Absolute Labeling and Simultaneous Speciation in Tracer Experiments with Multiple Stable Isotopes

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An enriched stable isotope is recognized as a label because of its high abundance relative to the corresponding endogenous natural abundance isotope in hosts, and hence is a relative label. Here, lowering the corresponding endogenous natural abundance element, multiple enriched stable isotopes were used as absolute labels for each. Thus, host animals were depleted of natural abundance stable isotopes by feeding a single enriched isotope, followed by administration of multiple precursors labeled with different homo-elemental stable isotopes. Metabolites of each labeled precursor were traced and speciated simultaneously by HPLC-inductively coupled argon plasma-mass spectrometry without interference from endogenous natural abundance isotopes. In the present experiments, rats with a single stable isotope (^{82}Se) were administered with ^{76}Se-selenite and ^{77}Se-selenomethionine simultaneously. The metabolites of ^{76}Se and ^{77}Se were speciated simultaneously as absolute labels, and independently from the endogenous selenium. The advantages of multiple absolute labeling coupled with simultaneous speciation, and general applications to metallomics of biometals and other wider applications, are proposed and discussed.

Key words ——— tracer experiment, stable isotope, speciation, metallome, metallomics, metallo-metabolomics

INTRODUCTION

An enriched stable isotope can be used as a tracer in metabolic and mechanistic studies where a highly enriched stable isotope of the lowest or lower natural abundance ratio among the homo-elemental stable isotopes is selected as a better tracer. Such an enriched stable isotope can be recognized as a tracer by the relative ratio over the corresponding natural abundance ratio. Therefore, an element that does not contain a stable isotope of low natural abundance ratio is not readily applicable to tracer experiments. For example, although low abundance ^{74}Se is a suitable isotope for tracer experiments in the case of selenium (Se), which exists in six stable isotopic forms [^{74}Se (0.89), ^{76}Se (9.37), ^{77}Se (7.63), ^{78}Se (23.77), ^{80}Se (49.61) and ^{82}Se (8.73%)], neither isotopes can be used as an effective tracer in the case of copper (Cu) that consists of two stable isotopes of high natural abundance ratios [^{63}Cu (69.2) and ^{65}Cu (30.8%)], rendering them ineffective as biological tracers. Thus, a highly enriched stable isotope of low natural abundance ratio can be used as a better tracer, especially when the label can be used as an absolute tracer by neglecting the corresponding endogenous natural abundance isotope. However, a stable isotope of a high natural abundance ratio may be the only choice for use as a relative tracer in some case, as illustrated in Fig. 1A and 1C.

Recent developments in the detection of stable isotopes by mass spectrometry (MS), particularly inductively couple argon plasma (ICP)-MS, has enabled speciation analysis much more efficiently when coupled with HPLC in a hyphenated technique (HPLC-ICP MS).[1–3] An ICP MS can be used, not only as a multiple elements-specific detector, but also as a detector for stable isotopes used as tracers.[4] Metabolites of a precursor labeled with an enriched stable isotope can be identified much more easily by tracing the label with HPLC-ICP MS, compared with tracing using a radioisotope. In addition to the merits of using stable isotopes as tracers, HPLC-ICP MS can be used to speciate metabolites of different precursors labeled with multiple homo-elemental and hetero-elemental isotopes simultaneously, in a single experiment. Thus, if multiple nutritional sources of selenium, for example, are labeled with different stable isotopes and are administered simultaneously to a host animal, metabolites of each enriched stable isotope can be traced simultaneously. This makes it possible to compare various factors

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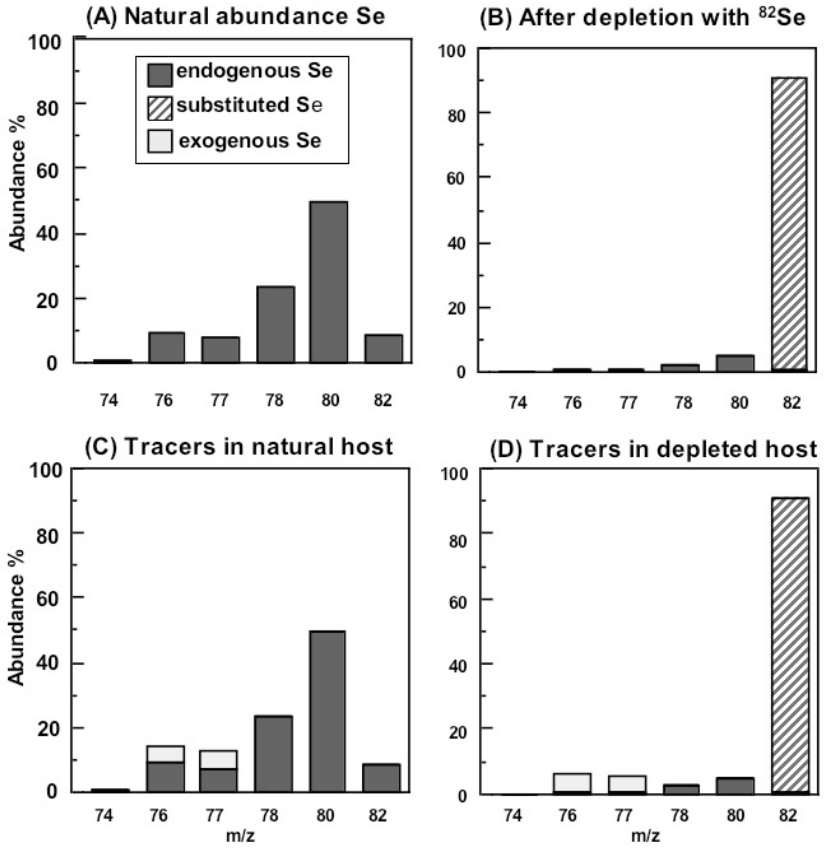

Fig. 1. Enriched Stable Isotopes as Relative and Absolute Tracers

A highly enriched stable isotope of a lower natural abundance ratio is recommended as a better tracer, *i.e.*, ^{74}Se in panel A. Enriched isotopes ^{76}Se and ^{77}Se used as tracers can be recognized as labels relative to the corresponding endogenous natural abundance isotopes (relative tracers) (panel C). Replacement of endogenous natural abundance isotopes with a single isotope lowers the original endogenous natural abundance isotopes (panel B), and the relative ratio of (labeled isotopes ^{76}Se and ^{77}Se) to (the corresponding endogenous isotopes) increases, whereby the endogenous isotopes decrease to a negligible level (absolute tracers) (panel D).

such as absorption, distribution, availability and excretion exactly under identical host and analytical conditions with a single experiment. However, tracer experiments using multiple enriched stable isotopes simultaneously have not been applied. This application can be undertaken with use of multiple homo-elemental stable isotopes according to the scheme illustrated in Fig. 2.

The simultaneous administration and speciation method (*i.e.*, simultaneous feeding of multiple precursors labeled with different enriched stable isotopes followed by simultaneous speciation with HPLC-ICP MS) can be carried out by feeding multiply enriched stable isotopes to host animals containing the corresponding natural abundance isotopes. However, the participation of the endogenous natural abundance isotopes must be subtracted to reveal the exogenous enriched stable isotopes as tracers. The data handling process is essential, particularly for stable isotopes with high natural abundance ratios. The process is theoretically simple. However, it is practically not easy to delete only data resulting from endogenous stable isotopes, especially for stable isotopes of high natural abundance ratios.

The present study was intended to develop, by lowering the endogenous natural abundance isotopes, a simple and efficient tracer method using simultaneous multiple stable isotopes. Typically, we employed two nutritional sources of selenium which were labeled with different stable isotopes [*i.e.*, ^{76}Se-selenite and ^{77}Se-selenomethionine (SeMet)]. To enhance sensitivity and maximize the detectable ratio, host animals (rats) were depleted of natural abundance selenium stable isotopes with a single enriched selenium isotope (^{80}Se). The depletion process lowered the endogenous natural abundance selenium, which made the labeled stable isotope equivalent to those of low natural abundance ratio. Thus, tracers were converted from relative to absolute labels. Multiple homo-elemental labels (^{76}Se and ^{77}Se) were

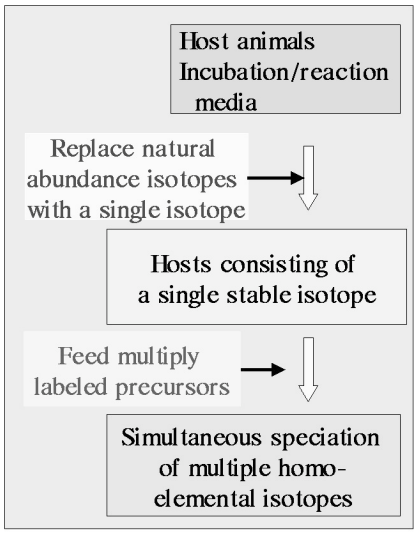

Fig. 2. Experimental Procedure for Absolute Labeling and Simultaneous Speciation

Hosts such as animals, plants and microorganisms are depleted of the endogenous natural abundance isotopes by feeding a single enriched stable isotope. Then, various precursors labeled with different homo- and/or hetero-elemental isotopes are administered to the depleted hosts, and then each isotope is determined simultaneously by HPLC-ICP MS.

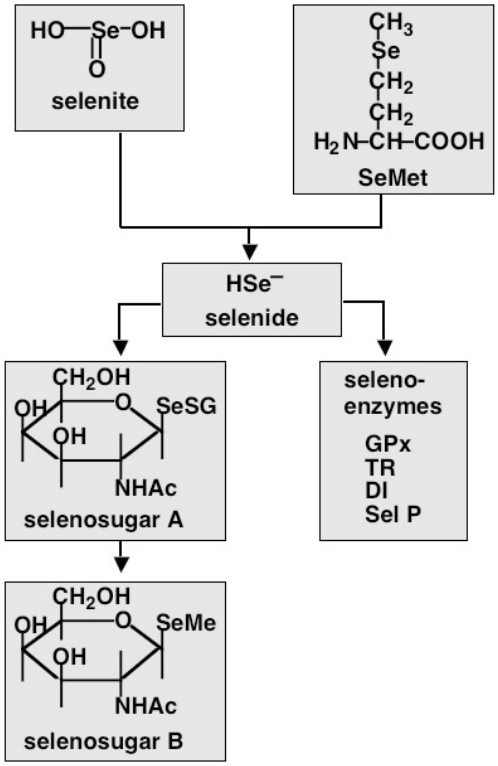

Fig. 3. Metabolic Pathway for Selenium: Nutritional Sources, Intermediate, Selenoenzymes for Utilization and Selenosugars for Excretion

Diverse selenium sources, typically presented as selenite and SeMet are transformed to the common intermediate, selenide, and then the selenide is utilized for the synthesis of selenoenzymes as gene products or surplus one is transformed to the major urinary metabolite, selenosugar. Glutathione peroxidase (GPx), thioredoxin reductase (TR), deiodinase (DI) and selenoprotein P (Sel P).

simultaneously speciated by HPLC-ICP MS, and the results were presented as independent absolute labels, as illustrated in Fig. 3. The potential merits are discussed and wide applications proposed.

MATERIALS AND METHODS

Preparation of ^{76}Se-, ^{78}Se- and ^{82}Se-Selenite, and ^{77}Se-SeMet ——— Metallic forms of ^{76}Se (99.8), ^{77}Se (99.9), ^{78}Se (97.8) and ^{82}Se (98.9% enriched) were purchased from Isoflex U.S.A., San Francisco, U.S.A. ^{76}Se-, ^{78}Se- and ^{82}Se-Selenite were prepared by dissolving each metallic form in nitric acid, followed by adjustment to neutral pH. ^{77}Se-SeMet was synthesized from (S)-(–)-α-amino-γ-butyrolactone by three steps,[5] and the chemical structure was confirmed by NMR and mass spectral data together with HPLC-ICP MS data on a GS 220 column.

Depletion of Natural Abundance Selenium in Rats ——— Male weanling Wistar rats of 3 weeks of age were purchased from Clea Japan Inc. (Tokyo, Japan), maintained in plastic cages at 22 ± 2°C with a light/dark cycle of 12/12 hr, and fed a selenium-deficient diet (Oriental Yeast Co., Ltd.; selenium concentration, < 0.02 μg/g diet) and drinking water containing ^{82}Se-selenite at a concentration of 1.0 μg Se/ml *ad libitum* for 14 days, and then the same diet and water without selenite for 10 days. The rats (n = 3) at 7 weeks of age were injected intravenously with a mixed solution of ^{76}Se-selenite and ^{77}Se-SeMet at the dose of 20 μg Se/kg body weight for each (Fig. 4B, 4D, 4F, and 4G). The rats were sacrificed by exsanguinations under light ether anesthesia, and livers were excised after whole body perfusion, homogenized with a Polytron homogenizer (Kinematica, Switzerland) in 4 volumes of 50 mM Tris-HCl buffer solution, pH 7.4, in an atmosphere of nitrogen. The homogenates were centrifuged at 105000 g for 60 min to obtain the supernatant fraction. Instead of ^{82}Se-selenite for the depletion, selenite of natural abundance ratio was used for the control in the rats for panels A–D. Labeled selenite and SeMet were not injected into the rats for panels A, C and E in Fig. 4. The experimental procedures used in the present study met the guidelines of the Animal Care and Use Committee of Graduate School

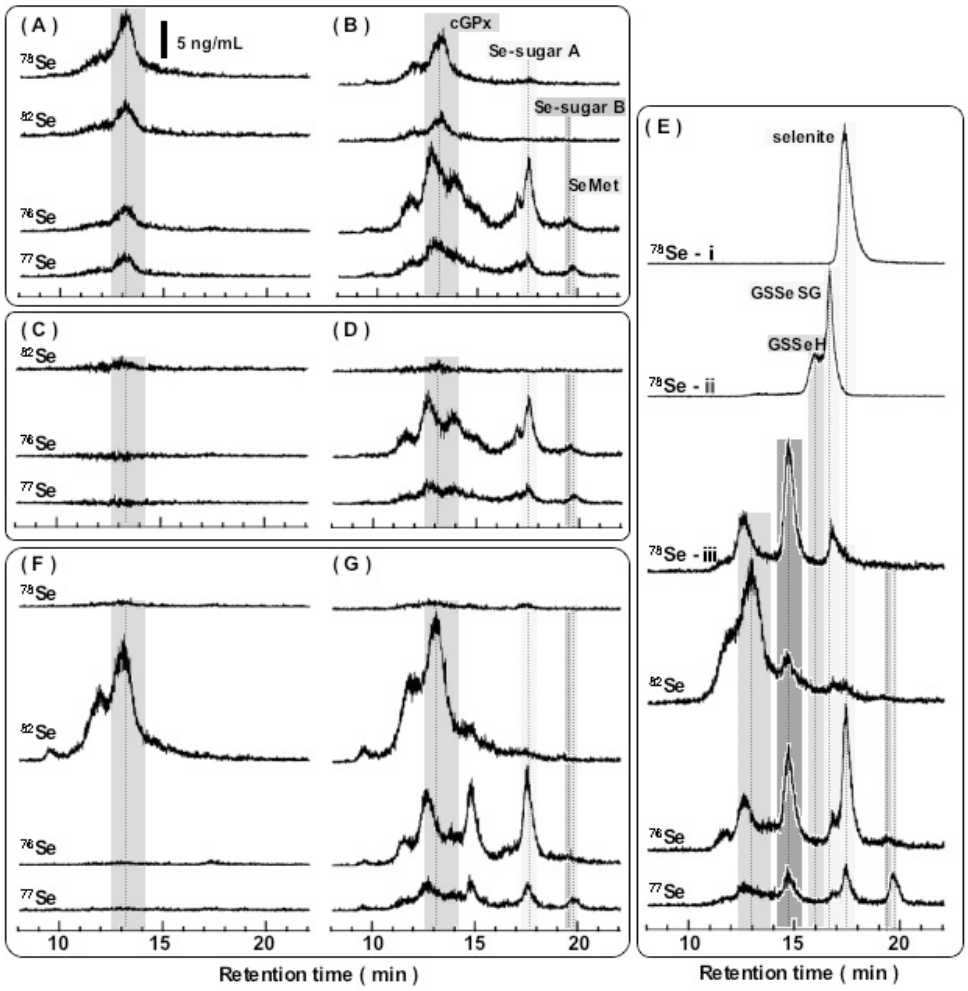

Fig. 4. Simultaneous Speciation of Multiple Homo-Elemental Isotopes: Selenium of ^{76}Se-Selenite and ^{77}Se-SeMet Origin in the Liver Supernatant of Rats by HPLC-ICPMS

Male Wistar rats of 3 weeks of age were fed a selenium-deficient diet to all groups, while they were fed drinking water containing natural abundance selenite (panels A–D) or ^{82}Se-selenite (panel F and G) *ad libitum*. Profiles in panels A and B show those before the subtraction, whereas profiles in panels C and D are presented after subtraction of the corresponding endogenous isotopes. Panels A, C, and E are the respective controls without administration of ^{76}Se-selenite and ^{77}Se-SeMet, and panels B, D, and F are the experimental groups administered with ^{76}Se-selenite and ^{77}Se-SeMet. Selenosugar A (Se-sugar A) and B (Se-sugar B), cGPx, GSSeH, GSSeSG.

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HPLC-ICPMS for Speciation of Selenium in Liver Supernatants —— An Agilent 7500cs ICPMS (Yokogawa Analytical Systems, Hachiouji, Japan) was coupled to an HPLC system as the detector. The HPLC system consisted of an on-line degasser, an HPLC pump (PU713; GL Science Co., Ltd., Tokyo), a Rheodyne six-port 8 injector with a sample loop, and a column. A gel filtration Shodex Asahipak GS-520 7G column (7.5 i.d. × 500 mm, with a guard column, 7.5 i.d. × 75 mm; Showa Denko, Tokyo) was used. A 200-μl aliquot of the liver supernatant was applied on a column, and then the column was eluted with 50 mM Tris-HCl, pH 7.4, at a flow rate of 1.0 ml/min. The eluate was introduced directly into the Babington nebulizer of the ICP MS.

RESULTS AND DISCUSSION

Conversion of Tracers from Relative to Absolute Labels in Tracer Experiments with Use of Enriched Stable Isotopes

Although the natural abundance ratio of each element is fixed in nature, endogenous natural abundance ratios can be lowered by depleting the endogenous natural abundance stable isotopes with a single

stable isotope. In the case of selenium, ratios of each stable isotope shown in Fig. 1A can be changed to those in Fig. 1B by depleting the endogenous natural abundance stable isotopes with one of the isotopes of selenium (*i.e.*, ^{82}Se). The exogenous to endogenous ratio will become greater by this depletion. Not only ^{74}Se, but also ^{76}Se and ^{77}Se, and possibly ^{78}Se and even ^{80}Se, can be used as absolute labels depending on the extent of depletion, as illustrated in Fig. 1C and 1D. Hence, this depletion process converts relative tracers to absolute ones. In addition, the endogenous and exogenous (labeled) selenium can be clearly separated as ^{82}Se and the other isotopes, respectively, and their interaction can be easily identified in the depleted hosts.

In tracer experiments, hosts can be animals, plants and microorganisms *in vivo*, or reaction and incubation media *in vitro*. Hosts can be depleted of natural abundance stable isotopes by feeding an enriched single stable isotope. In the present study, weanling rats (3 weeks of age) were depleted of endogenous natural abundance stable isotopes by feeding a selenium-deficient diet (less than 0.02 μg Se/g) and drinking water containing ^{82}Se-selenite (98.9% enriched) at a concentration of 1.0 μg/ml for two weeks. Replacement of natural abundance stable isotopes with one of the homo-elemental stable isotopes would likely be most efficient in younger animals, or by feeding the diet to dams starting at early gestation.

Simultaneous Feeding of Multiple Precursors Labeled with Different Homo-Elemental Stable Isotopes

Inorganic (selenite and selenate) and organic [selenocysteine (SeCys), SeMet, methylselenocysteine (MeSeCys) and their residues] selenocompounds are the usual forms of selenium in nutritional sources.[6–8] They are transformed to the assumed common intermediate (selenide) and then utilized for the synthesis of selenoenzymes and/or excreted into urine after being transformed to the major urinary metabolite selenosugar, as depicted schematically in Fig. 3. Therefore, only the element selenium out of nutritional selenochemicals can be incorporated into selenoenzymes and selenosugars from nutritional sources, and selenium sources can be traced only with selenium.[6–8] These diverse nutritional selenium sources can be labeled with different stable isotopes for each, and traced simultaneously by feeding all of them at a single trial in which experiments including hosts, sample preparations and analytical conditions can be carried out under controlled, identical conditions.

The present tracer method can be applied widely to metabolic and mechanistic studies in metallomics.[9–11] Any biometals consisting of multiple stable isotopes such as zinc [^{64}Zn (48.6), ^{66}Zn (27.9), ^{67}Zn (4.10), ^{68}Zn (18.8) and ^{70}Zn (0.62%)] and nickel [^{58}Ni (68.1), ^{60}Ni (26.2), ^{61}Ni (1.14), ^{62}Ni (3.63) and ^{64}Ni (0.93%)] can be applied as in the case of selenium. Furthermore, copper consisting of two stable isotopes of high natural abundance ratios is difficult to use in tracer experiments with stable isotopes. However, by depleting of natural abundance stable isotopes of copper with either of the isotopes, the other enriched isotope can be used as an absolute tracer.

Furthermore, the depletion makes it possible to use an isotope of high natural abundance ratio as a tracer. In the case of selenium, ^{80}Se of the highest natural abundance ratio in natural abundance SeMet can be a good (49.61% enriched) tracer in hosts after depletion with one of selenium isotopes. This application can be efficiently used when a labeled precursor is not easy to prepare but natural one is available, such as SeMet and selenosugar.[12] Thus, the present depletion method is applicable efficiently and widely to tracer experiments.

Simultaneous Speciation of Metabolites of Different Precursors in Metallomics and Metabolomics

Each precursor labeled with different homo-elemental isotopes can be traced as an absolute label in hosts depleted of natural abundance isotopes. A metallome in a biological system is now speciated with a hyphenated technique, presently most commonly by HPLC-ICP MS.[1–3] Thus, multiple metallomes of each precursor origin, in which they are labeled with different homo-elemental isotopes, can be speciated simultaneously by HPLC-ICP MS together with hetero-elemental isotopes under identical sampling and analytical conditions.

Application to the Metabolic Study of Selenium

Here, the present method was applied to the metabolic study of selenium by administering two typical selenium sources (^{76}Se-selenite and ^{77}Se-SeMet) into rats that had been depleted of endogenous natural abundance selenium with a single stable isotope (^{82}Se). The two nutritional sources labeled with two different stable isotopes were injected intravenously into depleted and control rats, and then livers were subjected to HPLC-ICP MS analysis.

Figure 4 demonstrates the results of the present application by showing the distribution profiles of selenium isotopes in the liver supernatant after feeding the two selenium sources (selenite and SeMet) labeled differently with ^{76}Se and ^{77}Se, respectively. Two sets of distribution profiles on a GS 520 gel filtration column are presented, one for the control without administering ^{76}Se and ^{77}Se sources, and the other for those administered with ^{76}Se and ^{77}Se sources at a single dose of 20 μg Se/kg body weight for each.

Figure 4A and 4B show the distributions of ^{78}Se, ^{82}Se, ^{76}Se and ^{77}Se in the liver supernatants of rats that had been fed the selenium-deficient diet and drinking water containing natural abundance selenite at 1.0 μg Se/ml, and then administered saline (A), or simultaneously with ^{76}Se-selenite and ^{77}Se-SeMet (B).

The four profiles in Fig. 4A show the similar distributions of broad (retention time, 12.0 min) and relatively sharp (13.0 min) peaks of selenoproteins reflecting the intensities of the natural abundance ratio (^{78}Se : ^{82}Se : ^{76}Se : ^{77}Se = 23.8 : 8.73 : 9.37 : 7.63). The latter peak seems to be cellular glutathione peroxidase (cGPx).

Figure 4B shows the distribution profiles of each selenium isotope in the liver supernatant of rats after a simultaneous intravenous injection of ^{76}Se-selenite and ^{77}Se-SeMet; ^{78}Se and ^{82}Se profiles are similar to those shown in Fig. 4A, while ^{76}Se and ^{77}Se profiles gave additional peaks reflecting the metabolites of ^{76}Se-selenite and ^{77}Se-SeMet, respectively. Although both profiles were similar, ^{77}Se peaks of SeMet origin were smaller than ^{76}Se peaks of selenite origin, suggesting that SeMet was distributed less efficiently in the liver than selenite. Although endogenous ^{76}Se and ^{77}Se may be overlapping on the broad exogenous ^{76}Se and ^{77}Se peaks between 11–15 min, the present profiles are different from those of endogenous peaks owing to the metabolites of ^{76}Se-selenite and ^{77}Se-SeMet, respectively. However, those peaks were not examined further in the present study. The sharp peak at 17.4 min in the ^{76}Se and ^{77}Se profiles was eluted at the same retention time as that of selenosugar A, while the small peak at 19.6 min in the ^{77}Se profile corresponded to selenosugar B.[12,13] Although the small peak was eluted at 19.3 min in the ^{76}Se profile, it did not coincide with selenosugar B, but was assignable to be SeMet from the same retention time as that of the authentic SeMet, suggesting the distribution of intact SeMet in the liver. These similar peaks were clearly separated on the separate profiles, demonstrating an additional merit of the present application with simultaneous multi-labeling for identification of metabolites.

Figure 4C and 4D are those of Fig. 4A and 4B, respectively, after each corresponding endogenous isotope being subtracted. The contribution of the endogenous natural abundance isotope was calculated from the ^{78}Se profile and subtracted from each profile according to the natural abundance ratio. After this data processing, the control panel C showed only broad noise peaks between 11–15 min. On the other hand, the ^{76}Se and ^{77}Se profiles in panel D revealed the distributions of metabolites of ^{76}Se-selenite and ^{77}Se-SeMet, respectively, without overlapping each corresponding endogenous natural abundance isotope.

Figure 4E and 4F show the distributions of ^{78}Se, ^{82}Se, ^{76}Se and ^{77}Se in the liver supernatant of the rats that had been depleted of natural abundance selenium, and then administered saline (E) or simultaneously with ^{76}Se-selenite and ^{77}Se-SeMet (F). Figure 4E demonstrates that the endogenous natural abundance selenium was replaced completely with ^{82}Se, and ^{82}Se was present only as single endogenous selenium stable isotope. No noises due to subtraction processes were observed. Feeding of the labeled selenite and SeMet to the depleted rats produced only ^{76}Se and ^{77}Se peaks of each label origin, together with demonstrating ^{82}Se profile as the new single endogenous selenium, as shown in Fig. 4F. Small but distinct peaks of selenosugar B and SeMet were detected together with distinct selenosugar A and unidentified peaks at 11.3, 12.5 and 14.6 min in both ^{76}Se and ^{77}Se profiles in Fig. 4F.

To gain an insight of the unidentified peaks in Fig. 4F, *in vitro* experiments were carried out with the liver supernatant used for panel F, as shown in Fig. 4G. Although the ^{78}Se-selenite was eluted at 17.3 min (profile G-i) as a single peak in the extraction buffer, it was reacted with glutathione (GSH) to give two peaks (profile G-ii). The two peaks were assumed to be S-selenoglutathione (GSSeH) (15.9 min) and S,S′-selenodiglutathione (GSSeSG) (16.6 min)[14] from the relative ratio of the two peaks depending on the relative ratio of GSH to selenite [*i.e.*, more GSSeH at a higher GSH to selenite ratio (data not shown)]. When ^{78}Se-selenite and GSH were incubated in the liver supernatant used for panel F, ^{78}Se was eluted as three peaks (12.5, 14.6 and 16.6 min), as shown in Fig. 4G-iii. The three peaks appearing by incubation with the liver supernatant ap-

pear to correspond to those in the ^{76}Se and ^{77}Se profiles in Fig. 4F. Although ^{76}Se-selenite can be transformed to the reduced forms of selenium (GSSeH and GSSeSG) even during the sampling procedure in the liver supernatant (*i.e.*, as *in vitro* products), ^{77}Se-SeMet cannot be transformed by incubation in the liver supernatant *in vitro* (data not shown). Therefore, ^{77}Se-SeMet taken up by the liver is deduced to be transformed *in vivo* to selenide by trans-selenation and β-lyase reactions.[6,8] Selenide be reacted with constituents in the liver supernatant as in the case of ^{76}Se-selenite. Thus, the present *in vitro* experiments also demonstrated potential merits of use of enriched stable isotopes as absolute tracers, not only in *in vivo* metabolic studies, but also in *in vitro* mechanistic studies.

Applications of the Use of Multiple Stable Isotopes as Each Absolute Label Followed by Simultaneous Speciation to Metallomics of Biometals

The present application (simultaneous labeling with different homo-elemental stable isotopes followed by simultaneous speciation) to metabolic and mechanistic studies of selenium *in vivo* and *in vitro* demonstrated several merits that can be widely applicable to metallomics of general biometals; Multiple homo-elemental stable isotopes can be used simultaneously as independent tracers without interference from the corresponding endogenous natural abundance isotope. Multiple homo-elemental stable isotopes used as tracers can be traced and speciated simultaneously by ICP MS and HPLC-ICP MS. Simultaneous multiple labeling and feeding of isotopic tracers to a host, followed by simultaneous speciation, makes it possible to evaluate biological data such as absorption, distribution and excretion accurately and sensitively under the same sampling and analytical conditions in hosts in a single procedure. The present absolute labeling and simultaneous detection method can be applied, not only to metabolic study *in vivo* but also to mechanistic studies *in vivo* and *in vitro*. The use of absolute stable isotopes as authentic samples simplifies the identification procedure of complex peaks on HPLC-ICP MS profiles with much more reliable comparison in the speciation analysis. Absolute labeling and simultaneous detection can be coupled, not only for homo-elemental isotopes, but also for hetero-elemental isotopes. An isotope of high natural abundance ratio can be used as a tracer in the depleted hosts. Thus, the present method will be a widely applicable powerful means to metallomics / metabolomics of various biometals.

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REFERENCES

1) Montes-Bayon, M., DeNicola, K. and Caruso, J. A. (2003) Liquid chromatography-inductively coupled plasma mass spectrometry. *J. Chromatogr., A*, **1000**, 457–476.
2) Szpunar, J., Lobinski, R. and Prange, A. (2003) Hyphenated techniques for elemental speciation in biological systems. A. *Appl. Spectrosc.*, **57**, 102A–111A.
3) Lobinski, R., Edmonds, J., Suzuki, K. T. and Uden, P. (2000) Species-selective analysis for selenium compounds in biological materials. *Pure Appl. Chem.*, **72**, 447–461.
4) Suzuki, K. T. (1996) Simultaneous speciation of endogenous and exogenous elements by HPLC/ICP-MS with enriched stable isotopes. *Tohoku J. Exp. Med.*, **178**, 27–35.
5) Plenevaux, A., Cantineau, R., Guillaume, M., Christiaens, L. and Tihange, G. (1987) Fast chemical synthesis of [^{75}Se]L-selenomethionine. *Int. J. Rad. Appl. Instrum. A*, **38**, 59–61.
6) Birringer, M., Pilawa, S. and Flohe, L. (2002) Trends in selenium biochemistry. *Nat. Prod. Rep.*, **19**, 693–718.
7) Whanger, P. D. (2002) Selenocompounds in plants and animals and their biological significance. *J. Am. Col. Nutr.*, **21**, 223–232.
8) Suzuki, K. T. (2005) Metabolomics of selenium: Se metabolites based on speciation studies. *J. Health Sci.*, **51**, 107–114.
9) Szpunar, J. (2004) Metallomics: a new frontier in analytical chemistry. *Anal. Bioanal. Chem.*, **378**, 54–56.
10) Haraguchi, H. (2004) Metallomics as integrated biometal science. *J. Anal. At. Spectrom.*, **19**, 5–14.
11) Suzuki, K. T. (2005) Metabolomics of arsenic based on speciation studies. *Anal. Chim. Acta*, **540**, 71–76.
12) Kobayashi, Y., Ogra, Y., Ishiwata, K., Takayama, H., Aimi, N. and Suzuki, K. T. (2002) Selenosugars are key and urinary metabolites for selenium excretion within the required to low-toxic range. *Proc. Natl. Acad. Sci. U.S.A.*, **99**: 15932–15936.
13) Francesconi, K. A. and Pannier, F. (2004) Selenium metabolites in urine: a critical overview of past work

and current status. *Clin. Chem.*, **50**, 2240–2253.
14) Ganther, H. E. (1999) Selenium metabolism, selenoproteins and mechanisms of cancer prevention: complexities with thioredoxin reductase. *Carcinogenesis*, **20**, 1657–1666.