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# Isolation of Selenoprotein-P and Determination of Se Concentration Incorporated in Proteins in Human and Mouse Plasma by Tandem Heparin Affinity and Size-exclusion Column HPLC-ICPMS

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Sel-P is considered to be the most important selenoprotein for evaluating the selenium (Se) status in the body. To isolate and determine Sel-P in plasma, we have developed an analytical method combining heparin affinity (AF) and size-exclusion column (SEC) high-performance liquid chromatography–inductively coupled plasma mass spectrometry (HPLC-ICPMS). We used this method to validate the adsorption efficiency of selenoproteins on a heparin AF column, and to then determine the Se concentrations incorporated in proteins in human and mouse plasma. The adsorption efficiency of Sel-P on a heparin column was more than 90% for both human and mouse plasma. Tandem AF and SEC separation proved to be useful for determining the Se concentrations incorporated in Sel-P in mouse plasma, but not in human plasma, because of nonspecific adsorption of plasma-extracellular glutathione peroxidase (eGPx) and albumin on the heparin AF column. Ultimately, we used the tandem AF and SEC separation method for mouse plasma and SEC separation alone for human plasma. The Se concentration incorporated in selenoproteins determined by our method showed good agreements with the total Se concentration determined following acid digestion.

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## Introduction

Selenium (Se) is now recognized to be an essential element for humans and other animals, and the importance of Se biochemistry has been widely reported. Se is incorporated into various proteins, and selenoproteins have many functions.[1-4] However, the appropriate Se concentration range in animal body is quite narrow, and insufficient,[5,6] or excess[7] intake of Se is implicated in disease. In biomedical applications for assessing the Se status, researchers have focused on the selenoproteins in the serum or plasma-extracellular glutathione peroxidase (eGPx) and selenoprotein P (Sel-P). In human blood, Se is also incorporated into albumin (Alb); Se containing albumin is formed through a nonspecified replacement of methionine with seleno-methionine,[8] or the binding of selenide to one of the protein's 17 disulfide bonds.[9] Therefore, Se-Alb is not a selenoprotein, but a Se-containing protein. However, these three proteins (Sel-P, eGPx, and Se-Alb) are referred to as selenoproteins in this study. Sel-P is a major selenoprotein in human plasma, and its concentration is a good index of the Se status in humans.[10,11]

High-performance liquid chromatography (HPLC) combined with inductively coupled plasma mass spectrometry (ICPMS) was often used for Se speciation analysis. Especially, size-exclusion column (SEC) HPLC combined with ICPMS is a versatile and nondenaturing method for analyzing biological samples,[9,12] but it is often time-consuming and shows low separation efficiency. Affinity (AF) column HPLC is the most efficient chromatographic approach for isolating one or more specific proteins with rapid elution. Because heparin interacts with Sel-P in plasma,[13] heparin AF HPLC has been used for the speciation analysis of Sel-P to distinguish it from other, non-heparin-binding, selenoproteins.[14,15] However, AF HPLC-ICPMS has many limitations. For example, only certain proteins can be separated from others with an AF-based column. In the case of Se analysis, quantification is difficult because neither chlorine (Cl) nor bromine (Br) is retained on AF HPLC columns; that is, they both coelute with certain selenoproteins. Consequently, when serum or plasma is analyzed by AF HPLC without prior removal of Br and Cl, the eGPx level is overestimated by 300% ($^{82}$Se) or 400% ($^{77}$Se).[16] To solve these problems, researchers have developed a tandem approach combining a heparin AF column with either a blue sepharose AF column or an anion exchange column.[17-20] In their papers, validation was performed by comparing the sum of the Se concentrations in Sel-P, eGPx, and Se-Alb with the total Se concentration in serum or plasma. However, the adsorption efficiencies on the affinity columns were not obtained for each selenoprotein. In this paper, the adsorption efficiencies of Sel-P and other selenoproteins on the heparin column were obtained precisely.

In addition to the adsorption efficiencies of selenoproteins, we determined the concentrations of Se in these selenoproteins in human and mouse plasma while using a separation system consisting of tandem AF and SEC columns and ICPMS detection to separate selenoproteins. Our method is a modification of a technique reported by Koyama *et al.*[21] The mobile phase was changed from phosphate buffer to Tris-$NO_3$ buffer, and ammonium acetate was used as an elution buffer instead of heparin. Also, the elution time was reduced from 60 to 30 min

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by changing the flow rate from 0.6 to 1.0 ml $min^{-1}$ and reducing the switching time of the buffer solutions from twice to once. Moreover, Koyama *et al*.[21] did not report the quantitative results of selenoproteins in mouse plasma, although they reported the quantitative results of selenoproteins in human plasma. In this study, we established a separation system for selenoproteins in human and mouse plasma, evaluated the adsorption efficiencies of the selenoproteins, and quantified the concentrations of Se in these proteins.

## Experimental

*Sample preparation*

Human plasma for transfusion was kindly provided by Atsuko Shinohara at Juntendo University. Mouse plasma was collected by a procedure approved by the Animal Experiment Ethical Committee, Juntendo University School of Medicine (approval number 210068). Blood samples were heparinized, centrifuged at 1500*g* for 10 min at 4°C, and separated into plasma and red blood cells. Prior to HPLC injection, plasma samples were filtered through a 0.45-μm filter (GL Chromatodisc 13A, GL Sciences Inc., Tokyo, Japan). Standards for human Alb (A9511-100MG, Sigma-Aldrich, Saint Quentin Fallavier, France) and mouse Alb (A3559-5MG, Sigma-Aldrich, France) were used for identification.

*Separation procedures*

The HPLC system consisted of a degasser (DG-158053, Jasco Co., Tokyo, Japan), an HPLC pump (PU-1580i, Jasco Co., Japan), and a sample injector (Model 9725i, Rheodyne Inc., Cotati, CA). A prepacked heparin AF column (AHR-894, 8.0 × 50 mm, Showa Denko K.K., Tokyo, Japan) and an SEC column (Asahipak GS-520 7G, 7.5 × 500 mm, Showa Denko K.K., Japan) were attached to the HPLC system in that order. We refer to this system as the tandem HPLC system. Two mobile phases were prepared: mobile phase A (50 mmol $L^{-1}$ Tris–$HNO_3$ buffer, pH 7.4) and mobile phase B (50 mmol $L^{-1}$ Tris–$HNO_3$ buffer, pH 7.4, in 1.4 M ammonium acetate). Before the separation, both columns were equilibrated with mobile phase A. A 100-μL aliquot of human or mouse plasma was injected with mobile phase A at a flow rate of 1.0 mL $min^{-1}$. After 30 min, the SEC column was removed from the tandem HPLC system, and the mobile phase was switched from A to B. The separation efficiency of this procedure was compared with that of a system consisting of only an SEC column (described above) and a guard column (Asahipak GS-2G 7B, 7.5 × 50 mm, Showa Denko K.K., Japan).

*ICPMS measurement*

The eluates from the SEC and the AF column were introduced directly to an ICPMS instrument (HP4500, Agilent Technologies Co., Santa Clara, CA). Mass/charge (*m*/*z*) ratios of 77 and 82 were monitored for Se detection. In addition, *m*/*z* ratios of 35 ($^{35}Cl$) and 79 ($^{79}Br$) were monitored for the discrimination of ghost peaks due to $^{40}Ar^{37}Cl$ and $^{82}Br^{1}H$; also, *m*/*z* ratios of 65 ($^{65}Cu$) and 66 ($^{66}Zn$) were monitored for identification of the Alb peak. For determining the Se concentration, the *m*/*z* ratio of 82 was used. The concentrations of Se were determined by means of a one-point standard calibration method with a 100-μL aliquot of analytical standards of selenite after passing through the column in both the mobile-phase conditions to correct for any signal enhancement due to coexisting carbon.[14] All analyses were performed in triplicate.

*Validation of Se quantification*

The Se concentrations incorporated in selenoproteins were verified by comparing the values with the total Se concentration determined after the sample was digested with nitric acid (70%) and hydrogen peroxide (30%) (both electronic laboratory grade, Kanto Chemical Co., Tokyo, Japan) in a microwave oven (MLS-1200 MEGA; rotor, MDR-300/s; Milestone General, Tokyo, Japan). The digestion procedure was described in a previous paper.[14] The digested samples were diluted with ultrapure water (18.4 MΩ, Milli-Q Element, Nihon Millipore K.K., Tokyo, Japan) after addition of In as an internal standard. Mathematical correction of the spectral interferences of $^{81}Br^{1}H$, enhancement effect by coexisting carbon, and internal standardization were performed in this order, as described elsewhere.[14]

## Results and Discussion

*Evaluation of the separation system*

SEC HPLC-ICPMS chromatograms of human plasma showed three Se peaks (retention time, 580, 700, 765 s; Fig. 1a), whereas the chromatogram of mouse plasma showed one major Se peak (retention time, 660 s; Fig. 2a) with small shoulder peaks (retention time, 580, 720 s) and minor Se peak (retention time, 820 s). Similar interspecies differences in the Se distribution in plasma were reported previously:[22,23] three Se peaks were observed in human plasma, whereas only one major Se peak was observed in the plasma of animals (monkey, rat, and sheep).

In the SEC HPLC-ICPMS chromatograms, the peak at a retention time of 765 s in human plasma was identified as Alb, because the retention time matched that of standard human Alb (Figs. 1b and 1c). Similarly, the peak at 820 s in mouse plasma was identified as Alb. Peaks at 1035 s in both human and mouse plasma were considered to be ghost peaks of $^{81}Br^{1}H$ because they coeluted with Br, and showed no $^{77}Se$ signal.

In tandem HPLC-ICPMS chromatograms for human plasma (Fig. 3), each peak was shifted to a longer retention time by 80 – 100 s relative to the corresponding peak in the SEC chromatograms, owing to passage through the AF column. That is, the peaks eluted from the SEC system (Fig. 1) at 700 and 765 s correspond to those eluted from the tandem system (Fig. 3a) at 780 and 850 s, respectively. The Se intensity of the peak eluted at 780 s from the tandem system was 10 ± 2% of the intensity of the corresponding peak eluted from the SEC system. This result showed that the peaks eluted at 780 s from the tandem system and eluted at 700 s from the SEC systems were Sel-P, and that the adsorption efficiency of human Sel-P under the conditions of this study was 90 ± 2%. The Se peak eluted at 850 s from the tandem system was identified as Alb because the retention time matched that of standard human Alb. The area of the Alb peak produced by the tandem system was 69 ± 16% of that produced by the SEC system. Using the method of elimination, the Se peak eluted at 580 s from the SEC system was assigned as eGPx. However, the signals of eGPx produced by the tandem system, which was expected to be eluted at a retention time of 670, was quite low to determine. Thus, eGPx and Alb were also nonspecifically adsorbed on the heparin AF column under this condition. In a Se chromatogram obtained after removal of the SEC column and change of the mobile phase from A to B (Fig. 3c), the area of the Se peak at 200 s was bigger than that shown in Fig. 1a because of enhancement effect by coexisting carbon from an eluent.

In the tandem HPLC-ICPMS chromatograms for mouse

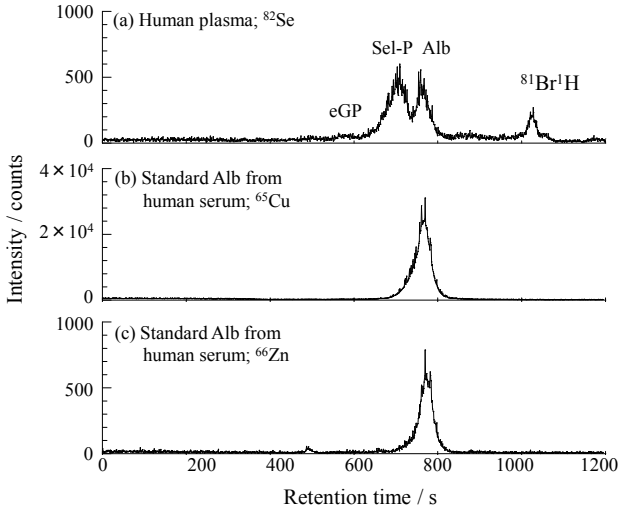


Fig. 1 SEC HPLC-ICPMS chromatograms for human plasma and standard human Alb.

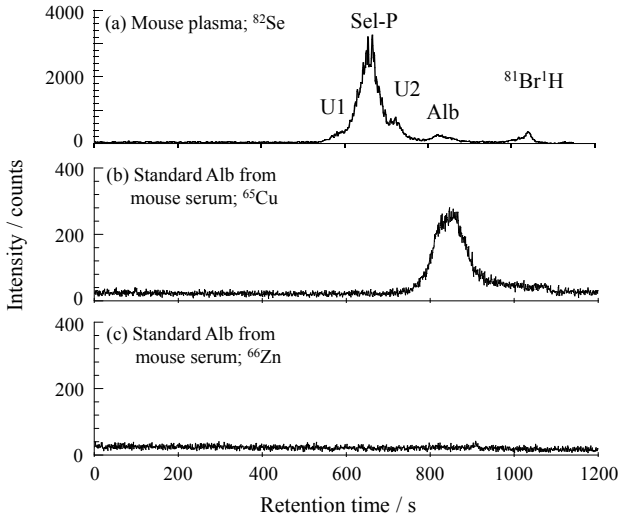


Fig. 2 SEC HPLC-ICPMS chromatograms for mouse plasma and standard mouse Alb.

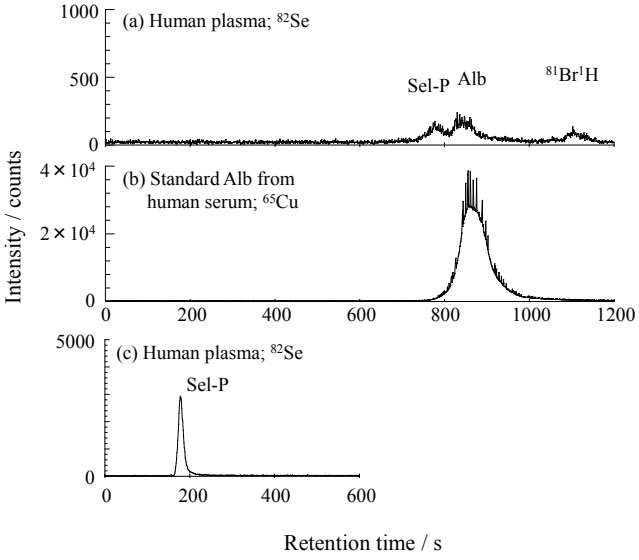


Fig. 3 Tandem AF and SEC HPLC-ICPMS chromatograms for human plasma and standard human Alb: (a) Se signals in human plasma before removal of the AF column and change of the mobile phase from A to B and (c) Se signals in plasma after column removal and mobile-phase change; (b) Cu signals in standard human Alb before removal of the AF column.

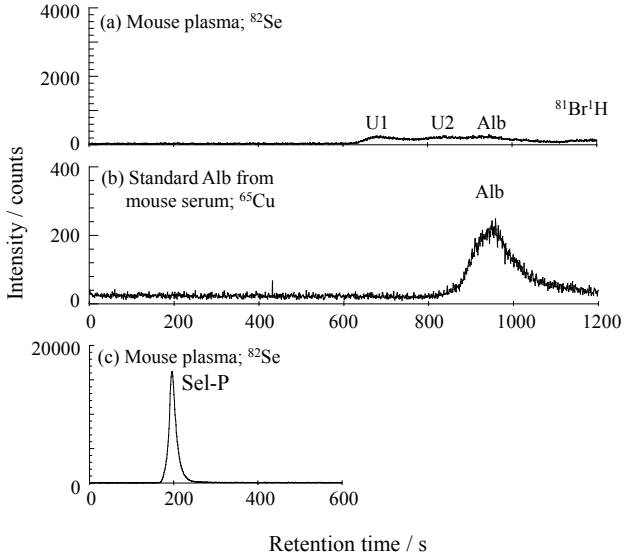


Fig. 4 Tandem AF and SEC HPLC-ICPMS chromatograms for mouse plasma and standard mouse Alb: (a) Se signals in mouse plasma before removal of the AF column and change of the mobile phase from A to B and (c) Se signals in plasma after column removal and mobile-phase change; (b) Cu signals in standard mouse Alb before removal of the AF column.

plasma (Fig. 4a), the main peak that eluted at 660 s from the SEC system (Fig. 2a) was almost completely eliminated. The adsorption efficiency of mouse Sel-P was $93.9 \pm 0.4\%$. Therefore, the main peak eluted at 660 s from the SEC system could be assigned to Sel-P. Two unidentified Se peaks appeared in chromatograms of the tandem system: U1 at 655 s and U2 at 815 s. U1 was eluted 95 s before the expected retention time of Sel-P (750 s), and U2 was eluted 65 s after the expected retention time of Sel-P. We tentatively assigned U1 as eGPx on the basis of the Se chromatogram for human plasma. A Se peak at a retention time of 900 s was identified as Alb because the retention time matched that of mouse standard Alb. The Se intensity of the peak eluted at 900 s from the tandem system was $85 \pm 4\%$ of the corresponding peak eluted from the SEC system. Unlike human Alb, mouse Alb was not retained on the heparin AF column. A Se chromatogram obtained after removal of the SEC column and change of the mobile phase from A to B showed one major Se peak, which was due to Sel-P (Fig. 4c).

*Quantification of Se concentrations*

In quantification of the concentrations of Se in selenoproteins, the combination of AF and SEC separation was useful for mouse plasma, but not for human plasma. In human plasma, unexpected adsorption in the tandem system caused errors in determining the amounts of eGPx and Alb. Also, the adsorption efficiency of human Sel-P on heparin AF column was slightly lower than that in mouse plasma. Therefore, the concentrations of Se

Table 1 Se concentrations incorporated in Se-containing proteins in human and mouse plasma ($n = 3$)

| | Se concentration/ng mL$^{-1}$ | | | | | | |
|---|---|---|---|---|---|---|---|
| | eGPx | Sel-P | Alb | U1 (eGPx) | U2 | Sum | Total Se[a] |
| Human plasma[b] | 3 ± 1 | 56 ± 3 | 30 ± 3 | | | 89 ± 4 | 94 ± 3 |
| Mouse plasma[c] | | 313 ± 5 | 18 ± 6 | 26 ± 1 | 20 ± 9 | 377 ± 12 | 361 ± 6 |

a. Flow injection.
b. SEC.
c. Tandem.

incorporated into selenoproteins in human plasma were determined by means of the SEC system, and those in mouse plasma were determined by means of the tandem system (Table 1). In human plasma, the sum of the Se concentrations incorporated in the three selenoproteins showed a good agreement (within 1$\sigma$) with the total Se concentration determined by flow injection following acid digestion. For mouse plasma, the analytical result by the tandem system also showed a good agreement (within 1$\sigma$) with the total measured Se concentration. The recovery percentages for human and mouse plasma were 95 ± 5 and 104 ± 3%, respectively. These percentages are slightly better than those reported by Jitaru *et al*. (113 ± 6%)[20] in a certified reference material (BCR-637, Institute for Reference Materials and Measurements, Geel, Belgium) and Reyes *et al*. (89 ± 4%)[17] in human plasma from five healthy volunteers and five patients on haemodialysis; their samples were different from ours.

The Se concentrations incorporated in Sel-P in the serum of 5 healthy volunteers have been reported to be 54 ± 11 ng mL$^{-1}$.[17] Our data (56 ± 3 ng mL$^{-1}$) showed a similar value. To our knowledge, this is the first report on Sel-P concentrations in mouse plasma. This value (313 ± 5 ng mL$^{-1}$) was quite similar to that in rat serum (about 4.0 μmol L$^{-1}$ = 316 ng mL$^{-1}$).[24] We observed an interspecies difference in the Sel-P concentration between mouse and human. The Sel-P concentration in the mouse plasma was 5.5-times that in human plasma.

## Conclusion

We established a tandem AF and SEC HPLC-ICPMS method, and used it to determine the Se concentrations incorporated in selenoproteins in human and mouse plasma. A heparin AF column was useful for Sel-P isolation from other selenoproteins. Because the major Se peaks obtained by SEC HPLC-ICPMS in both human and mouse plasma were adsorbed on the heparin AF column, these peaks were assigned to Sel-P. The adsorption efficiency of Sel-P on the heparin AF column was 90 ± 2% in the case of human plasma. In the case of mouse plasma, 93.9 ± 0.4% of Sel-P in mouse plasma was adsorbed on the heparin column. The usefulness of the heparin AF column to isolate Sel-P was confirmed for both human and mouse plasma in this study. The tandem AF and SEC separation technique was useful for mouse plasma, but not for human plasma, because of nonspecific adsorptions of eGPx and Alb. The Se concentration incorporated in selenoproteins determined by our method showed good agreements with the total Se concentrations determined following acid digestion. Finally, an interspecies difference was found in the plasma Sel-P concentration; the Sel-P concentration in mouse plasma was 5.5-times that in human plasma.

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