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Detection of selenium-containing biological constituents by high-performance liquid chromatography–plasma source mass spectrometry

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Abstract

Mass spectrometry with inductively coupled argon plasma excitation (ICP-MS) was used as a multi-element specific detection method for HPLC for the speciation of selenium (Se) in biological samples. Se-containing biological constituents were separated on a size-exclusion column and were detected Se-specifically at *m/z* 78 and 82 for natural abundance ^{78}Se and ^{82}Se, respectively. Se peaks not identical with known authentic samples were detected. Diets with different Se contents induced changes in the distributions of Se-containing constituents more in urine, kidney and liver samples than in plasma and red blood cells samples. The results indicate that HPLC–ICP-MS is a specific and sensitive means for the speciation of Se-containing biological constituents.

1. Introduction

Selenium (Se) in the body exists mainly into two forms, inorganic and organic. The valence state of the former Se is cationic (Se^{6+} and Se^{4+}), zerovalent (monomeric, Se^{0}) or anionic (Se^{2-}). The cationic Se behaves in the body as negatively charged oxo acids [selenate (SeO_4^{2-}) and selenite (SeO_3^{2-})], whereas the anionic Se forms inorganic selenides, the most typical being mercury (II) selenide (HgSe) [1]. On the other hand, the organic Se behaves as a neutral or positively charged moiety in diverse biomolecules of different sizes, from low-molecular-mass monomethylselenol (CH_3SeH) to high-molecular-mass selenoproteins. In the biological system, the inorganic Se is easily reduced by its intrinsic ionic potential from selenate to selenite, and then to the key form in Se metabolism, selenide (Se^{2-}). The selenide is consecutively methylated and detoxified to monomethylselenol, dimethyl selenide [$(CH_3)_2Se$] and finally to trimethylselenonium ion [$(CH_3)_3Se^+$] [2,3]. The selenide is incorporated into selenoproteins such as selenoprotein P [4–9] and cellular glutathione peroxidase (cGPx) [10,11] through selenocysteine [12–16]. Further, selenide reacts with a heavy metal, mercury, to form the less toxic mercury (II) selenide [1,17]. Se in the body is thus present in diverse chemical forms.

Although Se is an essential element [18,19], excessive exposure to it is toxic, and it is known that the dietary concentration range between deficiency and excess is extremely narrow [20].

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This suggests that the Se pool in the body may be relatively small and a deficient or excessive state of Se in the body would be reflected more sensitively by changes in the chemical forms of Se in body fluids and organs. Therefore, speciation of Se in biological samples should provide more information on the nutritional and biological states in Se in the body than only the total concentration of Se, and may be a good candidate for the development of sensitive and specific biological markers for the Se pool in the body. However, at present there are no sensitive analytical methods for specifying Se present in diverse chemical forms in biological samples.

Se can be detected by flame and flameless atomic absorption spectrometry and by atomic emission spectrometry. Recent developments in mass spectrometry with inductively coupled argon plasma excitation (ICP-MS) have made it possible to detect most elements with greater sensitivity than by absorption or emission spectrometry. However, Se is not as sensitive as other elements owing to its high ionization potential, even by ICP-MS.

In-line coupling of metal-specific detection with high performance liquid chromatography (HPLC) has been shown to be a powerful method for the determination of metal-binding proteins in biological samples. Atomic absorption spectrometry (AAS) and atomic emission spectrometry with inductively coupled argon plasma excitation (ICP-AES) have been used as effective speciation methods for elements by employing HPLC–AAS [21] and HPLC–ICP [22]. ICP-MS has also been used as a specific multi-element detection method for HPLC to speciate iron (Fe) and nickel (Ni) in the body [23] and for liquid chromatography (LC) to speciate Se [24,25].

The purpose of this study was to apply HPLC–ICP-MS techniques to Se speciation in biological samples. Conditions for the detection of Se by HPLC–ICP-MS were defined and the method was examined to develop a Se-specific biological marker for the Se pool in the body. In addition, the distribution of iron, copper, and zinc are also reported to show the feasibility of the simultaneous detection of these elements using the proposed technique.

2. Experimental

2.1. Reagents and diets

Selenite and selenate (Kanto Chemical, Tokyo, Japan) were of analytical-reagent grade. Trimethylselenonium iodide (TRI Chemical Laboratory, Kanagawa, Japan) was a gift from Dr. K. Nakamuro (Setsunan University). Constituents in a commercially available Se-free diet (Oriental Yeast, Tokyo, Japan) were as follows: torula yeast 30%, sucrose 56.7%, lard 5%, liver oil 2%, mineral mixture (g per 100 g of mixture: $CaHPO_4$ 14.56, KH_2PO_4 25.72, NaH_2PO_4 9.35, NaCl 4.66, Ca lactate 35.09, Fe citrate 3.18, $MgSO_4$ 7.17, $ZnCO_3$ 0.11, $MnSO_4 \cdot 4\text{–}5H_2O$ 0.12, $CuSO_4 \cdot 5H_2O$ 0.03, KI 0.01) 5%, vitamin mixture (per 100 g of mixture: vitamin A 46 600 IU, vitamin D_3 23 300 IU, vitamin E 1.2 g, vitamin K_3 6.0 mg, vitamin B_1 59 mg, vitamin B_2 59 mg, vitamin B_6 29 mg, vitamin B_{12} 0.20 mg, vitamin C 588 mg, biotin 1.0 mg, folic acid 2.0 mg, calcium pantothenate 235 mg, nicotinic acid 294 mg, inositol 1.176 g, choline chloride 15 g, adjusted to 100 g with lactose) 1%, DL-methionine 0.3%.

2.2. Animals

Female Wistar rats were purchased at 4 weeks of age from a breeder (Clea Japan, Tokyo, Japan). After feeding a normal diet (CE-2, Clea Japan) and tap water ad libitum for 1 week, each rat was fed an Se-deficient diet (Se-deficient diet), an Se-deficient diet fortified with sodium selenite to 0.2 μg/g (Se-adequate diet) or a 2.0 μg Se/g diet (Se-excessive diet) for 3 weeks. The animals were killed by exsanguination under ether anaesthesia.

2.3. Preparation of tissue samples for HPLC–ICP-MS

Blood was centrifuged at 1200 *g* for 10 min at 2°C to separate plasma and blood cells. Blood cells, liver and kidneys in each rat were homogenized in 3–4 volumes of extraction buffer (50 m*M* Tris–HCl buffer, pH 7.4) using a Polytron homogenizer (Kinematica, Lucerne,

Switzerland) in a nitrogen atmosphere. The homogenates were centrifuged at 105 000 *g* for 60 min at 0°C for the resultant supernatants.

2.4. Analyses by HPLC–ICP-MS

HPLC with an LC-10AD system (Shimadzu, Kyoto, Japan) was employed for the initial separation of biological species of elements. A 0.1- or 0.2-ml aliquot of plasma or tissue supernatant was applied to a size-exclusion column (Asahipak GS-520, exclusion limit M_r 300 000, particle size 9 ± 0.5 μm, 500×7.6 mm I.D.; Showa Denko, Tokyo, Japan) and the column was eluted with 50 m*M* Tris–HCl buffer (pH 7.4) at a flow-rate of 1 ml/min. The column used for similar aliquots of urine samples was Asahipak GS-320 (Showa Denko), with an exclusion limit of M_r 40 000. The eluate was introduced directly into the nebulizer tube of an ICP-MS instrument (PMS 2000; Yokogawa Analytical Systems, Musashino, Japan), operated under the following conditions: forward power 1300 W, reflected power <10 W, plasma gas (Ar) flow-rate 14.5 l/min, auxiliary gas (Ar) flow-rate 1.1 l/min, nebulizer gas (Ar) flow-rate 0.9 l/min, sampling cone (Cu) diameter 1.0 min and skimmer cone (Cu) diameter 0.5 mm. The parameters for setting the ion lens were adjusted daily to optimize the detection sensitivity.

3. Results

The purpose of this study was to develop an HPLC–ICP-MS method for the speciation of Se in biological samples. However, three additional elements were also determined together with Se and the distributions of iron, copper and zinc are also shown. Each element was detected by two atomic mass units (m/z) of the respective isotopes, but only one of the two chromatograms with fewer ghost peaks (see below) is shown for the three elements other than Se. The chromatograms of the three elements are shown only for samples from rats fed an Se-deficient diet because there were no differences in the distributions of these three elements among the three dietary groups.

Peaks detected in only one of the two chromatograms were designated as ghost peaks. For example, an Se peak at 17.2 min in the plasma profile (Fig. 1) was detected only on a chromatogram of m/z 82 (^{82}Se) and not in the chromatogram of m/z 78 (^{78}Se), and therefore was considered a ghost peak. For Se, the most abundant isotope, ^{80}Se (natural abundance 49.8%), could not be used for detection by MS, because of the presence of a molecular argon peak at m/z 80, hence ^{78}Se (23.5%) and ^{82}Se (9.2%) were used. As for the peaks due to the $^{40}Ar^{42}Ca^{+}$, we did not detect the ghost peaks as represented by their absence, where ghost peaks should have been present in the Ca profile.

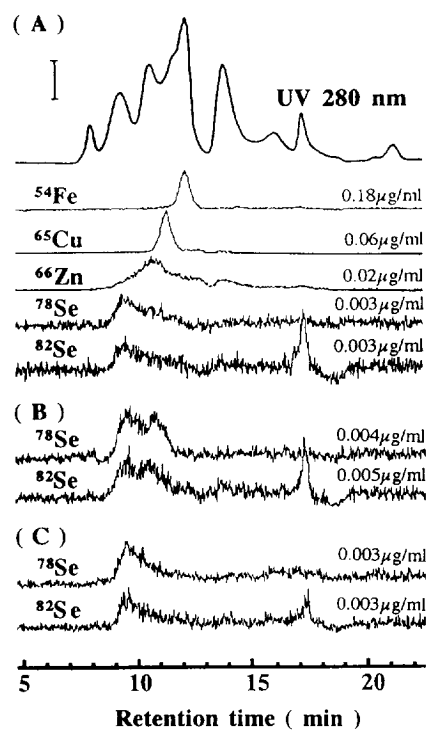

Fig. 1. Element-specific profiles obtained by HPLC–ICP-MS for Se, Fe, Cu and Zn in plasma. Plasma was obtained from rats fed an (A) Se-deficient, (B) Se-adequate or (C) Se-excessive diet for 3 weeks. Distribution profiles of Se and the other elements were determined on a GS-520 column by HPLC–ICP-MS. Profiles of the other three elements are shown only for plasma of the Se-deficient group. The vertical bar on the top left denotes the detection level for each metal with corresponding value indicated on the right-hand side of each chromatogram.

3.1. Distributions of Se-containing constituents in plasma

Plasma proteins were separated mainly into two fractions under the present conditions; globulins eluted faster than a retention time of 13.0 min, while the elution of albumin requires more than 13.0 min [26]. The peaks in Fig. 1 are assigned from the element-specific profiles of various macromolecules, e.g., mercaptoalbumin (MAlb, UV absorption at 14.0 min), transferrin (Tf, Fe peak at 12.2 min) and ceruloplasmin (Cp, Cu peak at 11.2 min) [26].

A broad Se peak was detected at 9.4 min in all chromatograms of plasma obtained from rats fed on the Se-deficient, -adequate and -excessive diets for 3 weeks (Fig. 1A, B and C, respectively). This broad Se peak was eluted at the same retention time as α_2-macroglobulin in the UV profile. Although a broad peak was detected at 10.5 min in Fig. 1B, it was not detected in the other two profiles. An Se peak at 17.2 min was detected only in the profile of ^{82}Se and not in that of ^{78}Se, indicating that this peak was a ghost peak. There were no apparent dose-dependent differences in the Se distribution profiles.

3.2. Distributions of Se-containing constituents in the soluble fraction of red blood cells (RBC)

An intense peak of haemoglobin was detected at 13.0 min from the Fe profile in the soluble fraction of RBC (Fig. 2). Two Cu peaks eluted, at 11.2 and 12.6 min; the former peak was detected at the same retention time as Cp in plasma (Fig. 1), while the latter was accompanied by Zn and eluted at a position corresponding to Cu,Zn-superoxide dismutase (SOD). The most intense Zn peak at 13.4 min represents carbonic anhydrase (CA).

A single peak was detected at 12.1 min on the two chromatograms of Se for the three diet groups (Fig. 2A, B and C). The biological half-life of RBC is far longer than the present experimental period, and therefore the effect of diet on the Se profile may not appear in the RBC in accordance with the present observations.

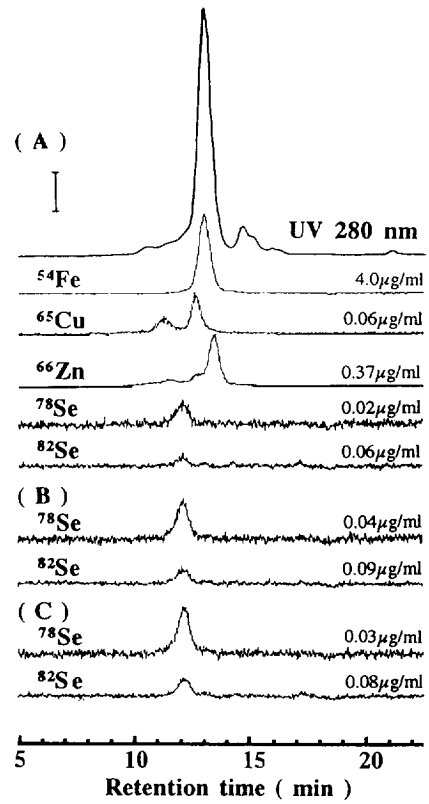

Fig. 2. Element-specific profiles obtained by HPLC–ICP-MS for Se, Fe, Cu and Zn in the supernatant fraction of red blood cells. RBC were obtained from rats fed on (A) Se-deficient, (B) Se-adequate or (C) Se-excessive diet for 3 weeks. For other details, see Fig. 1.

3.3. Distributions of Se-containing constituents in the soluble fraction of liver

An intense peak of haemoglobin was detected at 13.0 min on the Fe profile because the liver was used without perfusion. A broad Fe peak was detected at 10.5 min in the Fe profile, which was designated as ferritin in Fig. 3. A Cu peak of Cu,Zn-SOD was detected at 12.6 min together with a peak corresponding to metallothionein (MT) at 14.0 min. Both Cu peaks were accompanied by Zn, but the Zn peak of Cu,Zn-SOD was hidden by the Zn peaks of alcohol dehydrogenase (ADH) and other Zn proteins, whereas a Zn peak of MT was eluted as a small shoulder peak [27].

A dose-related change in the distribution pro-

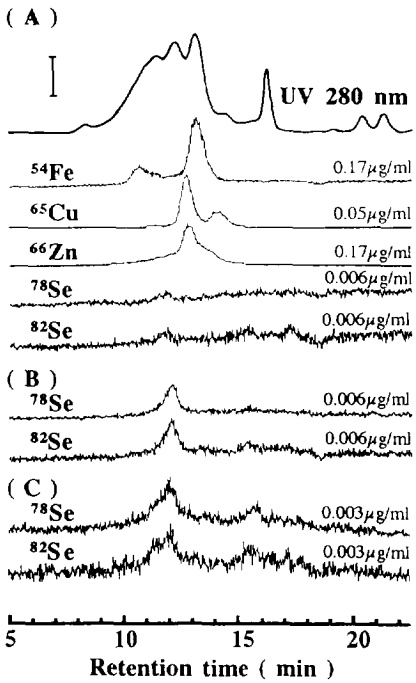

Fig. 3. Element-specific profiles obtained by HPLC–ICP-MS for Se, Fe, Cu and Zn in the supernatant fraction of livers. Livers were obtained from rats fed an (A) Se-deficient, (B) Se-adequate or (C) Se-excessive diet for 3 weeks. For other details, see Fig. 1.

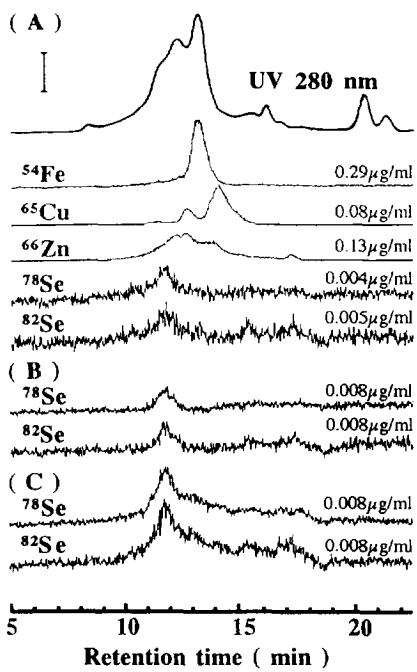

Fig. 4. Element-specific profiles obtained by HPLC–ICP-MS for Se, Fe, Cu and Zn in the supernatant fraction of kidneys. Kidneys were obtained from rats fed an (A) Se-deficient, (B) Se-adequate or (C) Se-excessive diet for 3 weeks. For other details, see Fig. 1.

files of Se in the liver was observed in Fig. 3A, B and C. The Se peaks at 11.8–12.0, 15.5 and 17.2 min increased in intensity with increasing Se concentration in the diets.

3.4. Distributions of Se-containing constituents in the soluble fraction of kidneys

An intense peak of haemoglobin was detected at 13.0 min in the Fe profile because the kidneys were used without perfusion (Fig. 4). A Cu peak of Cu,Zn-SOD was detected at 12.6 min together with the corresponding Zn peak. The Cu peak of MT at 14.0 min was larger in the kidney profile than in the liver profile, in accordance with the abundant Cu,Zn-MT in the kidneys [28].

A dose-dependent change in the broad peak at 11.8 min was observed in the Se distribution of the kidney profiles (Fig. 4).

3.5. Distributions of Se-containing constituents in urine

Urine was analysed on a column appropriate for the separation of low molecular-mass compounds (Fig. 5). The profiles were different from those in Figs. 1–4. An intense Fe peak was detected at 11.2 min, while Cu was eluted as a broad peak around 13.6 min and Zn was detected at the same retention time as Fe. None of these peaks were identified.

The pattern of Se in urine profiles, shown in Fig. 5A, B and C, changed with the Se concentration in the diet. A broad Se peak at 13.8 min increased in intensity with increasing Se dose. This peak seems to contain ghost peak(s) because the $^{78}Se/^{82}Se$ ratio was not of natural abundance. The tiny Se peak at 16.0 min in the ^{82}Se profile and at 15.5 min in the ^{78}Se profile appear to be ghost peaks. In Fig. 5C, the most

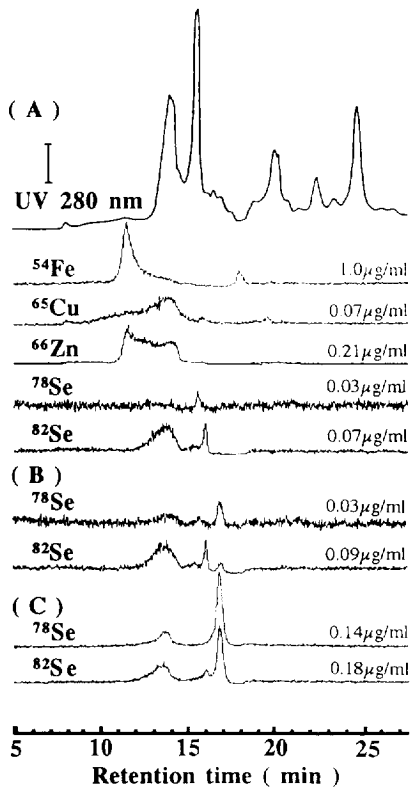

Fig. 5. Element-specific profiles obtained by HPLC–ICP-MS for Se, Fe, Cu and Zn in urine. Urine was obtained from rats fed an (A) Se-deficient, (B) Se-adequate or (C) Se-excessive diet for 3 weeks. Distribution profiles of Se and the other three elements were determined on a GS-320 column by HPLC–ICP-MS. Profiles of the other three elements are shown only for urine of the Se-deficient group. For other details, see Fig. 1.

intense peak was a sharp Se peak at 16.9 min, suggesting that this was a metabolite excreted into urine owing to excessive Se uptake. This sharp Se peak was absent from the profile of urine obtained from rats fed an Se-deficient diet (Fig. 5A).

4. Discussion

The presence of excessive ions causes the main interference in MS detection and buffers containing low concentrations of inorganic ions and organic substances that produce inorganic ions after burning by the ICP have to be selected for elution. Buffers showing less interactions with metals are necessary for the separation of metal-binding proteins, indicating that functional groups with efficient chelating abilities are not desirable. Further, the column materials should contain less functional groups for metals, and resins coated on copolymers rather than silica gels are therefore more suitable for the present purpose. From these considerations, two size-exclusion columns of a copolymer type were used for the separation of urine (GS-320 column) and the other samples (GS-520 column), according to the molecular sizes in the samples to be separated. The columns were eluted with Tris–HCl buffer of relatively low concentration because it showed less interactions with metals and low-concentration inorganic ions [21,22]. Although a better separation could probably be achieved under more sophisticated conditions, the present conditions were found to be satisfactory for the detection of Se-containing compounds in biological samples without any further treatment.

Se-containing biological constituents in body fluids and tissue supernatants were separated on a size-exclusion column and detected by in-line ICP-MS. ICP-MS was successful as a sensitive element-specific detection method for Se by HPLC. Ghost peaks arising from ion peaks of the same mass numbers were discriminated by the use of two isotopes for each element.

Although the Se peaks detected in this study were not completely identified, several peaks were assigned from their molecular sizes in the Se profiles for each biological sample. The major Se peak at 9.4 min in the plasma profile appears to be selenoprotein P of M_r 57 000 [4–9], whereas the single Se peak in the RBC profile is most likely cellular glutathione peroxidase (cGPx) [10,11]. The major Se peaks at 12.0 min in the liver and at 11.8 min in the kidneys also appears to be cGPx. An authentic trimethylselenonium ion [29,30] was eluted at 18.8 min on a GS-320 column but was not detected in the present urine profile (Fig. 5). An authentic selenate eluted at 12.3 min on a GS-320 column and at 15.2 min on a GS-520 column, while an authentic selenite

eluted at 14.0 and 16.2 min from these two columns, respectively. A more precise identification of these and additional Se peaks needs to be performed.

Dose-related changes in the distribution profiles of Se-containing constituents were detected with greater sensitivity in the profiles of urine, kidney and liver, while the profiles of plasma and RBC appeared to be less sensitive to the Se pool in the body. However, the RBC profile may reflect the former Se pool before the RBC were synthesized.

The present results indicate that HPLC–ICP-MS can be successfully applied to the detection of Se-containing compounds in biological samples. Chromatograms of Se in the urine, kidney and liver appeared to reflect the Se pool in the body and could be used as a sensitive biological marker specific for Se.

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