Determination of selenoprotein P in submicrolitre samples of human plasma using micro-affinity chromatography coupled with low flow ICP-MS

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Se-containing proteins play an important role in human metabolic processes such as antioxidant action. However, speciation analysis of Se-containing proteins in biological samples is challenging because of their small amounts. In order to determine selenoprotein P (Sel-P) in human plasma, separation performance was compared between size exclusion chromatography and affinity chromatography. It was found that affinity chromatography showed better separation performance for Sel-P. Micro-affinity chromatography coupled with low flow ICP-MS was developed, which enabled separation analysis of Sel-P in sub-μl samples. Standard solution flow rate ranges for low sample consumption and total consumption nebulizers were investigated. Moreover, the stability analysis for each nebulizer with the change in composition of the mobile phase was investigated. Finally, using the developed micro-affinity chromatography coupled with the low flow ICP-MS, Sel-P and the other Se-containing proteins in sub-μl samples of human plasma were determined.

Introduction

Selenium is now recognized as an essential element for both animals and humans, and the importance of Se biochemistry has been revealed by many recent works. It has been found that Se is the only trace element to be specified in the genetic code, such as ''the 21st amino acid'', selenocysteine.[1] Se-containing proteins are versatile, undertaking many functions such as antioxidant,[2] de-iodination of thyroxine,[3] maintenance of sperm[4] and detoxification of heavy metals.[5] On the other hand, inadequate or excess intake of Se is implicated in disease since the appropriate concentration range for effective participation in biological processes is not met. In fact, it has been reported that myocardosis,[6] an increased risk of cancer and virus infection[7] are induced by Se deficiency, whereas loss of hair and transformation of nail, skin and teeth are induced by Se excess.[8]

Se exists in human plasma as distinct chemical forms, *i.e.*, glutathione peroxidase (GPx), selenoprotein P (Sel-P) and albumin.[9–11] It is known that Sel-P is a major Se-containing protein in human plasma, and the concentration level is a good index of human Se status.[12,13] Therefore, the determination of Sel-P in human plasma is very important for the diagnosis of Se status, hence the focus of this work.

In the case of clinical diagnostic investigations, methods which utilize a small sample volume are required because available sample is limited. A simple and rapid measurement based on a small sample volume also has the benefit of reducing analysis cost through reduced maintenance of instrumentation and usage of reagents.

High-performance liquid chromatography-inductively coupled plasma mass spectrometry (HPLC-ICPMS) is one of the hyphenated techniques that is widely used for speciation measurement of trace elements in biological fluids. So far the speciation studies on Sel-P have been carried out using size exclusion chromatography[14] and affinity chromatography.[15] These previous studies required a relatively large sample volume (sub-ml).

Recently, a total consumption nebulizer has been developed for liquid sample introduction of small sample volume (sub-μl).[16,17] The sample introduction efficiency for a conventional nebulizer in ICP-MS is typically 1–2%,[18] whereas the fine aerosol mists produced by the total consumption nebulizer can result in a dramatic improvement in sample introduction efficiency, and thus good sensitivity for μl sample volumes can be realized.

In this study, operating conditions for both liquid chromatography (LC) and the interface between LC and ICP-MS were optimized. Then, micro-affinity chromatography coupled with the low flow ICP-MS system was applied to the determination of Sel-P in human plasma samples.

Experimental

HPLC-ICP-MS

In the case of normal flow rate (0.6–1.0 ml min^{-1}), a HPLC pump PU-1580i (Jasco Co., flow rate coverage 1 μl min^{-1}–10 ml min^{-1}) was used. For low flow rate studies (6–100 μl min^{-1}), a HPLC pump MP 701i (GL Sciences Co. Ltd, flow rate coverage 0.01–200 μl min^{-1}) was used. Injection valves used in this study were Model 9725i (Rheodyne Inc., sample

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Table 1 Operating conditions of ICP-MS

Nebulizer	AR-35	PFA-100	PFA-20	CEI-100
Spray chamber volume/cm^3	72 (Scott)	72 (Scott)	72 (Scott)	8 (Burgner)
RF power/W	1300	1300	1300	1300
Ar gas flow rate/L min^{-1}				
Plasma	15	15	15	15
Auxiliary	1.0	1.0	1.0	1.0
Nebulizer	1.08	0.95	0.65	1.15
Make up	—	0.18	0.48	—
Isotopes and molecular ions (integration time/s)	$^{138}Ba^{2+}$ (1.0 s)	$^{77}Se^{+}$ (0.1 s)	$^{138}Ba^{+}$ (0.1 s)	$^{138}BaO^{+}$ (1.0 s)

loops 2, 10, 20 or 100 μl) and Model C4-1344 (Valco International Co. Inc., sample volume 0.5 μl).

An ICP-MS (HP4500, Agilent Technologies Co.) was used for the measurement of Se. For nebulization, a conical concentric nebulizer (AR-35, Meinhard Glass Products, recommended flow rate 1 ml min^{-1}), two types of micro-flow nebulizers (PFA-100, Elemental Scientific, recommended flow rate 100 μl min^{-1} and PFA-20, Elemental Scientific, recommended flow rate 20 μl min^{-1}) and a capillary electrophoresis interface (CEI-100, CETAC Technologies, recommended flow rate $\leq$10 μl min^{-1}) were used. The AR-35 is a conventional nebulizer, whereas the PFA-100 and PFA-20 are low flow rate nebulizers, and the CEI-100 is a total consumption nebulizer. Out of these nebulizers, the CEI-100 provides 100% sample introduction efficiency. Consequently, there is no requirement for a spray chamber drain. ICP-MS operating conditions for each nebulizer are summarized in Table 1.

In the case of the CEI-100, a fused silica tube (GL Science, 30 μm id, 375 μm od) was used for connection of the low flow HPLC pump and the total consumption nebulizer. The adjustment of torch position and ion-lens voltages was conducted prior to measurement using a mixed solution of 20 ng ml^{-1} of Ge, Tl and Ba.

Reagents

The optimization of ICP-MS measurement conditions was performed using a mixed solution of Se and Ba, which was prepared from standard solutions (1000 μg ml^{-1}, AAS grade, Kanto Chemical Co. Inc.). Selenium was in the form of Se (IV). Sensitivity tuning of the ICP-MS was conducted using a mixed solution of Ge, Tl and Ba, which was prepared from standard solutions of 1000 μg ml^{-1} (AAS grade, Kanto Chemical Co. Inc.). A mixed solution of Cl and Br was prepared for spectral interference correction using HCl (special reagent grade, Kanto Chemical Co. Inc.) and KBr (special reagent grade, Kanto Chemical Co. Inc.). Dilution of standard solutions was achieved using 0.1 M HNO_3 (70% HNO_3, electric laboratory grade, Kanto Chemical Co. Inc.) and ultra-pure water (Milli-Q Element, Japan Millipore Co.). A Tris buffer solution was prepared by diluting tris(hydroxymethyl)aminomethane (Sigma Ultra grade, Sigma–Aldrich) with water and adjusting to pH 7.4 with HNO_3. Acetic acid (99.8 %, electric laboratory grade, Kanto Chemical Co. Inc.) and NH_3 (29%, electric laboratory grade, Kanto Chemical Co. Inc.) were mixed to prepare ammonium acetate as the buffer solution.

Chromatography

For the separation of Sel-P from GPx and albumin in human plasma, size exclusion chromatography and affinity chromatography were evaluated. The chromatographic conditions are presented in Table 2. The nebulizer used in this experiment was a conventional nebulizer AR-35.

The column used for size exclusion chromatography was an Asahipak GS-520 (Showa Denko K. K., 7.6 mm id × 300 mm long). The Asahipak GS-520 is a multi-mode column which functions not only as size exclusion but also partition, adsorption and ion exchange columns. The Asahipak column can be used in size exclusion mode by selecting an appropriate mobile phase. The exclusion limit is 300 kDa. It is known that the retention time for albumin using GS-520 is relatively long because the OH functional group of the stationary phase, poly-vinyl alcohol, has an interaction with albumin.[14]

The column used for affinity chromatography was AFpak AHR-894 (Showa Denko K. K.) with a stationary phase of heparin. The column size was 4.6 mm id × 150 mm long in the case of conventional flow rates and 0.5 mm id × 35 mm long in the case of low flow rates. An affinity column has specificity for a specific enzyme or protein, and thus it is used for fractionation and separation of physiologically active substances. It has been reported that only Sel-P among all of the Se-containing proteins in blood plasma is adsorbed on heparin.[10,15] For the elution of the adsorbed Sel-P, the pH and/or salt concentration of the mobile phase was changed. Sodium chloride, ammonium chloride and ammonium acetate are usually used for the elution of adsorbed substances on heparin.[15] In this study, ammonium acetate was chosen as eluent to prevent the deposition of salt on the sampling cone of the ICP-MS.

Table 2 Chromatographic conditions for size exclusion and affinity chromatography

Size exclusion chromatography	
Column	Asahipak GS-520 (7.6 mm id × 300 mm)
Sample volume	20 μl
Flow rate	0.6 ml min^{-1}
Mobile phase	50 mM Tris–HNO_3 (pH 7.4)
Affinity chromatography	
Column	AFpak AHR-894 (4.6 mm id × 150 mm)
Sample volume	100 μl
Flow rate	1.0 ml min^{-1}
Mobile phase (equilibration buffer)	20 mM Tris–HNO_3 (pH 7.4)
Mobile phase (elution buffer)	20 mM Tris–HNO_3 + 1.4 M ammonium acetate (pH 7.4)

Pre-treatment of human plasma

Human blood sample was collected from a single volunteer. After heparin (100 μl) had been added to human blood (5 ml) to prevent coagulation, the sample was centrifuged at 1500*g*, 4 °C, for 10 min. The supernatant was stored at −30 °C until analysed. In order to minimize any possible alteration of chemical forms, the human plasma was not diluted, and filtration (nitrocellulose membrane filter, Japan Millipore Co., pore size 0.45 μm) was performed just before analysis.

Determination of Sel-P in human plasma

Sel-P in a sub-μl sample of human plasma was separated and determined using micro-affinity chromatography coupled with a low flow ICP-MS system. Total Se in human plasma samples was determined by flow injection (FI) ICP-MS. Analyses for both experiments were based on integration of respective transient signals. From these experimental results, a summation of Se derived from the Se-containing proteins was compared with the total Se. For the determination of Se, standard solutions (0, 10, 30 and 50 ng ml^{-1} Se) were prepared by dilution of commercially available standards (1000 μg ml^{-1}, AAS grade, Kanto Chemical Co. Inc.) with 0.1 M HNO_3. Chloride ion (100 μg ml^{-1}) and bromine ion (10 μg ml^{-1}) standard solutions were used to correct for spectral interferences of $^{40}Ar^{37}Cl$ on ^{77}Se and $^{81}Br^{1}H$ on ^{82}Se, respectively.

Results and discussion

Initial chromatography studies

First, separatory performance for Sel-P in human plasma using both size exclusion chromatography and affinity chromatography was investigated. The chromatogram (^{77}Se) for human plasma (sample volume 20 μl) by size exclusion chromatography is shown in Fig. 1(a). The peaks eluting at 680, 790 and 840 s correspond to GPx, Sel-P and albumin according to a former study.[19] In addition, the peak eluting at 1140 s corresponds to $^{40}Ar^{37}Cl$, which is derived from chlorine in human plasma. It is clear that the peaks for GPx, Sel-P and albumin were not well resolved. Therefore, it can be said that the present size exclusion system was not able to achieve complete separation of Sel-P in human plasma.

The chromatogram (^{77}Se) for human plasma (sample injection 100 μl) by affinity chromatography is shown in Fig. 1(b). The first peak (eluting at 110 s) corresponds to Se-containing proteins which were not adsorbed by the stationary phase. After 300 s, the mobile phase was switched from the equilibration buffer to the elution buffer by step gradient. The second peak (eluting at 390 s) was judged to be Sel-P, which was selectively adsorbed by heparin.[10] Moreover, the analysis time for affinity chromatography was shorter relative to size exclusion chromatography. From the above results it was found that affinity chromatography was suitable for separation analysis of Sel-P. As the next step, a low flow ICP-MS system was developed using affinity chromatography.

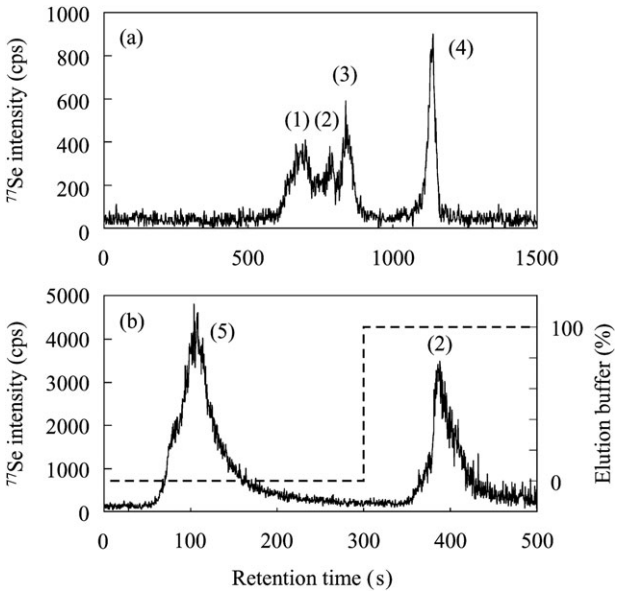

Fig. 1 Comparison of chromatograms (^{77}Se) for measurement of human plasma by (a) size exclusion chromatography (flow rate 0.6 ml min^{-1}, sample volume 20 μl) and (b) affinity chromatography (flow rate 1.0 ml min^{-1}, sample volume 100 μl). Peak identification: (1) GPx, (2) Sel-P, (3) albumin, (4) $^{40}Ar^{37}Cl$ and (5) Se-containing proteins. The broken line indicates mixing ratios for the elution buffer.

Development of the low flow ICP-MS system

In order to identify optimum flow rate conditions, a mixed solution of 500 ng ml^{-1} Se and 100 ng ml^{-1} Ba was continuously introduced into PFA-100 or PFA-20 low sample consumption nebulizers or the CEI-100 total consumption nebulizer using the low flow HPLC pump (MP 701i) at a controlled standard solution flow rate (1–200 μl min^{-1}). For evaluation, the signal intensity for ^{77}Se and the relative standard deviation (% RSD), together with the formation ratios of BaO^+/Ba^+ and Ba^{2+}/Ba^+, were acquired. The results are shown in Figs. 2–4. Since the % RSD of the ^{77}Se signal intensity was less than 5% in the range of ≥ 10 μl min^{-1} for the PFA-100 (Fig. 2(a)), it was judged that signal stability was acceptable. From Fig. 2(b), the formation ratio of Ba^{2+}/Ba^+ was less than 10% at <130 μl min^{-1} for the PFA-100. The formation ratio of BaO^+/Ba^+ was less than 0.05% in all investigated standard solution flow rates for the PFA-100. Consequently, the acceptable standard solution flow rate range for the PFA-100 was considered to be in the range of 10–130 μl min^{-1}.

In the range of ≥ 10 μl min^{-1} for the PFA-20 (Fig. 3(a)), the % RSD of the ^{77}Se signal intensity was less than 5%, and thus it was judged that signal stability was acceptable. From Fig. 3(b), for the PFA-20, the formation ratio of Ba^{2+}/Ba^+ was less than 10% at <50 μl min^{-1}, and the formation ratio of BaO^+/Ba^+ was less than 0.05% in all investigated standard solution flow rates. Consequently, the acceptable standard solution flow rate range for the PFA-20 was 10–50 μl min^{-1}.

In the case of the CEI-100 system, the % RSD of the ^{77}Se signal intensity was less than 5% at ≥ 5 μl min^{-1}, as shown in Fig. 4(a). Also, the formation ratio of Ba^{2+}/Ba^+ was less than

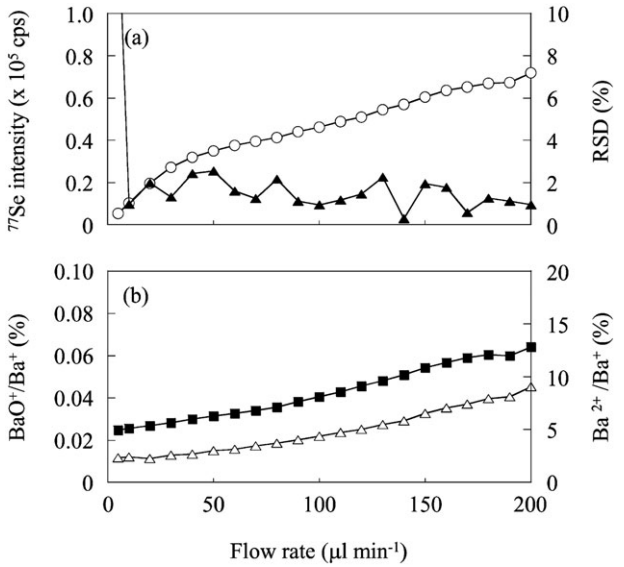

Fig. 2 (a) Signal intensity for ^{77}Se and the relative standard deviation (% RSD). (b) Formation ratios (BaO^+/Ba^+ and Ba^{2+}/Ba^+) as a function of standard solution flow rate for the PFA-100. ^{77}Se, ○; RSD (%), ▲; BaO^+/Ba^+, △; and Ba^{2+}/Ba^+, ■. A standard solution is a mixed solution of 500 ng ml^{-1} Se and 100 ng ml^{-1} Ba.

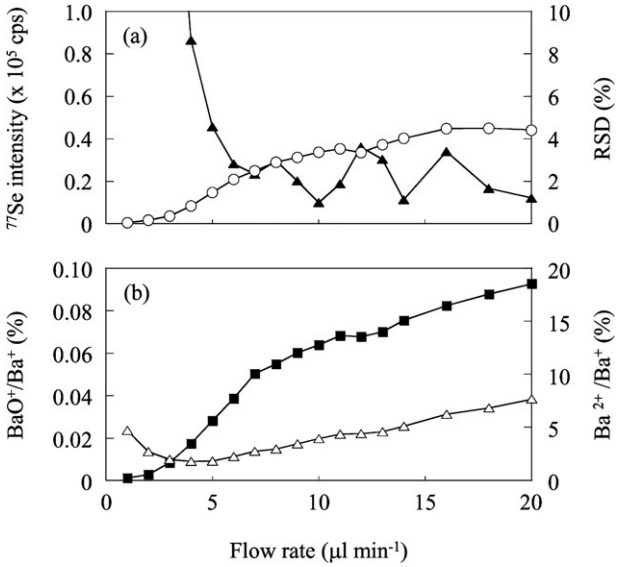

Fig. 4 (a) Signal intensity for ^{77}Se and the relative standard deviation (% RSD). (b) Formation ratios (BaO^+/Ba^+ and Ba^{2+}/Ba^+) as a function of standard solution flow rate for the CEI-100. ^{77}Se, ○; RSD (%), ▲; BaO^+/Ba^+,△; and Ba^{2+}/Ba^+, ■. A standard solution is a mixed solution of 500 ng ml^{-1} Se and 100 ng ml^{-1} Ba.

10% at <6 μl min^{-1} (Fig. 4(b)). Consequently, the acceptable standard solution flow rate range for the CEI-100 was 5–6 μl min^{-1}. Note that it has been reported[17] that the Se signal intensity showed maximum value at a standard solution flow rate of 7 μl min^{-1} when a CEI-100 system was coupled with a low dead volume spray chamber (spray chamber volume 4 ml) for ICP-MS.

In summary, measurement at low flow rate was found to be satisfactory using low sample consumption nebulizers or a total consumption nebulizer. Moreover, the formation ratios of oxides are sufficiently low to ignore interference from oxides in such conditions. Although the standard solution flow rate for the CEI-100 was 1/17 and 1/5 of those for PFA-100 and PFA-20, respectively, the ^{77}Se signal intensity for CEI-100 at 6 μl min^{-1} was approximately half of those for the PFA-100 at 100 μl min^{-1} and the PFA-20 at 30 μl min^{-1}.

In order to test the coupling of the micro-affinity chromatography with the developed low flow ICP-MS, the stability of the ^{77}Se signal during change in the composition of a mobile phase solution was investigated. Using low sample consumption nebulizers or a total consumption nebulizer, a discrete injection of 100 ng ml^{-1} Se solution was introduced into the ICP-MS *via* flow injection. The compositions of the used mobile phase solutions are the same as those listed in Table 2. In Table 3, the mobile phase flow rate and the sample volume for each nebulizer are listed. The mobile phase flow rates were set within the flow rate ranges previously established. The sample injection volumes were set at the values which are approximately proportional to the mobile phase flow rates. As is shown in Fig. 5, only the equilibration buffer

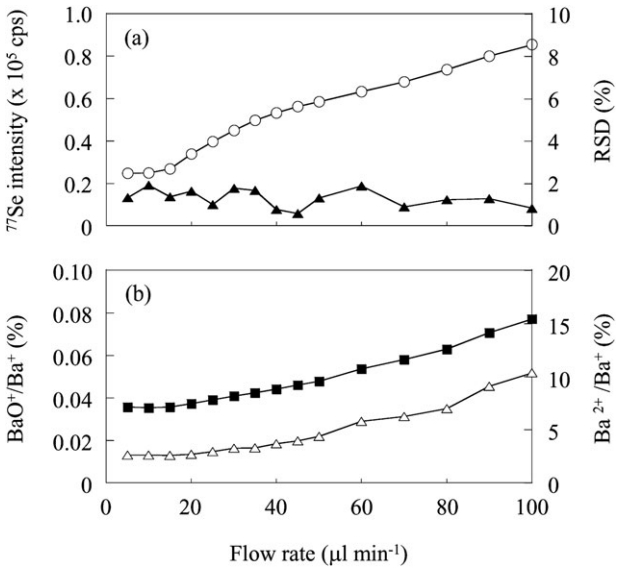

Fig. 3 (a) Signal intensity for ^{77}Se and the relative standard deviation (% RSD). (b) Formation ratios (BaO^+/Ba^+ and Ba^{2+}/Ba^+) as a function of standard solution flow rate for the PFA-20. ^{77}Se, ○; RSD (%), ▲; BaO^+/Ba^+, △; and Ba^{2+}/Ba^+, ■. A standard solution is a mixed solution of 500 ng ml^{-1} Se and 100 ng ml^{-1} Ba.

Table 3 Instrumental conditions, S/N (^{77}Se) and detection limit (Se) for each nebulizer when the equilibration buffer was replaced with the elution buffer (1800–2400 s)

Nebulizer	Flow rate/ μl min^{-1}	Sample volume/μl	S/N	Detection limit/ng ml^{-1}
PFA-100	100	10	400 (220)[a]	0.15 (0.27)[a]
PFA-20	30	2	84	0.72
CEI-100	6	0.5	120	0.49

[a] Obtained when the enhancement effect due to organic compounds did not occur (0–600 s).

was introduced between 0 and 600 s. From 600–1800 s, the equilibration buffer was replaced with the elution buffer with a linear gradient. At 2400 s, the elution buffer was replaced with the equilibration buffer with a step gradient.

The time profiles of the ^{77}Se signal intensities for the PFA-100, PFA-20 and CEI-100 nebulizers are shown in Fig. 5 (a), (b) and (c), respectively. In the case of the PFA-100, the ^{77}Se signal intensity increased with the concentration of ammonium acetate in the elution buffer. Larsen and Stürup reported the enhancement of Se and As signal intensities by the addition of carbon derived from organic compounds.[20] However, the same phenomenon was not observed for the PFA-20 and CEI-100 nebulizers. The reason for this is that the enhancement of the ^{77}Se signal intensity was eliminated because the mobile phase flow rate was small, and a considerable amount of ammonium acetate was not introduced into the ICP-MS.

Table 3 summarizes the signal to noise ratio (S/N) and the detection limit for Se for each nebulizer when the equilibration buffer was replaced with the elution buffer. The S/N and the detection limit were calculated from the peak area of the spiked sample. The detection limit is defined as the concentration when the S/N is 3. The S/N for the PFA-100 (when the equilibration buffer was replaced with elution buffer, 1800–2400 s) was 400, whereas the S/N for the PFA-100 (when the equilibration buffer was initially introduced, 0–600 s) was 220. This reason for the difference in performance is that the ^{77}Se signal intensity was enhanced by the organic compounds as mentioned above. When the CEI-100 was used, the S/N (120) was approximately 1/2, and the detection limit (0.49 ng ml^{-1}) was 2 times poorer compared with the PFA-100. If the sample introduction efficiency for each nebulizer is the same, the S/N should be proportional to the sample volume. In this case, the S/N for the CEI-100 (120) was only 1/2 less than that for PFA-100 (220), even though the sample volume was 1/20 of that for the PFA-100. Thus, it is said that the sample introduction efficiency was improved dramatically using CEI-100. When the PFA-20 was used, the S/N (84) was approximately 2/5, and the detection limit (0.72 ng ml^{-1}) was 2.5 times as much as that for PFA-100 even though the sample volume was 1/5 of that for the PFA-100. Thus, considerable improvement in the sample introduction efficiency in comparison with use of the CEI-100 was not realized.

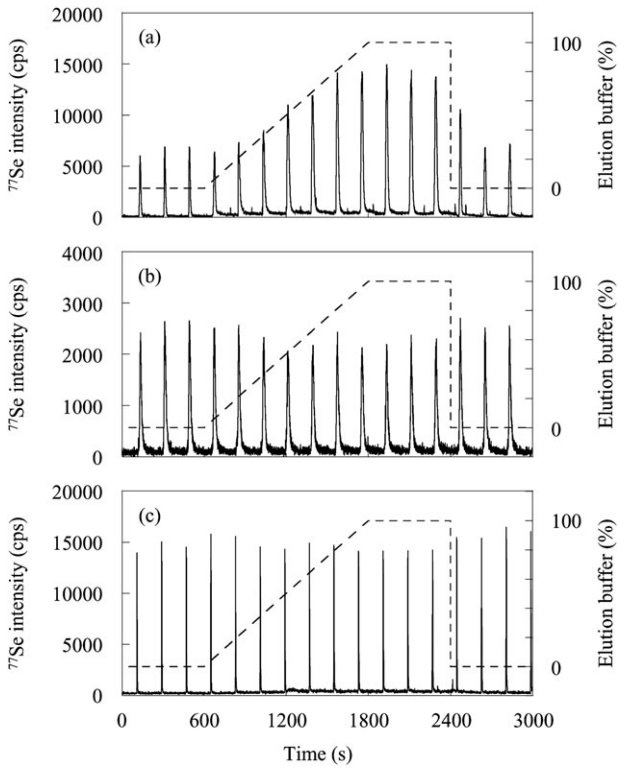

Fig. 5 Effect of elution buffer on signal stability (^{77}Se) for (a) PFA-100 (flow rate 100 μl min^{-1}, sample volume 10 μl), (b) PFA-20 (flow rate 20 μl min^{-1}, sample volume 2 μl) and (c) CEI-100 (flow rate 6 μl min^{-1}, sample volume 0.5 μl). Interval for injection of 100 ng ml^{-1} Se is 180 s. The broken lines indicate mixing ratios for the elution buffer.

Measurement of Sel-P in human plasma

In order to determine Sel-P in human plasma, micro-affinity chromatography coupled with the low flow ICP-MS system was used for analysis of human plasma. From the results in the previous section, it is expected that speciation measurement of sub-μl samples is feasible using the CEI-100 system. The compositions of the mobile phase solutions for micro-affinity chromatography are the same as those listed in Table 2. In order to operate at a low flow rate (6 μl min^{-1}), the micro-affinity column and the sample injector were scaled down to 0.5 mm id × 35 mm long (AFpak AHR-894) and 0.5 μl (Model C4-1344), respectively. The chromatogram obtained is shown in Fig. 6. The mobile phase was switched from equilibration buffer to elution buffer by a step gradient at 180 s. The first peak corresponds to GPx, albumin and other selenium proteins which were not adsorbed by the affinity column. The second peak corresponds to Sel-P as a result of elution.

Table 4 lists the selenium concentrations of Sel-P and the other Se-containing proteins in human plasma determined by the micro-affinity chromatography method coupled with the low flow ICP-MS system. The total selenium concentration in human plasma determined by FI-ICP-MS is also listed in Table 4. In this study, the total selenium concentration is considered as the internal control value. It can be seen that the summation of the selenium concentration of Sel-P and the other selenium proteins was effectively equivalent to the total

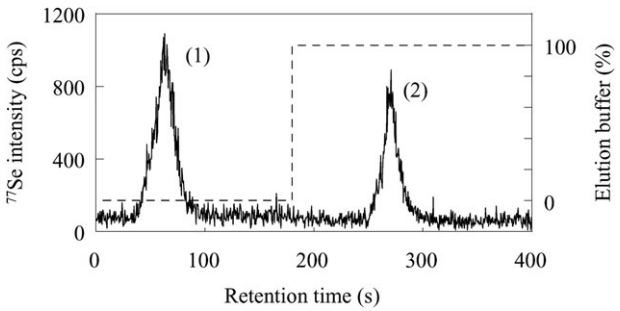

Fig. 6 Chromatogram (^{77}Se) for human plasma (sample volume 0.5 μl) using the developed micro-affinity chromatography coupled with CEI-100 nebulizer (flow rate 6 μl min^{-1}, sample volume 0.5 μl). Peak identification: (1) selenium-containing proteins and (2) Sel-P. The broken line indicates mixing ratios for the elution buffer.

Table 4 Determination of selenoproteins (micro-affinity chromatography-ICP-MS) and total selenium (FI-ICP-MS)

	Se-containing proteins	Sel-P	Total
Micro-affinity chromatography-ICP-MS			
Average (n = 3)/ng ml^{-1}	57 ± 4	35 ± 3	92 ± 5
RSD (%)	7.5	8.0	5.6
FI-ICP-MS			
Average (n = 3)/ng ml^{-1}	—	—	88 ± 7
RSD (%)	—	—	7.6

selenium concentration in human plasma within analytical error. Consequently, it is considered that there was no loss of analyte when human plasma samples were processed by the affinity column.

Conclusions

Affinity chromatography linked to ICP-MS was effective for the rapid separation and determination of Sel-P. When the standard solution flow rate ranges were investigated, analysis at low flow rate was found to be feasible using a total consumption nebulizer. When equilibration buffer was replaced with elution buffer the ^{77}Se signal intensity for the PFA-100 increased with the concentration of ammonium acetate. This phenomenon was observed with the addition of carbon derived from organic compounds. However, the same phenomenon was not observed for the PFA-20 and CEI-100 nebulizers because the mobile phase flow rate was small. The detection limits for Se for the tested nebulizers were at the sub-ng ml^{-1} level. The detection limit for the CEI-100 was only two times higher than that for PFA-100, even though the sample volume was 1/20 of that for the PFA-100. This is attributable to the high sample introduction efficiency for the CEI-100. Finally, it has been shown that it is possible to determine Sel-P in a sub-μl sample of human plasma by combining the micro-affinity chromatography with the low flow ICP-MS.

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