system were aspirated into the plasma of the ICP-MS (Elan 5000, Perkin Elmer, Norwalk, CT, USA) with an ultrasonic nebulizer (USN) equipped with a membrane desolvation unit (U-6000AT$^+$, Cetac Technologies, Omaha, NE, USA). For HPLC-hydride generation (HG)-ICP-MS measurements, a

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Table 1 Operating conditions for HPLC-ICP-MS with ultrasonic nebulisation or hydride generation

HPLC:	
Anion exchange columns	Dionex IonPac AS 14, 250 mm × 4 mm + Dionex IonPac AG 14, 4 mm (10–32), Dionex Corporation, Sunnyvale, CA, USA. Hamilton PRP-X100, 250 mm × 4 mm + guard column PRP 1, Reno, NE, USA ION-120, 120 mm × 4.6 mm + guard column, Cetac Technologies, Division of Transgenomics, Omaha, NE, USA
Flow rate	1.5 ml min^{-1}
Injection volume	100 μl
Ultrasonic nebulization:	
Nebulizer	U-6000AT$^+$ (Cetac Technologies, Omaha, NE, USA)
Sweep gas (argon)	2.35 l min^{-1}
Heating temperature	80 °C
Desolvation temperature	80 °C
Cooling temperature	2 °C
Hydride generation:	
$NaBH_4$ solution concentration	0.6% (w/v), stabilised with 0.04% (w/v) NaOH
$NaBH_4$ solution flow rate	4 ml min^{-1}
HCl solution concentration	0.5% (w/v)
HCl solution flow rate	6 ml min^{-1}
Carrier gas flow rate	Argon, 50 ml min^{-1}
ICP-MS:	
Forward Power	1200 W
Cones	Nickel
Plasma gas	15.0 l min^{-1}
Nebulizer gas	≈1.0 l min^{-1}, daily optimised to obtain maximum ^{121}Sb(v) signal intensity
Auxiliary gas	≈1.0 l min^{-1} daily optimized to obtain maximum ^{121}Sb(v) signal intensity
Data acquisition	^{121}Sb, peak hoping mode, replicate time 1 s, dwell time 20 ms, 50 sweeps per reading, 1 reading per replicate

commercial hydride generation system (FIAS 400, Perkin Elmer), already previously adapted for the online coupling of HPLC to HG-AAS,[4] was utilised. HPLC eluents were mixed with adequate solutions of HCl and $NaBH_4$, and the thus formed gaseous hydrides were transported by a stream of argon *via* a cross-flow nebulizer into the spray chamber of the ICP-MS. The signal (^{121}Sb) of the mass spectrometer was recorded with a sampling rate of 1 point s^{-1} with the time-resolved software of the ICP-MS. Chromatographic data were processed with Origin 5.0 (Microcal Software, Inc., Northampton, MA, USA). Detailed operating conditions of the HPLC-USN-ICP-MS and of the HPLC-HG-ICP-MS systems as well as specifications of the anion-exchange columns used in this study are summarised in Table 1.

Reagents and standards

For the preparation of all solutions, MilliQ water (Millipore, Milford, MA, USA) was used. The mobile phases for HPLC were prepared by dissolution of appropriate amounts of ethylenediamine tetraacetic acid (*puriss. p.a.*, Fluka, Neu-Ulm, Switzerland) or ammonium hydrogen carbonate (Fluka, MicroSelect) and tartaric acid (Fluka, MicroSelect) in MilliQ water. Adjustment of pH values was made with an ammonia solution (25%, suprapur®, Merck, Darmstadt, Germany). Corresponding chromatographic investigations have been extensively reported earlier.[4] Briefly, among the five anion-exchange columns tested, the Dionex AS14 provided best results for the separation of Sb(v) and Sb(III) with 1.25 mM EDTA at pH 4.7. The ION-120 column was used to separate TMSb and Sb(v) with 2 mM NH_4HCO_3 and 1 mM tartaric acid at pH 8.5.

Two 1000 mg l^{-1} stock solutions of Sb(III) and Sb(v) were prepared by dissolving appropriate amounts of potassium antimony tartrate (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and potassium hexahydroxyantimonate (Fluka) in high-purity water. A 100 mg l^{-1} stock solution of trimethylantimony dichloride ($TMSbCl_2$) was prepared similarly. $TMSbCl_2$ was kindly donated by H.J. Breunig, University of Bremen, Germany. All stock standard solutions were stored in polyethylene bottles in a refrigerator held at 6 °C. These solutions were stable for at least 12 months.

Sodium chloride (suprapur®) and silver nitrate (analytical-reagent grade) were purchased from Merck. The quality and type of acids employed for the open vessel digestion procedure, as well as of the reagents used for hydride generation, have been reported earlier.[7]

Urine samples

Spot urine samples from two unexposed persons and from two occupationally exposed workers in the battery producing industry were collected into Sb-free polyethylene tubes. Additionally, two lyophilised urine reference materials, namely SRM 2670 (NIST, Gaithersburg, MD, USA) and Seronorm Batch No. 403125 (Nycomed, Oslo, Norway) were analysed. Both freeze-dried reference materials were reconstituted with high-purity water, as recommended by the manufacturers. All urine samples were kept at 4 °C until analysis.

Analytical procedures

Determination of total Sb concentration. A slightly modified procedure in comparison to that developed previously for plant and lipid-rich materials[7] was used for the determination of total Sb in urine. It is based on open vessel digestion followed by hydride generation atomic absorption spectrometry (HG-AAS). Three ml of urine were mineralised with 0.5 ml sulfuric acid, 3 ml nitric acid, 0.5 ml perchloric acid and 0.1 ml hydrofluoric acid in digestion vessels made of glassy carbon in an aluminium heating block. Digestion solutions were diluted to 10 ml with 10% (w/v) HCl. After addition of 1 ml of the reduction solution (30% KI and 5% ascorbic acid) to the diluted digests, they were subjected to HG-AAS as described in detail elsewhere.[7,8]

Speciation analysis. The only sample pretreatment of urine samples involved plain dilution and filtration before the urine was injected into the HPLC-ICP-MS. Depending on the concentration of Sb species in urine, samples were diluted from 1 + 2 to up to 1 + 49 with the mobile phase employed for the chromatographic separation. Samples were filtered through 0.2 μm Minisart RC 25 filters (Satorius, Göttingen, Germany) before injection of the urine onto the chromatographic column.

The species $TMSbCl_2$ in urine samples cannot be unequivocally identified, because $TMSbCl_2$ is eluted in the void volume. So far signals for $TMSbCl_2$ can only be obtained using anion-exchange chromatography, giving rise to very broad peaks and unfavourable separations, otherwise it is eluted in the void volume.[1]

Results and discussion

This study has been devoted to the development of reliable speciation procedures for the determination of Sb compounds in urine by HPLC-ICP-MS. Total Sb concentrations in urine of occupationally non-exposed adults are very low and range from 0.19 to 1.1 μg l^{-1}.[9] In a previous study we established even lower Sb concentrations, *i.e.*, a median of 0.06 μg l^{-1}

(range: <0.01 to 0.53 $\mu g\,l^{-1}$), in the urine of 30 school children by USN-ICP-MS.[10] In the urine of workers occupationally exposed to Sb compounds (mainly to Sb_2O_3 and SbH_3) distinctly elevated Sb levels (normally 5 to 10 $\mu g\,l^{-1}$, up to several hundreds of $\mu g\,l^{-1}$) have been determined.[11] Therefore, HPLC-ICP-MS, providing the lowest detection limits currently available for Sb speciation,[5,6] was used for the development of Sb speciation procedures in urine.

Method development

Preliminary experiments with HPLC-USN-ICP-MS. Initial attempts to determine Sb species in urine obtained by plain 1+9 dilution with high-purity water or with the employed mobile phase (1.25 mM EDTA) on the Dionex AS14 column yielded insufficiently-reproducible chromatograms containing several peaks. Firstly, these peaks could not be identified by comparing their retention times with those of aqueous Sb(v) and Sb(iii) standards [Fig. 1(a)]. Spiking of the urine samples with adequate amounts of Sb(v) and Sb(iii) revealed that both Sb peaks were seriously broadened and, as a more severe problem, that the Sb(iii) peak was distinctly shifted to longer retention times. Besides, additional peaks which could not be identified occurred in the chromatograms [Fig. 1(b)–(d)].

As the chromatographic separation of Sb(v) and Sb(iii) is based on anion-exchange chromatography, it was assumed that the high concentration of sodium chloride present in the urine (physiological NaCl concentration 0.9%), in particular the high chloride concentration, might hamper the chromatographic separation of the anions. It should be noted that Sb(v) is mononegatively charged in aqueous solutions of pH 2.7 to 10.4 whereas Sb(iii) is not charged. Thus, a conversion of Sb(iii) to a soluble, stable, negatively charged complex, for example by reaction with EDTA, phthalic or tartaric acids, is desirable for retaining Sb(iii) on an anion-exchange column.

To investigate the potential influence of the high sodium chloride concentration in urine on the chromatographic separation, various amounts of NaCl (0.05–0.2%) were dissolved in 1.25 mM EDTA, which served as mobile phase. Higher NaCl concentrations were not studied because chromatograms had already shown strong signal depression and unacceptable separation with 0.2% NaCl. The results can be summarised as follows [Fig. 1(b)–(d)]. (1) The sharp peak eluting with the solvent front can be attributed to the elution of sodium present in the urine [Fig. 1(b)]. Sodium as a cation is obviously eluted in the void volume of the anion-exchange column, a fact that can also be easily detected by the colour of the inner plasma region turning to yellow when Na elutes. The peak height increased with increasing NaCl concentration in the investigated synthetic solutions. (2) With increasing NaCl concentration, the Sb(iii) peak was shifted to longer retention times, whereas the retention time of the Sb(v) peak remained constant. Both Sb signals in Fig. 1(b) represent blank levels of Sb(v) and Sb(iii) in NaCl of the highest commercially available purity (0.05% solution). However, both peaks distinctly broadened with increasing NaCl concentration. At an NaCl concentration of 0.2%, Sb(v) co-eluted with the Na-induced peak and the Sb(iii) peak split into two broad peaks starting at 650 s and covering a retention time of 500 s. (3) Addition of standard solutions containing Sb(v) and Sb(iii) to the sodium chloride test solutions revealed that signals for both Sb species were severely suppressed by the NaCl matrix in comparison to the peak areas obtained from aqueous standard solutions. For a comparison, the different concentrations of the Sb(iii) and Sb(v) standard solutions—as specified in detail in the figure legend—as well as the different scale of the *y* axis have to be considered. (4) The occurrence of additional peaks in the chromatograms could not be reproduced easily and was strongly dependent on the spiked or natural concentration of Sb(v) and Sb(iii) present in the urine [Fig. 1(d)].

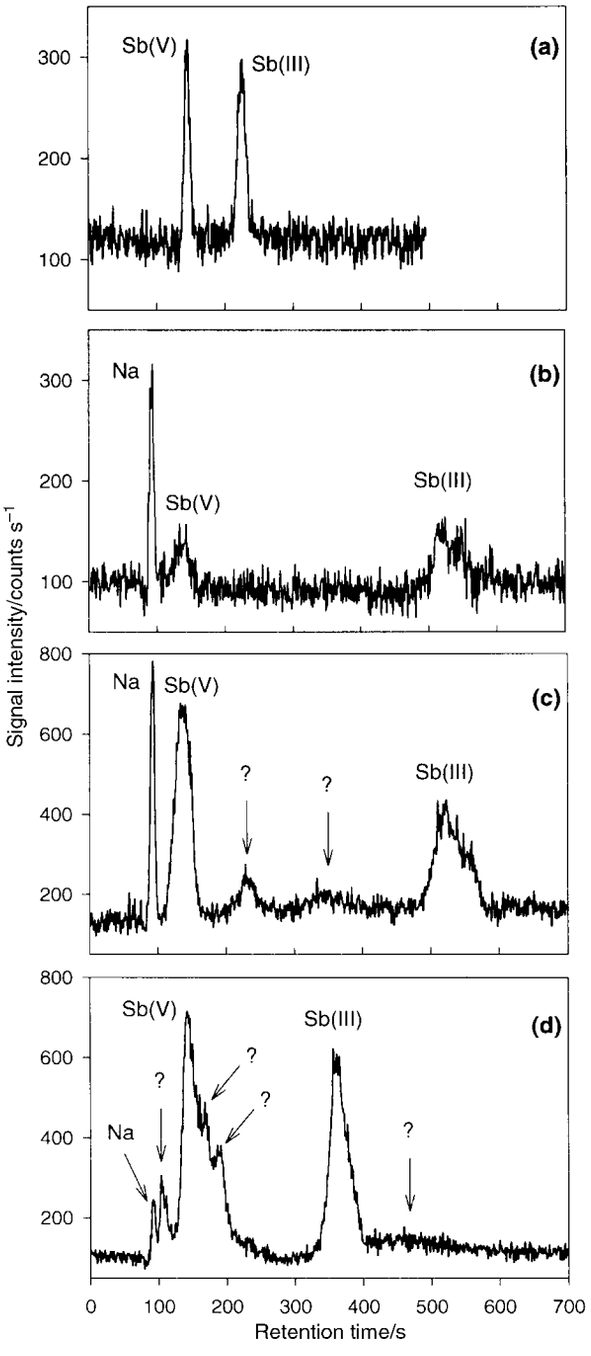

Fig. 1 Experimental conditions. HPLC: mobile phase 1.25 mM EDTA, pH 4.7; column Dionex AS14; detection: USN-ICP-MS. (a) Chromatogram of 50 $ng\,l^{-1}$ of each Sb(v) and Sb(iii). (b) Chromatogram of a 0.05% NaCl solution in 1.25 mM EDTA (mobile phase). (c) Chromatogram (b) spiked with 1 $\mu g\,l^{-1}$ of each Sb(v) and Sb(iii). (d) Chromatogram of a urine sample diluted 1+4 with 1.25 mM EDTA and spiked with 1 $\mu g\,l^{-1}$ of each Sb(v) and Sb(iii).

Matrix separation. It was evident from the results of the aforementioned experiments that the NaCl matrix is responsible for at least some of the problems initially observed in the speciation of Sb in urine by HPLC-ICP-MS. The involvement of chloride in the chromatographic separation of Sb(v) and Sb(iii) could be experimentally proven by simultaneous monitoring of the ICP-MS signal at *m/z* 37 (^{37}Cl). The ^{37}Cl intensity always increased when one of the unknown peaks shown in Fig. 1(b)–(d) appeared in the chromatograms. Consequently, a procedure to eliminate or to reduce the NaCl-based interferences had to be developed. The "classical" precipitation of chloride as AgCl was tested for its suitability in reducing the chloride concentration in urine thoroughly.

Therefore, $AgNO_3$ was added to the diluted urine samples until the precipitation of chloride was finished. Urine samples were then passed through 0.2 μm filters and subsequently analysed for Sb species by HPLC-USN-ICP-MS. In fact, the diminished concentration of chloride in the diluted urine samples changed the elution profile of both Sb and Cl species dramatically [Fig. 2(a)]. Sb(v) and Sb(III) co-eluted under these conditions, and the chloride trace in Fig. 2(a) indicates that the major amount of chloride was eluted after Sb(III). The chloride signal remained, without $AgNO_3$ addition, at baseline level during the elution of Sb(v) and only increased when Sb(III) was eluted [Fig. 2(b)]. At higher Sb(III) concentrations in urine, the Sb(III) peak divided into two peaks and similarly also the chloride pattern changed [Fig. 2(c)]. Obviously, competition between the formation of Sb-chloride and Sb-EDTA complexes led to these two Sb(III) peaks, and was in general responsible for the bad peak shapes of the chromatograms. Similarly, the significant tailing of the Sb(v) peak [Fig. 2(b) and (c)] or fractionation [Fig. 1(d)] can be attributed to the same complexation. Overall, an acceptable chromatographic separation of Sb(v) and Sb(III) was not possible under all aforementioned conditions.

Therefore, another anion-exchange chromatographic separation

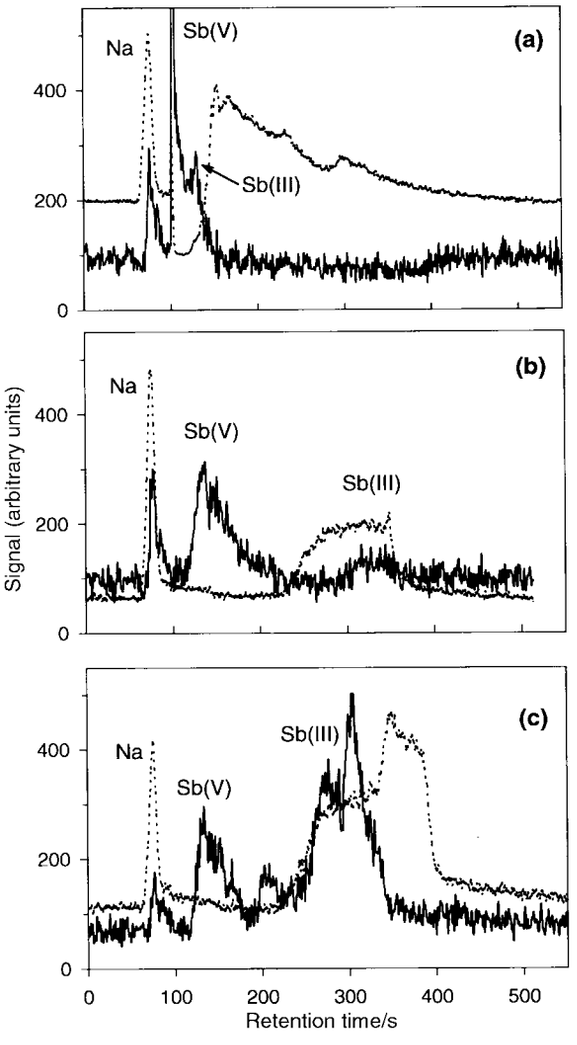

Fig. 2 Experimental conditions. HPLC: mobile phase 1.25 mM EDTA, pH 4.7; column Dionex AS14; detection: USN-ICP-MS. Monitored signals: *m/z* 121 (Sb), bold lines and *m/z* 37 (Cl), dotted lines. (a) Chromatogram of a urine sample diluted 1+9 with 1.25 mM EDTA followed by precipitation of chloride with $AgNO_3$. (b) Chromatogram of the urine sample in (a) after plain dilution with 1.25 mM EDTA and spiking with 1 μg Sb(v) l^{-1}. (c) Chromatogram of the urine sample in (a) after plain dilution with 1.25 mM EDTA and spiking with 2 μg Sb(III) l^{-1}.

of Sb(v) and Sb(III) with the PRP-X100 column was tested, which has been already successfully applied within a HPLC-HG-AAS set-up.[4] Compared to the previous separation with the Dionex AS14 column, the EDTA concentration in the mobile phase of the separation using the Hamilton PRP-X100 column was increased from 1.25 mM to 20 mM, thus favouring the formation of an Sb-EDTA complex. Stable signals of well-shaped peaks for both Sb(v) and Sb(III) were thus obtained. However, the Na-induced peak appearing at the solvent front, which had been already observed when using the Dionex AS14 column, was still noticed. Because Sb(v) eluted during the separation with the PRP-X100 column near the solvent front, the Na-induced peak and Sb(v) co-eluted. Consequently, quantification of Sb(v) was not easily achievable. In order to avoid the occurrence of the "Na-peak" in the void volume, the use of hydride generation was considered to convert the eluted Sb species into their volatile hydrides.

As well as analysing aqueous standard solutions, the Dionex column provides two important advantages over the Hamilton column: (a) Sb(v) is eluted in the void volume of the Hamilton PRP-X100 column, whereas Sb(v) is retained on the Dionex column; and (b) the concentration of EDTA in the mobile phase is 20 mM for the separation of Sb(v) and Sb(III) with the PRP-X100 column, whereas only 1.25 mM are needed when using the Dionex column. The reduced amount of EDTA needed with the Dionex column leads to a lower baseline in the chromatograms and to better signal-to-noise ratios.

HPLC-HG-ICP-MS. For the coupling of HPLC to hydride generation (HG)-ICP-MS, the USN was replaced by the HG-system. Identical parameters ($NaBH_4$ and HCl concentrations, flow rates, gas pressure), as optimised in previous work for HPLC-HG-AAS,[4] proved to give best results also for HPLC-HG-ICP-MS and were thus used throughout all further investigations. As expected, the Na-induced peak in the void volume of the PRP-X100 column disappeared, allowing the quantification of Sb(v) [Fig. 3(a)]. Signals for Sb(III) were higher than for Sb(v), due to the preferred kinetics of Sb(III) during hydride generation. When analysing urine samples, Sb(III) was slightly shifted to earlier retention times compared to standard solutions. Nevertheless, peaks for Sb(III) retained their good peak shape and the shifting effect could be avoided when an injection of a urine sample onto the column was made about every 12 min. The PRP-X100 column did not suffer from performance loss, even when urine samples were diluted only 1+2. To keep the dilution factor as low as possible, this 1+2 dilution has been routinely applied in the analysis of Sb(v) and Sb(III) in urine samples.

$TMSbCl_2$ cannot be eluted under the aforementioned chromatographic conditions, as alkaline pH values are required for that purpose. The ION-120 anion-exchange column provided a good separation between $TMSbCl_2$ and Sb(v) using 2 mM NH_4HCO_3 and 1 mM tartaric acid at pH 8.5 as mobile phase within less than 3 min [Fig. 4(a)]. When this chromatographic separation was applied to urine samples, severe peak broadening of Sb(v), but not of $TMSbCl_2$, was observed [Fig. 4(b) and (c)]. As Sb(v) can easily be determined in the set-up with the PRP-X100 column, the broad Sb(v) peak would only hamper the determination of $TMSbCl_2$ when urinary concentrations of Sb(v) exceed approximately 5 μg l^{-1}, which is normally not the case.

Analytical characterisation

The two successfully applied chromatographic separations using the PRP-X100 and ION-120 columns were thoroughly evaluated for their analytical performances, including the calculation of limits of detection (LOD) for all three investigated Sb species. To this end, at least 10 aliquots of standard solutions at low concentrations of the analytes [0.1 μg l^{-1} of each $TMSbCl_2$ and Sb(III), 0.2 μg l^{-1} of Sb(v)] were repetitively injected onto the columns. LODs were calculated from 3 times the standard deviation of the mean

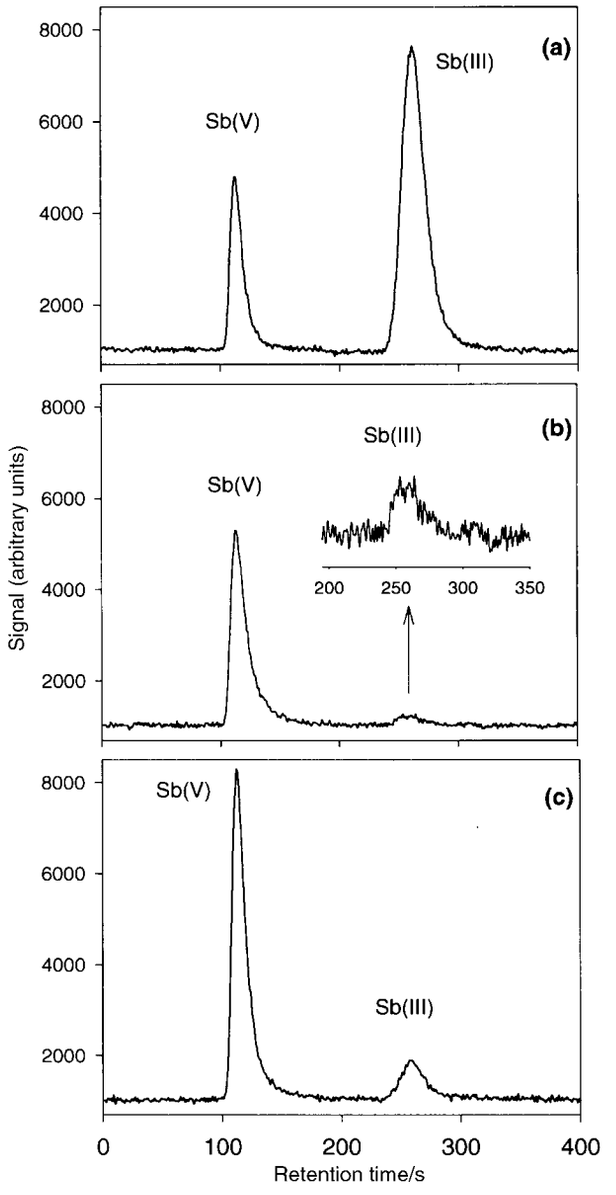

Fig. 3 Experimental conditions. HPLC: mobile phase 20 mM EDTA, pH 4.7; column Hamilton PRP-X100; detection: HG-ICP-MS. (a) Chromatogram of a standard solution containing 1 μg l^{-1} of both Sb(v) and Sb(III). (b) Chromatogram of the Seronorm reference urine diluted 1+49. (c) Chromatogram of the urine from (b) spiked with 1 μg Sb(v) l^{-1} and 0.1 μg Sb(III) l^{-1}.

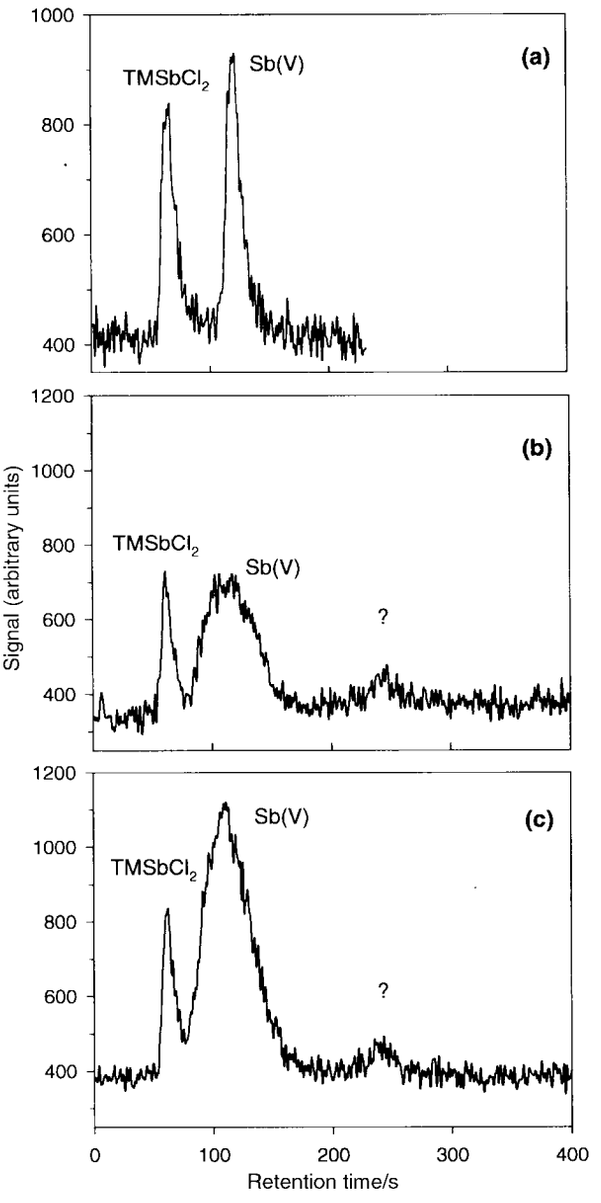

Fig. 4 Experimental conditions. HPLC: mobile phase 2 mM NH_4HCO_3 and 1 mM tartaric acid, pH 8.5; column ION-120; detection: HG-ICP-MS. (a) Chromatogram of a standard solution of 100 ng l^{-1} $TMSbCl_2$ and 200 ng l^{-1} Sb(v). (b) Chromatogram of a 1+4 diluted urine sample of an exposed person. (c) Chromatogram of the urine sample in (b) spiked with 250 ng l^{-1} Sb(v).

value of the thus obtained peak areas divided by the slope of the calibration curve. These investigations were carried out in solutions prepared from dilution of Sb stock standard solutions with the employed mobile phases, or by dilution of the standard solutions with 1+2 diluted urine. For the developed HPLC-HG-ICP-MS procedure, detection limits in the low ng l^{-1} range for all three Sb species, as specified in detail in Table 2, could be achieved. These LODs are comparable to those already obtained with an HPLC-USN-ICP-MS set-up and are about 100 times lower than LODs for HPLC-HG-AAS (Table 2). Although LODs for both ICP-MS approaches are similar, it should be stressed that the use of hydride generation allows the quantification of Sb compounds in urine, which is rather difficult, if not impossible, to realise by using the USN. Moreover, HG is much cheaper than USN. The reproducibility of repetitive injections/quantifications at the aforementioned concentrations levels of the analytes in the 1+2 diluted urine matrix ranged from 2.7% for Sb(III) to 5% for $TMSbCl_2$. For standard solutions the corresponding values amounted to approximately 3%. The slope of the calibration curves was equal for Sb(III), no matter whether standards were prepared with the mobile phase or with 1+2 diluted urine. On the contrary, sensitivities, *i.e.*, peak areas, for Sb(v) and $TMSbCl_2$ changed slightly depending on the urine matrix. In general, peak areas for Sb(v) and $TMSbCl_2$ were approximately 10 to 20% (depending on the urine matrix) lower in 1+2 diluted urine when compared to the peak areas obtained from standards prepared with the employed mobile phase. Therefore, quantification of all analytes was carried out by standard additions.

Table 2 Comparison of limits of detection (μg l^{-1}Sb) for Sb(v), Sb(III) and $TMSbCl_2$ using different spectrometric detection devices

	Sb(v)	Sb(III)	$TMSbCl_2$	Reference
Detection				
HG-AAS	1.0	0.7	0.4	4
USN-ICP-MS	0.012	0.014	0.009	6
HG-ICP-MS	0.020	0.008	0.012	This work

Table 3 Concentrations of total Sb and of Sb species ($\mu g\ l^{-1}$Sb) in urine samples of occupationally exposed and non-exposed subjects and in two lyophilised urine reference materials as determined by HG-AAS and HPLC-HG-ICP-MS

	Total Sb concentration	Speciation analysis				
Samples	Experimental value, $n=5$	Sb(v)	Sb(III)	$TMSbCl_2$	Sum of species	Recovery (%)
Non-exposed person A	<0.12	<0.060	<0.025	<0.036	<0.12	—
Non-exposed person B	<0.12	<0.060	<0.025	0.09 ± 0.02	<0.18	—
Exposed person A	8.3 ± 0.3[a]	5.9 ± 0.2	0.15 ± 0.05	0.40 ± 0.04	6.45	78
Exposed person B	5.1 ± 0.4[a]	2.0 ± 0.1	<0.025	0.57 ± 0.07	2.60	51
NIST SRM 2670	0.53 ± 0.05[b]	<0.060	0.07 ± 0.02	0.23 ± 0.03	0.36	68
Seronorm 403125	104 ± 3[c]	101 ± 0.5	1.7 ± 0.4	—[d]	102.7	99

[a]Results confirmed by independent analysis of the urine samples at the Institute of Environmental and Occupational Health, University of Erlangen-Nürnberg, Germany. [b]No certified value available, concentration determined in a previous study using USN-ICP-MS (ref. 10). [c]100 $\mu g\ Sb\ l^{-1}$ added by the manufacturer. [d]Not determined (see also text).

Speciation analysis of urinary Sb compounds

The developed HPLC-HG-ICP-MS procedure was applied to the determination of Sb(v), Sb(III) and $TMSbCl_2$ in selected urine samples. The corresponding results are summarised in Table 3. Total Sb concentrations in urine were determined by HG-AAS. All results are based on at least 3 independent determinations. In the two urine samples of non-exposed subjects, the total Sb concentration was below the method detection limit of 0.12 $\mu g\ Sb\ l^{-1}$. Concentrations for Sb(v) and Sb(III) were also below the method detection limits, except for $TMSbCl_2$, which was found in ultratrace amounts in one sample (Table 3). Total Sb urine concentrations of two persons working in the lead battery producing industry were about 100 times higher than for non-exposed persons. These workers are exposed to antimony trioxide and stibine (SbH_3). Surprisingly Sb(v), but not Sb(III), followed by $TMSbCl_2$ were the predominant Sb species determined in the two urine samples. Sb(III), if detectable, was only present at very low concentrations (Table 3). As Sb(III) is known to exert a 10-times higher toxicity than Sb(v), it seems that the human body can detoxify Sb(III) by oxidation to Sb(v), and possibly biomethylates Sb(III) and/or Sb(v) to $TMSbCl_2$. The biomethylation of both Sb(III) and Sb(v) by microorganisms and by fungi cultures of *Scopulariopsis brevicaulis* has been established experimentally during recent years.[1,12–14] Additionally, an unknown Sb species was detected in the urine of one exposed person [Fig. 4(b) and (c)]. Assuming an identical slope for the calibration of this compound as established for Sb(v), the concentration of this Sb compound amounts to 0.13 $\mu g\ l^{-1}$.

In the NIST urine reference material, $TMSbCl_2$ was the most abundant species with traces of Sb(III). Antimony added (100 $\mu g\ l^{-1}$) to the Seronorm urine reference urine was spiked by the manufacturer as Sb(v) [Fig. 3(b) and (c)]. The highest amount (1.7 ± 0.4 $\mu g\ l^{-1}$) of Sb(III) by far was identified in the Seronorm urine. $TMSbCl_2$ was not quantified in this sample because its determination was severely hampered by the extraordinarily high concentration of Sb(v) that had been added to the urine by the manufacturer. Generally, in non-spiked urine samples, the sum of the concentrations of Sb(v), Sb(III) and $TMSbCl_2$ ranged between 51 and 78% of the total Sb concentrations.

Conclusions

The high chloride concentration in urine can severely hamper the chromatographic separation of Sb(v) and Sb(III) by anion-exchange chromatography, especially that of Sb(III). The applied separation principle is based upon the *in situ* conversion of Sb(III) in urine, which is associated with chloride, into its corresponding EDTA-complex. Therefore, increasing the concentration of the complexing agent, *i.e.*, EDTA, in the mobile phase will enhance the stability and reproducibility of the separation of Sb(v) and Sb(III). Thus the chromatographic separation of Sb(v) and Sb(III) on the PRP-X100 column involving 20 mM EDTA at pH 4.7 is superior to that on the Dionex AS14 column with 1.25 mM EDTA at pH 4.7 as mobile phase when analysing matrices with high chloride content.

Sodium eluting in the void volume of the anion-exchange columns gives rise to a Na-induced peak which overlaps with signals of Sb species eluting in or near [Sb(v) on the PRP-X100 column] the void volume. To overcome this problem, hydride generation can be successfully employed, which adds further selectivity by converting only hydride-forming species into volatile compounds. Thus only hydride-forming Sb species can be detected by this approach. But Zheng *et al.*[15] demonstrated recently that at least four non-identified Sb species are HG-active besides Sb(III), Sb(v) and $TMSbCl_2$.

This first study on the elucidation of Sb species in urine indicates that people exposed to Sb(III) metabolise Sb(III) in the body mainly to Sb(v) as well as to $TMSbCl_2$, thus detoxifying the 10-times more harmful Sb(III). Although, generally, much more than 50% of the total Sb can be attributed to the investigated Sb species, further studies are needed to identify the remaining currently non-identified Sb species in urine.

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