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Determination of selenium compounds in urine by high-performance liquid chromatography–inductively coupled plasma mass spectrometry

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Abstract

Selenium species, selenite, selenate, selenomethionine (Semet), seneloethionine (Seet) and trimethylselenonium ion (TmSe) were separated in aqueous solution using a gel-permeation (polyvinyl alcohol-based resin) GS-220 column by eluting with 25 m*M* tetramethylammonium hydroxide and 25 m*M* malonic acid at pH 7.9. The GS-220 column coupled with inductively coupled plasma mass spectrometry was used for the separation, identification, and quantification of selenium compounds present in certified reference material (CRM) No. 18 human urine from the National Institute for Environmental Studies in Japan (NIES). Spiking of the authentic standard to the urine and use of a silica-based LC-SCX cation-exchange column validated the peak of selenium compounds. High concentrations of chloride and bromide in the urine eluted from the GS-220 column formed molecular ions $^{40}Ar^{37}Cl^{+}$ and $^{81}Br^{1}H^{+}$ in the plasma, and these molecular ions created additional peaks in the chromatograms when ^{77}Se and ^{82}Se isotopes were monitored respectively. Thus, both the isotopes were selected concurrently for signal monitoring to eliminate the interfering signals. On the LC-SCX column, chloride and bromide were eluted with selenate and complicated its determination, but the peak of TmSe was baseline separated from rest of the Se compounds. Two unknown Se compounds were detected in both the columns. An additional Se compound having the same retention time as that of Semet was detected on the LC-SCX column. Peaks of selenite, selenate, TmSe and unknown selenium compounds in the urine were baseline separated on the GS-220 column, and were free from interferences. Therefore, the GS-220 column was used for the determination of selenium compounds in NIES CRM No. 18. Unknown Se compounds were the predominant selenium species followed by selenite, TmSe and selenate. The estimated value of TmSe as Se, by the standard additions method using the GS-220 column, was 3.42 ± 0.17 μg l^{-1} and was in good agreement with the LC-SCX value [3.38 ± 0.21 ($n=5$) μg l^{-1}].

Keywords: Organoselenium compounds; Selenium compounds

1. Introduction

Selenium is an important micronutrient with a narrow range of intake. Outside this narrow range deficiency disease and toxicity occur. It is an essential element that exerts its biological effect through selenoproteins [1]. It has a composite behavior on

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man and animals depending on the concentration and the chemical form in which it enters into the organism [2].

Selenium exists in different chemical forms in the environment and in biota. It is found mainly as selenoproteins, Se-lipids, Se-peptides and Se-amino acids. The inorganic species selenite and selenate are very important for the biochemical cycle of selenium. Seleno amino acids are incorporated into proteins. Selenomethionine (Semet) is used for selenium supplementation. It is generally found in plants. Selenocystine (Secys) plays an essential role in the active site of several enzymes, including glutathione peroxidase. About two-thirds of the Se content in plasma is found as Secys in the proteins; the remaining part is probably found as Semet in albumin [3]. The trimethylselenonium ion (TmSe) is identified as a urinary metabolite of Se [4,5] and is measured in the urine of supplemented individuals [6]. TmSe is found to be an inactive Se compound that is easily and almost completely excreted in urine [7]. From the nutritional point of view, the necessary amount of this element is taken up by the organisms through these Se compounds, and the excess amount is removed via various pathways of excretion, urine being one of the most representative ones [8]. The chemical forms and quantities of selenium in urine are of interest because they may reflect the Se status in the body, either as a detoxified form or as the metabolites of an essential chemical form. It also provides information for evaluating the possible biochemical pathway for transformations arising due to digestion in the body. Thus the development of a reliable technique for the determination of Se compounds in urine [9–11] is a necessary step to understanding the biogeochemical cycle, mobility, transfer, and uptake of Se with toxicity [12].

Several procedures are adopted for the separation and determination of selenium compounds in urine. The common and available one is HPLC–inductively coupled plasma (ICP) MS. However, Ar-ICP-MS (quadruple) suffers from spectroscopic and nonspectroscopic interferences. Major isotopes of selenium $m/z = 76$ (9.0%), 78 (23.5%) and 80 (49.8%) are subject to severe interference from $^{40}Ar^{36}Ar^{+}$, $^{40}Ar^{38}Ar^{+}$, and $^{40}Ar_2^{+}$. The molecular ions $ArCl^{+}$ ($^{40}Ar^{37}Cl^{+}$) and BrH^{+} ($^{81}Br^{1}H^{+}$) strongly interfere with the m/z 77 and 82 of Se isotopes when the sample contains high chloride and bromide matrixes. Therefore, careful selection of Se isotopes is essential for the determination of Se compounds. Moreover, chromatographically the chloride and bromide should be separated from the investigated Se compounds [13].

A number of manuscripts have been published for the separation of different selenium compounds. Most of the separation methods have been developed using ion-exchange and ion-pair reversed-phase chromatographic columns. The separation of Se compounds using gel-permeation chromatographic columns is rather limited [14]. Halogens are determined using the GS-220 column [15]. However, the application of developed separation procedures for Se compounds to human urine samples is counted [16]. Only a number of the separation systems have been reported for human urine; most of them have been performed on spiked urine samples. Selenite [17], selenate and TmSe [18] have been detected in human urine. The concentration of TmSe and total Se is reported in urine using the gel-permeation chromatographic column [14]. However, the peak of TmSe has overlapped fully with the BrH^{+} (m/z 82) peak [14]. Nevertheless, reports for the identification of Se compounds in human urine are contradictory [16]. TmSe in human urine ranges from 8 to 70% of total Se [14,19], while other studies have detected a major inorganic contributor [18,20,21].

In order to tackle the present problems for the identification and confirmation of selenium compounds in biological samples, particularly in urine, there is an urgent need for reference urine, with certified selenium species. Hence, development of certified reference materials (CRM) for the speciation of Se compounds is imperative in order to validate newly developed speciation methods as well as to conduct quality assurance of the real analysis. As a preparatory step for this goal, development and evaluation of a separation method for Se compounds has been conducted on CRM No. 18 (having high chloride and bromide matrixes) from the National Institute for Environmental Studies (NIES; Ibaraki, Japan), by using the cation-exchange and gel-permeation chromatographic systems. Different chromatographic conditions have been used in order to confirm the identification of specific selenium compounds. Quantification of selenium compounds has

been performed using the standard additions technique.

2. Experimental

2.1. Reagents and solutions

NIES freeze-dried CRM No. 18 was used as a sample in the present study [22]. All solutions were prepared with Milli-Q (18.2 MΩ cm; Milli-QSP TOC Water System, Nihon Millipore, Japan) water. The mobile phases for the gel-permeation chromatography (LC-1) were prepared by dissolving 22.8 g tetramethylammonium hydroxide (TMAH), (Nacalai Tesque, Kyoto, Japan, GR grade) 10% in water + 2.60 g malonic acid (Nacalai Tesque, Kyoto, Japan) in Milli-Q water, adjusting the pH to 6.8, 7.2, 7.5 and 7.9 by adding 2.0 *M* aqueous ammonia solution (Kanto, Japan, analytical-reagent grade). Finally, the solution was made up to 1000 ml solution with Milli-Q water (25 m*M* TMAH + 25 m*M* malonic acid). The mobile phase for cation-exchange chromatography was prepared by dissolving 1.58 g pyridine (Merck, analytical-reagent grade) in Milli-Q water and adjusting the pH of this solution to 1.9 by adding formic acid (~98%, Fluka, ''puriss'' grade), then made up to 1000 ml solution (20.0 m*M*) with Milli-Q water (LC-2).

All reagents were of analytical grade and were used without further purification. Sodium selenate was purchased from Fluka, sodium selenite pentahydrate from Merck, Seleno DL-methionine, seleno-DL-cystine, seleno-DL-ethionine from Sigma and trimethylselenonium iodide from TRI Chemical Laboratory, Japan. Stock solutions were prepared with deionized Milli-Q-water (18.2 MΩ cm) from sodium selenate (1196 mg to 500 ml, 1000 μg Se ml^{-1}), from sodium selenite pentahydrate (1666 mg to 500 ml, 1000 μg Se ml^{-1}), from trimethylselenonium iodide (63.6 mg to 20 ml, 1000 μg Se ml^{-1}), from selenoethionine (133 mg to 100 ml, 500 μg Se ml^{-1}), from selenocystine (26.5 mg to 100 ml, 125 μg Se ml^{-1}) and from selenomethionine (124 mg to 100 ml, 500 μg Se ml^{-1}). The stock solutions were stored in a refrigerator at −20 °C before use. Solutions of the selenium compounds with concentrations in the range of 1.00–50.0 ng Se ml^{-1} were prepared by appropriate dilution of the stock solutions with Milli-Q water.

2.2. HPLC–argon (Ar) ICP-MS

The HPLC system consisted of a Shimadzu LC-6A solvent delivery unit (Shimadzu, Japan) and a Rheodyne 9725 six-port injection valve (Cotati, Ca, USA) with a 100 μl injection loop. Separations were performed on a Supelcosil LC-SCX cation-exchange column (Supelco, Bellefonte, Pa, USA; 25 cm × 4.6 mm I.D., 5 μm silica-based particles with propylsulfonic acid exchange sites), connected with a guard cartridge (Supelcosil, 2 cm), and an Asahipak GS-220 (Shodex, Showa Denko, Japan) gel-permeation column (I.D. 7.6 mm × 50 cm, 9 μm, polyvinyl alcohol-based resin), connected with a guard cartridge (GF 1G, 7B, Shodex). Columns were equilibrated by passing at least 100 ml (flow-rate 1.5/1.0 ml min^{-1}) of the mobile phase through the column before any injection of the selenium compounds. The outlet of the column was connected to the quartz concentric nebulizer of the ICP-MS system (HP 4500, Yokogawa, Tokyo, Japan) through a sample line two-way connector block (G1820-65347, HP) via 300 mm × 0.25 mm I.D. PEEK (polyether ether ketone) capillary tubing. Ion intensities at *m*/*z* 53, 77, 79, and 82 were recorded with the HP-4500 (Japan) time-resolved analysis software. For quantification ^{82}Se monitoring ion signals were used. Chromatograms were exported, peak areas were calculated, and the concentrations were determined with external calibration curves established for each of the Se compounds and with the standard additions technique. The operation conditions for the ICP-MS system are radiofrequency power: (a) forwarded 1.2 kW, (b) reflected <10 W; argon: plasma gas flow/l min^{-1} 14.4, auxiliary gas flow/l min^{-1} 1.04, nebulizer gas flow/l min^{-1} 1.04; data acquisition mode: time resolved analysis; integration time/mass/s 1.0; detector mode: pulse; sampling time/s 1.00090; nebulizer (Meinhard): concentric; temperature of spray chamber <5 °C; sample uptake rate/l min^{-1} 1.0 to 1.5; monitored signals: $^{37}Cl^{16}O^+$, $^{77}Se^+$, $^{40}Ar^{37}Cl^+$, $^{79}Br^+$, $^{81}Br^1H^+$, $^{82}Se^+$, at *m*/*z* 53, 77,79, 82; total analysis time/s 800 to 1600.

2.3. Urine preparation

The certified value for total selenium of NIES CRM No. 18 is 59±5 μg l^{-1} when it is dissolved in 9.57 ml water. A detailed description of the pretreatment of the sample for the analysis was given previously [22].

3. Results and discussion

3.1. Chromatographic separation of selenium compounds in NIES CRM No. 18

An injection of larger volumes of samples will help to achieve lower detection limits for selenium compounds. However, larger loop volumes introduce a larger matrix to the columns and ultimately disturb chromatographic equilibrium conditions on the column, causing loss of separation efficiencies, peak broadening and peak overlapping. In the present study, a gel permeation column based on polyvinyl alcohol-based resin, GS-220, was used for the separation and identification of selenium compounds present in NIES CRM No. 18 (Figs. 1 and 2). Because of the wide range of pK_a values and different ionic characters of selenium species (Table 1), the separation of selenium compounds by a single ion-exchange chromatographic condition is not easy to achieve. We have used a combination of several different chromatographic conditions with different principles of separation for the reliable and accurate speciation of arsenic in environmental samples [14]. A gel-permeation chromatographic system (GS-220 column) is found to be especially suitable for the speciation of Se compounds because of its high separation efficiency and durability against injection of large sample size. The column is stable in high matrix samples like urine as high as 100 μl (Fig. 2). We have tested the mobile phase with varying pH conditions (6.8, 7.2, 7.5 and 7.9) and concentrations (15 m*M* TMAH+15 m*M* malonic acid; 25 m*M* TMAH+25 m*M* malonic acid and 30 m*M* TMAH+30 m*M* malonic acid solutions) to optimize the separation efficiencies of Se compounds. The optimized separation conditions for selenous acid, selenic acid, Semet, Seet and TmSe are obtained at pH 7.9, with 25 m*M* TMAH+25 m*M* malonic acid solution (LC-1, retention times for selenous acid, selenic acid, Semet, Seet and TmSe are 738, 862, 950, 1016 and 1092 min respectively). Using these conditions one could obtain a good resolution of the peaks within a reasonable time (Fig. 1). This can be attributed to the different interaction processes between the stationary phase and selenium compounds at the pH chosen. Although GS-220 is made of polyvinyl alcohol, a neutral and hydrophilic compound, it has both negative charge and a hydrophobic character. Negative charge comes from carboxyl groups which were introduced during the polymerization procedure on the resin, while hydrophobicity comes from double bonds, added to harden the resin in order to resist mechanical stress during the HPLC stage. These two help to improve separation of various compounds. In other words, GS-220 separates compounds based on not only the size of the molecule but also the charge-state and hydrophobicity. Selenate at pH 7.9 carries two negative charges. Therefore, it is repelled by the negatively carboxylic group of the resin and elutes in the front of the chromatogram (Fig. 1). Increasing pH, repulsion between carboxylic group and selenate may increase. Hence, this anionic repulsion may be responsible for decreasing the retention time of

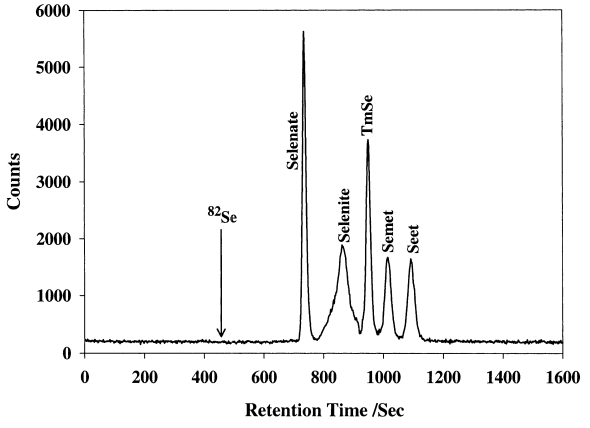

Fig. 1. HPLC–ICP-MS chromatogram obtained with a solution of selenate, selenite, selenomethionine (Semet), selenoethionine (Seet), and trimethylselenonium ion (TmSe) (24.0, 23.8, 24.4, 23.4 and 24.8 μgl^{-1} Se respectively) in distilled water on a GS-220 gel-permeating column using HPLC–ICP-MS (injection volume 100 μl, flow-rate 1.0 ml min^{-1}, monitoring signal ^{82}Se, mobile phase 25 m*M* TMAH+25 m*M* malonic acid, pH 7.9, LC-1).

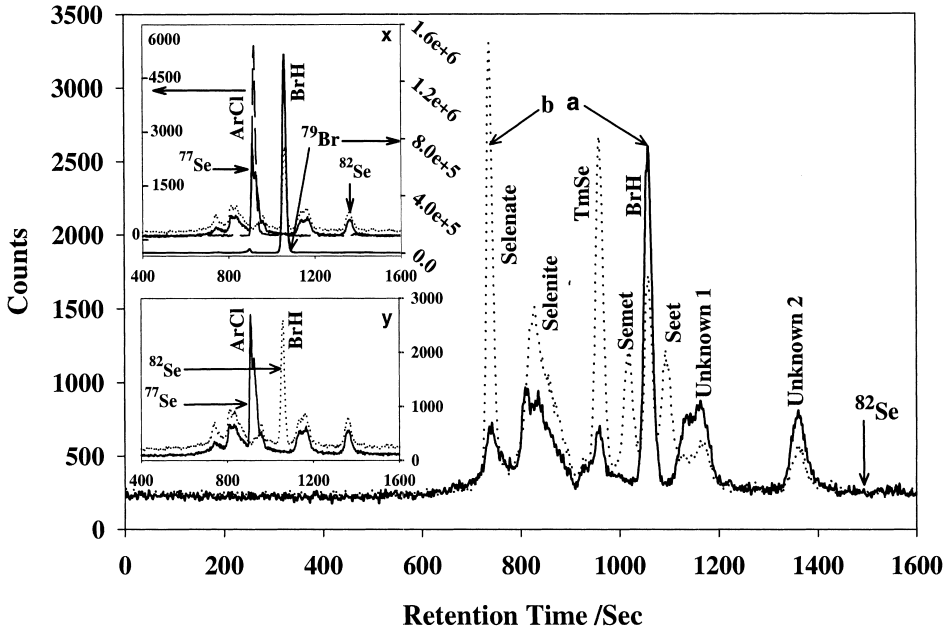

Fig. 2. HPLC–ICP-MS chromatograms obtained with (a) the reconstituted NIES freeze-dried CRM No. 18 (100 μl injected; monitoring signal ^{82}Se); (b) urine (1:1 dilution) spiked with 12.0, 11.9, 12.2, 11.7 and 12.4 μg l^{-1} of selenate, selenite, selenomethionine (Semet), selenoethionine (Seet), and trimethylselenonium ion (TmSe), respectively (100 μl injected, monitoring signal ^{82}Se); Inset (x) urine and 5000 mg l^{-1} chloride each (injection volume 100 μl, monitoring masses 77, 79 and 82), Inset (y) urine (100 μl injected, monitoring isotopes are ^{77}Se and ^{82}Se), on a GS-220 gel-permeating column (mobile phase 25 m*M* TMAH + 25 m*M* malonic acid, pH 7.9, LC-1).

selenate with increasing pH values. Selenous acid is about 25% mono-negatively and about 75% di-negatively charged at pH 7.9 (Table 1). The lower anionic repulsion with stationary phase compared to selenate may be accountable for the weak retention of this compound on the column; it elutes slowly after the selenate, and that generates a broad peak. The TmSe is positively charged irrespective of pH. The interaction between the anionic carboxylic group of the resin and cationic TmSe causes larger retention in the column, and it elutes after selenate and selenite (Fig. 1). Furthermore, TmSe has a greater hydrophobic interaction than selenate and selenite. Semet at pH 7.9 exists almost as a zwitterionic (ammonium group and carboxylic group) which does not interact with the negatively charged carboxylic group of the resin but may have a strong hydrophobic interaction. This hydrophobic interaction favors retention of Semet in the column; it elutes after TmSe but has a shorter retention time than Seet, which is more hydrophobic compared to Semet.

The GS-220 column is capable of separating Se compounds in NIES CRM No. 18 with LC-1 conditions. However, Secys, which has the same re-

Table 1
pK_a values of selenium compounds [29–32]

Selenium compound	pK_a value
Selenous acid	pK_1 2.46; pK_2 7.31
Selenic acid	pK_2 1.92
Selenomethionine (Semet)	pK_1 2.19; pK_2 9.05
Selenoethionine (Seet)	Not available
Trimethylselenonium ion (TmSe)	Cation
Selenocystine (Secys)	pK_1 1.68; pK_2 2.15; pK_3 8.07; pK_4 8.94

tention time as selenite, coelutes with selenite under the chromatographic conditions applied (LC-1). So, separation and identification of both of the compounds (selenite and Secys) under these conditions are not possible. Overlapping of Secys and selenite at different pH values (6.8, 7.2, 7.5 and 7.9) and different mobile phase concentrations (15, 25 and 30 m*M*) prevents the resolution of five Se species in one chromatographic run. Secys is separated from the selenite and other selenium compounds with the LC-SCX cation-exchange column using 20 m*M* pyridinium formate at pH 1.90 as mobile phase (Fig. 3, inset y_1). Secys is not detected in NIES CRM No. 18 (Fig. 3). Peaks of TmSe, selenous acid, selenic acid and other unknown Se compounds in the urine are well separated (Fig. 2) under the LC-1 conditions. Hence, in NIES CRM No. 18, selenite, selenate, TmSe and unknown Se compound peaks are detected. The unknown Se compounds are the predominant selenium species followed by selenite, TmSe and selenate. We have further confirmed the peak identity of Se compounds by spiking the urine with individual and mixtures of standard Se compounds (Fig. 2). The peak of TmSe is confirmed using the LC-SCX cation-exchange column (Fig. 3) and by spiking of standard TmSe in urine. Selenate, Semet, Seet, Secys and TmSe (standards prepared in Milli-Q water) are baseline separated on the cation-exchange LC-SCX column (Fig. 3, inset y_1). Selenite is eluted after 165 s as broad peak under the LC-2 conditions. Therefore, baseline ion signals are higher after 165 s. In urine matrix the baseline is enhanced. Chloride and bromide are eluted in the solvent front and they overlap with selenate (Fig. 3). Therefore, we are unable to separate and determine selenate using the LC-SCX column. TmSe is baseline separated from the rest of the Se compounds (Fig. 3). Individual as well as mixtures of Semet, Seet, and TmSe are spiked to the urine. The peaks are baseline separated. The mixtures of Semet, Seet,

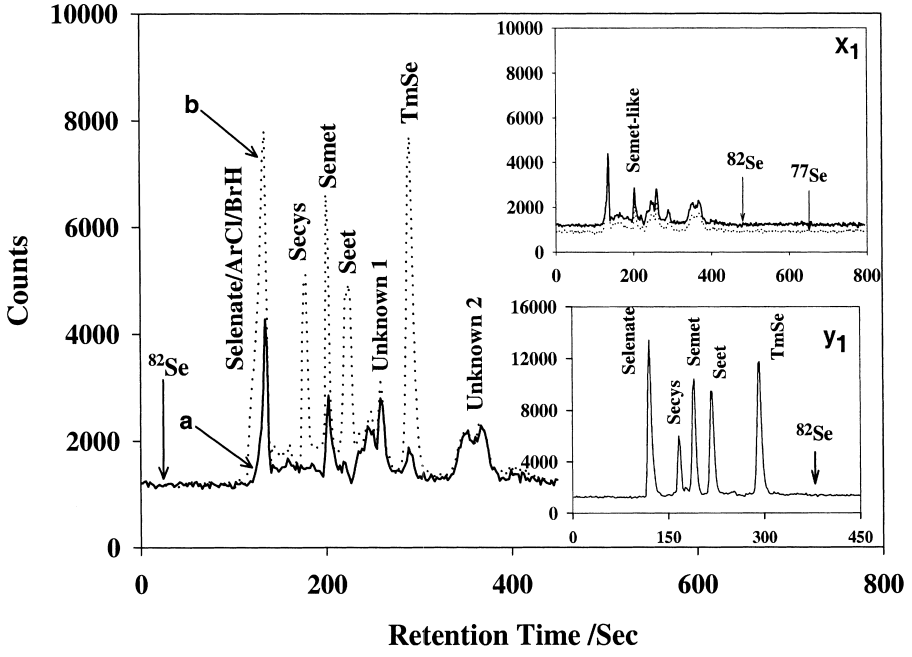

Fig. 3. HPLC–ICP-MS chromatograms obtained with (a) reconstituted NIES freeze-dried CRM No. 18 (1:1 dilution, 100 μl injected; monitoring signal ^{82}Se); (b) urine (1:1 dilution) spiked with 12.0, 11.9, 9.24, 12.2, 11.7 and 12.4 μg l^{-1} of selenate, selenite, selenocystine (Secys), selenomethionine (Semet), selenoethionine (Seet), and trimethylselenonium ion (TmSe) as Se respectively (100 μl injected, monitoring signal ^{82}Se). Inset (x_1), urine (100 μl injected), monitoring isotopes are ^{77}Se and ^{82}Se; Inset (y_1), a solution of selenate, selenite, selenocystine (Secys), selenomethionine (Semet), selenoethionine (Seet) and trimethylselenonium ion (TmSe) (24.0, 23.8, 10.5, 24.4, 23.4 and 24.8 μg l^{-1} Se respectively) in Milli-Q water (injection volume 100 μl, monitoring isotope ^{82}Se); on a LC-SCX cation-exchange column (flow-rate 1.5 ml min^{-1}; mobile phase 20 m*M* pyridine, pH 1.9, LC-2).

and TmSe spiked to the urine are baseline separated too (Fig. 3). Two unknown Se compounds are detected. The unknown Se compounds are probably cationic in nature. One of them even elutes after TmSe (Fig. 3), which is a cationic compound for all pH values. Both peaks of the unknown Se compounds are split (Fig. 3), suggesting that each unknown Se compound may be the mixture of two or more Se compounds which are not baseline separated with LC-2 chromatographic conditions. Moreover, on the GS-220 column we have detected a single broad peak of each unknown Se compound (Fig. 2). On the LC-SCX column under LC-2 conditions with both ion signal modes ^{77}Se and ^{82}Se (m/z 77 and 82), we have detected a peak having the same retention time as that of Semet (Fig. 3 and inset x_1). To confirm the peak identity, we have spiked Semet to the urine. Spiked urine has been injected to the LC-SCX column. Both the Semet-like Se compound and Semet elute with the same retention time in the spiked urine (Fig. 3). Surprisingly, we have not found the peak of Semet in the NIES CRM No. 18 on the GS-220 column under the LC-1 chromatographic conditions (Fig. 2). Further work will be needed to identify the Se compound having an identical retention time as that of Semet on the LC-SCX (LC-2) column in NIES CRM No. 18.

Urine contains numerous inorganic and organic substances that begin to decompose within time [23]. It has been reported that TmSe, Semet and selenate are stable for 2 days. However, Secys is unstable [23] in urine matrix. 60% of Secys is decomposed to other Se compounds (selenate and selenite). Therefore, absence of Secys in the NIES CRM No. 18 is due to the true absence of Secys, which seems most probable, or decomposition of Secys may occur during the preparation and preservation processes that are normally applied for the generation of CRM urine. Furthermore, Secys is not reported in Japanese human urine [14].

3.2. Choice of isotopes and interference study

Major isotopes of selenium are subject to severe interference from the plasma gas Ar in the Ar-ICP-MS. The isotopes of selenium at m/z 77 and 82 are commonly used to monitor the ion signals for the determination of Se. However, signals at m/z 77 and 82 are not free from interference. The molecular ions $ArCl^+$ ($^{40}Ar^{37}Cl^+$) and BrH^+ ($^{81}Br^1H^+$) strongly interfere with the m/z 77 and 82 Se isotopes when the sample contains high chloride and bromide matrixes [13]. Chromatographically, chloride and bromide can be separated from the investigated Se compounds. However, $ArCl^+$ ($^{40}Ar^{37}Cl^+$) and $^{81}Br^1H^+$ will form additional peaks in the chromatograms when m/z 77 and 82 are used for signal monitoring in ICP-MS, respectively. Hence, choice of isotopes to monitor ion signals of Se is important for urine analysis to detect and avoid interference peaks.

During urine analysis, monitoring ^{77}Se mode (m/z 77), an interfering peak appears at the retention time 871 s on the GS-220 column (Fig. 2, insets x and y). However, this peak does not appear simultaneously at m/z 82 (Fig. 2, inset y). Polyatomic interference from $^{40}Ar^{37}Cl^+$ strongly affects ^{77}Se determination in samples having a high chloride matrix such as urine. We have also detected $^{37}Cl^{40}Ar^+$ interference during our study of arsenic compounds in the same NIES CRM No. 18 [24]. To investigate the argon chloride interference with the detection of selenium species in this study, 5020 μg ml^{-1} of chloride (about the same concentration found in the urine) has been analyzed using the same chromatographic conditions. Chloride is monitored as $^{37}Cl^{16}O^+$ ion at m/z 53 and $^{37}Cl^{40}Ar^+$ ion at m/z 77 on the GS-220 column with LC-1 chromatographic conditions. It is eluted at 871 s. The peak of $^{37}Cl^{40}Ar^+$ ion at m/z 77 is shown in Fig. 2, inset x. So, with LC-1 chromatographic conditions, chloride causes an interference problem in the speciation of selenium in urine and ultimately generates an additional peak at 871 s by forming $ArCl^+$. This additional peak overlaps with the TmSe peak at m/z 77 mode (Fig. 2, inset x). Further, using ^{82}Se (m/z 82) ion signal mode, another interfering peak appears at 1162 s (Fig. 2). However, the same peak is not found simultaneously in the m/z 77 mode (Fig. 2, inset y). Several polyatomic interferences are reported for Se at m/z 82. Most of them are $^{81}Br^1H^+$, $^{40}Ca^{42}Ca^+$, $^{40}Ar^{42}Ca^+$, $^{66}Zn^{16}O^+$, $^{64}Zn^{18}O^+$, and $^{41}K^{41}K^+$. In NIES CRM No. 18 the concentrations of Ca, Zn, and K are 190, 0.62 and 1320 μg ml^{-1} respectively [22]. The concentration of Br is about 5.5 μg $ml.^{-1}$ Thus, the probability of interference from Zn is very low.

To identify the interfering signal we have injected separately 210, 1500, and 7.5 μg $ml^{-1}$of Ca, K and bromide solution in Milli-Q water and in urine matrix, respectively, and ion signals at m/z 82 have been monitored. We have found a peak at 1162 s when bromide has been injected. So, Br is eluted at 1162 s in the LC-1 chromatographic conditions and forms an additional peak in the chromatogram at m/z 82 mode by forming $^{81}Br^{1}H^{+}$ (Fig. 2). To confirm the additional peak at 1162 s we have also monitored the ion signals at m/z 79 in the urine (Fig. 2, inset x). A peak of Br at 1162 s, having the same retention time as that of the interference peak, at m/z 82 ($^{81}Br^{1}H^{+}$) is detected. Hence, in the NIES CRM No. 18, bromide causes a strong interference problem in the m/z 82 mode, forming a polyatomic species $^{81}Br^{1}H^{+}$ which generates an additional peak at 1162 s. It is also reported that in human urine [14], the peak of $^{81}Br^{1}H^{+}$ fully overlaps the peak of TmSe [14]. Therefore, careful selection of Se isotopes is essential for the determination of Se compounds in biological samples, especially in human urine having high chloride and bromide matrixes, and at least two isotopes (^{77}Se and ^{82}Se) should be monitored simultaneously so that interference peaks can be detected.

3.3. Quantification of Se compounds in NIES CRM No. 18

The peak of selenate, selenite, TmSe and two unknown Se compounds obtained from NIES CRM No. 18 on the GS-220 column are well separated from each other and also from the BrH^{+} peak (Fig. 2) with LC-1 conditions when the ^{82}Se mode has been monitored. The concentration of selenate, selenite and TmSe, estimated using the standard additions technique, are 2.08±0.14, 10.1±1.21 and 3.42±0.17 μg l^{-1} ($n=5$). Moreover, the verification of the inorganic selenium compounds requires the use of other methods and different chromatographic systems before certification. The unknown Se compounds are the major Se species followed by selenite, TmSe and selenate. The concentration of TmSe has been also calculated by using an external aqueous calibration curve. The estimated value of TmSe (3.68±0.18 μg l^{-1}) is in good agreement with respect to the standard additions technique. The external aqueous calibration curves for Semet, Seet, TmSe, selenate and selenite in Milli-Q water are linear ($r=0.998$–0.999) with concentrations in the range of 2.00–24.9 μg Se l^{-1}. The value of r is [calculated for standard additions methods (SAM)] better than 0.997–0.999, revealing the linear relationship between standards added to the urine and the HPLC–ICP-MS signals. The mean concentration of TmSe, estimated by SAM on the LC-SCX column, is 3.38±0.21 μg l^{-1} ($n=5$). This value of TmSe is consistent with the value that has been obtained using the GS-220 column (3.42±0.17 μg l^{-1}). The peak of TmSe overlaps with the peak of chloride on the GS-220 column when ^{77}Se (m/z 77) isotope has been monitored (Fig. 2, inset x and y). Therefore, we have used ^{82}Se isotope for signal monitoring. The LC-SCX column is used mainly for cross-examination of the value of TmSe that has been obtained using the GS-220 column. We have spiked the standard solution of selenate, selenite, TmSe, Semet and Seet in the urine matrix. Percentage recoveries of the above spiked Se compounds have been measured after separation on the GS-220 column. The recoveries of selenate (94–97%), selenite (101–109%), TmSe (99–106%), Semet (100–102%) and Seet (78–83%) have resulted from the measurements of ^{82}Se mode. This appears satisfactory to ensure that the injected selenium compounds are eluted completely from the column. Memory effects [25] of the column or any other part of the HPLC system may cause the surplus. The Seet peak is not baseline separated, and overlaps the peak of BrH^{+} at m/z 82 (Fig. 2). However, no interference from BrH^{+} at m/z 77 is possible. So, peak overlapping of Seet does not occur in m/z 77 mode, and percentage recoveries of Seet are in the range of (95 –103%). Hence, the BrH^{+} interference problem in m/z 82 mode is removed for m/z 77, and Seet can be determined without peak overlapping (Fig. 2, inset y).

4. Conclusions

A GS-220 (polyvinyl alcohol based resin) column has been used for the determination of Se compounds in NIES CRM No. 18. In NIES CRM No. 18 selenate, selenite, TmSe and unknown Se compounds are detected. The concentrations of selenate, selenite and TmSe are found to be 2.08±0.14, 10.1±1.21

and 3.42 ± 0.17 μg l^{-1}. Further verification of the Se compounds requires the use of other methods and different chromatographic systems before final certification. Chloride and bromide interferences are overcome by careful selection of the selenium isotopes. The GS-220 column is suitable for separation and determination of Se compounds in NIES CRM No. 18. The LC-SCX column is inappropriate with LC-2 conditions for the quantification of selenate. This is due to the selenate peak overlapping with those of chloride and bromide. On the LC-SCX column, a Se compound having the same retention time as that of Semet has been found. However, on the GS-220 column, Semet has not been detected at all. Se compounds are widely used for Se supplementation, anticancer reagents and prevention of heart diseases [26,27]. Determination of Se compounds in urine is the major concern in understanding the metabolic fate and biogeochemical cycling of Se. The growing international concern about environmental contamination by toxic trace elements has led to the implementation of monitoring programmes for controlling Se levels in terrestrial and aquatic environments. Analytical quality assurance of Se compounds in urine is an important and ongoing requirement nowadays. Accordingly, urine CRMs containing Se compounds are immediately needed. No CRMs are available for the quality control of Se species [28]. The results of the present study will be useful as preliminary data for the future certification of selenium species in NIES CRM No. 18.

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