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Article

Selenoneine is methylated in the bodies of mice and then excreted in urine as Se-methylselenoneine

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28 **Abstract**

29 Oral intake of purified selenoneine and seafoods has been reported to result in
30 selenoneine accumulation in erythrocytes in mice and human. In addition, Se-
31 methylselenoneine was suggested to be produced as a metabolite of selenoneine in the
32 urine and whole blood of humans. In order to confirm the molecular mechanism of
33 production of Se-methylselenoneine, a stable isotope (Se-76) labeled selenoneine was
34 biosynthesized using genetically modified fission yeast and administered to mice. The
35 Se-76-labeled Se-methylselenoneine was detected in urine but Se-78 and Se-80-labeled
36 Se-methylselenoneine arising from natural isotopes of Se was hardly detected. These
37 results suggest that Se-methylselenoneine was a metabolite and the excreted form of
38 selenoneine. The methylation of selenoneine in mice administered selenoneine
39 continuously was evaluated by the analyses of organs using an online liquid
40 chromatograph system with an inductively coupled plasma mass spectrometer (LC-
41 ICP-MS). These experiments indicate that selenoneine is methylated in the liver and
42 (or) kidneys.

43

44 **Keywords**

45 selenium, erythrocyte, urine methylation, isotope

46

Introduction

Selenoneine is an organic selenium (Se) compound discovered in the blood of bluefin tuna (Fig. 1a, b) [1]. It has stronger radical scavenging activity versus that found for ergothioneine, which is a sulfur analog of selenoneine [1]. This compound is contained in seafoods, such as tuna, mackerel and beluga skin, and is additionally found in erythrocytes of people who daily eat these seafoods [1-4]. Incorporation of selenoneine from these seafoods is due to the organic cation/carnitine transporter-1 (OCTN1), which is a known transporter in animals for ergothioneine [5, 6]. OCTN1 is expressed in many organs and cells, for example small intestine and kidney, and ergothioneine is distributed and accumulates in these organs and cells [7, 8]. Thus, while the distribution of selenoneine in the body is predictable, the actual specific distributions have yet to be experimentally determined *in vivo*.

In the human urine, Se-methylselenoneine (Fig. 1c) has been reported to be one of the major forms of excreted Se [9] in addition to selenosugars including methyl-2-acetamido-2-deoxy-1-seleno-ß-D-galactopyranoside (selenosugar 1) and methyl-2-amino-2-deoxy-1-seleno-ß-D-galactopyranoside (selenosugar 3) [10]. A study that examined a group of healthy volunteers reported finding that Se-methylselenoneine was consistently detected in the urine of all volunteers, with a significant metabolite found in one volunteer that contributed up to 24% of the total urinary Se [11]. Furthermore, another study stated that Se-methylselenoneine was predicted to be a metabolite of selenoneine and was generated in the liver and kidney by an unknown methyltransferase [9]. In addition, as Se-methylselenoneine excretion is not stimulated by the selenite supplementation in healthy volunteers [12], the pathway of Se-methylselenoneine synthesis is predicted to not be related to the main pathway of the Se excretion [13]. In contrast, Se-methylselenoneine could potentially be derived from food intake, as it has been detected in the muscles of fish such as, mackerel (*Scomber*

scombrus), sardine (*Sardina pilchardus*), and tuna (*Thunnus albacares*) [14]. A recent study detected Se-methylselenoneine in Caco-2 cells that had been cultured with selenoneine [15]. These results strongly support the supposition that Se-methylselenoneine is a metabolite of selenoneine. However, there has not been any experimental verification *in vivo* that Se-methylselenoneine is a metabolite of selenoneine.

In the present study, after mice were administered purified selenoneine, urine samples were analyzed by liquid chromatography coupled with a photodiode array and high-resolution mass spectrometer (LC-PDA-HRMS) and a liquid chromatograph system with an inductively coupled plasma mass spectrometer (LC-ICP-MS) in order to verify whether Se-methylselenoneine was excreted after the selenoneine administration. In addition, selenoneine labeled with Se-76, which is one of the lower abundant stable isotopes of Se, was synthesized by the genetically modified fission yeast and then administered to mice as a Se tracer in order to confirm whether or not Se-methylselenoneine was a metabolite of selenoneine. We also performed continuous administration of selenoneine in order to clarify the distributions of selenoneine in organs, and determine which organs in the bodies of mice methylate selenoneine.

Material and Methods

Reagents

Selenoneine was extracted from the genetically modified fission yeast strain FY25320 and purified as per a previous report [16, 17]. Selenium purity was determined using an online liquid chromatograph system (GL Science, Tokyo, Japan) with an inductively coupled plasma mass spectrometer (ICP-MS; ELAN DRC II, PerkinElmer, Inc., Waltham, MA, USA) using a system equipped with a concentric quartz nebulizer (WE02-4371, PerkinElmer, Inc.) and a sample injector (2 mm inner

diameter, quartz) as per a LC condition previously reported [17] and the ICP-MS conditions were described in supplementary information (Table S1). The purity of the selenoneine was greater than 99.5% based on Se. The selenoneine was dissolved in Milli-Q water and then stored at 4 °C.

Se-76 selenoneine synthesis

Selenoneine labeled by the stable isotope Se-76 was synthesized using the genetically modified fission yeast, FY25320, which was cultured with Se-76 selenate. Se-76 selenate was synthesized from Se-76 selenite (the isotope purity was 99.8 %) purchased from AMT Ventures Pte. Ltd. (Mackenzie Road, Singapore). Se-76 selenite was oxidized by H_2O_2 in 1.0 M NaOH at pH 11.0 that was heated at 70 °C resulting in conversion to Se-76 selenate, as per the details provided in the previous patent [18]. Residual H_2O_2 was decomposed by catalase (EC 1.11.1.6) from bovine liver (Wako, Tokyo, Japan), and the Se-76 selenate was extracted using ultrafiltration unit to remove substances with a molecular weight of 3000 (Vivaspin 500, Sartorius, Stonehouse, UK). Se-76 selenoneine was synthesized by the fission yeast in the media with 10 μM Se-76 selenate and then purified by HPLC as per a previous method [17].

LC-ICP-MS conditions for Se-76 selenoneine analysis

Se-76 selenoneine was analyzed by the LC-ICP-MS in line with the previously reported LC conditions [17]. Separation was achieved on an Ultrahydrogel-120 column (7.8 × 300 mm, Nihon Waters, Tokyo, Japan) equilibrated with 0.1 M ammonium acetate aqueous solution containing 0.1% IGEPAL (Sigma- Aldrich) at a flow rate of 1.0 mL/min at 40 °C using a column oven (Table S1). Se-76 and Se-78 were detected by monitoring *m/z* 76 and *m/z* 78 by introducing ammonia gas as the reaction gas into the dynamic reaction cell (DRC) at a flow rate of 0.45 L/min (Table S1). Other LC-ICP-MS

conditions were presented in Table S1. Using these LC-ICP-MS conditions, Se-76 selenoneine eluted with a retention time of 9.6 min.

LC-PDA-HRMS conditions for selenoneine and Se-methylselenoneine analysis

Selenoneine and Se-methylselenoneine were qualitatively analyzed by LC-PDA-HRMS using an Ultimate 3000 LC-PDA system (Thermo Fisher Scientific, Waltham, MA, USA) and a micrOTOFQ II time-of-flight MS system (Bruker, Bremen, Germany) in line with the parameters reported in a previous study [17]. Analyses of purified Se-76 selenoneine and mice urine samples were carried out on the Cosmosil PBr packed column (2.0 mm × 150 mm, 5 µm, Nacalai Tesque) equilibrated with 0.1 % acetic acid at a flow rate of 0.3 mL/min at 30°C using a column oven (Table S2). A PDA monitored the eluent from 195 to 800 nm (Table S2). HRMS survey scans in the positive ion mode detected ions from *m/z* 50 to *m/z* 800 (Table S2). The Se-76 selenoneine dimer mass was extracted from the measurement data as *m/z* 273.03 of doubly charged ion $[M+2H]^{2+}$. The Se-methylselenoneine (Se-80) mass was extracted from the measurement data as *m/z* 292.05 of singly charged ion of $[M+H]^{+}$, which was calculated from the composition formula for Se-methylselenoneine ($C_{10}H_{17}O_2N_3Se$), with an extraction width of ± 0.05.

Animal experiments

Animal experiments were approved by the Fisheries Technology Institute, Japan Fisheries Research and Education Agency, Kanagawa, Japan (Permission number: H30-1 and H31-1) and performed in accordance with the Guidelines for the Ethical Treatment of Laboratory Animals of the institute. Male 4-week-old mice (Balb/c) were purchased from Japan SLC, Inc. (Shizuoka, Japan). They were fed the standard rodent chow diet, MF containing 0.26-0.49 mg/kg selenium (Oriental Yeast Co., Tokyo, Japan), and kept in an environmentally controlled room at a temperature of 22 °C and

humidity of 55% with a 12-h light and dark cycle throughout the experiment.

Single dose administration of selenoneine

Male 5-week-old mice (n = 3) were administered 100 μM Se as selenoneine or 100 μM Se-76 as Se-76 selenoneine using the gastric sonde method. After the administration, each group of mice was moved into a metabolic cage with pooled urine samples then collected 480 minutes later. The urine samples were not diluted using water but just centrifuged (1021 × g, 10 min, 4°C), and the supernatants were injected into the LC-PDA-HRMS and LC-ICP-MS with the PBr column.

Long-term administration of selenoneine

Six male 5-week-old mice were divided into two groups of three mice, with the mice in each group moved to a metabolic cage. Mice in the metabolic cages in both groups were fed the control diet, with the mice in one group administered selenoneine via the drinking water, which contained 10 μM Se as selenoneine for 6 days. Pooled urine samples were collected every 24 hours, with the Se-methylselenoneine quantified using the LC-ICP-MS. Concentrations were standardized based on creatinine concentrations evaluated by colorimetry (Cayman Chemical, Ann Arbor, MI, USA). After 6 days, mice were dissected under isoflurane anesthesia (Pfizer, New York, NY, USA). Blood was collected from the inferior vena cava using a 1 mL syringe rinsed with heparin lithium (Wako, Tokyo, Japan). The blood was centrifuged (1000 × *g*, 10 min, 20 °C) and separated into plasma and erythrocytes. The collected liver, spleen, kidney and whole brain were weighed. Samples were stored at −80 °C until analysis. High molecular weight Se species (HMW-Se) like Se-protein, selenoneine and Se-methylselenoneine in the blood and tissue samples were analyzed by the LC-ICP-MS. Peaks around the retention time at 5.5 min were presumed and were designated as

high molecular weight Se species (HMW-Se) based on a previous study [1]. All the Se compounds in this section were quantified from the standard curve determined for purified selenoneine.

Sample preparation for LC-ICP-MS analysis

For the extraction of HMW-Se, selenoneine and Se-methylselenoneine in the blood and tissue samples, erythrocytes were diluted using a 2-fold volume of MilliQ water. Liver, kidney and spleen were diluted using a 2-fold volume of MilliQ water and homogenized by pestles. Brain was diluted 4-volume of MilliQ water and homogenized by pestles. The diluted erythrocytes and homogenates of tissues were centrifuged (1021 × g, 10 min, 4°C). Plasma and urine were not diluted using water but just centrifuged (1021 × g, 10 min, 4°C). The supernatants were injected into the LC-ICP-MS instrument. Recoveries were determined using bloods and tissues of a control mouse spiking with purified selenoneine. Bloods and tissues were spiked 2000 pmol Se as selenoneine and leaved in place for 3 min. Then, selenoneine was extracted from samples by the same procedures in this section and the supernatants were injected into the LC-ICP-MS.

Results

Determination of Se-methylselenoneine in the urine from a mouse administered selenoneine

Se compounds in the urine from a mouse administered selenoneine were analyzed by the LC-ICP-MS. In urine samples at 480 minutes after administration of selenoneine, an additional compound was detected at a retention time of 10.3 minutes (Fig. 2a). For the identification of the Se compound, the elution from 10.0 to 11.0 minutes was manually fractionated by switching the flow path in front of the ICP-MS

and then analyzed by the LC-PDA-HRMS. Extracted ion chromatograms (EICs) revealed that the fraction contained a compound having m/z 292.05 and being fragmented as m/z 278.04 (Fig. 2b). The m/z 278.04 and m/z 277.03 are the mass to charge ratios for detecting Se-80 selenoneine monomer and dimer, respectively [17]. A peak at 12.3 min was detected in the EIC monitoring m/z 278.04 and it was suspected to be selenoneine monomer contaminated in urine (Fig. 2b). However, selenoneine monomer is more hydrophilic than Se-methylselenoneine based on those structures and it is eluted faster than Se-methylselenoneine in the PBr column. Thus, the peak of m/z 278.04 detected at the same time of m/z 292.05 was suspected to be a demethylated fragment of m/z 292.05. The isotope pattern indicated that the unidentified compound contained one Se atom (Fig. 2c). The major mass containing Se-80 was extracted from the measurement data as *m/z* 292.0508 (Fig. 2c). This was identified as Se-methylselenoneine (Fig. 1c) from the calculated composition formula, $C_{10}H_{17}O_2N_3Se$, with an extraction width of ± 0.05 and the theoretical mass spectrum of Se-methylselenoneine (Fig. 2d).

Analysis of Se-76 selenoneine

Se-76 selenoneine labeled by the stable isotope, Se-76, was synthesized and purified in line with the results of a previous study [17]. The LC-ICP-MS analysis of Se-76 selenoneine revealed that compounds containing Se-78 were not detected in any of the chromatograms monitoring Se-78 while selenoneine was detected in the chromatogram that was monitoring Se-76 (Fig. 3a). The Se purity of isolated Se-76 selenoneine was 95.2 % calculated from the chromatogram of Se-76 (Fig. 3a). The LC-PDA-HRMS analysis determined that the Se-76 selenoneine was synthesized successfully from the EICs and the mass spectrum (Fig. 3b, c, Fig. S1b). The EICs not detected m/z 277.03 and m/z 278.04 derived from Se-80 selenoneine (Fig. 3b, Fig. S1a,

b) and extracting the major mass spectrum of Se-76 selenoneine dimer as doubly charged ion *m/z* 273.0360 (Fig. 3c).

Se-76 Se-methylselenoneine detection in urine

To confirm whether Se-methylselenoneine found in the urine was a metabolite of selenoneine, mice were administered Se-76 selenoneine and urine samples were collected. In urine samples from the mice administered unlabeled selenoneine, Se-76, Se-78 and Se-80 Se-methylselenoneine were detected at a retention time of 11.8 minutes, depending on the isotope ratio of Se, by the LC-PDA-HRMS with the PBr column analysis (Fig. 4). In urine samples collected from the mice administered Se-76 selenoneine, there was little if any detection of Se-78 and 80 Se-methylselenoneine, while Se-76 Se-methylselenoneine was clearly detected (Fig. 4). Se-76 Se-methylselenoneine was also detected using LC-ICP-MS with the PBr column monitoring Se-76 and Se-78 with the DRC mode but Se-78 Se-methylselenoneine was not detected in the urine sample from Se-76 selenoneine administered mice (Fig. S2). These findings demonstrated that Se-methylselenoneine was a metabolite of selenoneine.

Monitoring of the time course of the Se-methylselenoneine excretion

Monitoring of the time course of the Se-methylselenoneine excretion was performed by continuous administration of selenoneine in the drinking water study over six days. Body weights of mice were not affected by the continuous administration of selenoneine (Fig. 5a). Fig 5b shows that Se-methylselenoneine was hardly detected at 24 hours, but it was subsequently detected from 48 hours through 120 hours in the pooled urine sample from each group mice (Fig. 5b). Selenoneine would elute at 9.2 min between the two peaks detected from 48 hours through 120 hours in Fig. 5b because

selenoneine was detected at the retention time with the same LC-ICP-MS conditions in Fig 2a. The peak present at all times in all urine samples was considered to be a major metabolite of Se but unidentified (Fig. 5b). Se-methylselenoneine was detected only in the group administered selenoneine, with the increase in the concentration found to be time-dependent (Fig. 5c).

Accumulations of selenoneine and Se-methylselenoneine in the tissues

After 6 days, mice were dissected under isoflurane anesthesia (Pfizer) and tissues were weighed (Table 1). HMW-Se, selenoneine and Se-methylselenoneine accumulations in the tissues were analyzed by the LC-ICP-MS (Fig. 6, Table 2). The values for the limit of detection (LOD) and the limit of quantification (LOQ) for HMW-Se, selenoneine and Se-methylselenoneine were calculated from the ratios of the signal and noise (LOD: S/N = 3, LOQ: S/N = 10) of the purified selenoneine and they were 0.389 nmol/g and 1.30 nmol/g. Recoveries of selenoneine from plasma, erythrocyte, liver, kidney, spleen and brain were 57.2 %, 105 %, 111 %, 94.9 %, 96.2 % 107 %, respectively. The concentrations of selenoneine in erythrocyte, liver, kidney spleen and brain were corrected by the recoveries. HMW-Se was detected in all of the tissues (Table 2). The concentrations did not differ significantly between the control and the group administered selenoneine (Table 2). In the plasma of mice administered selenoneine, while Se-methylselenoneine was barely detected, it could not be quantified as the concentration at 0.45 ± 0.02 nmol/g was lower than the LOQ value (Table 2). In the erythrocytes, while selenoneine was detected at 3.0 ± 0.1 nmol/g, Se-methylselenoneine was not detected (Table 2). In the liver, selenoneine was quantified at 16.1 ± 3.3 nmol/g, while Se-methylselenoneine was barely detected (Table 2). In the kidney, selenoneine was quantified at 13.1 ± 1.9 nmol/g, while Se-methylselenoneine was barely detected (Table 2). In the spleen, selenoneine was detected at 2.8 ± 0.5

281 nmol/g, while Se-methylselenoneine was not detected (Table 2). In the brain, both
282 selenoneine and Se-methylselenoneine were not detected based on the LOD value,
283 although selenoneine was detected in a chromatogram in traces (Table 2, Fig. 6).
284
285 **Discussion**
286 In the present study, Se-methylselenoneine was verified to be a metabolite of
287 selenoneine through the administration of the stable isotope, Se-76, labeled
288 selenoneine. Moreover, after the continuous administration of selenoneine to the mice,
289 the chromatograms of the LC-ICP-MS demonstrated that selenoneine was shown to be
290 distributed in the erythrocytes, liver and kidney, which suggested that selenoneine was
291 methylated in the liver and/or kidney.
292 Se-methylselenoneine, which was detected in the blood and urine, has been
293 predicted to be a metabolite of selenoneine [9]. This prediction is supported by the
294 findings of a previous study that showed Se-methylselenoneine was detected in the
295 Caco-2 cells after the addition of selenoneine [15]. In the present study, Se-
296 methylselenoneine was detected in the urine after the administration of selenoneine to
297 the mice (Fig. 2). Furthermore, the Se in Se-methylselenoneine was derived from
298 selenoneine after the administration of the stable isotope, Se-76, labeled selenoneine
299 and acted as a tracer (Fig. 4). At 480 minutes after a single dose administration of 100
300 μM of the selenoneine dimer, a peak was detected at a retention time of 10.3 minutes in
301 the LC-ICP-MS chromatogram (Fig. 2a). The peak was fractionated and identified as
302 Se-methylselenoneine based on the measurement data as *m/z* 292.05 and the
303 calculated composition formula, $C_{10}H_{17}O_2N_3Se$ using the LC-PDA-HRMS (Fig. 2b, c). In
304 the EICs, m/z 278 was detected at a retention time of 12.0 minutes (Fig. 2b). This peak
305 was considered as selenoneine monomer based on the mass but the retention time of
306 selenoneine monomer was 3.2 minutes referring our previous study [17]. Thus, it was

speculated to be a fragmented ion generated from demethylation of Se-methylselenoneine (Fig. 2b). These results indicated that mice generated Se-methylselenoneine and excreted it in urine after the intake of selenoneine.

To try and identify whether the Se-methylselenoneine was the metabolite of the administered selenoneine, we synthesized the stable isotope, Se-76, labeled selenoneine (Fig. 3, Fig S1) and then administered it to mice. Se is an element with multiple isotopes, and their abundance ratios vary according to the isotopes. The major isotope is Se-80, with an abundance of 49.61%, while the other isotope, Se-78 was at 23.78%. Se-76 is the third major isotope and had an abundance of 9.36%. We used this isotope for synthesizing the labeled selenoneine that was used as a tracer. Se-76 selenoneine was successfully synthesized through use of the genetically modified fission yeast and subsequent purification by HPLC (Fig. 3, Fig. S1). Although after administration of Se-76 Se-methylselenoneine it was detected in the urine, there was little detection of Se-78 and Se-80 Se-methylselenoneine (Fig. 4). If Se-methylselenoneine was the metabolite of both selenoneine and other Se compounds, Se-78 and Se-80 Se-methylselenoneine derived from endogenous Se should have been detected to a greater degree in the urine. In actuality, Se-78 and Se-80 Se-methylselenoneine was not detected in the urine of mice administered Se-76 selenoneine. Thus, these results suggest that Se-methylselenoneine is a metabolite of selenoneine.

Our results that showed selenoneine was absorbed in the body and methylated for excretion, indicating that the selenoneine dimer was absorbed and reduced, or vice versa, in the body, as the administered Se-76 selenoneine was the oxidized dimer. Although the transporter of selenoneine, OCTN1, can efficiently absorb the reduced selenoneine, it does not oxidize selenoneine [19,20]. Therefore, oxidized selenoneine was predicted to be reduced in the digestive tract and absorbed into the body via small-intestinal epithelial cells that express OCTN1. Although the selenoneine dimer is

reduced by glutathione or other reductants in vitro [19], the reduction mechanism *in vivo* has yet to be definitively determined.

Continuous administration of 10 μM purified selenoneine for 6 days did not exhibit any negative effects on the bodies of the mice (Fig. 5a, Table 1). A previous study using NAFLD model mice reported that 0.3 mg Se/kg purified selenoneine in a standard rodent chow did not change the body weight and suppressed the normally seen increase in the liver weight during the 4 months of feeding [21]. In the present study, we used a higher concentration but shorter-term administration as compared to that of the previous study, and found that selenoneine did not affect the liver, kidney, spleen and brain weight (Table 1). This is important information, as there have been few reports on the safety of purified selenoneine. The variations in urinary concentrations of Se compounds over time were evaluated after correcting the compound concentrations for creatinine concentrations, as the rate of concentration and dilution of urine is variable. Se-methylselenoneine was not excreted in the urine until 24 hours later and was excreted up until 48 hours after the initial administration (Fig. 5b, d). A transwell test using Caco-2 cells demonstrated that selenoneine was detected after 24 hours on the basolateral side after the addition of selenoneine on the apical side [15]. Se-methylselenoneine was detected at 72 hours after the addition of selenoneine [15]. Although our results were comparable to the absorption time of selenoneine in the small intestine, the methylation of selenoneine was faster than that seen in a cellular experiment. These findings suggest that other cells or organs might more efficiently methylate selenoneine.

There was an increase in the Se-methylselenoneine excretion in a time-dependent manner (Fig. 5c). These results suggested that the continuous administration of 10 μM selenoneine for 6 days was excessive, as mice began to accumulate it in their body, which then led to it being actively methylated for the excretion in order to

359 autonomously adjust the selenoneine concentration.

360 To identify which tissues metabolized selenoneine, HMW-Se, selenoneine and Se-
361 methylselenoneine in the blood and tissues were evaluated by the LC-ICP-MS (Table 2,
362 Fig. 6). HMW-Se was detected in all of the tissues (Table 2, Fig. 6) and the
363 concentrations did not differ significantly between the control and the group
364 administered selenoneine (Table 2). Most of HMW-Se is presumed to be selenoproteins
365 like glutathione peroxidase based on a previous study [1] and their expressions were
366 generally promoted by Se compounds [22] because Se compounds relate to the central
367 metabolic pathway of Se for the selenoproteins synthesis [23]. This result indicated
368 that selenoneine was not related to the central metabolic pathway of Se but further
369 studies are needed like measuring selenoproteins by types precisely.

370 As seen in Table 2, selenoneine was distributed in erythrocytes, liver, kidney and
371 spleen as in our previous study [24]. In the liver and the kidney, the accumulation of
372 selenoneine was 16.1 ± 3.3 and 13.1 ± 1.9 nmol/g, respectively (Table 2). Se-
373 methylselenoneine was also detected in the liver and kidney at 0.40 ± 0.19 and 0.48 ±
374 0.10 nmol/g, respectively, although these concentrations were below the LOQ value
375 (Table 2, Fig. 6). In the erythrocytes and the spleen, accumulation of selenoneine was
376 3.0 ± 0.1 and 2.8 ± 0.5 nmol/g, respectively (Table 2), although Se-methylselenoneine
377 was not detected (Fig. 6). The results indicate that selenoneine was methylated in the
378 liver and/or the kidney. Moreover, Se-methylselenoneine was detected in the plasma
379 (Fig. 6), but not detected in the erythrocytes. Previous studies have reported that Se-
380 methylselenoneine was detected in human whole blood [9] and a study detect it in
381 human erythrocytes [25]. In the present study, selenoneine was not detected in plasma
382 (Fig. 6). Thus, it was not considerable Se-methylselenoneine contaminated in plasma
383 by the hemolysis of erythrocytes. It remains unclear whether selenoneine is methylated
384 in plasma or erythrocyte. Moreover, these findings suggest that selenoneine was likely

methylated in the liver and/or kidney and/or plasma enzymatically or non-enzymatically and relatively rapidly exported for excretion. Methylation is a major process in organisms and occurs via enzymes such as methyltransferases and the methyl donors like S-adenosylmethionine (SAM). For example, thiopurine S-methyltransferase (TPMT; EC 2.1.1.67) is known to be a methyltransferase against thiol compounds and also is involved in selenocysteine methylation [26-28]. Although TPMT is expressed in erythrocytes [26,27], Se-methylselenoneine could not be detected in the erythrocytes in the present study (Fig. 6). While ergothioneine, the sulfur analog of selenoneine is methylated and excreted in urine, the mechanism of ergothioneine methylation has yet to be elucidated [29]. Se-methylergothioneine was detected in whole blood, liver, kidney, brain, but not in the spleen and heart [7]. Selenoneine was suspected to be methylated by the same mechanism as that for ergothioneine due to the similarity of the distributions of Se-methylselenoneine and Se-methylergothioneine (Fig. 6 and Table 2). As it is important that the mechanism of Se-methylselenoneine synthesis be understood, further studies that examine the selenoneine metabolism will need to be conducted.

In conclusion, Se-methylselenoneine in the urine was identified as the metabolite of selenoneine after the administration of selenoneine in mice. Moreover, selenoneine was predicted not to affect synthesis of selenoproteins. These results demonstrate that selenoneine is metabolized by a unique pathway unlike that observed for ordinary Se compounds.

Declarations

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Competing Interests

The authors declare no competing interests.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection, analysis and visualization were performed by Takuya Seko, Hajime Uchida, Yoko Sato, Shintaro Imamura, Kenji Ishihara and Yumiko Yamashita. The first draft of the manuscript was written by Takuya Seko and all authors commented on the previous versions of the manuscript. Michiaki Yamashita conducted the final review and editing of the manuscript. All authors read and approved the final manuscript.

Data Availability

Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

Ethics Approval

Animal experiments were approved by the Fisheries Technology Institute, Japan Fisheries Research and Education Agency, Kanagawa, Japan (Permission number: H30-1 and H31-1) and performed according to the Guideline for the Ethical Treatment of Laboratory Animals of the institute.

Consent to participate

Not applicable.

Consent to publish

Not applicable.

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547 **Table 1** Ratios of tissue weights according to body weights

	Ratio (%)			
	Liver	Kidney	Spleen	Brain
Control	4.75 ± 0.07	1.75 ± 0.07	0.384 ± 0.051	2.17 ± 0.21
Selenoneine	4.73 ± 0.12	1.75 ± 0.02	0.370 ± 0.015	2.40 ± 0.03

548

549 **Table 2** Concentrations of selenium compounds in tissues of mice

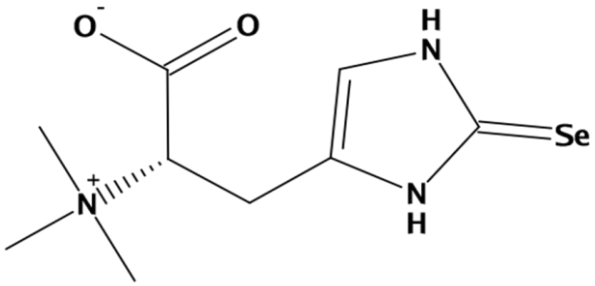
	HMW-Se (nmol Se/g)		Selenoneine (nmol Se/g)		Se-methylselenoneine (nmol Se/g)	
	Control	Selenoneine	Control	Selenoneine	Control	Selenoneine
Plasma	6.2 ± 1.8	7.8 ± 0.8	-	-	-	(0.45 ± 0.02)
Erythrocytes	15.7 ± 0.2	18.5 ± 3.3	-	3.0 ± 0.1	-	-
Liver	43.1 ± 2.6	43.7 ± 2.5	-	16.1 ± 3.3	-	(0.40 ± 0.19)
Kidney	12.8 ± 1.5	12.9 ± 1.2	-	13.1 ± 1.9	-	(0.48 ± 0.10)
Spleen	1.7 ± 0.4	1.5 ± 0.1	-	2.8 ± 0.5	-	-
Brain	(0.77 ± 0.06)	(0.89 ± 0.22)	-	-	-	-

550 Values are expressed as mean ± standard deviation. " - " means not detected, and a
551 value within parentheses means the value is lower than the limit of quantification but
552 higher than the limit of detection.

553

554 **Fig. 1**

555 (a)



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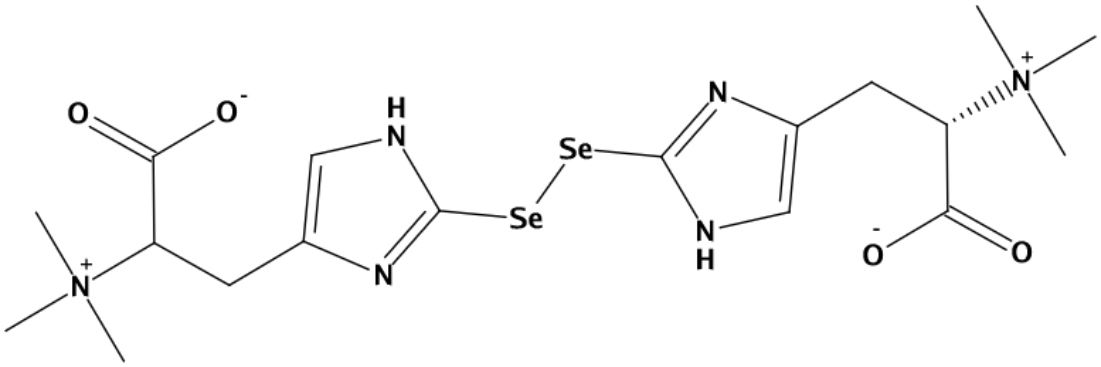
557 (b)



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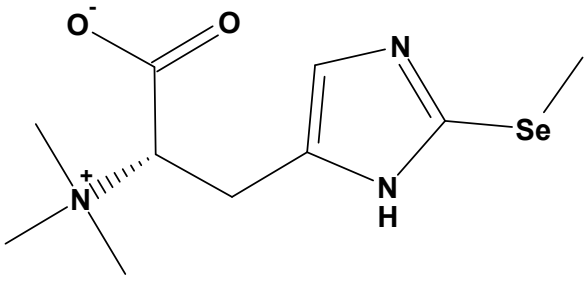
559 (c)



560

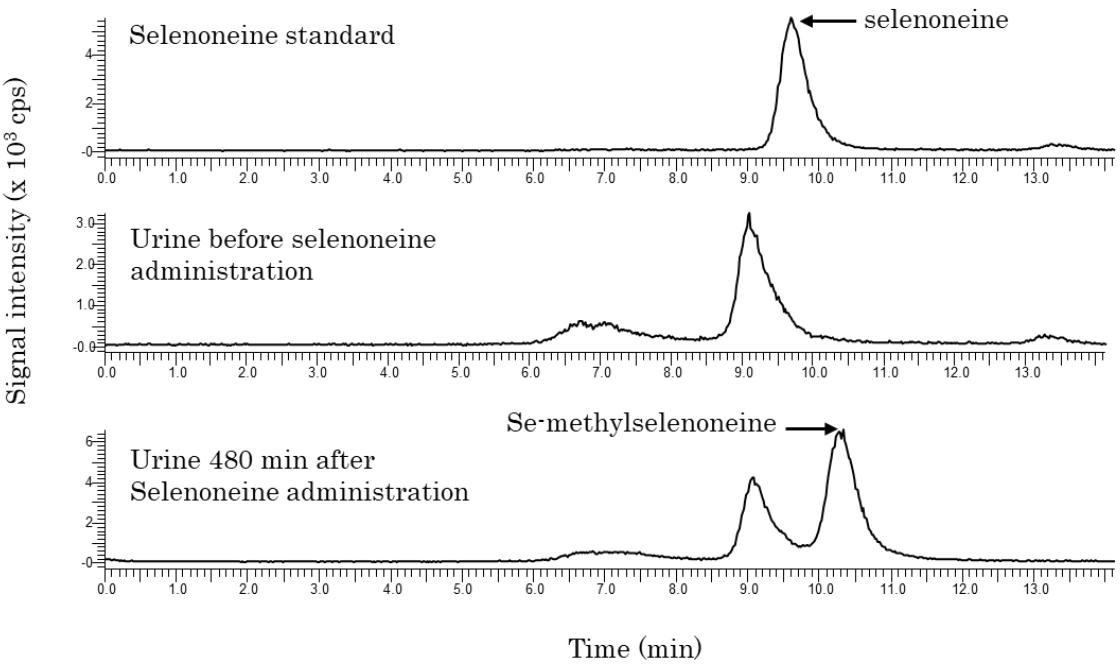
561 **Fig. 1** Structures of selenoneine and Se-methylselenoneine. (a) a reduced monomeric

562 form of selenoneine. (b) oxidized dimeric form of selenoneine. (c), Se-methylselenoneine

563

564 **Fig. 2**

565 (a)



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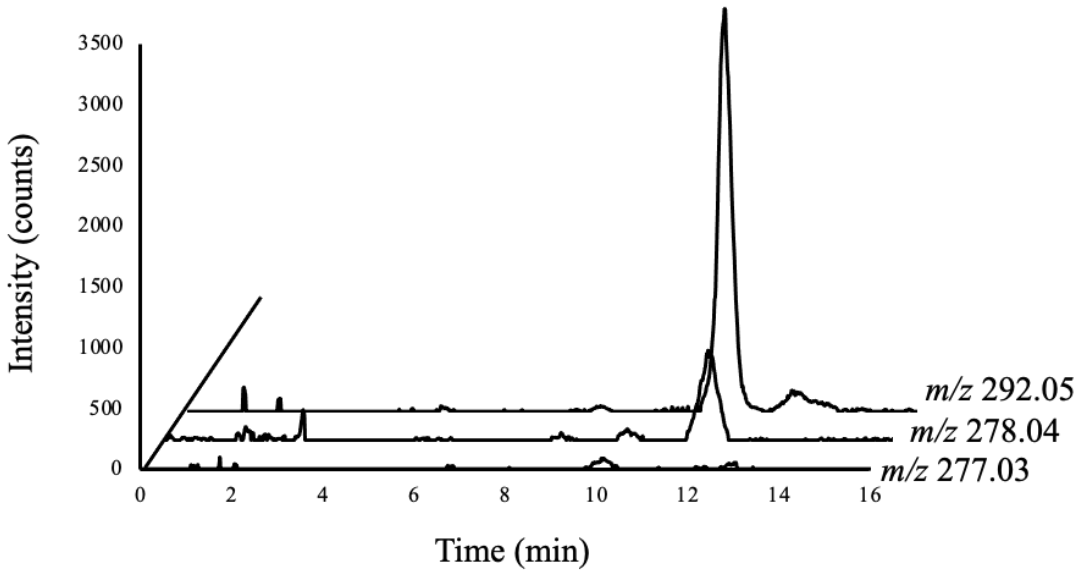

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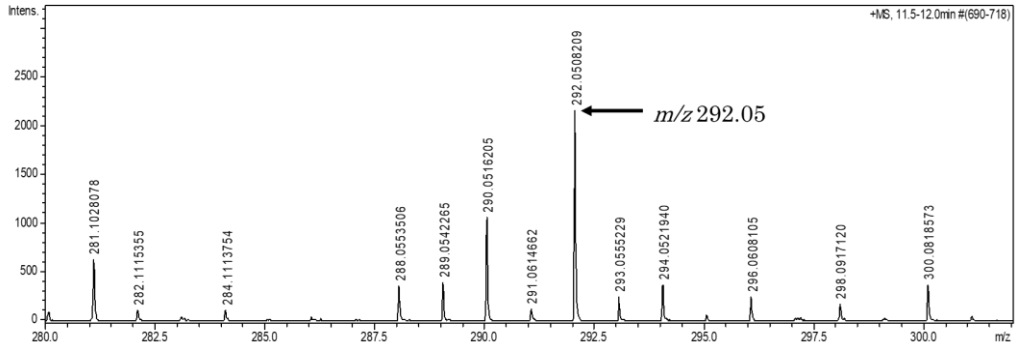
568 (c)



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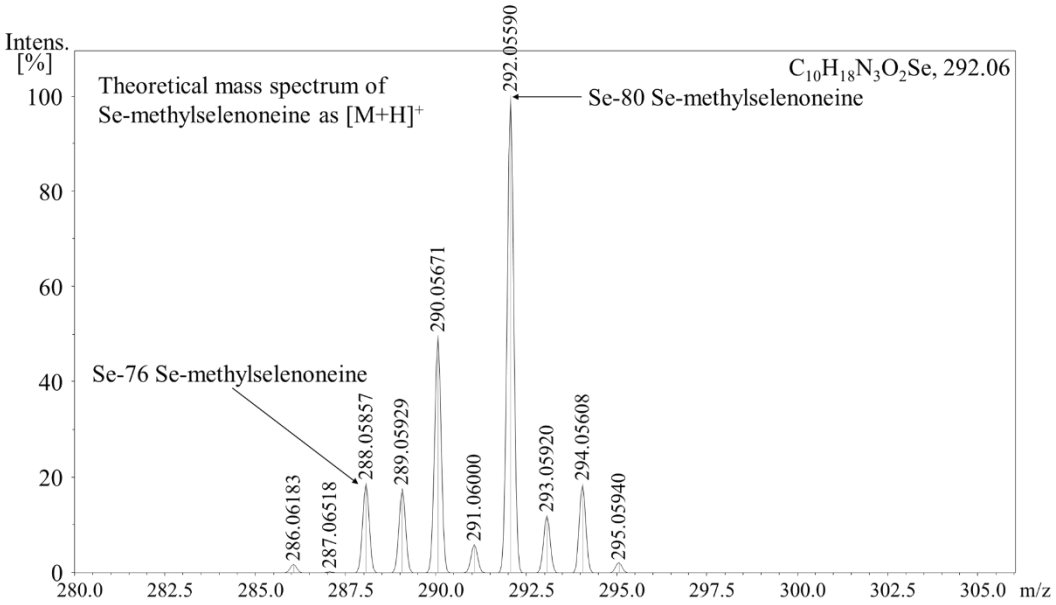
570 (d)



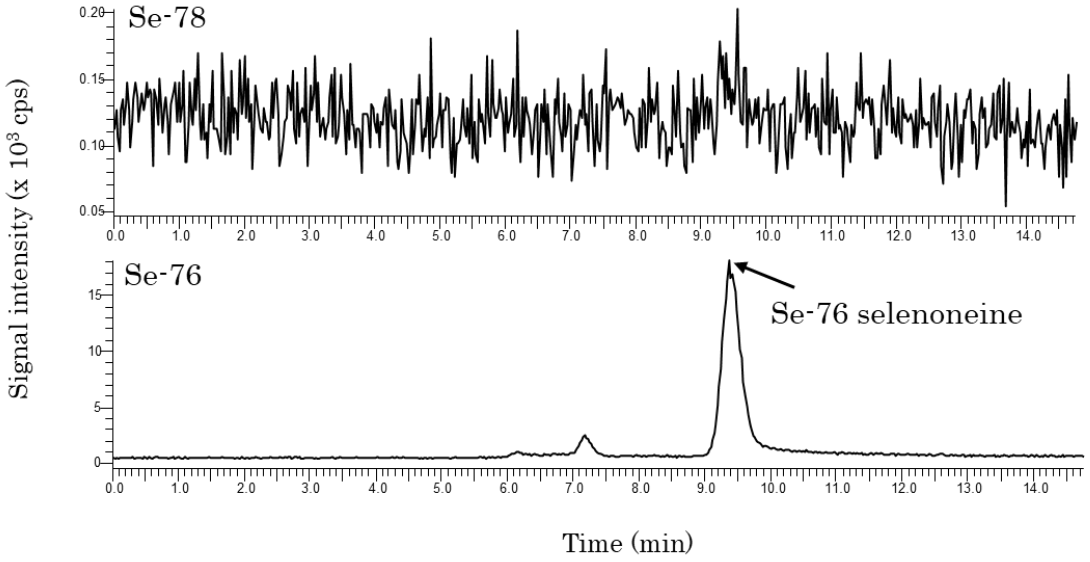
571

572 **Fig. 2** (a) LC-ICP-MS chromatograms monitoring Se-82 of the standard selenoneine
573 and the urine sample of a mouse before administration of selenoneine and at 480 min
574 after the administration. (b) EICs using LC-PDA-HRMS of the fraction from the urine.
575 The chromatograms exhibited ions, m/z 277.03 (selenoneine dimer), m/z 278.04
576 (selenoneine monomer) and m/z 292.05 (Se-methylselenoneine) with extraction widths
577 of ± 0.05 relating to Se-78 selenoneine dimer mass, Se-78 monomer mass and Se-
578 methylselenoneine mass, respectively. (c) Mass spectrum using LC-PDA-HRMS of the
579 Se-methylselenoneine fractionated from the urine sample at 480 min after

administration. (d) The theoretical mass spectrum of Se-methylselenoneine as $[M+H]^+$; (a) column: Ultrahydrogel-120 column 7.8 × 300 mm; mobile phase: 0.1 M ammonium acetate aqueous solution containing 0.1% IGEPAL; flow rate: 1.0 mL/min. (b, c) column: Cosmosil PBr packed column 2.0 × 150 mm; mobile phase: 0.1 % acetic acid; flow rate: 0.3 mL/min. Details provided in Material and Methods.

586 **Fig. 3**

587 (a)



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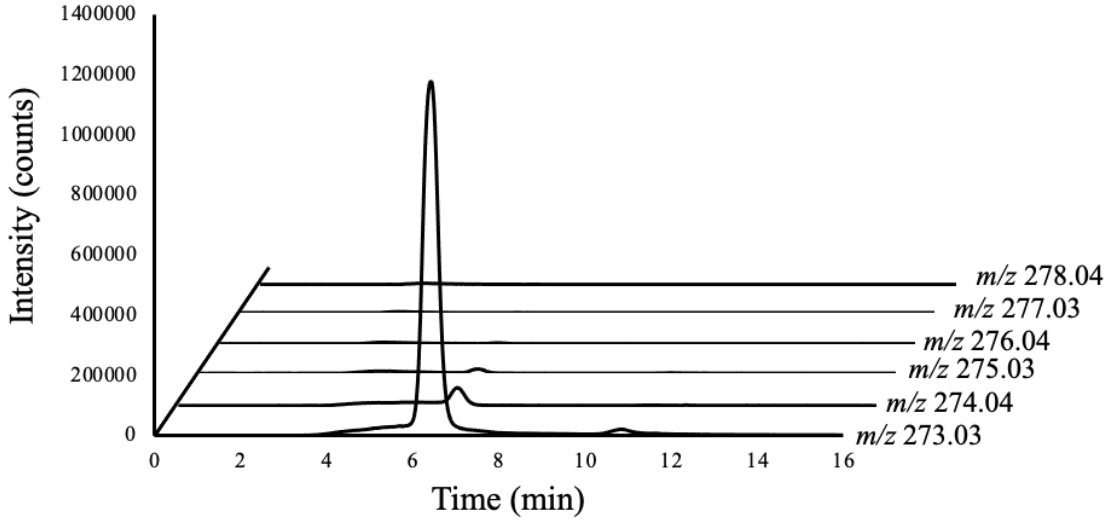
589 (b)



590

591

(c)

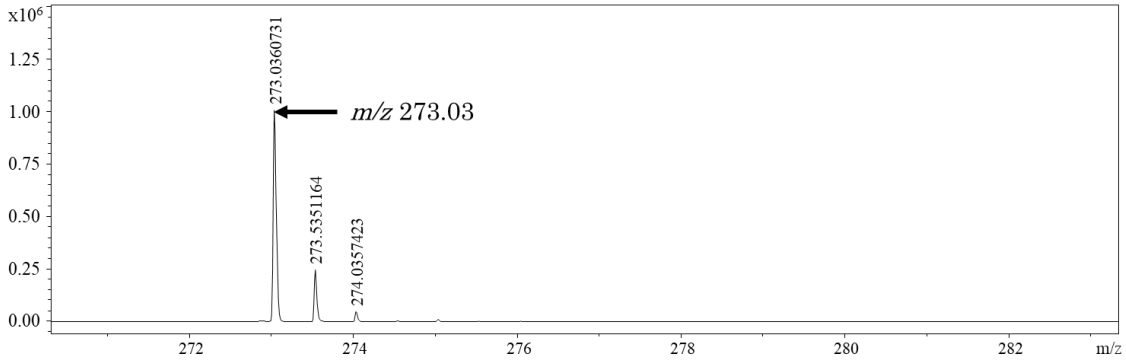
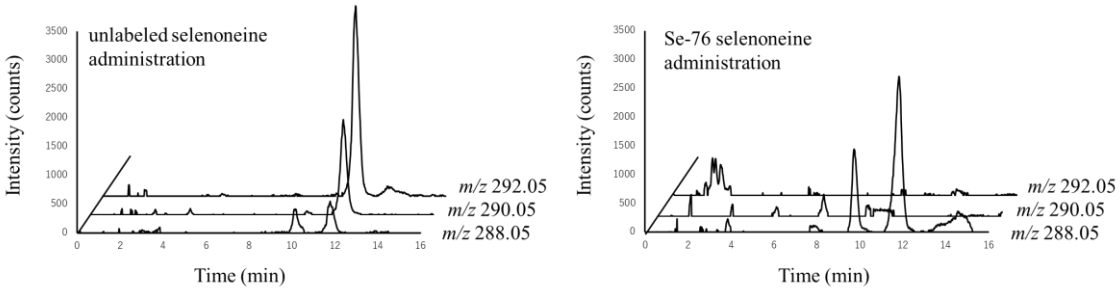


Fig. 3 (a) LC-ICP-MS chromatograms of synthesized Se-76 selenoneine. Se-78 was monitored in the upper chromatogram and Se-76 was monitored in the lower chromatogram. (b) EICs using LC-PDA-HRMS of synthesized Se-76 selenoneine. The chromatograms exhibited ions, m/z 273.03, 274.04, 275.03, 276.04, 277.03 and 278.04 with extraction widths of ± 0.05 relating to Se-76 selenoneine dimer mass, Se-76 selenoneine monomer mass, Se-78 selenoneine dimer mass, Se-78 selenoneine monomer mass, Se-80 selenoneine dimer mass and Se-80 selenoneine monomer mass, respectively. (c) Mass spectrum using LC-PDA-HR-MS of oxidized dimeric Se-76 selenoneine; (a) column: Ultrahydrogel-120 column 7.8 × 300 mm; mobile phase: 0.1 M ammonium acetate aqueous solution containing 0.1% IGEPAL; flow rate: 1.0 mL/min. (b, c) column: Cosmosil PBr packed column 2.0 × 150 mm; mobile phase: 0.1 % acetic acid; flow rate: 0.3 mL/min. Details provided in Material and Methods.

606 **Fig. 4**



607

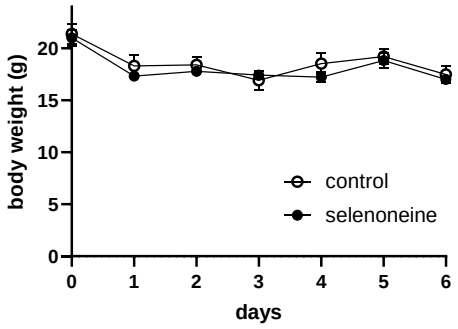
608 **Fig. 4** EICs using LC-PDA-HRMS of the urine samples from the mice after 480 min
609 administered 100 μM Se as unlabeled selenoneine or Se-76-labeled selenoneine. The
610 left EICs exhibit the urine sample from the mice administered unlabeled selenoneine
611 and the right EICs exhibited that from the mice administered Se-76 selenoneine. Se-
612 methylselenoneine is eluted at 11.8 min and peaks at 11.8 min in the EICs monitoring
613 m/z 288.05, m/z 290.05 and m/z 292.05 are Se-76, 78 and 80 Se-methylselenoneine. The
614 peak at 10.0 min in the EIC of m/z 288.05 was not identified; column: Cosmosil PBr
615 packed column 2.0 × 150 mm; mobile phase: 0.1 % acetic acid; flow rate: 0.3 mL/min.
616 Details provided in Material and Methods.

617

618

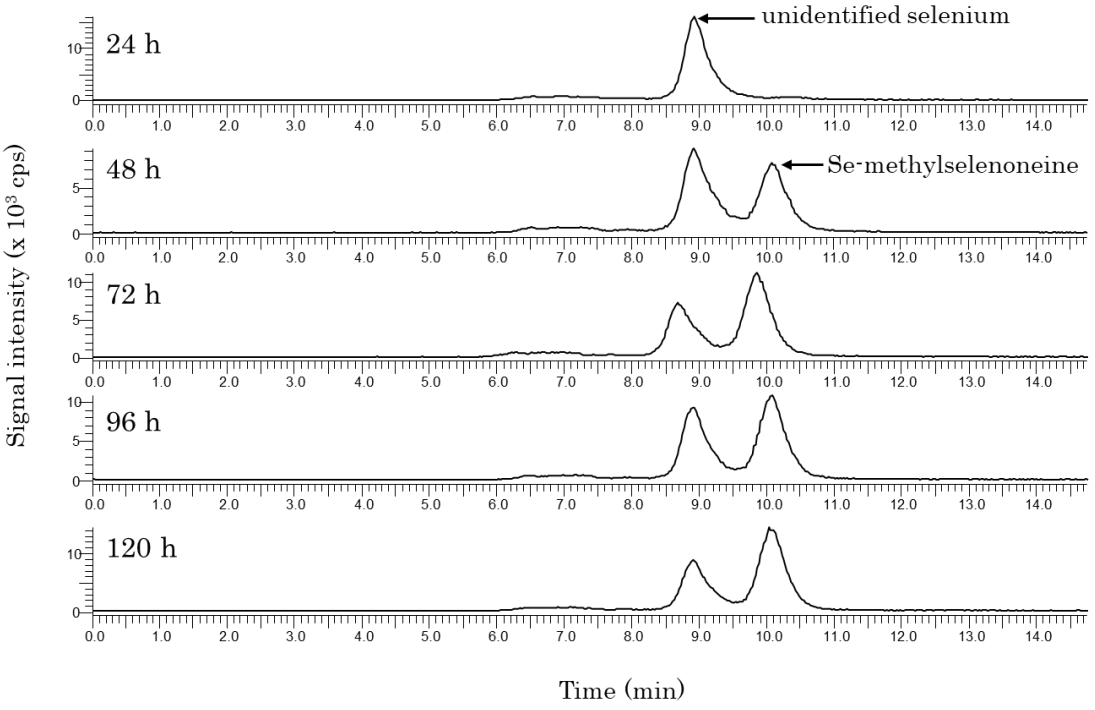
619 **Fig. 5**

620 (a)



621

622 (b)



623

624

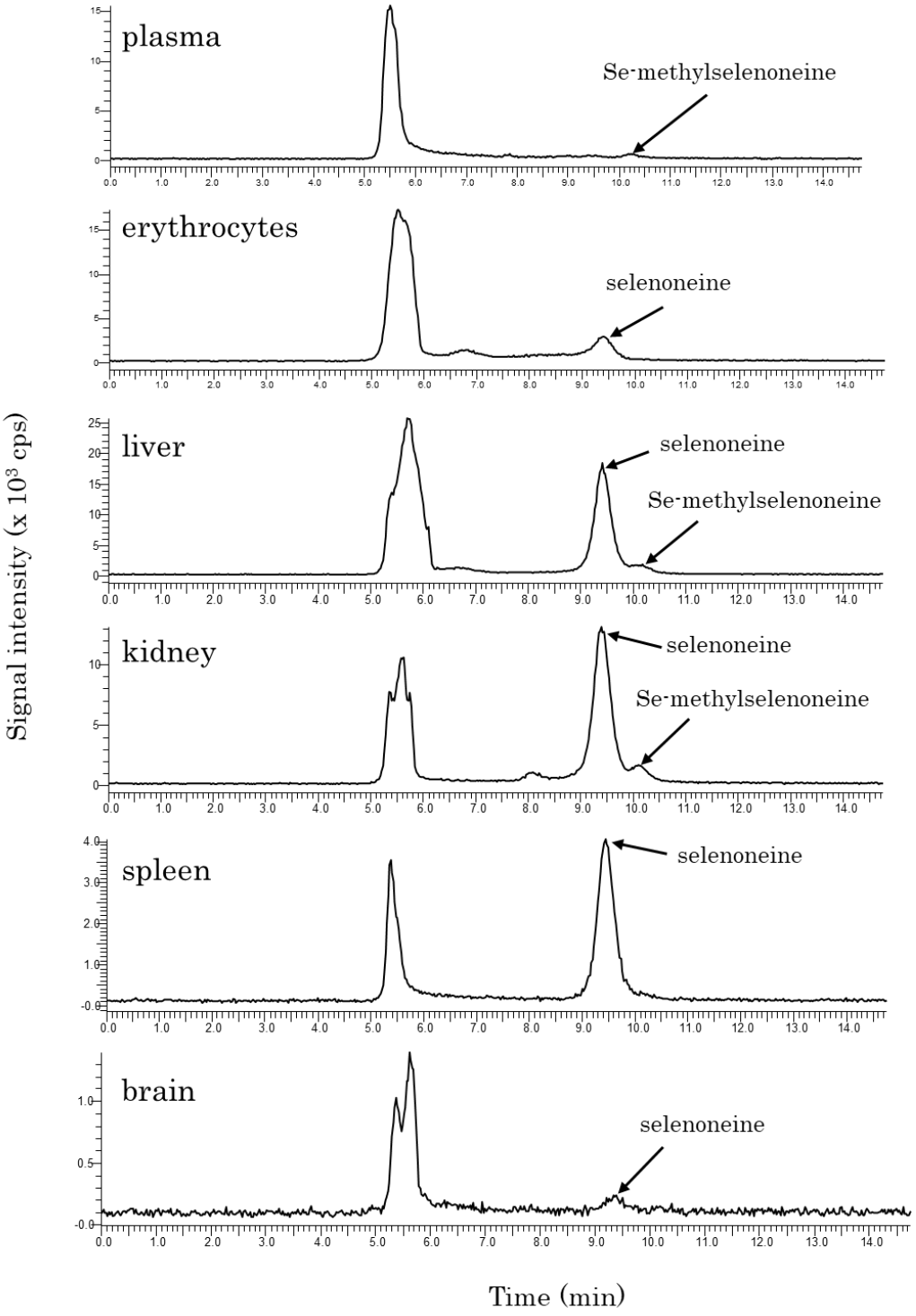
625 (c)

626

627 **Fig. 5** (a) Body weight changes of mice over 6 days. (b) LC-ICP-MS chromatograms
628 monitoring Se-82 of the urine sample from mice administered selenoneine. The peak
629 eluted at 10.3 min contains Se-methylselenoneine from the results of Fig. 2a and b. (c)
630 The time course of Se-methylselenoneine concentrations in the pooled urine during the
631 intake of selenoneine; column: Ultrahydrogel-120 column 7.8 × 300 mm; mobile phase:
632 0.1 M ammonium acetate aqueous solution containing 0.1% IGEPAL; flow rate: 1.0
633 mL/min. Details provided in Material and Methods.

634

635 **Fig. 6**



636

637 **Fig. 6** LC-ICP-MS chromatograms monitoring Se-82 of tissues of a mouse administered

638 selenoneine. The 5.5 min peak has been designated as high molecular weight Se

639 species (HMW-Se) based on previous results [1]. The second peak detected in the

640 erythrocytes, liver, kidney, spleen and brain contains selenoneine. The third peak
641 detected in the plasma, liver and kidney contains Se-methylselenoneine. The peaks
642 containing selenoneine or Se-methylselenoneine were identified from the results of
643 Fig.2a and b; column: Ultrahydrogel-120 column 7.8 × 300 mm; mobile phase: 0.1 M
644 ammonium acetate aqueous solution containing 0.1% IGEPAL; flow rate: 1.0 mL/min.
645 Details provided in Material and Methods.
646