Determination of Selenomethionine by High-Performance Liquid Chromatography-Fluorescence Detection Coupled with On-line Oxidation

Chiaki AOYAMA, Makoto TSUNODA,[†] and Takashi FUNATSU

Graduate School of Pharmaceutical Sciences, The University of Tokyo,
7-3-1 Hongo, Bunkyo, Tokyo 113-0033, Japan

A simple and sensitive determination method for selenomethionine (Se-Met) using an HPLC-fluorescence detection system coupled with an on-line electrochemical reactor has been developed. NBD-F (4-fluoro-7-nitro-2,1,3-benzoxadiazole) was used as the fluorescent derivatization reagent for Se-Met. NBD-Se-Met was separated from NBD-derivatives of 22 other amino acids within 35 min. Applying an optimized oxidation potential enhanced the fluorescence intensity of NBD-Se-Met 10-fold. The calibration curve was linear in the range of 300 fmol to 30 pmol with a correlation coefficient of 0.997. Detection limit ($S/N = 3$) was calculated to be 50 fmol, which is comparable to that of inductively coupled plasma mass spectrometry. This simple and sensitive method should be useful for the determination of Se-Met in physiological samples, such as serum or urine.

(Received September 24, 2008; Accepted November 14, 2008; Published January 10, 2009)

Introduction

Selenium (Se) is an essential trace element for human beings.¹⁻³ Some Se-containing proteins such as glutathione peroxidase and selenoprotein P are known to lose activity when Se intake is insufficient.⁴ Moreover, many studies suggest that selenium supplementation reduces the incidence of prostate cancer.^{1,5-7} In recent years, how selenium compounds contribute to the anticancer effect is of increasing interest.

Among several chemical forms, selenomethionine (Se-Met) has been reported to be responsible for the chemopreventive property of Se.⁸⁻¹⁰ Se-Met, which is biologically indistinguishable from methionine, is randomly incorporated into methionine-containing proteins in the place of methionine. Se-Met is a major natural food-form of selenium and thus is often employed for human dietary supplementation of Se.¹¹ Accurate determination of Se-Met is necessary for a better understanding of mechanisms governing the anticancer effect produced by selenium as well as for the monitoring of selenium status in the body.

The determination methods for Se-Met are mainly based on HPLC coupled with inductively coupled plasma mass spectrometry (ICP-MS) detection.¹²⁻¹⁸ Although ICP-MS enables selective detection of Se, several problems are mentioned by Francesconi *et al.*² First, interference by polyatomic species (*e.g.*, $^{40}\text{Ar}^{37}\text{Cl}^+$, $^{38}\text{Ar}^{40}\text{Ar}^+$, $^{40}\text{Ar}^{40}\text{Ar}^+$, $^1\text{H}^{79}\text{Br}^+$ and $^1\text{H}^{81}\text{Br}^+$) around the major selenium species (m/z 77, 78, 80, 82) are pointed out. The second point is inappropriate use of addition experiments. For example, when either the standard or the sample compound is in excess, distinguishing two different but chromatographically similar compounds becomes quite difficult. The third point is

assignment of the peaks at or near the void volume. Therefore, experiments performed with only one HPLC/ICP-MS system are thought in general to be insufficient to confirm the identity of a compound.

Consequently, it is significant to develop a determination method for Se-Met using HPLC with fluorescence detection because it provides high sensitivity and shows high accuracy in quantification. Hammel *et al.*¹⁹ demonstrated a determination method for Se-Met and selenocysteine using *o*-phthalaldehyde (OPA) as a fluorescence derivatization reagent. The absolute detection limit obtained from on-line detection is 30 pmol for Se-Met. To improve the sensitivity, they also performed off-line detection by atomic absorption spectrometry, which provides 100-fold higher sensitivity than on-line fluorescence detection.

In this paper, using 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F)²⁰ as a derivatization reagent, a sensitive determination method for Se-Met is described. Since the derivatives of NBD-F emit longer-wavelength fluorescence, NBD-F provides higher sensitivity than OPA in most cases.^{21,22} Furthermore, we found out that the fluorescence intensity of NBD-Se-Met was enhanced by oxidation reaction. Utilizing an on-line electrochemical reactor, our method enabled highly sensitive determination of Se-Met.

Experimental

Materials

Se-Met was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Type-H amino acid standard solution was obtained from Wako Pure Chemical Co. (Osaka, Japan). Asparagine was from Sigma Chemicals Co. (St Louis, MO). Glutamine, citrulline, and ornithine were from Kyowa Hakko Kogyo (Tokyo). NBD-F was purchased from Dojindo Laboratories (Kumamoto, Japan). Trifluoroacetic acid (TFA) was from Pierce (Rockford, IL).

[†] To whom correspondence should be addressed.
E-mail: makotot@mol.f.u-tokyo.ac.jp

Fig. 1 Scheme for derivatization of selenomethionine by NBD-F.

Acetonitrile and 2-propanol of HPLC grade were from Kanto Chemical Co., Inc. Water was purified using a Milli-Q system (Millipore, Bedford, MA). All other chemicals were of reagent grade.

Fluorescence derivatization procedure

Procedures of fluorescence derivatization were based on our previous method.²¹ The reaction scheme for the derivatization is shown in Fig. 1. To 45 μ L of Se-Met solutions, 25 μ L of 200 mM borate buffer (pH 8.5) and 30 μ L of 10 mM NBD-F in acetonitrile were added. After 3 min at 60°C, 100 μ L of 1 M L-tartrate buffer (pH 2.0) was added to stop the reaction. Twenty microliters of the resultant solution were subjected to HPLC analysis.

HPLC conditions for the determination of Se-Met

The system consisted of an L-7100 pump (Hitachi, Tokyo), a 7125 manual sample injector (Rheodyne, Rohnert Park, CA) with a 20- μ L sample loop, an ODS-80TsQA (250 $\times$ 4.6 mm i.d., 5 μ m, TOSOH Co., Tokyo) as a separation column, a Coulchem III (ESA Bioscience, Chelmsford, MA) as an electrochemical reactor, an F-1080 fluorescence detector (Hitachi) and a D-7500 integrator (Hitachi). The mobile phase consisted of water-acetonitrile-2-propanol-TFA (70:30:2:0.02, v/v/v/v). Flow rate was set at 1 mL/min. Fluorescence detection was performed at 540 nm with excitation at 470 nm. A 30-pmol volume of NBD-Se-Met was injected to optimize the oxidation potential.

Results and Discussion

First, to find out proper conditions for the chromatographic separation of NBD-derivative of Se-Met from 22 other amino acids, we tried the separation with a mobile phase that consisted of water, acetonitrile and TFA. However, sufficient separation was not obtained at any compositions of the three solvents. Hence, we added 2-propanol to the mobile phase because we have already succeeded in simultaneous determination of 22 amino acids with 2-propanol.²¹ As a result, the addition of 2% of 2-propanol improved the separation. Finally, a mobile phase that consisted of water-acetonitrile-2-propanol-TFA (70:30:2:0.02, v/v/v/v) was chosen as the optimum condition (Fig. 2(a)).

However, the peak area of NBD-Se-Met was about 30-fold smaller when compared to other amino acids. It was likely due to the quenching of fluorescence by intramolecular photo-induced electron transfer (PeT). PeT is a well-known

Fig. 2 Chromatogram obtained from standard solution (a) without any oxidation reaction and (b) with applied oxidation potential. Injected amount of NBD-derivatives was 30 pmol for Se-Met, and 16 pmol for other 22 amino acids. Peaks in the chromatogram: 1, NBD-methionine; 2, NBD-valine; 3, NBD-Se-Met; 4, NBD-cystine; 5, NBD-ornithine.

mechanism through which the fluorescence of a fluorophore is quenched by electron transfer.²³ The electron transfer from CH_3Se group of Se-Met to the excited state of benzoxadiazole skeleton might cause the quenching of fluorescence. The peak area of NBD-methionine, in which CH_3S group is expected to cause PeT less often than CH_3Se group, was between the areas of NBD-Se-Met and of NBD-valine, whose side chain does not cause PeT. This fact also supports our hypothesis.

Based on our idea mentioned above, we expected that the fluorescence intensity of NBD-Se-Met could be enhanced by chemical modification of CH_3Se group. In a previous report, utilizing an on-line electrochemical reactor enabled sensitive determination of NBD-tryptophan, which does not fluoresce, by HPLC-fluorescence detection system.²⁴ The electrochemical oxidation of the indole ring of tryptophan, which often quenches fluorescence of dyes, seemed to recover the fluorescence. Therefore, highly sensitive fluorescence detection of NBD-Se-Met should be achieved by utilizing a similar system.

We examined the effect of coulometric electrochemical reactor on the fluorescence intensity of NBD-Se-Met at various oxidation potentials. As shown in Fig. 3, the maximum response of the fluorescence was obtained at +0.6 V (vs. Pd reference). The fluorescence intensity was 10 times higher than without the reactor. Based on the result, we selected +0.6 V as the optimum potential. From the measured current through the electrochemical reactor, it was determined that the reaction of NBD-Se-Met is one-electron oxidation at +0.6 V. Mishra *et al.* presented the chemical structures of radical cations in one-electron oxidation of Se-Met.²⁵ In the mobile phase, NBD-Se-Met should be converted to a monomer radical cation forming a stable five- or six-membered ring as shown in Fig. 4(b). As a result, PeT from CH_3Se group to the excited state of benzoxadiazole skeleton

Fig. 3 Effect of applied potential on the fluorescence intensity of NBD-Se-Met.

Fig. 4 Scheme for PeT in the chemical structures of (a) Se-Met and (b) its expected products of oxidation reaction.

(Fig. 4(a)) was diminished and the fluorescence intensity was increased.

A representative chromatogram obtained from the standard solution containing Se-Met and 22 other amino acids with coulometric electrochemical reactor is shown in Fig. 2(b). The peak of NBD-methionine was also four-fold enhanced, which also supports our hypothesis. On the other hand, the peak areas of NBD-cystine and NBD-ornithine got smaller. This result may be due to the differences in the chemical structures of the NBD-derivatives. NBD-derivatives of cystine and ornithine, which contain two benzoxadiazole rings, are less stable than other derivatives containing one benzoxadiazole ring.

The calibration curve was linear in the range of 300 fmol to 30 pmol with a correlation coefficient of 0.997. The detection limit ($S/N = 3$) was calculated to be 50 fmol. The value was about 600-fold lower than that of previous methods for Se-Met using HPLC-fluorescence detection, and was comparable to those of methods using ICP-MS detection. This highly sensitive method should be useful for determination of Se-Met in samples where only very low concentrations of Se-Met are present.

Intra-day and inter-day precisions were 2.2 and 6.4%, respectively ($n = 3$). This data shows good reproducibility of the system.

In conclusion, we developed a determination method for Se-Met using an HPLC-fluorescence detection system coupled with an on-line electrochemical reactor. By applying an optimized oxidation potential, we found that the sensitivity was highly enhanced. This simple and sensitive method should be useful for the determination of Se-Met in physiological samples, such as serum or urine.

Acknowledgements

The authors thank ESA Bioscience for kindly providing Coulochem III.

References

1. G. F. Combs Jr., *Br. J. Nutr.*, **2001**, 85, 517.
2. K. A. Francesconi and F. Plannier, *Clin. Chem.*, **2004**, 50, 2240.
3. E. Dumont, F. Vanhaecke, and R. Cornelis, *Anal. Bioanal. Chem.*, **2006**, 385, 1304.
4. J. Chen and M. J. Berry, *J. Neurochem.*, **2003**, 86, 1.
5. L. C. Clark, G. F. Combs Jr., B. W. Turnbull, E. H. Slate, D. K. Chalker, J. Chow, L. S. Davis, R. A. Gliver, G. F. Graham, E. G. Gross, A. Kongrad, J. L. Leshner Jr., H. K. Park, B. B. Sanders Jr., C. L. Smith, and J. R. Taylor, *J. Am. Med. Assoc.*, **1996**, 276, 1957.
6. E. Meuillet, S. Stratton, D. P. Cherukuri, A. C. Goulet, J. Kagey, B. Porterfield, and M. A. Nelson, *J. Cell. Biochem.*, **2004**, 91, 443.
7. L. Letavayová, V. Vlčková, and J. Bromžmanová, *Toxicology*, **2006**, 227, 1.
8. C. Redman, J. A. Scott, A. T. Baines, J. L. Basye, L. C. Clark, C. Calley, D. Roe, C. M. Payne, and M. A. Nelson, *Cancer Lett.*, **1998**, 125, 103.
9. D. G. Menter, A. L. Sabichi, and S. M. Lippman, *Cancer Epidem. Biomarkers Prev.*, **2000**, 9, 1171.
10. H. Zhao and J. D. Brooks, *J. Urol.*, **2007**, 177, 743.
11. G. N. Schrauzer, *Rec. Adv. Nutr. Sci.*, **2000**, 130, 1653.
12. D. W. Nyman, M. S. Stratton, M. J. Kopplin, B. L. Dalkin, R. B. Nagle, and A. J. Gandolfi, *Cancer Detect. Prev.*, **2004**, 28, 8.
13. A. Chatterjee, H. Tao, Y. Shibata, and M. Morita, *J. Chromatogr. A*, **2003**, 997, 249.
14. J. R. Encinar, D. Schaumlöffel, Y. Ogra, and R. Lobinski, *Anal. Chem.*, **2004**, 76, 6635.
15. Z. Pedrero, J. R. Encinar, Y. Madrid, and C. Cámara, *J. Chromatogr. A*, **2007**, 1139, 247.
16. P. Moreno, M. A. Quijano, A. M. Gutiérrez, M. C. Pérez-Conde, and C. Cámara, *Anal. Chim. Acta*, **2004**, 524, 315.
17. J. Zheng, Y. Shibata, and N. Furuta, *Talanta*, **2003**, 59, 27.
18. P. C. Uden, H. T. Boakye, C. Kahakachchi, and J. F. Tyson, *J. Chromatogr. A*, **2004**, 1050, 85.
19. C. Hammel, A. Kyriakopoulos, U. Rösick, and D. Behne, *Analyst*, **1997**, 122, 1359.
20. Y. Watanabe and K. Imai, *J. Chromatogr.*, **1982**, 239, 723.
21. C. Aoyama, T. Santa, M. Tsunoda, T. Fukushima, C. Kitada, and K. Imai, *Biomed. Chromatogr.*, **2004**, 18, 630.
22. S. Nonaka, M. Tsunoda, K. Imai, and T. Funatsu, *J. Chromatogr. A*, **2005**, 1066, 41.
23. A. P. Silva, H. Q. N. Gunaratne, T. Gunnlaugsson, A. J. M. Huxley, C. P. McCoy, J. T. Rademacher, and T. E. Rice, *Chem. Rev.*, **1997**, 97, 1515.
24. N. Watanabe, T. Toyo'oka, and K. Imai, *Biomed. Chromatogr.*, **1987**, 2, 99.
25. B. Mishra, K. I. Priyadarsini, and H. Mohan, *J. Phys. Chem. A*, **2006**, 110, 1894.