have anti-tumor activity [8]. However, they are more toxic than SeMet. Thus, chemically synthesized L-SeMet and selenized yeast are mainly used as Se supplement [9]. In addition to its use as a supplemental source, the cancer-preventive effect of SeMet is also expected [10]. Indeed, SeMet was subjected to a clinical trial for the prevention of prostate cancer, called SELECT (the Se and Vitamin E Cancer Prevention Trial). However, no significant effects on the prevention of prostate cancer were observed and instead, a non-significant increase in the risk of diabetes mellitus compared with placebo was noted in the group that received SeMet [11]. On the other hand, it is known that SeMet accumulates in the pancreas [12] and ^{75}Se-labeled SeMet has been used as a radiocontrast medium for pancreas [13]. This may indicate that the chemical form of SeMet is not always suitable for use as a supplement. Consequently, the development of a third-generation Se source that is safer than currently available species, such as selenite and SeMet, is highly awaited.

Recently, a novel selenoamino acid, selenohomolanthionine (SeHLan), was identified in selenized Japanese pungent radish (*Raphanus sativus* L. cv. "Yukibijin") [14] and selenized yeast [15]. SeHLan (4,4′-selenobis[2-aminobutanoic acid] or *Se*-(3-amino-3-carboxypropyl)-homocysteine) has unique structural features, e.g., lack of a CH_3Se moiety in its molecule and symmetry with respect to Se. Homolanthionine (HLan), the sulfur analog of SeHLan, was converted into cysteine (Cys) in rats [16]. Thus, SeHLan is expected to be a third-generation Se source. We have already evaluated the toxicity of SeHLan to rats and compared it with that of SeMet when either SeHLan or SeMet was administered at a bolus dose (1.0 mg Se/kg body weight) [17]. SeMet at the bolus dose was preferably accumulated in the pancreas, increasing the serum amylase level, an index of pancreatic damage. SeHLan was preferably accumulated in the kidneys in a time-dependent manner, raising the serum creatinine level, an index of kidney damage. Hence, SeHLan is expected to be a potential supplemental source of Se without inducing the onset of pancreatic damage. The specific toxicity of SeHLan to the kidneys may be avoided if its dose is lower than the one used in the previous study (1.0 mg Se/kg body weight). The metabolism of SeHLan should be evaluated at a supplemental dose using a stable isotope-labeled SeHLan.

Either radio or enriched stable isotopes are required in tracer experiments. Stable isotopes have some advantages in tracer experiments over radio isotopes [18]. For instance, stable isotopes can be handled under conventional laboratory conditions because they are not toxic. The inductively coupled plasma mass spectrometer (ICP-MS) is one of the most powerful instruments for the atomic mass-specific detection of elements and is also used as an atomic mass-specific detector for the speciation of elements. For essential elements, in particular, the utilization of a stable isotope with ICP-MS detection can simultaneously reveal the metabolism of endogenous and exogenous elements [19].

In this study, ^{77}Se-labeled SeHLan was synthesized and administered to rats at a supplemental dose of 25 μg Se/kg body weight to reveal the metabolic fate of SeHLan. The metabolism of [^{77}Se]-SeHLan and the kinetics of ^{77}Se were monitored by ICP-MS. Finally, we discussed the possibility of using SeHLan as a novel supplemental source of Se in place of SeMet.

Experimental

Chemicals

The metallic form of ^{77}Se (99.9% enriched) was purchased from Isoflex USA (San Francisco, CA, USA). (*S*)-(+)-2-amino-4-bromobutyric acid hydrobromide was purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). Amberlite® 120-R, phosphate-buffered saline, Trizma® hydrochloride, and Trizma® base were purchased from Sigma-Aldrich (St. Louis, NJ, USA). Other chemicals and reagents were purchased from Wako Pure Chemical Ind. (Osaka, Japan).

Synthesis of ^{77}Se-labeled selenohomolanthionine

The metallic form of ^{77}Se (powder, 9.5 mg) was suspended in 0.25 mL of *N,N*-dimethylformamide dehydrate (DMF) under nitrogen atmosphere. Then, sodium tetrahydroborate (72 mg) in DMF (1.5 mL) was added and the mixture was stirred at room temperature for 60 min. (*S*)-(+)-2-amino-4-bromobutyric acid hydrobromide (68.2 mg) dissolved in DMF (0.25 mL) was gradually added and the reaction mixture was left to stand at room temperature for 48 h. The reaction was stopped by the addition of 1 M HCl and the reaction mixture was evaporated in vacuo. The residue was dissolved in deionized water and purified by cation exchange (Amberlite 120-R) and ODS (Wakogel 100C18) columns. Finally, SeHLan was purified on an Asahipak GS-520P (20.0 i.d.×500 mm; Showa Denko, Tokyo, Japan) column and its purity was confirmed by HPLC-ICP-MS as described below.

Animal experiments

All animal experiments were carried out according to the "Principles of Laboratory Animal Care" (NIH version, revised 1996) and the Guidelines of the Animal Investigation Committee, Graduate School of Pharmaceutical Sciences, Chiba University, Japan.

Male Wistar rats (Clea Japan Co., Tokyo, Japan) were purchased at 5 weeks of age and housed in a humidity-controlled room maintained at 22–25 °C with a 12-h light–dark cycle. The rats were fed a commercial diet (MF; Oriental Yeast, Tokyo, Japan) and tap water ad libitum. After a 1-week acclimation period, rats weighing 160–180 g were used. The rats were individually housed in a metabolic cage to collect urine and feces, and fed a standard diet and tap water ad libitum. [^{77}Se]-SeHLan at the dose of 25 μgSe/kgbody weight was administered intravenously into the rat tail vein. Each group of three rats was killed at 1, 6, and 24 h after the injection by exsanguination under ether anesthesia. Control rats were injected with an equivalent volume of saline vehicle. Non-heparinized blood was collected and clotted blood was centrifuged at 1600×*g* for 10 min to obtain serum. Then, the liver, kidneys, pancreas, muscle, brain, and testes were excised. The tissues and serum were preserved at −20 °C prior to use.

Determination of Se concentration in samples

A 200-mg or 200-μL portion of tissue, serum, or urine was digested with 1 mL of a mixture of concentrated nitric acid (HNO_3) and 30% H_2O_2 (*v*/*v*=1:1) in a test tube at room temperature for more than 1 day and then heated at 160–180 °C for several days until complete digestion was achieved. Se concentrations in the samples were determined with an ICP-MS (Agilent 7500ce, Agilent Technologies, Tokyo, Japan) equipped with an octopole reaction system at *m/z* of 76, 77, 78, 80, and 82. D_2 gas was used at a flow rate of 2.0 mL/min. Mass calibration and parameter optimization for ICP-MS were performed daily using a tuning solution containing 10 ng/mL each of lithium, yttrium, cerium, and thallium (Agilent Technologies) prior to use. The concentration of each Se isotope was obtained by an external calibration method. The concentration of endogenous Se was calculated from the signal intensity at *m/z* 76, whereas the concentration of exogenous Se was calculated from the signal intensity obtained by the following equation: [the signal intensity of exogenous Se] = [the signal intensity of ^{77}Se] − [the signal intensity of ^{76}Se] × (0.0763/0.0936). To validate our Se measurement, the dogfish muscle (DORM2, National Research Council, Ottawa, Canada) was used as a standard reference material.

HPLC-ICP-MS analysis for selenium speciation

The HPLC system consisted of an online degasser, an HPLC pump (PU713; GL Science Co., Ltd., Tokyo, Japan), a Rheodyne six-port injector with a sample loop, and a column. A multimode gel filtration column, Shodex Asahipak GS-520 HQ (7.5 i.d.×300 mm, with a guard column, measuring 7.5 i.d.×75 mm; Showa Denko) and GS-320 HQ (7.5 i.d.×300 mm, with a guard column measuring 7.5 i.d.×75 mm), was used for the separation of serum and tissue supernatants, and urine, respectively. The former column was injected with a 200 μL aliquot of sample and then eluted with 50 mM Tris–HCl, pH 7.4, at a flow rate of 0.6 mL/min. The latter one was injected with a 20 μL aliquot of sample and then eluted with 50 mM ammonium acetate, pH 6.5, at a flow rate of 0.6 mL/min. The eluate was introduced directly into a Babington nebulizer of the ICP-MS, and Se in the eluate was monitored at *m/z* 76, 77, 78, 80, and 82. Elution profiles of endogenous Se were represented by ^{76}Se chromatogram. Exogenous Se were expressed after subtracting the ^{77}Se count of natural abundance origin from the observed ^{77}Se chromatogram.

Statistical analysis

All statistical analyses were performed with JMP7.0 (SAS, USA). Differences from the control group were tested using the Dunnett method. A p value <0.05 was considered to be statistically significant.

Results and discussion

Distribution of exogenous Se (^{77}Se in SeHLan) in organs and serum

Se consists of six isotopes: ^{74}Se (0.89%), ^{76}Se (9.36%), ^{77}Se (7.63%), ^{78}Se (23.8%), ^{80}Se (49.6%), and ^{82}Se (8.73%). The Se with the lowest abundance, ^{74}Se, is the best choice as a tracer because the highest ratio of the corresponding endogenous natural abundance Se isotope to the tracer can be obtained. However, enriched ^{74}Se is too expensive to be used in animal experiments. Thus, ^{77}Se, which has the second lowest abundance, was selected as the labeling isotope in the study. We would like to cite another reason for choosing ^{77}Se: The deuterium (D_2) reaction mode of ICP-MS can effectively remove polyatomic interference originating from argon, such as $^{38}Ar^{38}Ar^+$, $^{40}Ar^{37}Cl^+$, $^{38}Ar^{40}Ar^+$, and $^{40}Ar^{40}Ar^+$, and avoid the generation of $^{79}Br^1H^+$ and $^{81}Br^1H^+$ when hydrogen (H_2) is used as the reaction gas [20]. Although the D_2 reaction mode has certain advantages over the H_2 reaction mode, it generates subtle but specific interference of $^{76}Se^2D^+$, $^{78}Se^2D^+$, and $^{80}Se^2D^+$ on ^{78}Se, ^{80}Se, and ^{82}Se, respectively. Hence, from the viewpoint of analytical chemistry, we used the second lowest isotope, ^{77}Se, to label SeHLan (Fig. 1).

The concentration of total Se, i.e., endogenous Se plus exogenous Se, in the kidneys was significantly increased 1 h after the administration of [^{77}Se]-SeHLan and then was restored rapidly 6 h after the administration (Fig. 2a). The

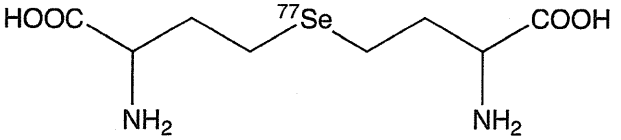

Fig. 1 Structure of [^{77}Se]-labeled selenohomolanthionine

concentration of exogenous Se in the kidneys was ten times higher than those in the other organs at 1 h after the administration (Fig. 2b). The major part of exogenous Se in the kidney supernatant that was eluted at the retention time of 18.6 min corresponded to SeHLan (Fig. 3b). This observation is coincident with the previous result that SeHLan showed renal accumulation when it was administered at a bolus dose of 1 mg/kg body weight [17]. However, the peak of exogenous Se corresponding to SeHLan disappeared within 24 h. These indicate that intact SeHLan is preferably accumulated but rapidly cleared in the kidney at the nutritional dose of 25 μg/kg body weight. The peak of exogenous Se corresponding to methyl-2-acetamido-2-deoxy-1-seleno-β-D-galactopyranoside (MeSeSug) began to be detected at 1 h after the injection, suggesting that SeHLan metabolism to MeSeSug started immediately. In addition, exogenous Se in the protein fraction was detected at the retention time of 10–16 min. Cellular glutathione peroxidase (cGPx, GPx1) is ubiquitously expressed in various organs, and thus, the major peak of endogenous Se may correspond to cGPx. A part of exogenous Se was also immediately used for the selenoprotein synthesis. Consequently, the experiments using [^{77}Se]-SeHLan indicated that SeHLan was preferably incorporated into the kidneys beyond the metabolic capacity to result in the accumulation of SeHLan in the intact form, but SeHLan was promptly utilized for the syntheses of selenosugars and selenoproteins.

Although the total Se concentrations in the other organs and serum were not altered by the administration (Fig. 2a), the concentrations of exogenous Se started to be actually increased in the organs and serum 1 h after the administration (Fig. 2b). This suggests that [^{77}Se]-SeHLan is rapidly assimilated, and assimilated ^{77}Se replaced endogenous Se in the organs and serum. In particular, the liver, pancreas, and serum assimilated ^{77}Se in SeHLan and replaced it with endogenous Se more efficiently than the other organs, such as the testes, muscle, and brain. The efficient assimilation of Se in the liver and pancreas was also observed in the case of other stable-isotope-labeled selenoamino acids, such as SeMet and MeSeCys [21]. Hence, it is speculated that these organs have specific metabolic pathways to assimilate these selenoamino acids. In contrast to the kidney supernatant, intact SeHLan was poorly detected in the liver supernatant even at 1 h after the injection (Fig. 4b). However, the precursor of MeSeSug, glutathionyl-2-acetamido-2-deoxy-1-seleno-β-D-galactopyranoside (GSSe-

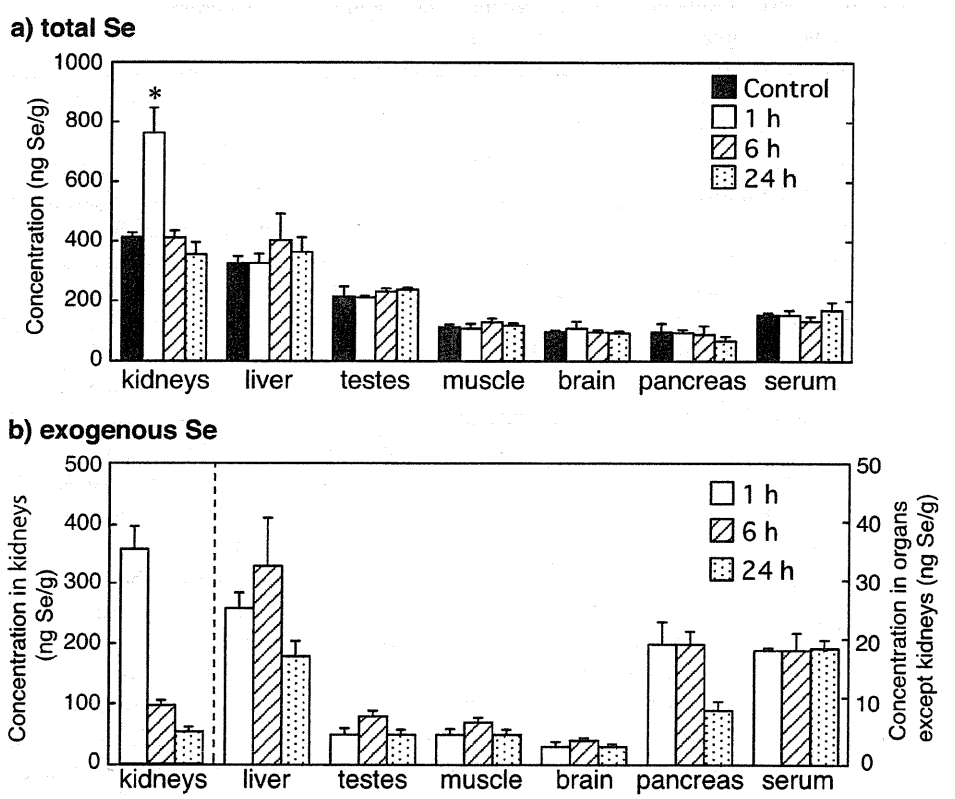

Fig. 2 Time-dependent changes in total Se (**a**) and exogenous Se (**b**) concentrations in the kidneys, liver, testes, muscle, brain, pancreas, and serum of rats injected with [^{77}Se]-SeHLan. The tissues and serum were collected at 0 (control), 1, 6, and 24 h after the intravenous administration of [^{77}Se]-SeHLan. Values are expressed as means ± SD for three rats. *Asterisk* indicates significant difference compared to the control group at $p<0.05$

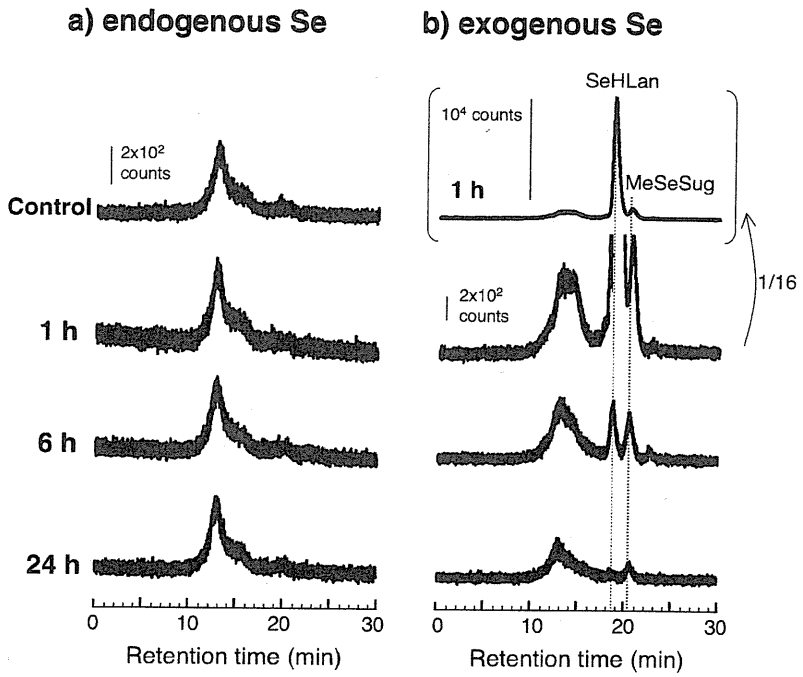

Fig. 3 Elution profiles of endogenous Se and exogenous Se in the kidney supernatants of rats injected with [^{77}Se]-SeHLan. Vertical bars indicate detection levels. The elution profile in parenthesis is a 1/16 image of the elution profile of exogenous Se in 1-h kidney supernatant. *MeSeSug* methyl-2-acetamido-2-deoxy-1-seleno-β-D-galactopyranoside

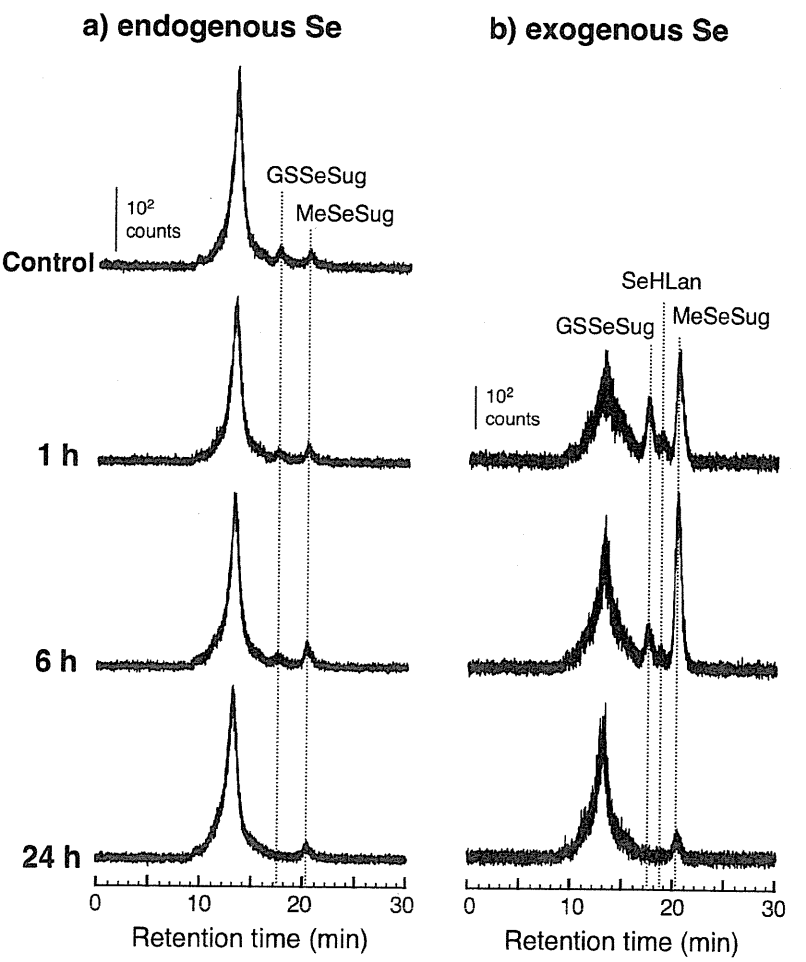

Fig. 4 Elution profiles of endogenous Se and exogenous Se in the liver supernatants of rats injected with [^{77}Se]-SeHLan. *Vertical bars* indicate detection levels. *GSSeSug* glutathionyl-2-acetamido-2-deoxy-1-seleno-β-D-galactopyranoside, *MeSeSug* methyl-2-acetamido-2-deoxy-1-seleno-β-D-galactopyranoside

Sug), was detected at a retention time of 17.3 min, in addition to MeSeSug. Although the total concentration of Se in the liver was comparable to that in the kidneys (Fig. 2a), the amount of soluble selenoproteins in the liver was larger than that in the kidneys (Figs. 3a and 4a). Thus, the amount of exogenous Se that assimilated into the hepatic soluble selenoproteins was also larger than the amount that assimilated into the renal soluble selenoproteins (Figs. 3b and 4b). These results suggest that the amount of SeHLan incorporated into the liver can be assimilated within metabolic capacity, and the liver has a larger metabolic capacity for Se than the kidneys.

Although the concentration of exogenous Se in the serum was almost steady throughout the experimental period (Fig. 2b), its components drastically changed as shown in the elution profiles of exogenous Se (Fig. 5). The major form of exogenous Se at 1 h after the injection was the intact form of SeHLan, and SeHLan disappeared from the serum within 6 h and was used for the biosynthesis of selenoprotein P (Sel P). The amount of Sel P decreased at 24 h after the injection, which marked the start of the biosynthesis of extracellular glutathione peroxidase (eGPx or GPx3). It is known that Sel P and eGPx are biosynthesized in the liver and kidneys, respectively [22, 23]. As mentioned above, the higher assimilation capacity and the more efficient assimilation of the liver than the kidneys may reflect the time lag for the biosynthesis of these serum selenoproteins after the injection of SeHLan. This result supports the idea that Sel P plays the role of an Se transport carrier in the bloodstream to deliver Se to specific organs. Indeed, an Sel P receptor has been identified on the cell surface, and it has been shown that the lack of the receptor results in Se deficiency [24, 25]. The time lag for the biosynthesis of serum selenoproteins was also observed when other selenoamino acids and inorganic Se salts were administered to the experimental animals [12, 21]. Hence, SeHLan shows the same metabolic fate as hitherto known selenoamino acids, such as SeMet and inorganic Se salts, and can therefore act as a nutritional source of Se. The metabolic pathway of SeHLan is only a speculation [17], while that of HLan, the sulfur analog of SeHLan, has been proven because HLan has been

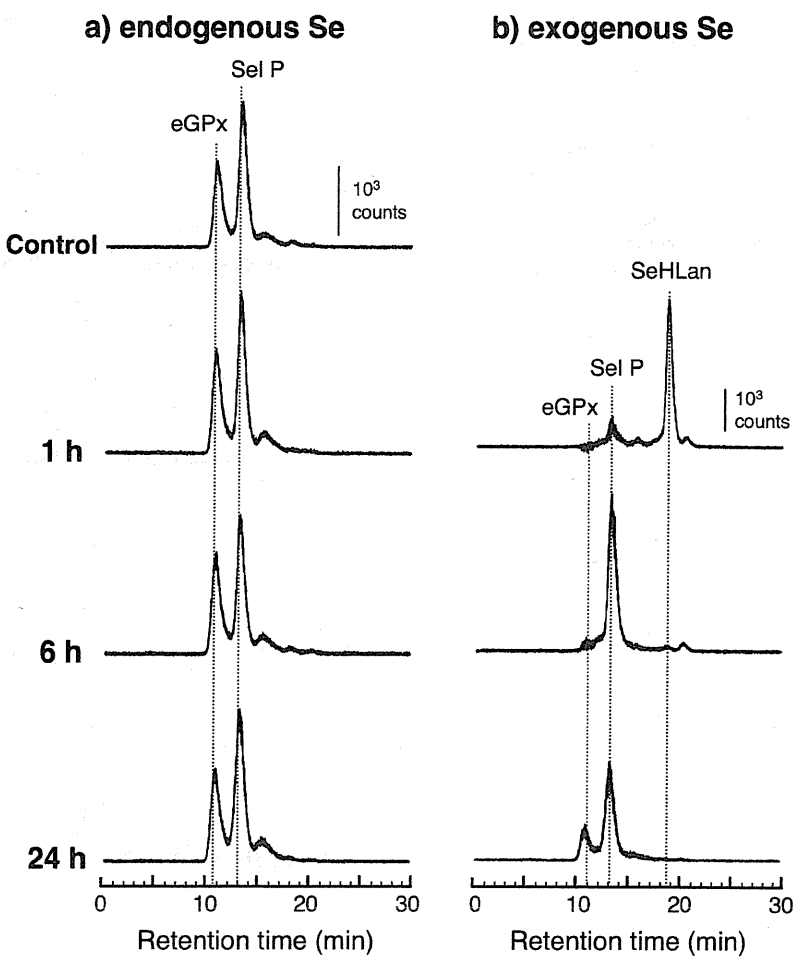

Fig. 5 Elution profiles of endogenous Se and exogenous Se in the serum of rats injected with [^{77}Se]-SeHLan. *Vertical bars* indicate detection levels. *eGPx* extracellular glutathione peroxidase, *Sel P* selenoprotein P

detected in the urine of homocystinuria patients [26]. HLan is biosynthesized via a γ-replacement reaction of two cystathionines catalyzed by cystathionine γ-lyase [27]. Although the inverse reaction of the γ-replacement has not been verified in vivo, Stekol and Weiss [16] indicated that radioisotope-labeled HLan was metabolized to Cys in rats and suggested a metabolic pathway from HLan to Cys via homocysteine and cystathionine. Based on the metabolic pathway of HLan, we suggest that SeHLan is metabolized to selenohomocysteine (SeHCys) and selenocysteine (SeCys). Then, SeHCys and SeCys are converted into selenide by γ- and β-lyase, respectively. As a result, the selenide that is converted from SeHLan is used as a metabolic intermediate for the biosyntheses of selenoproteins, selenosugars, and TMSe.

Time-dependent changes and speciation of exogenous Se (^{77}Se in SeHLan) and endogenous Se in urine

As the major urinary selenometabolite, MeSeSug was detected in the elution profiles of endogenous Se (Fig. 6). During the 6 h after SeHLan injection, the amount of endogenous Se increased, suggesting that exogenous Se pushed out endogenous Se from the Se pool in the body. As speculated in the literature [28], an Se pool may exist in the body. The suggestion that exogenous Se pushed out endogenous Se is explainable by the existence of the Se pool. A large part of exogenous Se that was excreted into urine was MeSeSug (Fig. 6). TMSe was detected in the initial 6-h urine sample, while an apparent peak corresponding to an intact form of SeHLan was not detected. Although substantial amounts of intact SeHLan existed in the kidney supernatant (Fig. 3), SeHLan was not excreted into urine. The intact form of SeHLan was excreted into urine when the rats were administered SeHLan at the bolus dose of 1.0 mg/kg body weight [17]. This suggests that SeHLan is not filtered by glomeruli or efficiently reabsorbed by renal tubules at the dose of 25 μg/kg body weight. The peaks of exogenous Se that was eluted at the retention times of 15–17 min could not be assigned yet despite their frequent detection in urine [12, 17, 21]. Actually, endogenous Se was also detected at the same retention times (Fig. 6). Although ESI-MS-MS is required to identify minor urinary selenometabolites, the trace amounts of the targets and the high matrix interfere with the access to the compounds by the molecular mass spectrometer.

Conclusion

The specific tissue distribution of SeHLan was observed by injecting [^{77}Se]-labeled SeHLan. SeHLan was preferably distributed to the kidneys and remained in the serum in the intact form at the nutritional dose. However, the intact form of SeHLan was not detected in urine, suggesting that the kidney was able to metabolize SeHLan at that dose. Indeed, exogenous Se was converted into urinary metabolites, MeSeSug and TMSe. Exogenous Se was also used for the biosynthesis of selenoproteins, such as Sel P, eGPx, and cGPx. In contrast to SeMet and MeSeCys, SeHLan did not show accumulation in pancreas; thus, SeHLan is expected

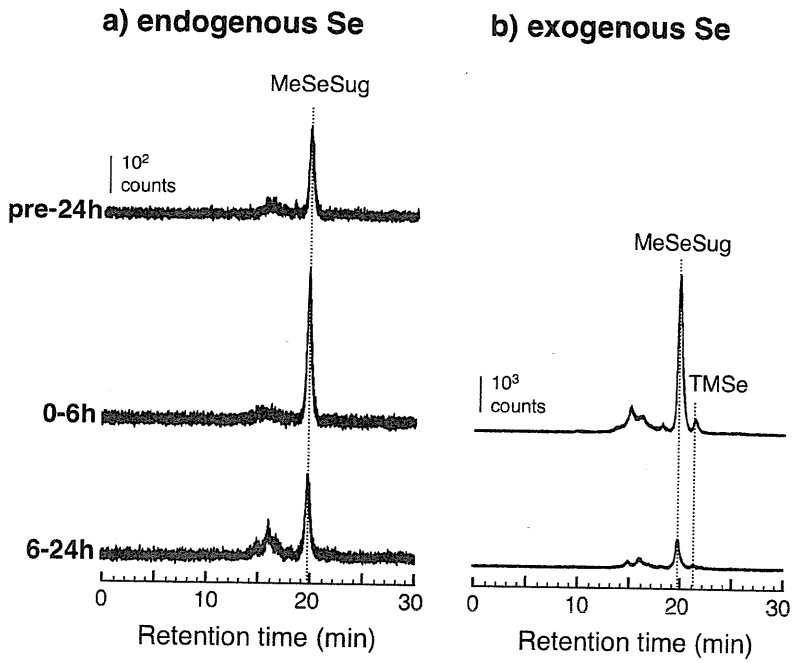

Fig. 6 Speciation of selenometabolites in urine samples. *Vertical bars* indicate detection levels. *MeSeSug* methyl-2-acetamido-2-deoxy-1-seleno-β-D-galactopyranoside, *TMSe* trimethylselenonium

to be used as a nutritional supplement of Se in place of SeMet.

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