http://dx.doi.org/10.5012/bkcs.2013.34.12.3817

Quantitative Speciation of Selenium in Human Blood Serum and Urine with AE- RP- and AF-HPLC-ICP/MS

Ji-sun Jeong, Jonghae Lee,† and Yong-Nam Pak*

Department of Chemistry Education, Korea National University of Education, Chung Buk 363-791, Korea
†Division of Metrology for Quality of Life, Korea Research Institute of Standards and Science, Daejeon 305-340 , Korea
**E-mail: pakyn@knue.ac.kr*
Received August 16 2013, Accepted October 15, 2013

Various separation modes in HPLC, such as anion exchange (AE), reversed-phase (RP), and affinity (AF) chromatography were examined for the separation of selenium species in human blood serum and urine. While RP- and AE-HPLC were mainly used for the separation of small molecular selenium species, double column AF-HPLC achieved the separation of selenoproteins in blood serum efficiently. Further, the effluent of AF-HPLC was enzymatically hydrolyzed and then analyzed with RP HPLC for selenoamino acid study. The versatility of the hybrid technique makes the in-depth study of selenium species possible. For quantification, post column isotope dilution (ID) with ^{78}Se spike was performed. ORC ICP/MS (octapole reaction cell inductively coupled plasma/mass spectrometry) was used with 4 mL min^{-1} Hydrogen as reaction gas. In urine sample, inorganic selenium and SeCys were identified. In blood serum, selenoproteins GPx, SelP and SeAlb were detected and quantified. The concentration for GPx, SelP and SeAlb was 22.8 ± 3.4 ng g^{-1}, 45.2 ± 1.7 ng g^{-1}, and 16.1 ± 2.2 ng g^{-1}, respectively when ^{80}Se/^{78}Se was used. The sum of these selenoproteins (84.1 ± 4.4 ng g^{-1}) agrees well with the total selenium concentration measured with the ID method of 87.0 ± 3.0 ng g^{-1}. Enzymatic hydrolysis of each selenium proteins revealed that SeCys is the major amino acid for all three proteins and SeMet is contained in SeAlb only.

Key Words : Selenium speciation, Seleno-proteins, HPLC-ICP/MS, Blood serum, Post column isotope dilution

Introduction

Selenium is a very interesting and surprising element because it is a nutritionally required element in a very narrow range; it is essential but becomes toxic at its high concentration in biological systems.[1,2] Moreover, its bio-availability and toxicity are greatly dependent on existing forms.[3] The total selenium content is thus not sufficient to assess its biological and environmental behaviors. The study on the transformation and metabolism facilitates the better understanding of the bio-efficiency and bio-availability for different selenium species. Speciation analysis of selenium is therefore becoming critical. The separation techniques with high efficiency in combination of elemental detectors with high sensitivity are still the mainstream methods for the purposes. While high performance liquid chromatography (HPLC), gas chromatography (GC), and capillary electrophoresis (CE) are the popular separation techniques, ICP/MS,[4-6] ICP-atomic emission spectrometry (ICP/AES),[7,8] atomic fluorescence spectrometry (AFS),[9,10] atomic absorption spectrometry (AAS)[11] are often used as the detection mode for the chromatographic or electrophoretic effluent. Different to the need of chemical derivatization for GC and the low loading of CE, HPLC can be used for the separation of almost all of analytes because there are various choices for the stationary and mobile phase with mL level sample loading (a few orders of magnitude higher than CE).[12] The operation at ambient temperature makes the analysis of thermally-instable species possible. ICP/MS has become popular due to its high sensitivity, multi-element and multi-isotope detection capacity.[13] Thus, HPLC-ICP/MS has become dominating for the speciation studies in the last decade.[3,14]

The other issue for the determination of selenium using ICP/MS is the interference from ploy-atomic ions, such as argon dimmers ([^{40}Ar$_2$] and [^{40}Ar^{38}Ar]) on the determination of the most abundant ^{80}Se and ^{78}Se isotopes with quadrupole mass analyzers.[2,3] Fortunately, a collision/reaction cell (CRC) system provides a solution on the issue through the removal of some spectral interference by addition of reaction cell with reaction gases, such as H_2, O_2, NH_3, and CH_4.[15,16] The reaction gases eliminate or decrease polyatomic interferences. Octapole reaction cell was used to eliminate the isobaric interference on the determination of ^{80}Se and ^{78}Se isotopes through the introduction of H_2 or/and He to suppress the formation of Ar dimmers as well as other isobaric interfering species.[15,17]

In urine sample, small molecules of selenium species are the target and anion exchange (AE) or reversed pahse (RP) HPLC can provide enough information on selenium metabolism without the use of affinity (AF) column because there is no selenoprotein existing in urine. In biomedical applications, blood serum is frequently monitored more often to assess the Se nutritional status. Three selenoproteins in blood serum, Glutathione peroxidase (GPx), Selenoprotein P

(SelP) and selenoalbumin (SeAlb) are known up to this date. GPx and SelP are selenoproteins synthesized by genetic code while SeAlb is a selenium containing protein *i.e.*, sulfur is randomly replaced by selenium so that methionine becomes selenomethionine (SeMet). In this paper, all three proteins are termed as selenoproteins. GPx is an antioxidizing playing a very important role in the reductive detoxification of peroxidases and reducing genotoxic effects. The level of selenium concentration in human blood serum is directly related to the concentration of GPx. Low level of selenium leads to deficiency of GPx in the blood plasma. SelP is produced in the liver and low level of SelP results in selenosis.[18] GPx and SelP levels are known to remain constant with age while SeAlb tend to decrease.[19] SeAlb plays the role of biomarker to indicate the proper level of selenium in human body. Thus, several researches[20-23] have been conducted to quantify these selenoproteins accurately in recent years.

One of the techniques to quantitate SeAlb is to determine SeMet species using enzymatic hydrolysis of the whole serum. If there is no free SeMet, the SeMet determined from the whole serum is from SeAlb alone because there is no SeMet existing in GPx nor SelP. Though this method is simple, it could show the level of SeAlb and consequently the nutritional level of selenium for any patients. However, this method does not provide information on GPx or SelP. The whole blood serum was enzymatically hydrolyzed to determine the concentration of SeMet by the standard addition method with a good precision and consequently SeAlb level could be determined.

Precise determination of selenoproteins is a challenging task especially in the complex matrix like blood serum. AF Chromatography has been the most efficient technique to isolate selenoproteins from the complex serum matrix. SelP and SeAlb are retained by heparin and sepharose respectively, while GPx is not retained at all. In this study, a combination of two AF columns is employed for the determination of GPx, SelP and SeAlb. Jitaru *et al.*[20,22-24] has been performing extensive researches on Se species in blood serum. They measured SeMet in blood serum with species specific ID to determine SeAlb concentration. Also, they used AE solid phase exchange to remove Cl and Br matrices that interferes on Se for the accurate deteremination of selenoproteins in human blood serum.[23,24] Pure selenoproteins were purchased and used to quantiy selenoproteins in blood serum with the external calibration method. However, even pure selenoproteins were found to contain some other impurities. Thus, it could be more accurately when ID is applied in blood serum study. In this research, post column ID method has been applied for the accurate determination of selenoproteins.

ID provides a simple platform for precise calibration to the effects of matrix and from the plasma and nebulizer.[25] Various ID-ICP/MS techniques were developed for the quantification of species in the real sample with complex matrix.[25,26] Though species-specific ID provides higher accuracy, species-unspecific (post column) mode has been applied in this research because isotopically labled standards are not available for all selenoproteins. The effluent from HPLC was mixed with an enriched ^{78}Se spike. The isotopic ratio from ^{80}Se/^{78}Se or ^{76}Se/^{78}Se was used to evaluate the concentration of selenium species in blood serum.

The selenoproteins separated by AF HPLC were collected and each portion was enzymatically hydrolyzed for the further analysis with the combination of RP HPLC-ICP/MS. This is the first research to report on the analysis of selenoproteins in human blood serum with the combination of AF HPLC and RP HPLC. Enzymatic hydrolysis study of each selenoprotein can provide more information on the nature of proteins and their purities. Accurate determination of selenoprotein in human blood serum has been applied as the diagnostic tool for prostrate cancer[27] and expected to be used in many more studies even in Korea in the near future.

Experimental Section

Apparatus. The HPLC coupled to post column ID ICP/MS was schematically illustrated in Figure 1. Separations were carried out on HPLC columns with various modes, including reversed-phase, anion exchange, and affinity. The operation conditions for each mode are listed in Table 1 in detail. Model 626 dual pumps (Alltech, USA) were used for HPLC separation with a 100 μL sample loop. The separated Se compounds were quantified with on-line post-column continuous mixing with an enriched ^{78}Se spike solution supplied using Model M312 peristaltic pump (Gilson Minipuls3, Gilson, France). The mixture of effluent from HPLC and enriched ^{78}Se spike solution was monitored on Agilent Model 7500ce ICP/MS (Agilent Technologies, Tokyo, Japan) with an octapole ion guide operated in RF-only mode and concentric nebulizer. 4 mL min^{-1} Hydrogen was introduced into the ORC as reaction gas. The intensity of chromatogram obtained was converted to mass flow chromatogram (*via* adequate mathematical corrections) and the total mass was obtained by integration of such chromatographic peaks. Selenium isotopic ratio was used to quantify each selenocompound. Before determination, the operating conditions for ICP/MS were tested with 10 ng g^{-1} tuning solution (Li, Y, Tl, and Ce) and the optimal conditions are listed in Table 2.

Reagents. Sodium selenite (Se(IV)), seleno-DL-methionine, seleno-L-cystine (SeCys), Se-(Methyl)selenocysteine hydrochloride (MSeCys), sodium selenate (Se(VI)) in 2% HNO_3 was obtained from Sigma-Aldrich (St. Louis, MO, USA).

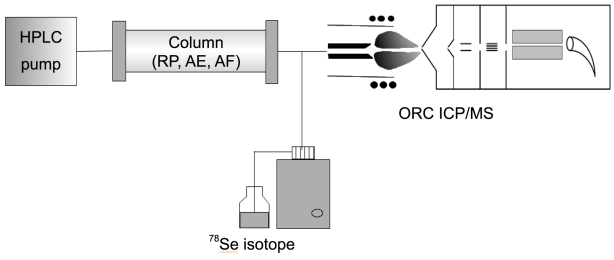

Figure 1. Schematic diagram of HPLC-ICP/MS with post-column isotope dilution.

Table 1. Experimental conditions for RP-, AE- and AF-chromatography used for the separation of selenium species in blood serum and urine

Reversed-phase chromatography	
Column	Symmetryshield^{TM} RP_8 (3.5 μm, 4.6 × 150 mm)
Mobile phase	5% MeOH, 0.05% nonafluoropentanoic acid, pH 2.5
Injection volume	100 μL
Flow rate	0.9 mL min^{-1}
Anion exchange chromatography	
Column	Allsep anion exchange column (100 mm × 4.6 mm, 7 μm)
Mobile phase	5% MeOH, 1 mM ammonium citrate, pH 5.3
Injection volume	100 μL
Flow rate	1.0 mL min^{-1}
Affinity chromatography	
Column	Heparin Sepharose and Blue Sepharose (1 mL each)
Mobile phase A (equilibration buffer)	0.02 M Tris-HCl buffer, pH 7.4
Mobile phase B (elution buffer)	buffer A + 1.4 M ammonium acetate, pH 7.4
Injection volume	300 μL
Flow rate	1 mL min^{-1}

The abundance of ^{78}Se in enriched isotope spike solution is ^{78}Se > 97.9 ± 0.3%. Protease type XIV from *Streptomyces griseus*, protease from bovine pancreas type I, proteinase K from Tritirachium album, trypsin from bovine pancreas, lipase from porcine pancreas type II, and glutathione peroxidase from bovine erythrocytes (all of proteases from Sigma-Aldrich) was used to extract the seleno-amino acids. Urea (98%) and albumin from human serum (96-99%, Sigma-Aldrich) were used as the sample for HPLC test.

Sample Preparation. Urine sample was collected from 7 healthy adults. Without any pretreatment, the sample was mixed with water 1:1, filtered and then analyzed. Since selenosugars could be degraded within a few days after collection, the sample was stored in the dark in a refrigerator at 4 °C. Blood serum samples were donated by 50 healthy adults who were residing in local areas. Sample was centrifuged for 10 min. (1790 g) and the supernatant was collected

Table 2. Octapole reaction cell ICP/MS operation conditions

Plasma conditions	
RF power	1570 W
Carrier gas	0.9 L min^{-1}
Ar/O_2 auxiliary gas	0.18 L min^{-1}
Sampling and Skimmer cone	Pt, 1.0 mm and 0.4 mm id
Nebulizer	concentric nebulizer, Agilent
Collision/reaction cell parameters	
H_2 flow rate	4.0 mL min^{-1}

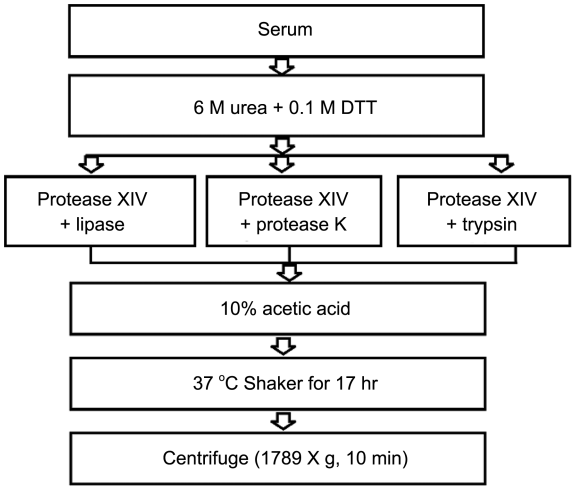

Figure 2. Enzyme extraction procedure for human blood serum sample preparation.

for the analysis. In case of storage, sample was kept at −70 °C. For enzymatic hydrolysis, 300 μL of 6 M urea and 60 μL of 6 M DTT (in 0.1 M Tris pH 7.5) were added. After being shaken for 1 h, 15 mg mL^{-1} protease XIV and 8 mg mL^{-1} lipase were added for the digestion of the protein *via* shaking the mixture for 17 h at 37 °C. After being mixed with 20 μL of 10% acetic acid, the mixture was submitted to be centrifuged and filtered. The supernatant was injected into HPLC for the analysis. Enzymatic extraction process for human serum is illustrated in Figure 2.

Results and Discussion

Separation of the Selenium Species with HPLC. The good separation of different selenium species is the prerequisite for the purpose of speciation analysis. AE HPLC[15,28] and RP HPLC[29,30] are the popular separation technique for selenium species. In this work, we test the application of the different HPLC modes in selenium separation. Although the organo-selenium species tested were separated with each other, the two inorganic species were co-eluted in RP mode as shown in Figure 3(a). AE column made the separation of inorganic selenium anions possible (Figure 3(b)). Because of their difference in apparent charge, selenite and selenate were separated easily, but the organic species were eluted quickly due to their low apparent charge. Considering the short separation time, RP HPLC was mainly used for the speciation analysis of small molecular selenium species but AE HPLC achieved the quantification of the two inorganic species.

Selenium Species in Urine. RP HPLC-ICP/MS and AE HPLC-ICP/MS were used to analyze selenium species in urine samples. The results are shown in Figure 4, inorganic selenium, selenocystine (SeCys) and unknown peaks U_1, U_2, U_3 were observed by RP HPLC-ICP/MS in the urine sample. This result agrees well with other report.[31] Because of strong Br interference, inorganic selenium and SeCys peaks could be buried under $[BrH]^+$ as shown in Figure 4(b) when ^{80}Se

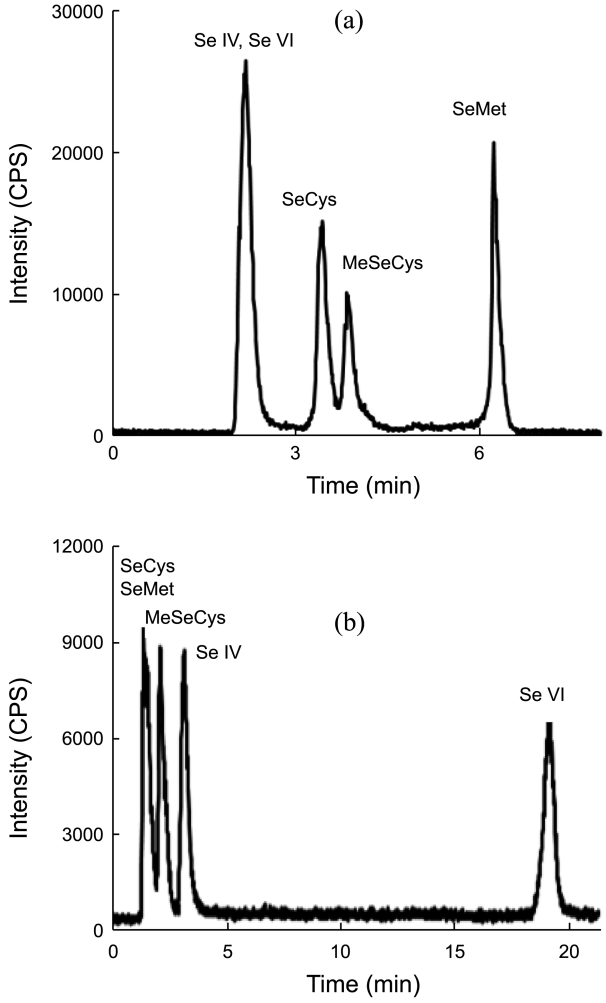

Figure 3. Separation of selenium standards mixture by (a) RP HPLC-ICP/MS (column: C_8, Symmetryshield^{TM} RP_8 3.5 μm, 4.6 × 150 mm) with mobile phase (5% MeOH, 0.05% nonafluoropentanoic acid, pH 2.5) and (b) AE HPLC-ICP/MS (column: Allsep column, 7 μm, 100 mm × 4.6 mm) with mobile phase (5% MeOH, 1 mM ammonium citrate, pH 5.3). Sample loop of 100 μL and concentration of 20 ng g^{-1} for each standard species were used.

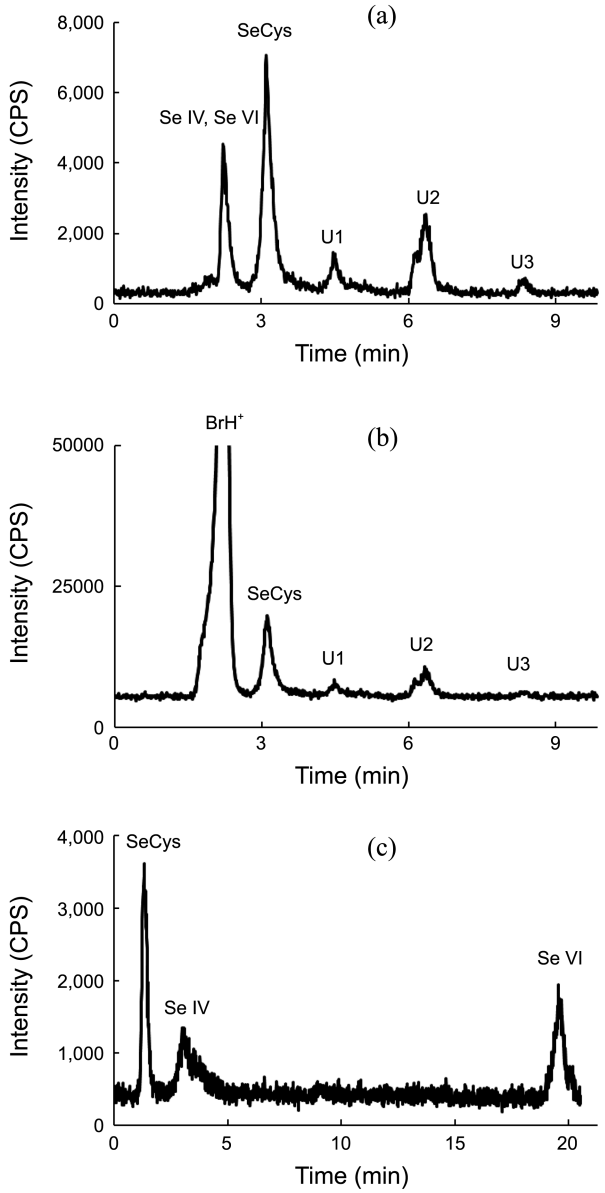

Figure 4. Separation of selenium species in human urine by RP HPLC-ICP/MS (a) with ^{78}Se (b) with ^{80}Se. Inorganic selenium are validated with (c) AE HPLC-ICP/MS.

was monitored. $[^{79}BrH]^+$ was produced by the reaction between urine matrix Br and H_2 in ORC. When ^{78}Se was monitored as shown in Figure 4(a), Br interference did not appear. Inorganic selenium, selenite (SeIV) and selenate (SeVI) were validated by AE HPLC-ICP/MS (Figure 4(c)). In AE HPLC-ICP/MS, SeCys showed a relatively large peak when compared with the one in RP HPLC-ICP/MS, which means that selenosugars were not separated but eluted along with SeCys in AE HPLC. Those unknown peaks could be trimethylselenonium ion or selenosugars. Organic mass spectrometry (such as ESI-MS, MALDI-TOF-MS) will be useful supplement for the speciation analysis and protein quantification purposes.[32,33] The unknown peaks will be studied with organic mass spectrometry in our further work.

Proteolytic Hydrolysis of Blood Serum: The developed HPLC-ICP/MS method was also used to study the hydrolysates of human serum sample with different hydrolyzing enzymes. Correspondingly, the chromatograms obtained from hydrolysates with different enzyme systems were observed in Figure 5. Three enzyme systems, protease X IV + lipase, protease X IV + protease K, and protease X IV + trypsin were selected to investigate the proteolysis efficiency to human serum. The main seleno-amino acids found in serum were SeCys and SeMet. Moreover, it could be found that the three enzyme systems had almost the same proteolytic hydrolysis efficiency from the signal intensity of SeCys and SeMet. The results indicated that RP or AE HPLC can be used to separate and detect low molecular selenium species in seleno-proteins after enzymatic proteolysis.

Determination of SeMet in Blood Serum After Proteolytic Hydrolysis: The level of SeAlb in blood serum can be determined by examining concentration of SeMet because

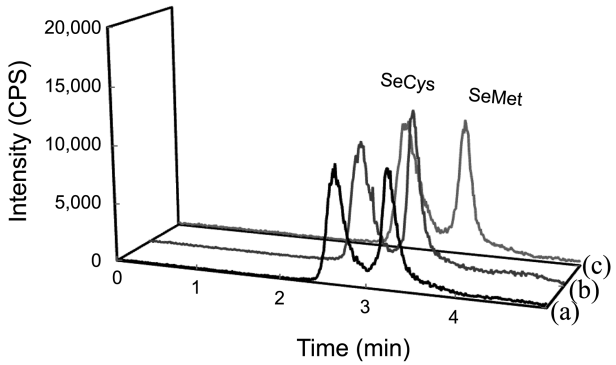

Figure 5. Proteolytic hydrolysis of human blood serum using different protease systems with (a) protease X IV + lipase, (b) protease X IV + protease K, and (c) protease X IV + trypsin. All systems show the same result.

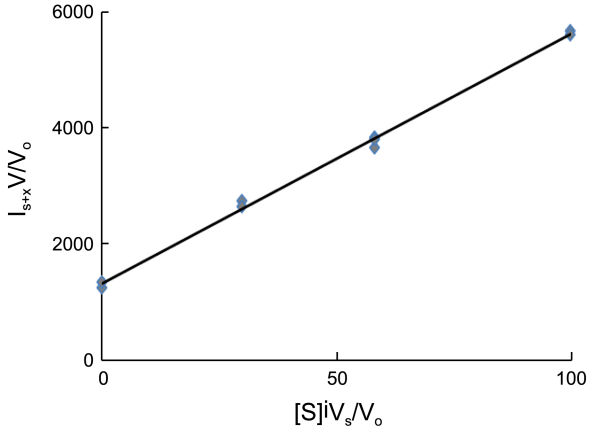

Figure 7. Calibration curve obtained for the standard addition method used in RP HPLC-ICP/MS. The curve shows good linearity (R^2 = 0.9993) and the concentration found is 30.68 ± 2.30 ng g^{-1} (n = 3).

only SeAlb contain SeMet providing that there is no free SeMet in serum. The validity of this assumption was checked and discussed later. The level of SeAlb based upon SeMet concentration was monitored for colorectal cancer (CRC) patients and it was found out that there was a significant difference between healthy control group and CRC group.[27] In our study, the standard addition method was applied for the quantification of SeMet in blood serum instead of SSID. SeMet standards (30 ng g^{-1}, 60 ng g^{-1}, and 100 ng g^{-1}) were added to the sample blood serum and were hydrolyzed using protease XIV(in 0.1 M Tris buffer, pH 7.5) and the results are shown in Figure 6 and Figure 7. The standard addition method for human blood serum showed good linearity for the curve (Fig. 7). Concentration of SeMet (from the pool of n = 35) found was 30.68 ± 2.30 ng g^{-1}, which is comparable to the other research.[34] The concentration in terms of selenium is 12.35 ± 0.92 ng g^{-1} when it is converted from SeMet to selenium. Other reports on SeAlb in blood serum[20,21,27] vary from 13 ng g^{-1} to 19 ng g^{-1} depending on samples and our result lies in the range studied by others. The level of SeMet is directly co-related with SeAlb but not with GPx or SelP. Thus, additional study is required if all three selenoproteins in the serum are to be determined.

AF-HPLC-ICP/MS for the Determination of Selenoprotein in Human Serum with post-column ID. An affinity (AF) chromatographic method with Heparin Sepharose (HEP) and Blue Sepharose (BLUE) columns was developed for the separation and quantification of seleno-proteins in serum. HEP and BLUE columns were connected with 6-way valve.[35] The optimum experimental conditions were set using the standard GPx and albumin. The effluent was directly connected to ICP/MS. Glutathione peroxidase was not retained in these AF columns, thus appeared on the first. SelP was retained on HEP column and SeAlb was on BLUE column. To elute SelP, mobile phase was changed from A to B and flowed through HEP column only. Then, both HEP and BLUE were flushed to elute SeAlb. All three selenoproteins in human blood serum were separated well as shown in

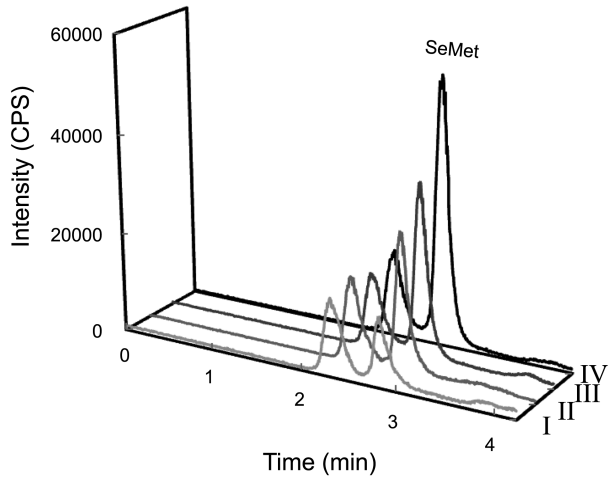

Figure 6. Standard addition method for the quantification of SeMet in human blood serum (from the pool of n = 35). ^{78}Se was monitored and standard SeMet was added (I; sample, II; sample + 30 ng g^{-1}, III; sample + 60 ng g^{-1}, IV; sample + 100 ng g^{-1}).

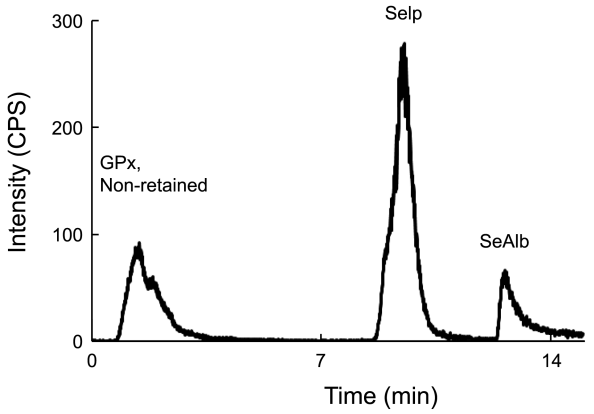

Figure 8. Separation of selenoproteins in human serum by AF HPLC-ICP/MS (column; HEP & BLUE, mobile phase A: 0.02 M Tris pH 7.4 mobile phase B: buffer A + 1.4 M ammonium acetate pH 7.4, ^{78}Se is monitored). GPx is eluted by equilibrium mobile phase A (0-6 min) while SelP and SeAlb are eluted with eluting mobile phase B (6-14 min).

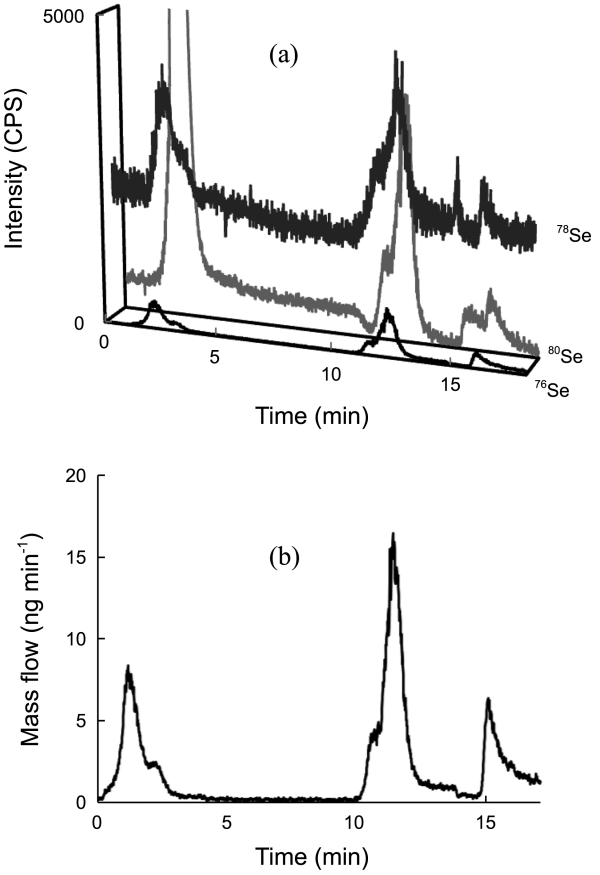

Figure 9. (a) Separation of selenoproteins in human serum by AF HPLC-ICP/MS monitoring with different isotopes. ^{78}Se is continually spiked. (b) Mass flow chromatogram obtained for ^{76}Se/^{78}Se.

Figure 8.

Species unspecific or post column ID has been developed because isotopically-labled standards are not available for all selenoproteins. The spike was mixed after HPLC effluent. Thus this technique does not correct for any events before the column such as sample extraction and chromatographic separation processes. However, it corrects for the changes in sample nebulization and ICP/MS detection. ^{78}Se was used as an isotope spike and both *m/z* 76 and 80 were used to measure isotopic ratio changes in the chromatogram (Fig. 9(a)). GPx peak was co-eluted with other matrix such as Br, which interfered severely on ^{80}Se due to $[^{79}BrH]^+$. It showed a strong false signal when *m/z* 80 was monitored while *m/z* 76 showed no interference. The intensity chromatogram was converted to the mass flow chromatogram for ^{76}Se/^{78}Se (Fig. 9(b)) and the peak was integrated over the peak width to get the total selenium mass.

The total content of selenoprotein in human serum measured (Table 3) using the isotope ratio of ^{76}Se/^{78}Se and ^{80}Se/^{78}Se is 88.2 ± 4.2 ng g^{-1} and 84.1 ± 4.4 ng g^{-1}, respectively. The results are comparable with each other.

The concentrations for each selenoprotein GPx, SelP, and SeAlb are 22.8 ± 3.4 ng g^{-1}, 45.2 ± 1.7 ng g^{-1}, and 16.1 ± 2.2 ng g^{-1}, respectively when ^{80}Se/^{78}Se is used. In case of ^{80}Se/^{78}Se, GPx could not be directly determined because of the interference from Br. The signal of ^{80}Se was mathematically corrected by monitoring ^{79}Br as described elsewhere.[36] The concentrations of selenoproteins determined by the two isotopic ratios agree well with each other within the 95% confidence interval. Though the signal intensity was large, the precision was low for GPx in case of ^{80}Se/^{78}Se. For SelP and SeAlb, the use of ^{80}Se/^{78}Se showed slightly better precision over ^{76}Se/^{78}Se due to high signals. The consistent results obtained with different isotopic ratios validated the possibility of determination of total selenoprotein with post-column ID technique.

Table 3. Quantification of selenoprotein concentrations in human blood serum (from the pool of 20 samples) by AF HPLC-ICP/MS using post column ID

	Se concentration (Se)	
	^{76}Se/^{78}Se	^{80}Se/^{78}Se
GPx	23.6 ± 2.5	22.8 ± 3.4
SelP	46.9 ± 2.5	45.2 ± 1.7
SeAlb	17.4 ± 2.2	16.1 ± 2.2
Total	88.2 ± 4.2	84.1 ± 4.4

average ± SD (n = 3). concentration in ng g^{-1}

Analysis of Total Selenium Concentration in Human Blood Serum. The total selenium concentration of selenium in blood serum was also determined with external curve calibration (EC) and ID methods using ICP/MS. As shown in Table 4, total selenium concentration determined by EC and ID was 112.6 ± 2.9 ng g^{-1} and 87.0 ± 3.0 ng g^{-1}, respectively. The result of ID should be more accurate and was closer to that of AF HPLC-ICP/MS post column ID, as expected. Those results validated the practicability of AF HPLC-ICP/MS with post-column ID technique for the analysis of selenoprotein in serum. Because of detection capacity of ng g^{-1} level, this work provides a sensitive protein quantification technique.

Rappel *et al.*[16] developed a HPLC-ID-ICP/MS for peptide quantification with lutetium labeling technique. The isotope with the element that existed originally in the protein or peptide provides a much simpler strategy for protein or peptide quantification in comparison. ESI-MS, MALDI-TOF-MS will be useful supplement for the analysis of protein structure and quantification purposes.[32,33]

Table 4. Comparison of total selenium concentrations in human serum (from the pool of 20 samples) determined by AF HPLC-ID-ICP/MS and ICP/MS using EC and ID

	ICP/MS		AF HPLC-ICP/MS[b]
	EC[a]	ID[a]	
Human serum	112.6 ± 2.9	87.0 ± 3.0	88.2 ± 4.2 (^{76}Se/^{78}Se) 84.1 ± 4.4 (^{80}Se/^{78}Se)

average ± SD (n=3). concentration in ng g^{-1}. [a]using ICP/MS external curve calibration (EC) and isotope dilution (ID). [b]sum of selenoproteins determined by post-column ID

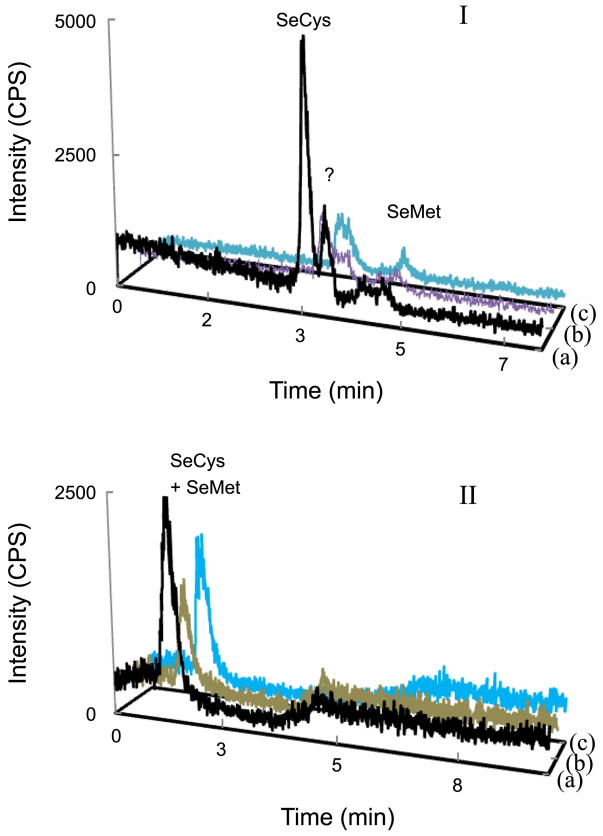

Figure 10. Separation of seleno-aminoacid with (I) RP HPLC-ICP/MS and (II) AE HPLC-ICP/MS after proteolytic digestion of selenoproteins (A; GPx, B; SelP, C; SeAlb). ^{78}Se was monitored. Hydrolysate of effluent A (mostly GPx) in RP HPLC shows free SeMet and other selenium species peaks that are not retained by double AF columns. SeAlb (C) shows SeMet as expected.

Selenium Species After Proteolytic Hydrolysis of Selenoproteins with AE and RP HPLC-ICP/MS. Selenoproteins in blood serum was separated and then each protein was hydrolyzed using protease XIV (in 0.1 M Tris buffer pH 7.5). The chromatograms for the hydrolysates of GPx, SelP, SeAlb were obtained and shown for RP HPLC-ICP/MS (Fig. 10-I) and AE HPLC-ICP/MS (Fig. 10-II). In all three selenoproteins, SeCys was the major species. In RP HPLC-ICPMS, GPx (A) showed mostly SeCys and small peaks probably due to some free SeMet and metabolites because they were not retained in the AF columns. SelP (B) showed SeCys only and while SeAlb (C) showed SeCys and some SeMet, as expected. In AE HPLC-ICP/MS, all three selenoproteins showed a single peak, probably a mixture of SeCys and SeMet. Broad peaks at around 5 minutes were suspected to be inorganic selenium. However, when RP HPLC was checked, there was no peak before SeCys concluding that there was no inorganic selenium. Since there was no inorganic selenium, determination of GPx concentration with AF HPLC-ICP/MS could give an accurate result. Still, it should be pointed out that GPx might contain free SeMet and it should be proved that there is no free SeMet in the serum sample or free SeMet should be removed for the precise measurement of GPx. A previous study[20] revealed that there is not much free SeMet in human blood sample. RP or AE HPLC study for the hydrolysates of each selenoprotein that has been separated by double AF columns, which has not been reported before, could show in-depth information for selenoproteins in human blood serum.

Conclusion

HPLC with various modes, such as RP, AE, and AF chromatography, were applied for the successful separation of different selenium species in urine and human blood serum. Accurate quantitative determination for selene-containing species with post column ID was performed with ICP/MS detection. Inorganic selenium and small molecule seleno-amino acids were observed in human urine. GPx, SelP, and SeAlb selenoproteins were the major proteins in human blood serum. The concentrations for each selenoprotein GPx, SelP, and SeAlb were 22.8 ± 3.4, 45.2 ± 1.7 ng g^{-1}, and 16.1 ± 2.2 ng g^{-1}, respectively. The sum of these concentrations, 84.1 ± 4.4 ng g^{-1}, agreed well with the total selenium concentration of 87.0 ± 3.0 ng g^{-1} in whole blood serum before the separation.

The selenoproteins were enzymatically hydrolyzed and the hydrolysates of GPx, SelP, SeAlb were analyzed with RP HPLC-ICP/MS. In all three selenoproteins, SeCys was the major species and GPx showed mostly SeCys and some free SeMet as well as metabolites. The results show the potential of the hybrid technique for study on the transformation and metabolism of selenium and can be applied to biomedical areas such as cancer diagnostics.

References

1. Tolu, J.; Hécho, I. L.; Bueno, M.; Thiry, Y.; Potin-Gautier, M., *Anal. Chim. Acta* **2011**, *684*, 126-133.
2. Pyrzyñska, K. *Microchim. Acta* **2002**, *140*, 55-62.
3. Chen, Y. W.; Belzile, N. *Anal. Chim. Acta* **2010**, *671*, 9-26.
4. Giusti, P.; Schaumlöffel, D.; Preud'homme, H.; Szpunar, J.; Lobinski, R. *J. Anal. At. Spectrom*. **2006**, *21*, 26-32.
5. Duan, J.; Hu, B. *J. Mass Spectrom*. **2009**, *44*, 605-612.
6. B'Hymer, C.; Caruso, J. A. *J. Chromatogr. A* **2006**, *1114*, 1-20.
7. Emteborg, H.; Bordin, G.; Rodriguez, A. R. *Analyst* **1998**, *123*, 245-253.
8. Harwood, J. J.; Su, W. *J. Chromatogr. A* **1997**, *788*, 105-111.
9. Mazej, D.; Falnoga, I.; Veber, M.; Stibilj, V. *Talanta* **2006**, *68*, 558-568.
10. Dietz, C.; Landaluze, J. S.; Ximénez-Embún, P.; Madrid-Albarrán, Y.; Cámara, C. *J. Anal. At. Spectrom*. **2004**, *19*, 260-266.
11. Shrivas, K.; Patel, D. K. *Food Chem.* **2011**, *124*, 1673-1677.
12. Ordonez, Y. N.; Montes-Bayón, M.; Blanco-Gonzalez, E.; Sanz-Medel, A. *Anal. Chem*. **2010**, *82*, 2387-2394.
13. Zoorob, G. K.; McKiernan, J. W.; Caruso, J. A. *Mikrochim. Acta* **1998**, *128*, 145-168.
14. Montes-Bayón, M.; DeNicola, K.; Caruso, J. A. *J. Chromatogr. A* **2003**, *1000*, 457-476.
15. Reyes, H. L.; García-Ruiz, S.; Tonietto, B. G.; Godoy, J. M.; Alonso, J. I. G.; Sanz-Medel, A. *J. Braz. Chem. Soc*. **2009**, *30*, 1878-1886.
16. Rappel, C.; Schaumlöffel, D. *Anal. Chem*. **2009**, *81*, 385-393.
17. Díaz Huerta, V.; Fernández Sánchez, M. L.; Sanz-Medel, A. *J. Anal. At. Spectrom*. **2004**, *19*, 644-651.

18. Pedrero, Z.; Madrid, Y. *Analytica Chimica Acta* **2009**, *634*, 135-152.
19. Andoh, M.; Hirashima, H.; Maeda, K.; Hata, O.; Inatomi, T.; Tsujikawa, M.; Sasaki, K.; Takahashi, Y,; Fujiyama, Y. *Nutrition* **2005**, *21*, 573-581.
20. Jitaru, P.; Goenaga-Infante, H.; Vaslin-Reimann, S.; Fisicaro, P. *Anal. Chim. Acta* **2010**, *657*, 100-107.
21. Suzuki, Y.; Sakai, T.; Furuta, N. *Anal. Sci*. **2012**, *28*, 1-5.
22. Jitaru, P.; Cozzi, G.; Gambaro, A.; Cescon, P.; Barbante, C. *Anal. Bioanal. Chem*. **2008**, *391*, 661-668.
23. Jitaru, P.; Cozzi, G.; Seraglia, R.; Traldi, P.; Cescon, P.; Barbante, C. *Anal. Methods* **2010**, *2*, 1382-1387.
24. Jitaru, P.; Prete, M.; Cozzi, G.; Turetta, C.; Cairns, W.; Seraglia, R.; Traldi, P.; Cescon, P.; Barbante, C. *J. Anal. At. Spectrom*. **2008**, *23*, 402-406.
25. Rodríguez-González, P.; Marchante-Gayón, J. M.; Alonso, J. I. G.; Sanz-Medel, A. *Spectrochim. Acta B* **2005**, *60*, 151-207.
26. Wallschläger, D.; London, J. *J. Anal. At. Spectrom*. **2004**, *19*, 1119-1127.
27. Roman, M.; Jitaru, P.; Agostini, M.; Cozzi, G.; Pucciarelli, S.; Nitti, D.; Bedin, C.; Barbante, C. *Michrochem. Journal* **2012**, *105*, 124-132.
28. Kotrebai, M.; Tyson, J. F.; Block, E.; Uden, P. C. *J. Chromatogr. A* **2000**, *866*, 51-63.
29. Bierla, K.; Szpunar, J.; Lobinski, R. *Anal. Chim. Acta* **2008**, *624*, 195-202.
30. Afton, S.; Kubachka, K.; Catron, B.; Caruso, J. A. *J. Chromatogr. A* **2008**, *1208*, 156-163.
31. Kuehnelt, D.; Kienzl, N.; Juresa, D.; Kevin, A. F. *J. Anal. At. Spectrom*. **2006**, *21*, 1264-1270.
32. Stewart, I. I. *Spectrochim. Acta B* **1999**, *54*, 1649-1659.
33. Rosenberg, E. *J. Chromatogr. A* **2003**, *1000*, 841-889.
34. Yang, X.; Tian, Y.; Ha, P.; Gu, L. *Wei Sheng Yan Jiu* **1997**, *26*, 13-16.
35. Reyes, H.; Marchante, L.; Gayón, J. M.; García Alonso, J. I.; Sanz-Medel, A. *J. Anal. At. Spectrom*. **2003**, *18*, 1210-1216.
36. Cho, H.; Pak, Y. *J. Korean Chem. Soc*. **2011**, *55*, 472-477.