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Reversed-phase liquid chromatography with mixed ion-pair reagents coupled with ICP-MS for the direct speciation analysis of selenium compounds in human urine

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A sensitive and robust method for the determination of five inorganic and organic Se species in human urine by reversed-phase liquid chromatography with mixed ion-pair reagents coupled with inductively coupled plasma mass spectrometry (HPLC-ICP-MS) is described in this paper. A good separation for anionic, cationic and neutral Se species, namely selenate [Se(VI)], selenourea (SeUr), selenomethionine (SeMet), selenoethionine (SeEt), and trimethylselenonium ion ($TMSe^+$), was achieved within 15 min on a LiChrosorb RP 18 reversed-phase column by using mixed ion-pair reagents of 2.5 mM sodium 1-butanesulfonate and 8 mM tetramethylammonium hydroxide, with isocratic elution at a flow rate of 1.0 mL min^{-1}. The detection limits of the five Se species obtained by HPLC-ICP-MS ranged from 0.6 to 1.5 ng Se mL^{-1} using an injection volume of 20 μL. It is noteworthy that the urine sample can be directly injected into the analytical system without any pre-treatment, except a filtration with a 0.45 μm membrane filter, and that the determination of Se species was free from chloride-induced matrix interference. In addition, no serious deterioration in column performance or decrease in the sensitivity of ICP-MS was observed for the experimental period of three months. In Japanese urine samples, no detectable SeMet, SeUr, and SeEt could be found, even if the total Se concentration was higher than 100 ng Se mL^{-1}. On the contrary, $TMSe^+$ and two unknown Se species (U1 and U2) were detected in the urine. The major unknown, U1, was found in all of the measured urine samples, suggesting that it might be one of the important Se metabolites.

Introduction

Selenium (Se) is an essential trace element at low levels of intake and produces toxic symptoms when it is ingested at levels higher than those required for adequate nutrition.[1] Selenium deficiency has been associated with several diseases, *e.g.*, heart diseases and cancer.[2] Recently, it was reported that sufficient selenium supplement can protect against cancer.[3] A variety of Se-enriched materials, such as garlic, yeast, and lactic acid bacteria, have been studied for the purpose of selenium supplementation. With the increased use of Se for dietary supplementation in animals and humans, the chemical forms and quantities of Se in urine are of interest because urinary excretion is the main route of Se elimination and more than 50% is excreted this way. Moreover, the urinary Se speciation may provide information about the Se status of the body, either as a detoxified form or as the metabolite of an essential chemical form.[4] To date, besides the inorganic Se compounds, the only organic Se compound identified conclusively in human urine has been $TMSe^+$.[5,6] Evidence for the presence of selenourea (SeUr) has also been reported.[7] Selenoamino acids, such as selenomethione (SeMet) and selenocystine (SeCys) have been hypothesized as metabolites in the biological pathways of Se.[8,9]

Due to the complex urine matrix and low concentration of Se (normally less than 100 ng mL^{-1}), analytical methods for urinary speciation of Se are very scarce. Earlier methods often required a large volume of urine samples (1–2 l) and employed a series of chemical pretreatment steps, such as precipitation/coprecipitation,[10] thermal decomposition and derivatization of selenoamino acids.[11,12] Such sample treatments may have changed the original Se species present in urine, thus resulting in erroneous results. In recent years, the development of analytical chemistry methods based on the hyphenation of HPLC with ICP-MS have been reported in the literature. In order to separate Se species, different separation modes, such as ion-exchange,[4,13–15] reversed-phase,[16–19] ion-pair reversed-phase[20] and vesicle-mediated chromatography,[19,21] have been examined. Some difficulties, however, have been encountered when the different HPLC methods were applied to urine samples, *e.g.*, the inability to separate inorganic Se species together with organic ones. Some reported methods can only separate two or three Se compounds, either inorganic or organic species, and, in some methods, the conclusively identified metabolite $TMSe^+$ was not considered at all in the separation system.[18,19,21] On the other hand, many reported methods are not robust enough to maintain their separation performance in urine samples. Furthermore, due to the lack of sensitivity, some methods can only work with spiked urine samples.[21,22] In order to minimize urine matrix interference in both the chromatographic separation and the ICP-MS detection steps, solid-phase extraction,[18] ethanolic precipitation[5] and dilution[14–16,19,20,23] sample pretreatment steps have been carried out. However, there is a strong chance that these sample preparations alter the composition of the original sample[24] or cause loss of the analytes of interest. Therefore, analytical methods that are sensitive and highly robust for direct urinary Se speciation analysis are urgently required.

In this paper, we report a sensitive and robust HPLC-ICP-MS method for urinary Se speciation analysis. This method was developed to meet the demand of our ongoing research project on the study of Se metabolism of Se–yeast supplements.

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By using a unique reversed-phase chromatographic separation system with mixed ion-pair reagents,[25,26] in which both anionic (sodium 1-butanesulfonate) and cationic (tetramethylammonium hydroxide) ion-pairing reagents were added simultaneously into an aqueous solution, five Se species, Se(VI), SeUr, $TMSe^+$, SeMet, SeCM, and SeEt, could be directly analyzed in human urine samples. Due to its remarkable analytical characteristics, such as low detection limits and highly matrix tolerance, this method provides a powerful tool for the study of Se metabolism and for the dose control of ingested Se nutritional supplements.

Experimental

Instrumentation

The ICP-MS instrument used was a HP 4500 (Yokogawa Analytical Systems, Tokyo, Japan). The sample introduction system used included a Scott-type spray chamber fitted with a concentric nebulizer. The chromatographic system consisted of a JASCO PU-1580I (metal-free) intelligent HPLC pump (Japan Spectroscopic Corporation), a syringe-loading injector (Model 9725i, Rheodyne six-port injection valve) with a 20 μL loop and a LiChrosorb RP 18 reversed-phase column (Seibersdorf, Austria, 250 × 4.6 mm id, 5 μm particle size). The chromatographic system was interfaced with the ICP-MS instrument using 120 mm of PEEK (polyether ether ketone) capillary tubing (0.25 mm id) to connect the column outlet to the inlet hole of the nebulizer. The column was conditioned by passing at least 100 mL of the mobile phase through the column before injection of the Se standards and samples. The chromatographic results were processed using Chromatosoftware (Yokogawa Analytical Systems, Tokyo, Japan). Quantifications were performed in the peak area mode. For the determination of total Se concentration, flow injection analysis (FIA) with the same instrumentation used for HPLC-ICP-MS, but without a HPLC column, was employed. A solution of 1% HNO_3 was used as a carrier with a flow rate of 1.5 mL min^{-1} and a sample loop of 100 μL was used for FIA-ICP-MS. The optimized operating conditions for HPLC-ICP-MS were the same as those previously described.[26] To achieve the best sensitivity, the nebulizer gas flow rate was optimized specifically with 5 ng mL^{-1} Se(IV) standard solution. Two selenium isotopes, ^{77}Se and ^{82}Se, were monitored.

Chemical and reagents

All commercial chemicals were of analytical-reagent grade and were used without further purification. Sodium selenate [Se(VI)] was purchased from Nacalai Tesque Inc. (Kyoto, Japan) and seleno-DL-ethionine (SeEt), seleno-DL-methionine (SeMet), seleno-DL-cystine (SeCys), selenocystamine dihydrochloride (SeCM), and selenourea (SeUr) from Sigma. Trimethylselenonium iodide ($TMSe^+$) was obtained from Tri Chemical Laboratory (Yamanashi, Japan). Sodium 1-butanesulfonate (98%) was obtained from Aldrich. Tetramethylammonium hydroxide (10% in water) was purchased from Merck. Malonic acid, HNO_3 (70%), NH_4OH (29%) and methanol (99.9%) were obtained from Kanto Chemical, Co. (Tokyo, Japan).

Stock solutions were prepared with Milli-Q water (18.3 MΩ cm) (Mill-Q SP ICP-MS, Millipore, Tokyo, Japan) according to the procedures described previously.[26] A Spex (Metuchen, NJ, USA) plasma standard solution (1000 g mL^{-1}) of selenite [Se(IV)] was also used as a stock solution. The stock solutions were stored in a refrigerator at −20 °C before use. No degradation of the compounds was observed over 3 months of storage.[27] Working solutions in the ng mL^{-1} range were prepared daily by appropriate dilution of the stock solutions. The pH of the mobile phases was adjusted by dropwise addition of dilute nitric acid or 20% NH_4OH. All mobile phases were filtered through 0.45 μm nitrocellulose membrane filters (Millipore, Bedford, USA) and degassed before use.

Urine samples

The urine samples of nine non-exposed laboratory members (seven males and two females) were collected in the morning of the day when the speciation analysis was carried out. The samples were filtered using a 0.45 μm GL chromatodisc filter (Kurabou, Okayama, Japan) and 20 μL of the filtrates were injected for HPLC-ICP-MS analysis. For the determination of total Se concentration by FIA-ICP-MS, an aliquot of the filtered urine sample was diluted (1 + 4) with Milli-Q water, and then a standard of Se(IV) was added to the dilute urine with concentrations of 5, 15, and 30 ng mL^{-1} (10, 30, and 50 ng mL^{-1} for NIST SRM 2670 elevated urine). Freeze-dried urine standard reference material (NIST SRM 2670) was analyzed for validation of the developed analytical system. This standard reference urine was reconstituted by the addition of 20.0 mL of Milli-Q water and used immediately.

Results and discussion

Analytical method development for urinary Se speciation analysis

A powerful separation system is indispensable for urinary Se speciation analysis. We developed a reversed-phase chromatographic separation system with mixed ion-pair reagents for the speciation analysis of eight Se compounds of environmental and biological importance, namely, Se(IV), Se(VI), SeCys, SeUr, $TMSe^+$, SeMet, SeCM, and SeEt (Fig. 1(a)). Details of the separation mechanism and optimization of chromatographic parameters were described in our previous work.[25,26] Due to its wide range of pK_a values (2.83, 5.69),[28] malonic acid was selected as a buffer solution in our chromatographic system. An investigation of the pH dependence of the retention time of Se compounds[25] revealed that the retention time of Se compounds were stable in the pH range from 3.8 to 5.5 and, therefore, a pH of 4.5 for the mobile phase was selected. This chromatographic system was characterized for the separation of eight Se compounds in a single run by good resolution, high efficiency (symmetrical peak with high theoretical plate number) and high stability (the relative standard deviation of the peak retention times was always less than 1%).[26]

Due to the fact that the major isotopes of Se at *m*/*z* 78 (23.8%) and 80 (49.6%) are subject to severe interferences from the argon dimers $^{40}Ar^{38}Ar^+$ and $^{40}Ar_2{}^+$, Se isotopes ^{77}Se and ^{82}Se are normally used for ICP-MS determination. When the chromatographic system was coupled with ICP-MS, a better signal-to-background ratio was obtained by using *m*/*z* 77 detection instead of the commonly used *m*/*z* 82. This is because sulfur and the impurity bromine, a result of the synthetic procedure of using an ion-pair reagent, were included in the mobile phase, resulting in a background increase at *m*/*z* 82 ($^{34}S^{16}O_3{}^+$ and $^{81}Br^1H^+$). Therefore, the signal obtained with *m*/*z* 77 was used for all quantification processes.

A high concentration of Cl^- in human urine (average concentration 5000 μg mL^{-1})[24] is the most troublesome problem for HPLC-ICP-MS speciation analysis. To evaluate the possible influence of Cl^- on the chromatographic separation and on ICP-MS detection ($^{40}Ar^{37}Cl^+$ and $^{12}C^{35}Cl_2{}^+$ interfere in the detection of Se at *m*/*z* 77 and 82, respectively), Se species standards were prepared in a series of NaCl solutions (with concentrations of 125, 250, 500, 1000, 2000 and 5000 μg mL^{-1} Cl^-) and in a urine sample collected from a volunteer in our laboratory. They were then injected into the HPLC-ICP-MS analytical system. The chromatograms obtained are shown in Fig. 1. It was found that there was no effect on the separation of eight Se species when the Cl^- concentration was less than

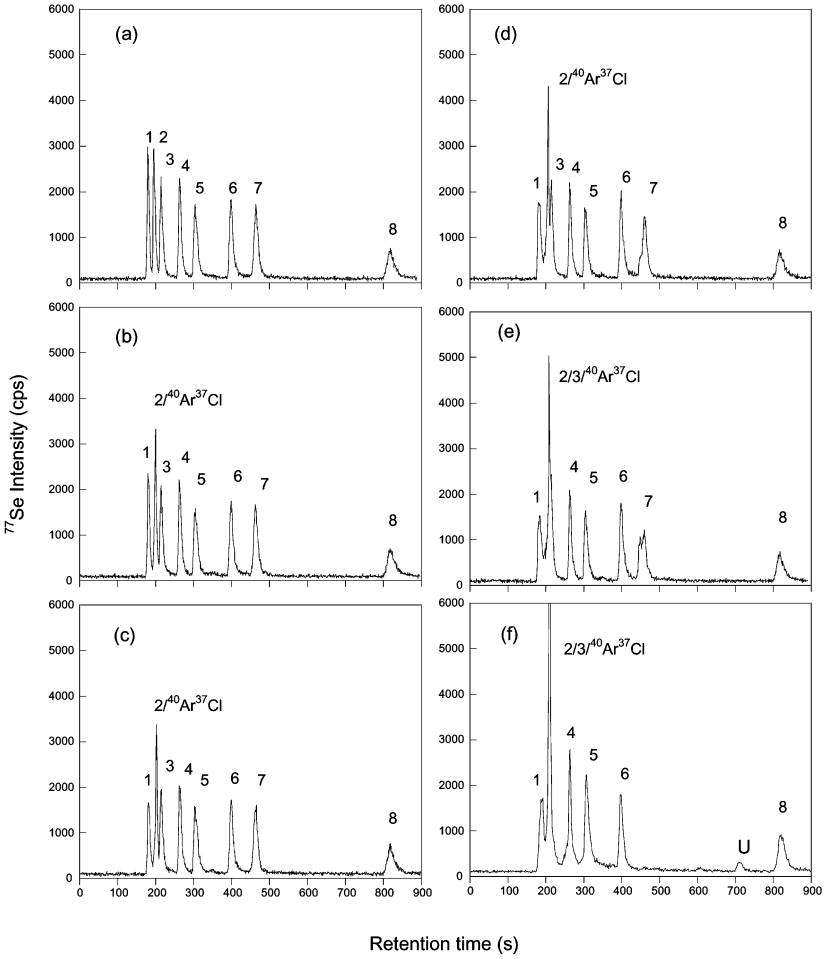

Fig. 1 Matrix effect of NaCl on the determination of Se species in a urine sample. Eight Se standards (25 ng mL^{-1} each) were prepared in: (a) aqueous solutions; (b) 500 μg mL^{-1} (NaCl); (c) 1000 μg mL^{-1} Cl^- (NaCl); (d) 2000 μg mL^{-1} Cl^- (NaCl); (e) 5000 μg mL^{-1} Cl (NaCl); and (f) undiluted urine. A Se isotope of *m*/*z* 77 was monitored by ICP-MS. Peaks labelled 1–8 correspond to Se(VI), Se(IV), SeCys, SeUr, $TMSe^+$, SeMet, SeCM and SeEt, respectively. U = Unknown Se species in a native urine sample.

1000 μg mL^{-1}; no retention time shift was observed for the eight Se species. Chloride was found to be co-eluted with Se(IV), which resulted in an increase of the peak area of Se(IV) signal due to the contribution from the $^{40}Ar^{37}Cl^+$ signal. When the Cl^- concentration increased to 2000 μg mL^{-1} (Fig. 1(d)) the retention time of SeCys decreased, and shifted towards Se(IV); when the Cl^- concentration increased further up to 5000 μg mL^{-1} (Fig. 1(e)), SeCys completely co-eluted with Se(IV) and Cl^-. Due to the high Cl^- concentration the peak shape of SeCM became deformed, and the peak of Se(VI) became broader. However, for the species SeUr, $TMSe^+$, SeMet, and SeEt, the results indicated that they were free from interference in the whole concentration range of Cl^- investigated and in the real urine matrix. It was noted that the SeCM signal disappeared in the urine matrix (Fig. 1(f)) due to the decomposition of SeCM. In order to evaluate the reliability of the developed HPLC-ICP-MS system, recovery tests in urine were also carried out. The obtained recoveries [mean ± *s* (%), *n* = 3] for the added 50 ng mL^{-1} Se species were: Se(VI) = 97.3 ± 6.5; SeUr = 93.6 ± 4.5; $TMSe^+$ = 98.4 ± 7.2; SeMet = 104 ± 8; and SeEt = 101 ± 6. Due to the co-elution of Se(IV) and SeCys with Cl^-, and the instability of SeCM in a urine matrix, the recovery tests were not performed for these three Se species. Therefore, the developed HPLC-ICP-MS system was found to be suitable for the determination of Se(VI), SeUr, $TMSe^+$, SeMet and SeEt in basal human urine samples. After filtration on a 0.45 μm filter, there was no need for any sample pretreatment, which makes it possible to obtain original Se species information in human urine. Under optimum conditions, the analytical characteristics of the developed HPLC-ICP-MS system with respect to the detection limits, the linear range of calibration curve and the repeatability were evaluated using Se standard solutions. The results are summarized in Table 1. The calibration curves (in the range of 0–100 ng Se

Table 1 Analytical characteristics of the developed method

Species	Concentration DL[a]/ng mL^{-1}	Absolution DL/pg	Repeatability[b] (% RSD)	Correlation coefficient[c] (R^2)
Se(VI)	0.6	12	3.8	0.9999
SeUr	1.1	22	1.5	0.9948
$TMSe^+$	0.9	18	3.2	0.9999
SeMet	1.2	24	3.3	0.9999
SeEt	1.5	30	2.3	0.9999

[a]Detection limits were determined as the elemental concentration that provides a signal three times the standard deviation (n = 7) of a 5 ng mL^{-1} standard solution. [b]Repeatability was determined by calculating the relative standard deviation (% RSD) of three successive measurements; concentration of each analyte was 50 ng mL^{-1} as Se. [c]Correlation coefficients (R^2) were evaluated based on peak area.

Table 2 Determination of total Se and Se metabolites in human urine

Sample[a]	$TMSe^+$/ ng Se mL^{-1}	U1/ng Se mL^{-1}	U2/ng Se mL^{-1}	Total Se[c]/ ng Se mL^{-1}	$TMSe^+$/ total Se (%)	U1/total Se (%)
1 (M)	5.4 ± 0.9	10.1 ± 0.5	ND[b]	92 ± 6	5.9	11
2 (F)	15.4 ± 2.2	44.3 ± 1.2	10.1 ± 1.5	156 ± 7	9.9	28.4
3 (M)[d]	8.0	7.0	ND	73 ± 13	11.0	9.6
4 (M)	26.5	5.1	ND	120 ± 6	22.1	4.3
5 (M)	12.6	14.7	4.5	140 ± 9	9.0	10.5
6 (M)	7.6	2.9	ND	32 ± 2	23.6	9.1
7 (M)	11.3	7.3	ND	85 ± 6	13.3	8.6
8 (F)	15.8	18.4	2.9	109 ± 12	14.5	16.9
9 (M)	8.9	9.9	ND	118 ± 12	7.5	8.4

[a]M = male; F = female. [b]ND, not-detected. [c]Total Se was determined by FIA-ICP-MS with the three-points standard addition method. [d]The speciation analysis results of samples 3–9 are the mean of duplicate determinations.

mL^{-1}) gave good linearity with correlation coefficients from 0.9948 to 0.9999. The repeatability for the five Se species at a concentration level of 50 ng Se mL^{-1} ranged from 1.5% to 3.8%. Detection limits ranged from 0.6 to 1.5 ng Se mL^{-1} for concentration detection limits (conc. DL) and from 12 to 30 pg for absolute detection limits (abs. DL). These detection limits are believed to be sufficiently low for direct speciation analysis of Se in urine samples. The reported concentrations (mean ± standard deviation, ng mL^{-1}) of Se in human urine from population studies are 38.6 ± 7.2 from a German population,[29] 28.7 ± 8.3 and 65.8 ± 21 from US studies[30,31] and 104.7 ± 51.7 from Japan.[32]

Determination of Se species in human urine samples

In order to evaluate the applicability for practical analysis, the developed HPLC-ICP-MS method was applied to the speciation of Se in Japanese urine samples. To establish the mass balance of Se, the total Se concentration in the studied urine samples was determined by an FIA-ICP-MS method similar to that reported by Yang and Jiang.[20] As a measure of quality control for the analysis, a human urine reference material, NIST SRM 2670 normal and elevated levels, was analysed. This reference material is certified for total Se concentrations (ng Se mL^{-1}) as 30 ± 8 for normal level and 460 ± 30 for elevated level, respectively. Our results, 31 ± 3 for normal level and 464 ± 23 for elevated level, are consistent with the certified values. From the results summarized in Table 2, it can be seen that the total Se concentration (ng Se mL^{-1}) ranged from 32 to 156, resulting in a mean (ng Se mL^{-1}) of 103 ± 37 for nine urine samples. These results are in good agreement with those reported by Hojo[32] for the Japanese general population. For speciation analysis, 20 μL of urine was injected into the HPLC-ICP-MS analytical system. The results obtained are summarized in Table 2 and the representative chromatograms obtained with sample 2 are shown in Fig. 2. The peak identification was made according to the retention time of the authentic Se standards and the standard spiking experiment. At least five Se species were observed in sample 2, although $TMSe^+$ was the only Se species that could be identified in the urine sample. Two trace unknown Se species with retention times of 255 s and 486 s (indicated with arrows) and two major unknown species U1 (with a retention time of 728 s) and U2 (with a retention time of 937 s) were found in sample 2. Due to the low concentrations of the trace unknown Se species, quantification was not carried out for them. The major U1 species was detected in all nine urine samples, while U2 was seen in 3 out of 9 samples. Although we could not identify the U1 species at this stage, it seems to be a major Se metabolite, and the final identification of this species should provide a better understanding of the metabolism of Se, especially when a high content of Se is ingested through the food chain. It should be noted that no detectable Se(VI), SeMet, SeUr and SeEt were observed in any of the urine samples. These results indicate that the unknown Se species in urine samples reported previously[12,20] could not be simply attributed to selenoamino acids, although selenoamino acids, such as SeMet and SeCys, were hypothesized as being the metabolites in the biological pathways of Se,[8,9] and excreted in urine if ingested in excess.

As $TMSe^+$ was the only conclusively identified organic Se metabolite in human urine, it is proposed as a bioindicator to provide information about the status of Se in the body.

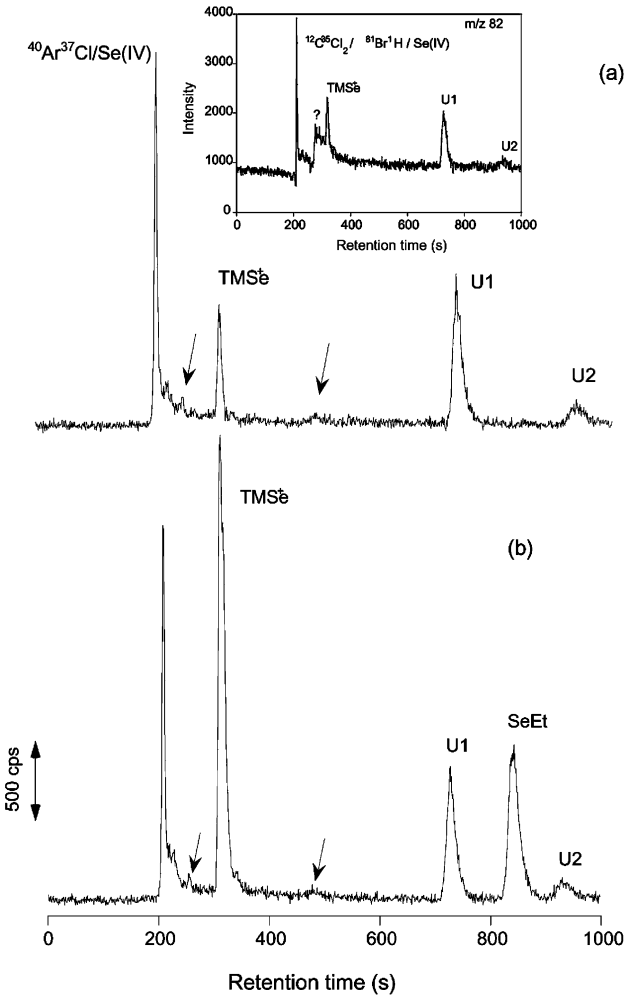

Fig. 2 Representative chromatograms of a urine sample: (a) native urine sample; (b) native urine sample spiked with $TMSe^+$ and SeEt. A Se isotope of *m/z* 77 was monitored by ICP-MS. The chromatogram of the same sample obtained at *m/z* 82 is shown as an inset in (a). The determination of Se(IV) was hampered by spectral interferences induced by $^{12}C^{35}Cl_2^+$ and $^{81}Br^1H^+$. A high background level and a sudden rise in the chromatographic baseline before the elution of $TMSe^+$ were observed due to the urinary matrix and the sulfur-containing mobile phase.

However, it is still a matter of discussion whether $TMSe^+$ is a major or minor metabolite in urine. It has been reported that $TMSe^+$ contents in urine vary between less than 1 and 80% of the total selenium content.[4,14,31] In our study, it was found that the urinary $TMSe^+$ content ranged from 5.9 to 23.6% of the total Se content, while the content of the major unknown species, U1, ranged from 4.3 to 28.4% of the total Se content. Apparently, $TMSe^+$ is not a major Se compound in normal human urine. No significant relationship could be established between the total Se level, the $TMSe^+$ level or the U1 level.

The urine reference material, NIST SRM 2670 (normal and elevated levels) was also analyzed for the Se species profile although it is only certified for total Se concentration. Fig. 3 shows the chromatograms obtained. Besides the peak for $^{40}Ar^{37}Cl$/Se(IV), three trace Se peaks could be detected in the normal level; one of these trace peaks was identified as $TMSe^+$, while the other two could not be identified. According to the certificate of analysis issued by NIST, the elevated level urine is normal human urine spiked with selected metals, and therefore a similar species profile as for the normal level could be expected except for the additional spiked inorganic Se. Surprisingly, four new unknown Se compounds (indicated with question marks in Fig. 3(b)) were observed, besides the same Se species detected in the normal level (indicated with arrows in Fig. 3(b)). Considering that the concentration of $TMSe^+$ increased from a concentration close to the detection limit in the normal level urine to 7.4 ± 0.8 ng mL^{-1} in the elevated level urine, it was obvious that the observed unknown species in the elevated level were not the native Se species in human urine, but the *in-vitro*-formed products of reactions between the spiked Se and the urine matrix. These results strongly indicated that the stability of native Se species in urine should be considered and the speciation analysis should be performed as early as possible after sample collection.

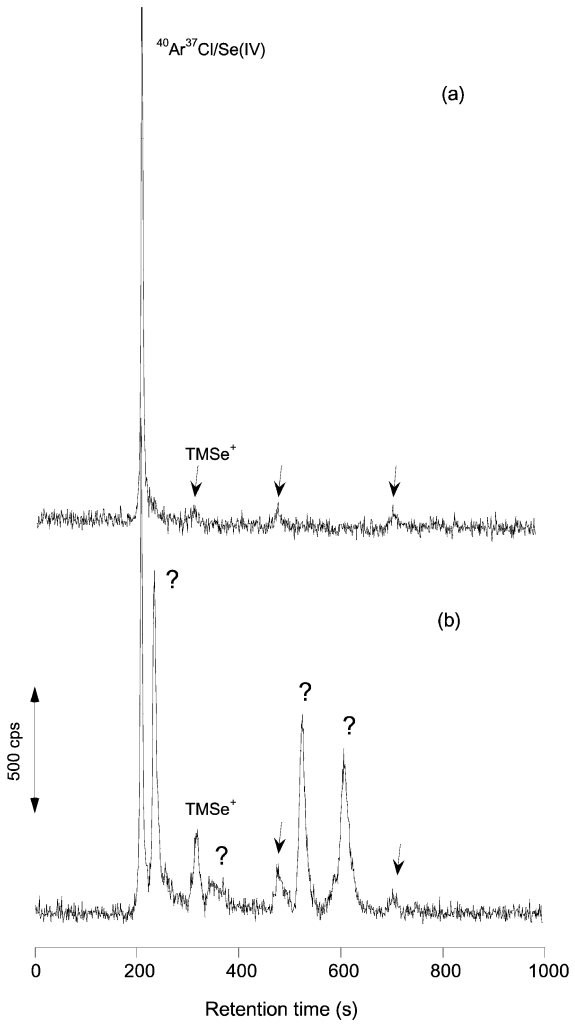

Fig. 3 Chromatograms of the urine reference material (NIST SRM 2670): (a) normal level; (b) elevated level. The Se isotope *m/z* 77 was monitored by ICP-MS.

Conclusion

The analytical challenge in urinary elemental speciation is to develop methods with excellent sensitivity and selectivity, good matrix tolerance, and good chromatographic resolution in combination with a reasonable analysis time. In this work, a method based on the hyphenation of reversed-phase chromatography with mixed ion-pair reagents and ICP-MS was developed to meet the analytical challenge for Se speciation analysis in human urine. This method is capable of the determination of Se(VI), SeUr, $TMSe^+$, SeMet, and SeEt in a native urine matrix. It is remarkable for the robustness of the developed method; no deterioration of column separation performance, decrease in sensitivity of ICP-MS, or clogging of the sampling cone was observed for the experimental period over three months. After filtration with a 0.45 μm disk, there is no need for any sample pretreatment prior to analysis. In the Japanese urine samples, although the total concentration of Se is high due to the frequent ingestion of seafood, no detectable selenoamino acids such as SeMet and SeEt, and SeUr, could be observed. In addition, $TMSe^+$ was found not to be the major Se metabolite in the urine. It is of interest that two major unknown Se species (U1 and U2) were detected. U1 was detected in all of the measured urine samples, suggesting that this Se species is an important Se metabolite. The final identification of this unknown Se metabolite will provide a better understanding of Se metabolism. The structural identification of the U1 species with electrospray mass spectrometry (ES-MS) is in process. This developed HPLC-ICP-MS method has been applied to the study of the metabolism of Se–yeast supplements and to the dose control of ingested Se supplements.

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