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TRENDS

Integrated strategies for identification of selenometabolites in animal and plant samples

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Introduction

Selenium (Se) is an essential micronutrient in animals as it is required by certain redox-regulating enzymes, such as glutathione peroxidases and thioredoxin reductase. In plants, Se is not essential and exists as a "bystander" mineral. However, its beneficial effects on plant growth have been reported [1]. Some Se-containing compounds (selenocompounds), such as methylselenocysteine (MeSeCys) and γ-glutamylmethylselenocysteine (GluMeSeCys), have anti-tumor activity, and selenomethionine (SeMet), a naturally occurring selenoamino acid, has cancer-preventing effects. Indeed, SeMet has been used in the selenium and vitamin E cancer prevention trial (SELECT) in the United States, Puerto Rico, and Canada to determine if taking selenium (as SeMet) and/or vitamin E supplements can prevent prostate cancer [2]. These pharmacologically available selenoamino acid derivatives are biosynthesized in selenium-enriched (selenized) plants and yeast. Therefore, novel selenized food materials are being actively developed for use in cancer chemotherapy and chemoprevention. On the other hand, Se is widely used in industry, such as glass and ceramic manufacturing and electronics, and is thus also known as an environmental contaminant. From the viewpoint of environmental chemistry, Se-hyperaccumulating plants are applicable to the phytoremediation of Se-contaminated water and soil.

As a non-metallic element, Se is utilized in the metabolic pathways of animals and plants to form Se-containing compounds having carbon–Se covalent bond(s) (organic selenometabolites). Therefore, it is necessary to identify selenocompounds to determine the metabolic pathway of Se and understand the beneficial or toxicological effects of these compounds. However, as Se is a micronutrient, selenometabolites exist at extremely low concentrations in animals. The difficulty of detecting Se in each selenometabolite after separation on the basis of chemical properties (chemical speciation) has been overcome with the emergence of inductively coupled plasma-mass spectrometry (ICP–MS) as the most sensitive and robust Se detector available to date. Easily hyphenated with HPLC, HPLC–ICP–MS is the technique of choice for speciation of Se in biological samples.

However, there remains a critical disadvantage in the identification of selenometabolites by HPLC–ICP–MS. Although ICP–MS is sensitive to target elements and is robust to matrices, it provides little molecular information about selenometabolites. Thus, identification by HPLC–ICP–MS is limited to the situation where certified or authentic Se species are available. As an alternative to HPLC–ICP–MS, electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI)–tandem mass spectrometry (MS–MS) are used to identify unknown selenometabolites. The fact that Se has a characteristic cluster of signals that reflect its isotopic pattern (^{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 %) has facilitated the scanning of Se-containing species by MS. Because of this, Se is the preferred target of ESI–MS–MS analyses. ESI is a softer technique than ICP, and can provide molecular information. In addition, MS–MS enables structure elucidation. However, ESI–MS–MS has a number of weak points compared with ICP–MS. First, the detection limit of ESI–MS–MS for selenocompounds is inferior to that of ICP–MS. Second, ESI is severely affected by the sample matrix. To counter this

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problem, ESI–MS–MS is coupled with HPLC. Therefore, the complementary use of HPLC–ICP–MS and HPLC–ESI (APCI)–MS–MS is a powerful tool for speciation and identification of selenometabolites.

In this communication, the complementary use of ICP–MS, ESI–MS–MS, and NMR is discussed from the viewpoint of identification of unknown selenometabolites in biological samples. In particular, as the identification of selenometabolites in selenized yeast is well documented [3, 4], identification in animal and plant samples is focused on. As plant and animal samples have more complex matrices and contain less selenocompounds than yeast samples, sample pretreatment is the key to successful identification. Pretreatment methods and limitations of currently used techniques are also discussed.

Sample pretreatment for ESI–MS–MS

The basic strategy for the complementary use of ICP–MS, ESI–MS–MS, and NMR is depicted in Fig. 1. As the first step in the speciation of selenometabolites, the screening of selenometabolites is performed with HPLC–ICP–MS (indicated as HPLC–ICP–MS (I) in Fig. 1). In this step, since native samples from animals and plants contain complex matrices, a column that is robust to such complex matrices is required. The identification of urinary selenometabolites is discussed below, together with the following strategy.

Identification of the major selenometabolite in urine is one of the major concerns of Se research. Some minor urinary selenometabolites whose standards are available were identified by HPLC–ICP–MS (indicated as HPLC–ICP–MS (II) in Fig. 1; validation level 1) and other techniques [5]. Since the major urinary selenometabolite was not identical to any of the authentic Se species as shown by HPLC–ICP–MS, its structure had been unclear before the advent of ESI–MS–MS. However, high matrix concentration and low selenometabolite concentration in urine made structure elucidation by ESI–MS–MS difficult.

Urine contains large amounts of salt and water-soluble organic compounds, such as urea. These matrices severely interfere with the detection of selenocompounds by ESI–MS–MS. Even with the removal of the matrices, preconcentration is needed so that the selenocompounds would exceed the detection limit of ESI–MS–MS. Three research groups have reported detection of the major selenometabolite in urine by appropriate pretreatments.

Ogra et al. were the first to report that the major urinary selenometabolite in rat was a selenosugar [6]. Urea, one of the major matrix species, was decomposed by treatment with urease, and salts were removed by the addition of methanol. After removal of these major matrices, the sample was concentrated and subjected to preparative LC to remove remaining matrices. The eluate was the subjected to HPLC–ESI–MS–MS, and Se-methyl-*N*-acetylselenohexosamine (a selenosugar) was identified as the major urinary selenometabolite.

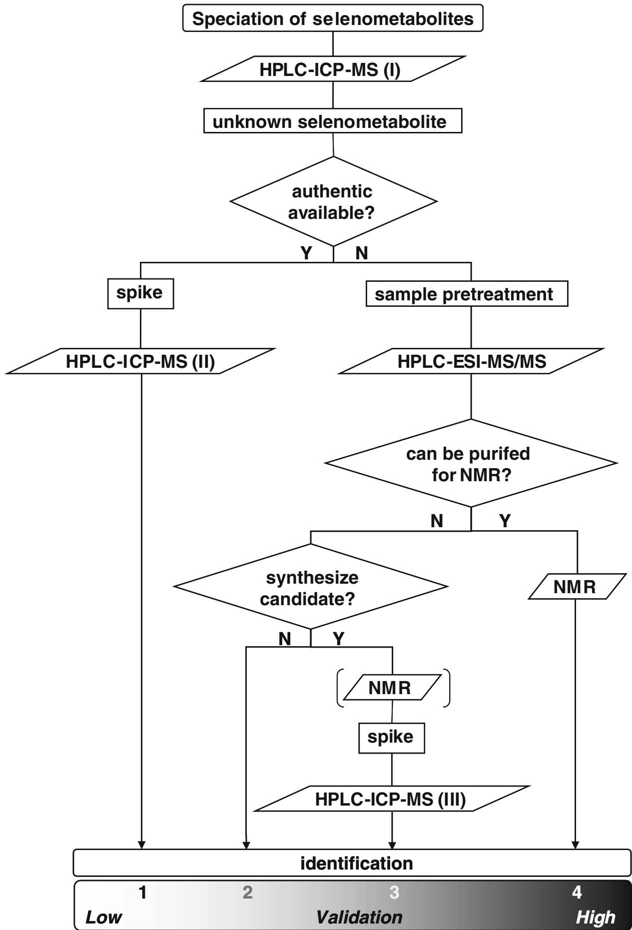

Fig. 1 Basic strategy for identification of selenometabolites by complementary use of HPLC–ICP–MS and HPLC–ESI–MS–MS. The validation level is defined on the basis of the suitability of strategies for identification of selenometabolites

Immediately after publication of the results mentioned above, Gammelgaard et al. reported that the major urinary selenometabolite in humans was the same as the selenosugar detected in rat urine [7]. Their sample pretreatments included solid-phase extraction, reversed-phase chromatography on a preparative scale, and fractionation by size-exclusion HPLC. The final eluate was concentrated and directly injected into APCI–MS^n (an ion-trap mass spectrometer). Although the authors attempted to detect the selenometabolite by ESI, they could not do so. However, they were able to detect it by APCI. In this case, the selenometabolite was dissolved in 50% methanol; thus, the metabolite was difficult to ionize by electrospray due to its neutral charge in the solvent.

Díaz Huerta et al. evaluated the sample preparation procedure for selenometabolites in urine [8]. They used

Table 1 Recently identified selenometabolites in animals and plants by ESI (APCI)–MS–MS

Compound	Acronym	Structure	Validation[1]
Animals			
Se-methyl-*N*-acetylselenogalactosamine	**MeSeGalNAc**		**4**
Se-methyl-selenogalactosamine	**MeSeGal**		**3**
Se-methyl-*N*-acetylselenoglucosamine	**MeSeGlcNAc**		**3**
Se-glutathionyl-*N*-acetylselenogalactosamine	**GSSeGalNAc**		**2**
Plants			
Se-methylselenomethionine selenonium	**MeSeMet**		**3**
selenohomolanthionine	**SeHLan**		**4**

[1] The validation level is defined in Fig. 1

crown ether extraction to remove sodium and potassium from urine samples. Although this method was effective for human urine, it was less effective than methanol desalting for rat urine, because rat urine has higher salinity than human urine. Their results also suggested that the desalting efficiency affected the mass spectra of urinary metabolites. Indeed, in the preceding two papers the selenometabolite was detected at *m*/*z* 300 as $[M + H]^+$. However, the selenometabolite was detected at *m*/*z* 322 and 338 as $[M + Na]^+$ and $[M + K]^+$, respectively, in the poorly desalted sample.

In contrast to animal samples, because plant samples contain highly insoluble cellular components, such as polysaccharides and lignins, extraction efficiency is important to detect selenometabolites in plants. Thus, enzyme extraction is always considered for Se detection.

Consequently, we must know the disadvantages of ESI–MS–MS for the complementary use of ICP–MS and ESI–MS–MS, particularly because ESI–MS–MS is more susceptible to the matrix effect and is relatively less sensitive for Se detection. Although the determination of selenosugars by HPLC–APCI–MS–MS without pretreatment has been reported, this method was suitable for the determination of "known" selenosugars [9]. Therefore, sample pretreatment, i.e., removal of matrices and preconcentration of selenometabolites, is important for identification of "unknown" species by ESI–MS–MS.

Limitations of ESI–MS–MS and overcoming them by use of NMR

The limitations of ESI–MS–MS should be discussed prior to its complementary use with ICP–MS. First, it is difficult to discriminate enantiomers and diastereomers with ESI–MS–MS. In a previous study that used HPLC–ESI–MS–MS to identify selenosugars in urine, the urinary selenometabolite was identified only as Se-methyl-*N*-acetylselenohexosamine [6]. This suggested that ESI–MS–MS could not unambiguously determine the configuration of the selenosugar, i.e., which anomer, α or β, and which epimer, glucopyranoside or galactopyranoside. Indeed, a candidate selenocompound chemically synthesized (Se-methyl-*N*-acetylselenoglucosamine) had different chromatographic behavior from the urinary selenometabolite in HPLC–ICP–MS [6]. The configuration of the urinary selenosugar was finally elucidated by NMR as methyl 2-acetamido-2-deoxy-1-seleno-β-d-galactopyranoside (Se-methyl-*N*-acetylselenogalactosamine, MeSeGalNAc) (shown as validation level 4 in Fig. 1 and Table 1) [10].

In the identification of selenocompounds in plants, the limitation mentioned above was pointed out. Recently, a novel selenocompound in radish, 4,4′-selenobis(2-aminobutanoic acid) (selenohomolanthionine, SeHLan), was identified [11]. According to the results of ESI–MS–MS, two candidate isomers, i.e., SeHLan and Se-methylselenocystathionine selenonium, were suggested because these two compounds gave identical molecular and fragment ions in the mass spectra. Although the target selenocompound was a minor Se species in radish, the matrix of the radish extract was not as complicated as that of animal samples. Thus, the selenocompound could be purified by two-dimensional HPLC and concentrated for NMR analysis. This identification also meets validation level 4.

As mentioned above, NMR can rigorously define the structure and/or configuration of a chemical species, so it is the most valuable technique for identification. However, NMR requires more stringent purification methods and a higher concentration of sample than ESI–MS–MS. Thus, this technique seems to be not always applicable to the identification of trace selenometabolites in animal and plant samples. As an alternative technique, ICP–MS coupled with HPLC or capillary electrophoresis (CE), having high resolution and/or a unique separation mode is used if a synthesized standard is available (indicated as HPLC–ICP–MS (III) in Fig. 1; validation level 3). For instance, Bendahl and Gammelgaard reported identification of additional selenosugars, for example Se-methyl-*N*-acetylselenoglucosamine (MeSeGlcNAc) and Se-methylselenogalactosamine (MeSeGalN), in human urine by HPLC–ICP–MS and CE–ESI–$(MS)^2$, using their synthesized standards [12]. Se-methylselenomethionine selenonium was also identified on the basis of this concept by Grant et al. [13].

The enantiomeric separation of naturally occurring selenoamino acids has been reported by Kápolna et al. [14]. Selenoamino acids in selenized chives were analyzed by HPLC–ICP–MS with a chiral column. Not surprisingly, the results revealed the presence of the L enantiomer of selenoamino acids, for example L-MeSeCys and L-SeMet, in the sample. This suggests that HPLC–ICP–MS equipped with a high-performance column overcomes the disadvantage of ESI–MS–MS in the identification of selenocompounds.

Second, there is a substantial difference in sensitivity to selenocompounds between ICP–MS and ESI–MS–MS. It has been reported that some selenometabolites, being precursors of urinary selenometabolites, were detected in liver by HPLC–ICP–MS, whereas only the most abundant hepatic selenometabolite, a glutathionylselenosugar, was identified by ESI–MS–MS without use of NMR, due to its low sensitivity (validation level 2) [10]. Other minor selenometabolites in liver remain unknown because their amounts are below the detection limit of ESI–MS–MS. On the other hand, as the amounts of urinary selenometabolites can be increased by Se supplementation, identification of urinary selenosugars by ESI–MS–MS and NMR is possible. However, it is difficult to increase the amounts of

selenometabolites in organs by Se supplementation because Se homeostasis is strictly maintained in the body. Thus, the amounts of upstream selenometabolites (metabolic intermediates) of selenosugars are still below the detection limits of ESI–MS–MS and NMR.

Outlook

As mentioned above, Se-containing compounds having carbon–Se covalent bond(s), i.e., selenometabolites, are formed in the metabolic pathways of animals and plants. Further improvement of the sensitivity of ESI–MS–MS or utilization of a high-sensitivity mass spectrometer, such as a Fourier-transform ion cyclotron resonance mass spectrometer (FT-ICR-MS) [15], is expected to lead to identification of the upstream selenometabolites of selenosugars, thereby paving the way to revealing the entire metabolic pathway of Se.

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References

1. Freeman JL, Lindblom SD, Quinn CF, Fakra S, Marcus MA, Pilon-Smits EA (2007) New Phytol 175:490–500
2. http://www.crab.org/select/
3. McSheehy S, Pohl P, Lobinski R, Szpunar J (2001) J Anal At Spectrom 16:1055–1062
4. McSheehy S, Szpunar J, Haldys V, Tortajada J (2002) J Anal At Spectrom 17:507–514
5. Francesconi KA, Pannier F (2004) Clin Chem 50:2240–2253
6. Ogra Y, Ishiwata K, Takayama H, Aimi N, Suzuki KT (2002) J Chromatogr B 767:301–312
7. Gammelgaard B, Grimstrup Madsen K, Bjerrum J, Bendahl L, Jøns O, Olsen J, Sidenius U (2003) J Anal At Spectrom 18:65–70
8. Díaz Huerta V, Szpunar J, Lobinski R, Fernández Sánchez ML, Sanz-Medel A (2003) J Anal At Spectrom 18:1471–1476
9. Letsiou S, Nischwitz V, Traar P, Francesconi KA, Pergantis SA (2007) Rapid Commun Mass Spectrom 21:343–351
10. Kobayashi Y, Ogra Y, Ishiwata K, Takayama H, Aimi N, Suzuki KT (2002) Proc Natl Acad Sci USA 99:15932–15936
11. Ogra Y, Kitaguchi T, Ishiwata K, Iwashita Y, Suzuki N, Suzuki KT (2007) J Anal At Spectrom 22:1390–1396
12. Bendahl L, Gammelgaard B (2004) J Anal At Spectrom 19:950–957
13. Grant TD, Montes-Bayón M, LeDuc D, Fricke MW, Terry N, Caruso JA (2004) J Chromatogr A 1026:159–166
14. Kápolna E, Shah M, Caruso JA, Fodor P (2007) Food Chem 101:1398–1406
15. Pickford R, Miguens-Rodriguez M, Afzaal S, Speir P, Pergantis SA, Thomas-Oates JE (2002) J Anal At Spectrom 17:173–176