ELSEVIER

Journal of Chromatography B, 767 (2002) 301–312

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Identification of a novel selenium metabolite, Se-methyl-*N*-acetylselenohexosamine, in rat urine by high-performance liquid chromatography–inductively coupled plasma mass spectrometry and –electrospray ionization tandem mass spectrometry

Yasumitsu Ogra[a], Kazuya Ishiwata[a], Hiromitsu Takayama[b], Norio Aimi[b], Kazuo T. Suzuki[a,*]

[a]*Department of Toxicology and Environmental Health, Graduate School of Pharmaceutical Sciences, Chiba University, Inage, Chiba 263-8522, Japan*

[b]*Department of Molecular Structure and Biological Function, Graduate School of Pharmaceutical Sciences, Chiba University, Inage, Chiba 263-8522, Japan*

Received 15 October 2001; accepted 26 November 2001

Abstract

The major urinary metabolite of selenium (Se) in rats was identified by HPLC–inductively coupled argon plasma mass spectrometry (ICP–MS) and –electrospray tandem mass spectrometry (ESI–MS/MS). As the urine sample was rich in matrices such as sodium chloride and urea, it was partially purified to meet the requirements for ESI–MS. The group of signals corresponding to the Se isotope ratio was detected in both the positive and negative ion modes at m/z 300 ($[M+H]^+$) and 358 ($[M+CH_3COO]^-$) for ^{80}Se, respectively. These results suggested that the molecular mass of the Se metabolite was 299 Da for ^{80}Se. The Se metabolite was deduced to contain one methylselenyl group, one acetyl group and at least two hydroxyl groups from the mass spectra of the fragment ions. The spectrum of the Se metabolite was completely identical to that of the synthetic selenosugar, 2-acetamide-1,2-dideoxy-β-D-glucopyranosyl methylselenide. However, the chromatographic behavior of the Se metabolite was slightly different from that of the synthetic selenosugar. Thus, the major urinary Se metabolite was assigned as a diastereomer of a selenosugar, Se-methyl-*N*-acetyl-selenohexosamine.

Keywords: Speciation; Selenium; 2-Acetamide-1,2-dideoxy-β-D-glucopyranosyl methylselenide

1. Introduction

Selenium (Se) is known to function as the active center of selenoproteins for redox enzymes such as glutathione peroxidase, 5′-iodothyronine deiodinase and thioredoxine reductase [1–4]. Contrary to other essential metals such as copper and zinc, Se is not bound to proteins as a ligand but incorporated into selenoproteins in the form of the selenol group of selenocysteine (SeCys). Both inorganic and organic Se compounds are nutritionally available for animals [5–8], and both of them are incorporated into

*Corresponding author. Tel./fax: +81-43-290-2891.
E-mail address: ktsuzuki@p.chiba-u.ac.jp (K.T. Suzuki).

1570-0232/02/$ – see front matter
PII: S1570-0232(01)00581-5

selenoproteins as SeCys residues via common metabolic intermediates, selenide (HSe^-) and selenophosphate [9,10]. Namely, inorganic Se compounds, selenite and selenate are simply reduced to HSe^- in red blood cells and liver, respectively [11–17]. Organic Se compounds SeCys, selenomethionine and their derivatives are converted to SeCys, and then HSe^- is released from SeCys by β-lyase [18]. HSe^- forms selenophosphate with ATP, and then the selenol group is transferred to selenoproteins as the gene-products through selenocysteinyl-tRNA [19, 20].

On the other hand, although the mechanisms underlying the incorporation and utilization of Se compounds, i.e. from inorganic and organic Se to selenoproteins, have been well documented, including the chemical forms of Se mentioned above, the mechanisms underlying the excretion of Se and the chemical reactions leading to its excretion are not known. Only a few methylated Se are known to be metabolites when Se is loaded to mammals at toxic levels. With excessive loading of Se, trimethylselenonium (TMSe) and dimethylselenide are excreted as metabolites in the urine and breath, respectively [21,22]. In our recent paper, monomethylselenol (MMSe) was assigned as the major urinary metabolite in rats under physiological conditions [23]. However, MMSe is not an appropriate form of Se as a metabolite, judging from its chemical properties. Although some additional minor Se species can also be detected in urine by utilizing recently developed speciation techniques, major Se metabolites other than TMSe have not been identified yet.

One of the hyphenated techniques, high-performance liquid chromatography–inductively coupled argon plasma mass spectrometry (HPLC–ICP MS), is a powerful and unique technique for elemental speciation from the viewpoints of sensitivity and specificity [24–26]. Another hyphenated technique, HPLC–electrospray ionization mass spectrometry (HPLC–ESI MS) is useful for molecular speciation [27–31]. However, the former technique does not provide any molecular information, and the latter one is less sensitive and less acceptable as to high matrix conditions than the former one. As a result, with the former technique it is difficult to identify unknown compounds, and the latter one is limited to low matrix samples. In fact, the latter has only been applied for yeast and plant cell extracts, which have lower matrix contents than urine.

In the present study, we attempted to identify the major urinary metabolite of Se in rats by adopting the HPLC–ESI tandem MS (HPLC–ESI MS/MS) method for molecular speciation beside the HPLC–ICP MS method. Then, a candidate compound was chemically synthesized.

2. Materials and methods

2.1. Apparatus

A HP4500 inductively coupled argon plasma mass spectrometer (ICP–MS; Yokogawa Analytical Systems, Musashino, Japan) and an API3000 triple quadrupole mass spectrometer (ESI–MS/MS; Applied Biosystems, Tokyo, Japan) equipped with a Turbo Ion Spray® ion source were used. Instrumental conditions of the ICP–MS were as follows: radio frequency (RF) forward power, 1250 W; plasma flow-rate, 15.0 l/min; auxiliary flow-rate, 1.15 l/min; nebulizer flow-rate, 1.0 l/min; dwell time, 300 ms. The ESI–MS/MS was operated in both the positive and negative ion modes. Instrumental conditions of the ESI–MS/MS were as follows: spray voltage, 4200 V; turbo-gas temperature, 450 °C; collision gas (N_2) pressure, 4 units. The two mass spectrometers were also used as HPLC detectors. The HPLC system consisted of an on-line degasser, a HPLC pump, a Rheodyne six-port injector and an appropriate column. The eluate was introduced directly into the nebulizer of the ICP–MS to detect Se at m/z 77 and 82 (HPLC–ICP MS method), or was introduced into the spray of the ESI–MS/MS to obtain a molecular mass spectrum (HPLC–ESI MS/MS method). A Z-8000 atomic absorption spectrophotometer (Hitachi, Tokyo) was used to determine the concentration of sodium in urine.

2.2. Reagents

Dimethyldiselenide and 2-acetamide-2-deoxy-α-D-glucopyranosyl chloride 3,4,6-triacetate were pur-

chased from Acros Organics (Geel, Belgium) and Tokyo Kasei Organic Chemicals (Tokyo), respectively. Urease from Jack beans and a urea nitrogen determination kit were purchased from Wako Pure Chemical Industries, Osaka, Japan. *N*-Acetylgalactosamine (GalNAc) and *N*-acetylglucosamine (GlcNAc) were purchased from Sigma (St. Louis, MO, USA). Other reagents were of the highest grade available.

2.3. Animals

Three male rats of the Wistar strain were purchased from Clea Japan, Tokyo, at 8 weeks of age, and each rat was housed in a plastic metabolic cage. The animals were maintained in an animal facility at a room temperature of 22±2 °C with a light/dark cycle of 12 h/12 h. The rats were fed a standard diet (CE-2; Clea Japan) and tap water ad libitum, and urine was collected each day for 1 week as a control. Then the rats were fed water containing sodium selenite at the concentration of 2 μg Se/ml, and urine was collected after 1 week for 5 weeks.

2.4. Preparation of a partially purified urine sample for HPLC–ESI MS

The major urinary matrices comprising salts and urea were reduced for HPLC–ESI MS/MS analysis as follows. A 12-ml portion of the Se-enriched urine was incubated with 10 mg of urease at 45 °C for 30 min, and then completely dried with centrifuging evaporator (EC-57C; Sakuma, Tokyo). The residual portion was extracted with 5 ml of cold methanol twice, and the resulting extract was dried up with a rotary evaporator (RE-46; Yamato, Tokyo). The residual portion was dissolved in 1 ml of deionized water, and the resulting solution was applied to a preparative HPLC column, Shodex Asahipak GS-520 21F (21.5×500 mm; Showa Denko, Tokyo), after centrifugation at 8000 *g* for 5 min, and then the column was eluted with deionized water at the flow-rate of 3.0 ml/min. The eluate was collected for each 30 s, and the Se concentration in each fraction was determined by ICP–MS. The Se-containing fractions were combined and concentrated to around 10 μg Se/ml with the centrifuging evaporator.

2.5. Synthesis of the candidate selenosugar found in rat urine

A candidate selenosugar indicated by the HPLC–ESI MS/MS data was synthesized as follows. Commercially available 2-acetamide-2-deoxy-α-D-glucopyranosyl chloride 3,4,6-triacetate (**1**) was treated with monomethylselenol, which had been generated in situ by the sodium borohydride reduction of dimethyldiselenide in acetonitrile at room temperature to give the 1-methylseleno derivative (**2**) in a 98% yield. The O-acetyl groups in (**2**) were removed by treatment with a catalytic amount of sodium methoxide in dry methanol at room temperature to produce 2-acetamide-1,2-dideoxy-β-D-glucopyranosyl methylselenide (**3**) (selenosugar) in a 97% yield. The structure was confirmed by the fast atomic bombardment–mass spectrometer (FAB–MS), and ^{1}H- and ^{13}C-NMR spectra.

2.6. Analytical procedures

Twenty-μl aliquots of urine and the partially purified urine metabolite were applied to a Shodex Asahipak GS-320 7G column (7.6×500 mm, with a guard column; Showa Denko), and then the column was eluted with 10 m*M* Tris–HNO_3, pH 7.4, at a flow-rate of 1.0 ml/min. The eluate was introduced into the ICP–MS to monitor Se at *m*/*z* 77 and 82. Ten-μl aliquots of urine and the partially purified urine metabolite were applied to a Shodex Asahipak GS-320HQ column (7.6×300 mm, with a guard column; Showa Denko), and then the column was eluted with 10 m*M* ammonium formate, pH 7.4 or 9.5, at the flow-rate of 0.5 ml/min. The eluate was introduced into the nebulizer of the ICP–MS or Turbo Ion Spray® of the ESI–MS/MS without splitting. The introduction into ESI–MS was time-separated for Se elution using a four-way valve, i.e. the eluate was introduced from 22.5 to 25.0 min and from 22.5 to 27.0 min for the positive and negative modes, respectively. For the ESI–MS/MS analysis of commercial available or synthetic compounds, a 10-μl aliquot of each 10 μg/ml of the samples was infused into the ESI source at the flow-rate of 0.5 ml/min with the same eluent as for the HPLC. Mass calibration and optimization of parameters of both

mass spectrometers were performed daily prior to use.

3. Results

3.1. Partial purification of the Se metabolite in urine

In the urine of rats fed the standard diet and tap water, the major urinary Se metabolite was observed at the retention time of 18.0 min on a GS-320 7G column (Fig. 1, bottom), being tentatively named the peak B material in relation to its precursor metabolite in the liver mentioned in our previous communications [11,32]. The peak B material increased to 20 times in the urine of rats on feeding of water enriched with selenite at the concentration of 2 μg Se/ml ad libitum, although Se metabolites other than the major one did not increase on the feeding of Se-enriched water up to 2 μg Se/ml (Fig. 1, top). These peaks including TMSe appeared for the urine above 2 μg Se/ml (data not shown). Therefore, the urine of rats fed water containing 2 μg Se/ml was used for partial purification of the major metabolite for ESI–MS/MS analysis.

Since massive urinary matrices, especially sodium salts and urea, have to be removed for ESI–MS/MS analysis, the urine was treated with urease, and then the Se metabolite was extracted with methanol and

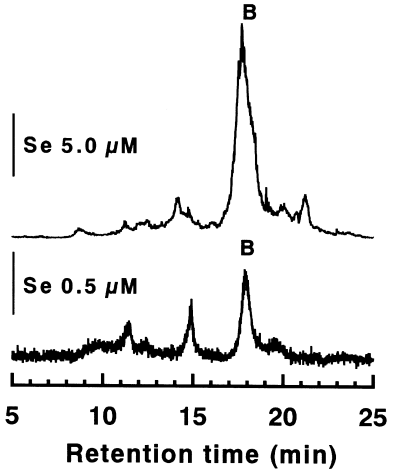

Fig. 1. Elution profiles of Se in urine samples. Urine samples were collected from male Wistar rats drinking deionized water containing 2.0 μg Se/ml (upper) or tap water (lower) ad libitum. A 20-μl aliquot of each sample was applied to a GS-320 7G column, and Se in the eluate was detected with an ICP–MS at m/z 82. Vertical bars indicate the detection level of Se. B, the major urinary Se metabolite.

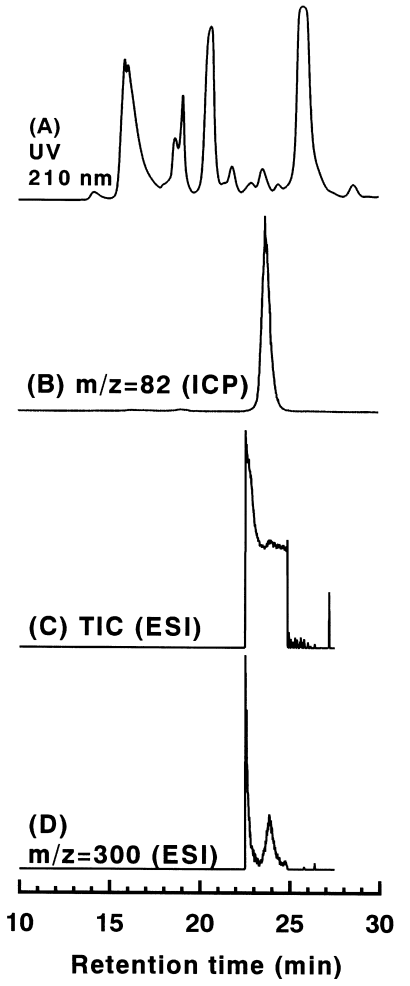

Fig. 2. Chromatograms of partially purified urine with the HPLC–ICP MS and HPLC–ESI MS. Urine was collected from male Wistar rats drinking deionized water containing 2.0 μg Se/ml ad libitum. The urine was partially purified according to our method (see Section 2). Twenty- and 10-μl aliquots of the sample were applied to a GS-320HQ column, and the eluate was directly introduced into the ICP–MS and ESI–MS, respectively. UV absorption of the eluate was monitored at 210 nm (A). Se in the eluate was detected at m/z 82 by ICP–MS (B), and total positive ions (TIC) were monitored with the ESI–MS from 22.5 to 25.0 min (C). The m/z 300 containing ^{80}Se was extracted (D).

separated on a HPLC column, 92% urea and 88% sodium being removed, and the Se concentration being increased to 6.1 times. Furthermore, the peak B material among the urinary Se compounds was specifically concentrated (Fig. 2B).

3.2. HPLC–ESI MS of the major urinary Se metabolite in the positive ion mode

As less matrix was detected around the retention time of the major urinary Se metabolite on elution with the buffer solution of pH 9.5 than of 7.4, the former buffer solution was chosen for the detection in positive ion mode (Fig. 2A). Although the detector of the ICP–MS was open throughout the elution, ions were introduced into the detector of the ESI–MS only during the period when Se was eluted (Fig. 2B and C). Se consists of six isotopes, i.e. ^{74}Se (0.89%), ^{76}Se (9.36%), ^{77}Se (7.63%), ^{78}Se (23.78%), ^{80}Se (49.61%), and ^{82}Se (8.73%). This unique isotope pattern is helpful for searching for signals concerning Se in mass spectra. Signals comprising the isotope pattern of Se were observed at around m/z 300, i.e. the most intensive peak at 300 for the ^{80}Se-containing peak and other peaks on scanning from the retention time of 23.6 to 24.3 min when Se was detected with the HPLC–ICP MS (Fig. 3). The retention time of the peak with m/z 300 in the ESI–MS chromatogram was identical to that of the major urinary Se metabolite detected at m/z 82 in the ICP–MS chromatogram (Fig. 2B and D). Furthermore, although the signals were not well separated from noise, the group of peaks comprising the Se isotope pattern was also detected at around m/z 317 for ^{80}Se. The parent ion at m/z 317 is assignable to the adduct of the major urinary Se metabolite and the ammonium ion of eluent origin. These findings suggest that m/z 300 and 317 correspond to $[M+H]^+$ and $[M+NH_4]^+$, respectively. Therefore, the molecular mass of the major urinary Se metabolite was estimated to be 299.

3.3. HPLC–ESI MS of the major urinary Se metabolite in the negative ion mode

The column was eluted at pH 7.4 in negative ion mode because of less noise than in the positive ion mode and the easier detection of acetylated anions at the neutral pH than the basic one. As expected, signals comprising of the isotope pattern of Se were detected at around m/z 358 for ^{80}Se (Fig. 4). These

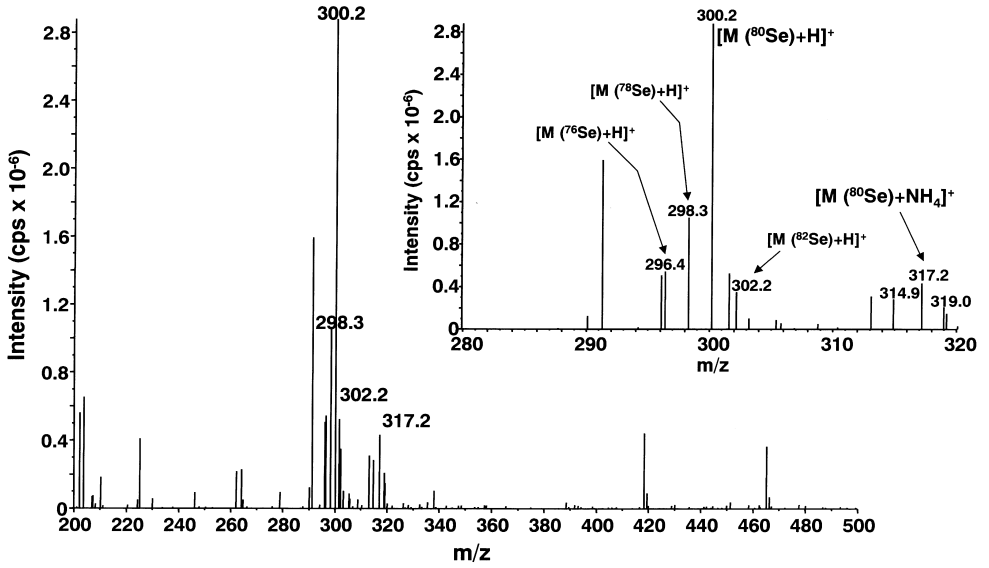

Fig. 3. Electrospray positive mass spectrum of the eluate at retention times from 23.6 to 24.3 min for the partially purified urine sample. A 10-μl aliquot of the sample was applied to a Shodex Asahipak GS-320HQ column and then eluted with 10 m*M* ammonium acetate, pH 9.5. The part of the spectrum containing the Se-characteristic isotope pattern is enlarged in the inset.

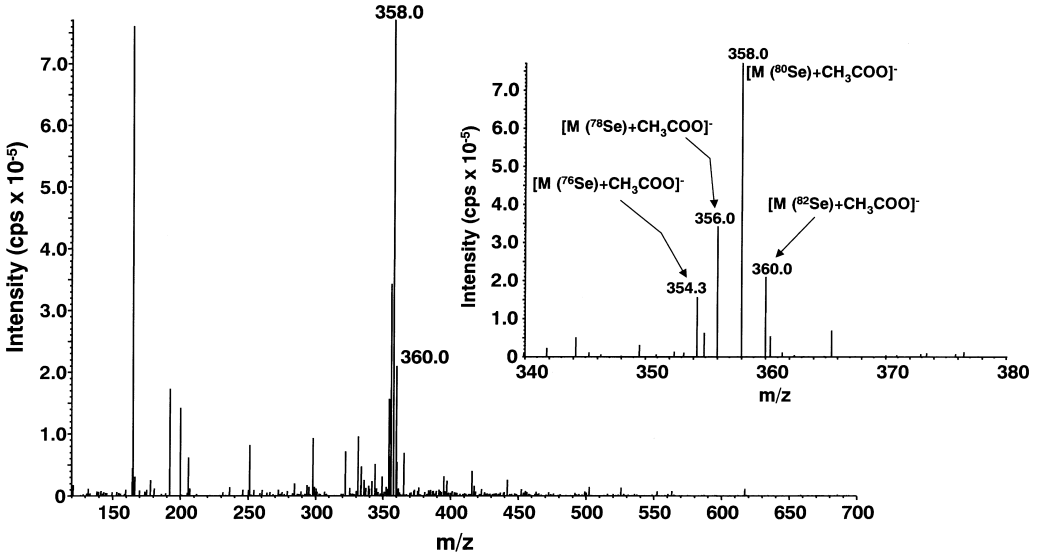

Fig. 4. Electrospray negative mass spectrum of the eluate at retention times from 23.7 to 24.2 min for the partially purified urine sample. A 10-μl aliquot of the sample was applied to a Shodex Asahipak GS-320HQ column and then eluted with 10 m*M* ammonium acetate, pH 7.4. The part of the spectrum containing the Se-characteristic isotope pattern is enlarged in the inset.

signals correspond to $[M+CH_3COO]^-$, confirming that the molecular mass of the major urinary Se metabolite is 299.

3.4. HPLC–ESI MS/MS of the major urinary Se metabolite

To obtain further structural information on the major urinary Se metabolite, HPLC–ESI tandem mass spectrometry was performed in both the positive and negative ion modes. Three kinds of parent ions, 298, 300 and 302, representing the Se isotopes, ^{78}Se, ^{80}Se and ^{82}Se, respectively, were extracted with the first quadrupole (Q1) and then introduced into the second quadrupole (collision cell, Q2). The fragmentation of the parent ions was induced in Q2, and *m/z* of the fragments were determined with the third quadrupole (Q3).

At lower collision energy, all parent ions produced the same major product ion at *m/z* 204, suggesting that a Se-containing moiety was removed from each parent ion (Fig. 5A–C). Since the difference in *m/z* between the parent ion and the fragment ion was 96, the common fragment ion was assumed to result in the removal of CH_3SeH from the parent ion, suggesting a methylselenyl group (CH_3Se–) in the major urinary Se metabolite. Another minor product ion appeared at *m/z* 186 from all parent ions (Fig. 5A–C). This was simply assigned as the fragment ion resulting from loss of H_2O for the major one at *m/z* 204.

At higher collision energy, common fragment ions derived from the parent ion appeared at *m/z* 186, 144, 138 and 126. Although three of the four fragment ions, i.e. 186, 144 and 126, can be assigned as $[186-H_2O]^+$, $[204-CH_3COOH]^+$ and $[144-H_2O]^+$, respectively, the fragment ion at *m/z* 138 has not been assigned yet (Fig. 5a–c, and Table 1). The fragment ion at *m/z* 108 was also detected with 40 eV collision energy (data not shown), and was assigned as $[126-H_2O]^+$.

In the negative ion mode, molecular ions of the major urinary Se metabolite were produced with 20 eV collision energy at *m/z* 356, 358 and 360 as the corresponding adduct ions of acetate with *m/z* 296, 298 and 300, respectively. The fragment ion at *m/z* 202 was assigned as a fragment of the parent ion resulting from removal of the CH_3SeH moiety (Fig. 6A–C). The signals at *m/z* 93, 95, and 97 correspond to $[CH_3^{78}Se]^-$, $[CH_3^{80}Se]^-$, and $[CH_3^{82}Se]^-$, respectively.

Thus the major urinary Se metabolite consists of two hydroxyl groups, one acetyl group and one methylselenyl group. Furthermore, as the molecular

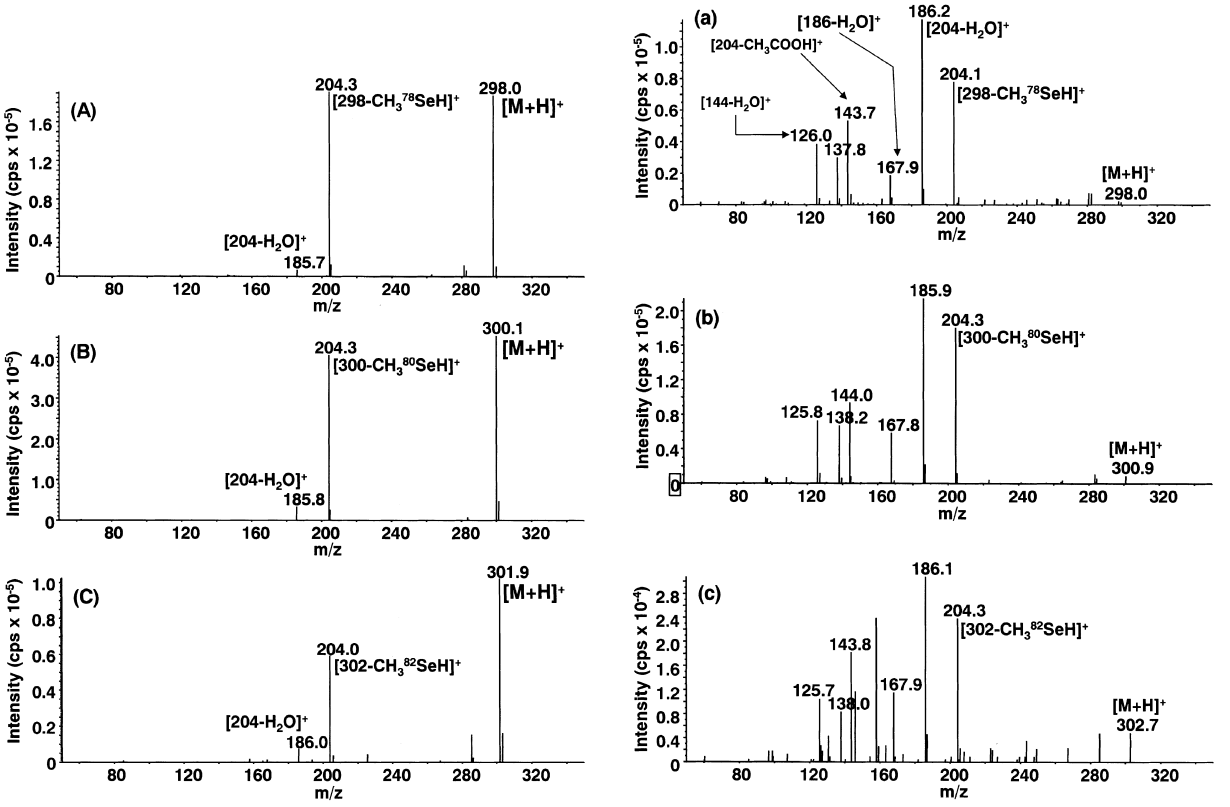

Fig. 5. Collision-induced dissociation mass spectra (ESI–MS/MS) of the Se-containing positive molecular ions in the eluate at retention times from 23.6 to 24.3 min for the partially purified urine sample. Dissociation of each Se-containing molecular ion, i.e. 298 (^{78}Se, panels A and a), 300 (^{80}Se, panels B and b), or 302 (^{82}Se panels C and c), was induced in the collision cell (Q2) with 10 (A, B and C) and 20 eV (a, b and c) collision energy, and then each product ion was detected with the second mass spectrometer (Q3).

Table 1
Assignment of molecular and fragment ions of the urinary Se metabolite and authentic samples

m/z[a]	Urinary Se metabolite[b]	GalNAc[c]	GlcNAc[d]	Synthetic selenosugar[b,e]
358	$[M + CH_3COO]^-$			
317	$[M+NH_4]^+$			$[M+NH_4]^+$
300	$\mathbf{[M+H]^+}$			$[M+H]^+$
298	$\boldsymbol{[M-H]^-}$			
222	n.d.	$[M+H]^+$	$[M+H]^+$	n.d.
204	$[M+H-CH_3SeH]^+$	$[M+H-H_2O]^+$	$[M+H-H_2O]^+$	$[M+H-CH_3SeH]^+$
186	$[204-H_2O]^+$	$[204-H_2O]^+$	$[204-H_2O]^+$	$[204-H_2O]^+$
168	$[186-H_2O]^+$	$[186-H_2O]^+$	$[186-H_2O]^+$	$[186-H_2O]^+$
144	$[204-CH_3COOH]^+$	$[204-CH_3COOH]^+$	$[204-CH_3COOH]^+$	$[204-CH_3COOH]^+$
138	n.a.	n.a.	n.a.	n.a.
126	$[144-H_2O]^+$	$[144-H_2O]^+$	$[144-H_2O]^+$	$[144-H_2O]^+$
108	$[126-H_2O]^{+,f}$	$[126-H_2O]^+$	$[126-H_2O]^+$	$[126-H_2O]^{+,f}$

[a] The molecular ions of the Se metabolite and the negative ions are presented in bold and italics, respectively.
[b] Only ions containing the ^{80}Se-isotope are indicated.
[c] *N*-acetylgalactosamine.
[d] *N*-acetylglucosamine.
[e] 2-Acetamide-1,2-dideoxy-β-D-glucopyranosyl methylselenide.
[f] Data not shown in figures; n.d., not detected; n.a., not assigned.

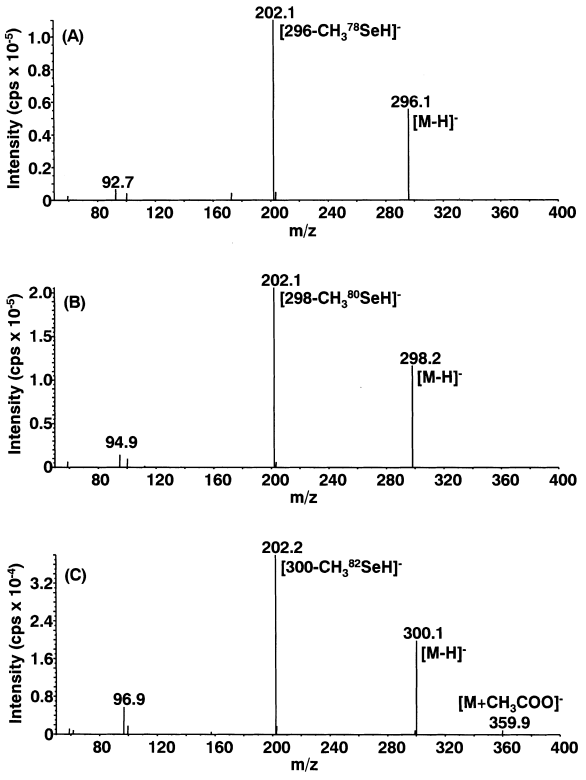

Fig. 6. Collision-induced dissociation mass spectra (ESI–MS/MS) of the Se-containing negative molecular ions in the eluate at retention times from 23.7 to 24.2 min for the partially purified urine sample. Dissociation of each Se-containing molecular ion, i.e. 356 (^{78}Se, panel A), 358 (^{80}Se, panel B), or 360 (^{82}Se, panel C), was induced in the collision cell (Q2) with 20 eV collision energy, and each product ion was detected with the second mass spectrometer (Q3).

mass was an odd number (299 for ^{80}Se), the major urinary Se metabolite was assumed to consist of an odd number of nitrogen atoms. As a result, a methylselenyl *N*-acetylhexosamine derivative was nominated as a candidate compound, although even the primary structure of a methylselenyl *N*-acetylhexosamine derivative has not been determined naturally or artificially. Therefore, several authentic *N*-acetylhexosamine derivatives and our synthetic selenosugar were compared with the major urinary Se metabolite as to the ESI–MS/MS spectra.

3.5. ESI–MS/MS of N-acetylhexosamines and HPLC–ESI MS/MS of the synthetic selenosugar

The fragment ions of GalNAc and GlcNAc were completely identical to those of the major urinary Se metabolite after removal of H_2O or CH_3SeH (Figs. 5 and 7, and Table 1). The HPLC–ESI MS/MS spectrum of the synthetic selenosugar (2-acetamide-1,2-dideoxy-β-D-glucopyranosyl methylselenide) clearly showed identical patterns of molecular and fragment ions to those of the urinary selenosugar (Figs. 5, 8 and 9, and Table 1). These results strongly suggested that the major urinary Se metabolite is a methylselenyl *N*-acetylhexosamine derivative.

The synthetic selenosugar was eluted at a retention time of 24.8 min from a GS-320HQ column with the HPLC–ESI MS (Fig. 8A). This retention time was slightly different from that of the major urinary Se metabolite at 23.9 min (Fig. 2B).

4. Discussion

A selenosugar was shown, for the first time, to be the major Se metabolite in the urine of rats fed a diet containing Se at a non-toxic level. The HPLC–ESI MS/MS data suggested that the major urinary Se metabolite is a derivative of *N*-acetylhexosamine and contains a methylselenyl group. Although the synthetic selenosugar (2-acetamide-1,2-dideoxy-β-D-glucopyranosyl methylselenide) coincided with the major urinary Se metabolite as to the HPLC–ESI MS/MS data, the chromatographic behavior of the synthetic selenosugar was slightly different from that of the major urinary metabolite, suggesting a difference in their configurations. The fragment ion at *m*/*z* 138 was dominant among all fragments for GlcNAc and the synthetic selenosugar at 20 eV collision energy, but not for GalNAc or the urinary selenosugar. In the case of *N*-acetylmannosamine, the dominant fragment ion at 20 eV collision energy appeared at *m*/*z* 126 (data not shown), suggesting the dependence of the dominant fragment ion on the molecular configuration of *N*-acetylhexosamines.

2-Acetamide-2-deoxy-α-D-glucopyranosyl chloride 3,4,6-triacetate was used as a starting material for selenosugar synthesis due to its commercial availability. Since the substitution of the α-chloro group to a β-methylselenyl group was fairly specific, the reaction product was assumed to be of the β-GlcNAc type. Therefore, the distinct chromatographic

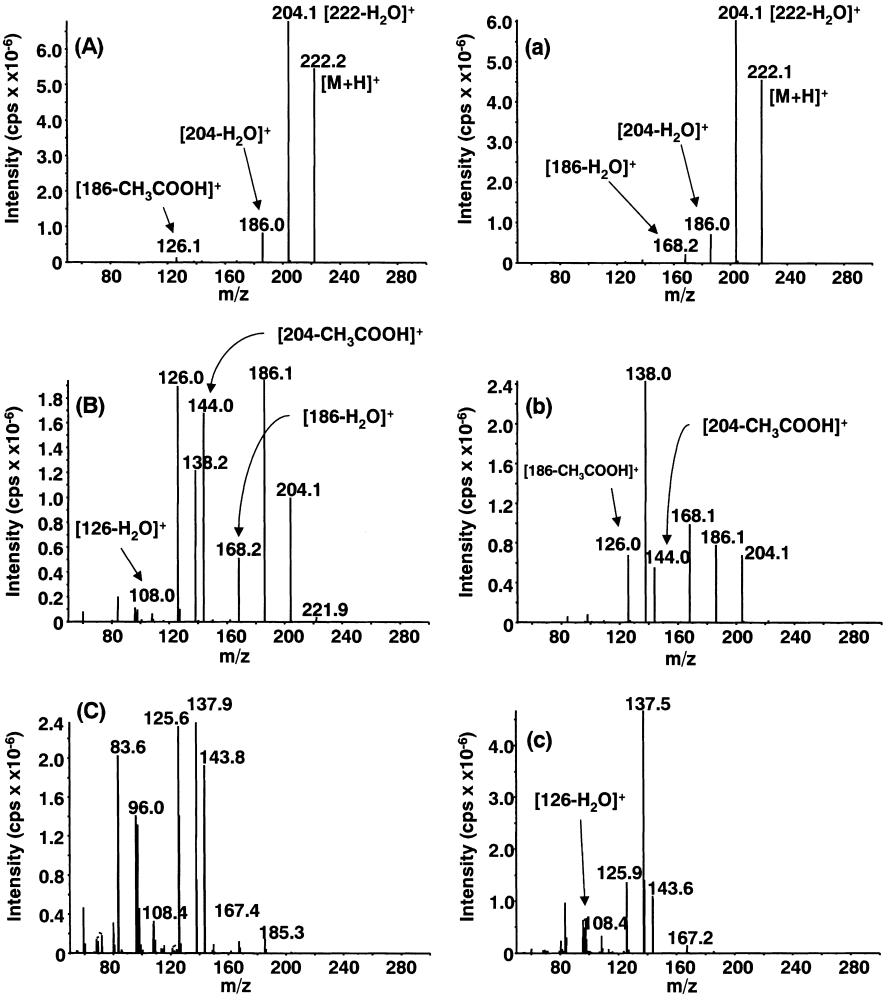

Fig. 7. Collision-induced dissociation mass spectra (ESI–MS/MS) of *N*-acetylgalactosamine and *N*-acetylglucosamine. The dissociation of *N*-acetylgalactosamine (panels A, B and C) and *N*-acetylglucosamine was induced in the collision cell (Q2) with 10 (panels A and a), 20 (panels B and b), and 30 eV (panels C and c) collision energy.

behavior of the urinary selenosugar compared to that of the synthetic selenosugar could be explained by that the urinary selenosugar might be a diastereomer of the synthetic selenosugar. Consequently, the configuration of the urinary selenosugar was speculated to be of the α-GalNAc type.

How can the selenosugar be synthesized biologically? The glucuronide conjugates of hydroxyphenylselenol and phenylselenol are excreted into the urine of mice in response to the administration of diphenyldiselenide at an acute toxic dose (at maximum 500 mg/kg body weight) with the deprivation of glutathione [33]. This metabolic pathway was explained by diphenyldiselenide being reduced to phenylselenol, and then conjugated with uridine diphosphate (UDP) glucuronide with or without hydroxylation. However, this metabolism is quite different from that of the urinary selenosugar in the dose and concentration of glutathione. Furthermore, the ESI–MS/MS data do not support that the urinary selenosugar is a glucuronide conjugate.

Arsenosugars have only been identified as individual metalloidal sugars in nature, and a methylated arsenic, i.e. trimethylarsine oxide, is known to react with *S*-adenosylmethionine to produce a methylated arsenic-containing ribo-

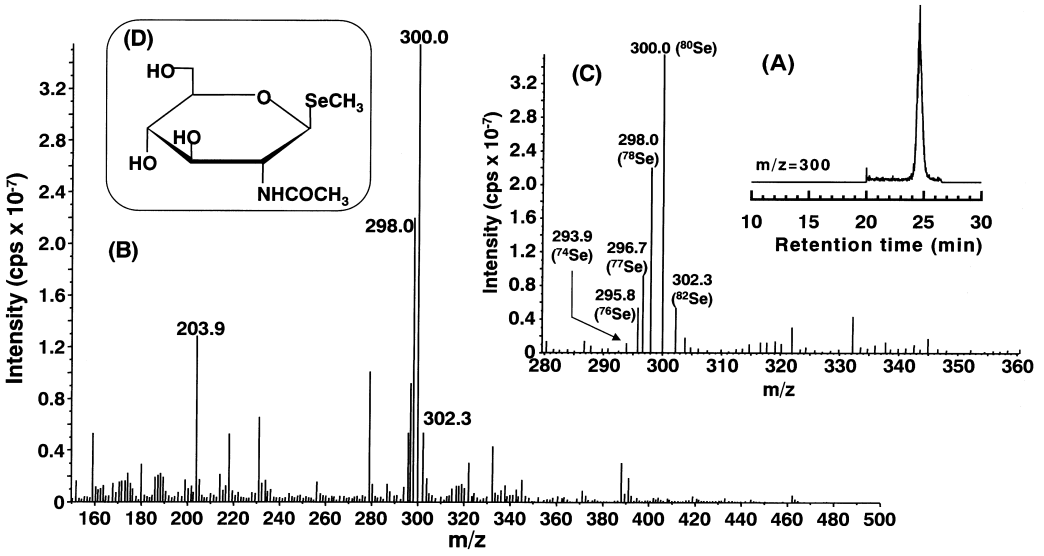

Fig. 8. Elution profile and mass spectrum of the synthetic selenosugar. A 10-μl aliquot of the synthetic selenosugar was applied to a GS-320HQ column and then eluted with 10 m*M* ammonium acetate, pH 9.5, and then the positive ion at *m*/*z* 300 in the eluate was detected with the ESI–MS (A). A 10-μl aliquot of the synthetic selenosugar was directly introduced into the ESI–MS, and scanned at *m*/*z* 150–500 (B). The part of the spectrum containing the Se-characteristic isotope pattern is enlarged in the inset (C). The structure of the synthetic selenosugar, 2-acetamide-1,2-dideoxy-β-D-glucopyranosyl methylselenide (D).

furanoside [34–36]. However, arsenosugars synthesized biologically have not been found in animals, and the sugar moiety (ribofuranose) is quite different from that of the urinary selenosugar (*N*-acetylhexosamine). Therefore, the metabolic pathway leading to the urinary selenosugar may not function for other pathways such as arsenosugars in rats. Similar to other biomolecules containing *N*-acetylhexosamines, UDP-*N*-acetylhexosamines may act as the donor of the sugar and form the urinary selenosugar.

Both inorganic (selenite and selenate) and organic (selenoamino acid derivatives) are metabolized to a common intermediate, selenide, and then utilized for the synthesis of selenoproteins and/or excreted into the urine. When inorganic Se compounds selenite and selenate were injected into rats at non-toxic levels, two major Se metabolites were detected in the liver cytoplasm [11]. One was named the peak B material and identical with the major urinary Se metabolite, while the other was named the peak A material and shown to be the precursor of the peak B material. The peak A material was metabolized to the peak B material with time after the administration, and it was converted chemically to the peak B material with methyliodide [32]. These observations suggest that selenide binds to the sugar at first, and then a methyl group is introduced into the selenosugar. Since selenide is reactive and highly toxic, animals detoxify selenide by transforming it into methylated metabolites or glucuronide [33]. The transformation of the urinary selenosugar may be another way of overcoming the toxicity of selenide as a urinary metabolite. TMSe, which is the ultimate methylated compound of Se, is apparently different from the urinary metabolite under non-toxic conditions. Therefore, the urinary selenosugar may have a biological role other than the detoxification of Se generated via normal metabolism.

Summarizing the present observations, Se-methyl-*N*-acetyl-selenohexosamine (selenosugar) was deduced to be the major urinary Se metabolite by the HPLC–ESI MS/MS method. Further studies for identification of the urinary selenosugar, i.e. determination of the actual configuration of the selenosugar, followed by the metabolic pathway for Se and the biological significance are needed.

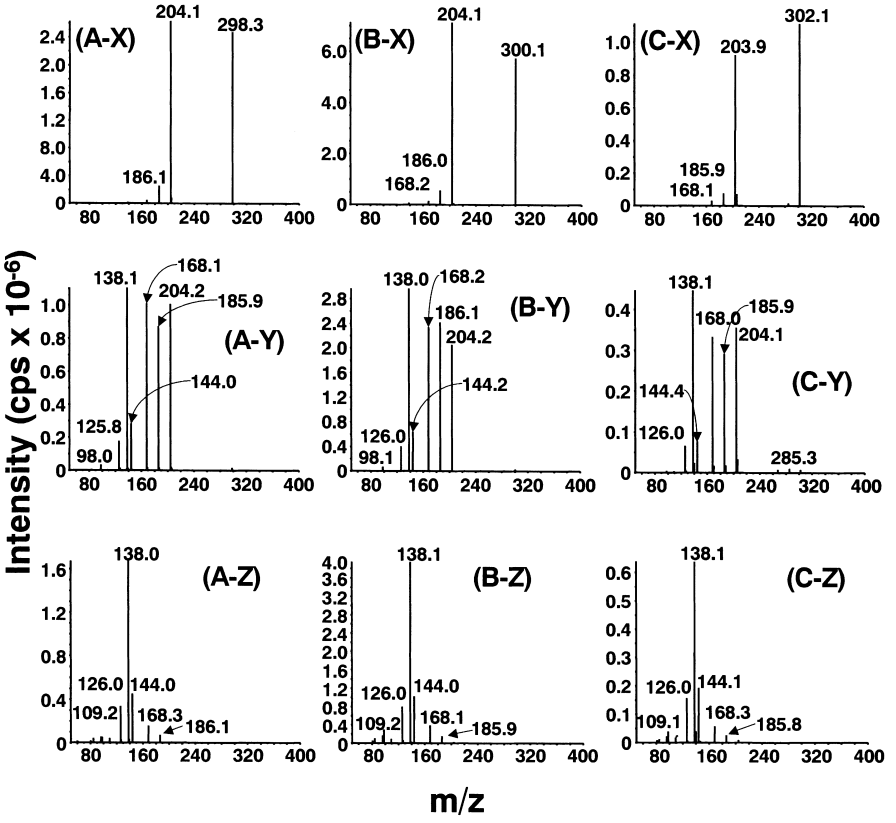

Fig. 9. Collision-induced dissociation mass spectra (ESI–MS/MS) of 2-acetamide-1,2-dideoxy-β-D-glucopyranosyl methylselenide (the synthetic selenosugar). Dissociation of each Se-containing molecular ion, i.e. 298 (^{78}Se, panel A), 300 (^{80}Se, panel B), or 302 (^{82}Se panel C), was induced in the collision cell (Q2) with 10 (panel X), 20 (panel Y), and 30 eV (panel Z) collision energy, respectively, and each product ion was detected with the second mass spectrometer (Q3).

5. Nomenclature

ESI–MS/MS	electrospray ionization tandem mass spectrometry
GalNAc	*N*-acetylgalactosamine
GlcNAc	*N*-acetylglucosamine
HPLC	high-performance liquid chromatography
ICP–MS	inductively coupled argon plasma mass spectrometry
MMSe	monomethylselenol
SeCys	selenocysteine
TMSe	trimethylselenonium

Acknowledgements

The present work was supported by a Grant-in-Aid from the Ministry of Education, Science, Technology, Sports and Culture (grant no. 12470509).

References

[1] W.H. Cheng, G.F. Combs Jr., X.G. Lei, Biofactors 7 (1998) 311.
[2] P.Y. Gasdaska, M.M. Berggren, M.J. Berry, G. Powis, FEBS Lett. 442 (1999) 105.

[3] A.M. Michelson, J. Environ. Pathol. Toxicol. Oncol. 17 (1998) 233.
[4] D.L. St Germain, V.A. Galton, Thyroid 7 (1997) 655.
[5] M.A. Beilstein, P.D. Whanger, J. Nutr. 116 (1986) 1711.
[6] M.A. Beilstein, P.D. Whanger, J. Inorg. Biochem. 33 (1988) 31.
[7] J.A. Butler, P.D. Whanger, A.J. Kaneps, N.M. Patton, J. Nutr. 120 (1990) 751.
[8] N. Esaki, T. Nakamura, H. Tanaka, T. Suzuki, Y. Morino, K. Soda, Biochemistry 20 (1981) 4492.
[9] S.C. Low, J.W. Harney, M.J. Berry, J. Biol. Chem. 270 (1995) 21659.
[10] Z. Veres, L. Tsai, T.D. Scholz, M. Politino, R. Balaban, T.C. Stadtman, Proc. Natl. Acad. Sci. USA 89 (1992) 2975.
[11] Y. Kobayashi, Y. Ogra, K.T. Suzuki, J. Chromatogr. B 460 (2001) 73.
[12] Y. Shiobara, Y. Ogra, K.T. Suzuki, Analyst 124 (1999) 1237.
[13] Y. Shiobara, K.T. Suzuki, J. Chromatogr. B 710 (1998) 49.
[14] Y. Shiobara, T. Yoshida, K.T. Suzuki, Toxicol. Appl. Pharmacol. 152 (1998) 309.
[15] K.T. Suzuki, Y. Shiobara, M. Itoh, M. Ohmichi, Analyst 123 (1998) 63.
[16] K.T. Suzuki, M. Itoh, J. Chromatogr. B 692 (1997) 15.
[17] K.T. Suzuki, M. Itoh, M. Ohmichi, Toxicology 103 (1995) 157.
[18] N. Esaki, T. Nakamura, H. Tanaka, T. Suzuki, K. Soda, J. Biol. Chem. 257 (1982) 4386.
[19] B.J. Lee, S.G. Kang, D.L. Hatfield, J. Biol. Chem. 264 (1989) 9696.
[20] B.J. Lee, M. Rajagopalan, Y.S. Kim, K.H. You, K.B. Jacobson, D.L. Hatfield, Mol. Cell. Biol. 10 (1990) 1940.
[21] J.L. Hoffman, K.P. McConnell, Arch. Biochem. Biophys. 254 (1987) 534.
[22] H.S. Hsieh, H.E. Ganther, Biochim. Biophys. Acta 497 (1977) 205.
[23] M. Itoh, K.T. Suzuki, Arch. Toxicol. 71 (1997) 461.
[24] R. Łobinski, J. Edmonds, K.T. Suzuki, P.C. Uden, Pure Appl. Chem. 72 (2000) 447.
[25] K.T. Suzuki, Analusis 26 (1998) M57.
[26] K.T. Suzuki, S. Yoneda, M. Itoh, M. Ohmichi, J. Chromatogr. B 670 (1995) 63.
[27] H. Chassaigne, V. Vacchina, R. Łobinski, Trends Anal. Chem. 19 (2000) 300.
[28] M. Kotrebai, J.F. Tyson, E. Block, P.C. Uden, J. Chromatogr. A 866 (2000) 51.
[29] J. Szpunar, Analyst 125 (2000) 963.
[30] M. Kotrebai, S.M. Bird, J.F. Tyson, E. Block, P.C. Uden, Spectrochim. Acta B 54 (1999) 1573.
[31] R. Łobinski, J. Szpunar, Anal. Chim. Acta 400 (1999) 321.
[32] K.T. Suzuki, K. Ishiwata, Y. Ogra, Analyst 124 (1999) 1749.
[33] W.J. Adams Jr., J.J. Kocsis, R. Snyder, Toxicol. Lett. 48 (1989) 301.
[34] W.R. Cullen, X.C. Li, S.A. Pergantis, G.K. Eigendorf, L.G. Harrison, Chemosphere 28 (1994) 1009.
[35] J.S. Edmonds, K.A. Francesconi, Experientia 43 (1987) 553.
[36] J.S. Edmonds, M. Morita, Y. Shibata, J. Chem. Soc. Perkin Trans. 1 (1987) 577.