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Enriched stable isotopes of elements used as tracers: methods of presenting high-performance liquid chromatographic–inductively coupled argon plasma mass spectrometric data

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Abstract

High-performance liquid chromatography (HPLC) of biological fluids and tissue cytosolic preparations was employed in conjunction with argon-induced inductively coupled plasma mass spectrometry (ICP-MS) to investigate the distribution of stable isotopes as tracers. The common way of presenting the data from the ICP-MS is by plotting the count rates versus the retention time of HPLC fractions. Additional information can be derived, e.g., the composite peaks can be further resolved, and the level of enrichment in various biological components can be expressed by alternative ways of presenting these data. The two additional approaches described here involve presenting the ratios of enriched tracer with a suitable naturally abundant mass number of the same element, and by expressing the extent of enrichment by the tracer isotope in a given fraction to that of the same mass number in the fraction derived from an untreated source. Each method of presentation has different merits and drawbacks. The data therefore may be best presented in more than one way to emphasize the conclusions from a given experiment. Observations are presented after simultaneously injecting stable isotopes of three essential elements, copper, selenium and zinc, into mice. Plasma and liver cytosolic fractions were analysed and data represented in different ways as indicated above.

1. Introduction

Mass spectrometry with inductively coupled argon plasma excitation (ICP-MS) has several advantages over the conventional atomic absorption spectrometry (AAS) and atomic emission spectrometry (AES) for the detection of metals. In addition to simultaneous multi-element detection with extremely high sensitivity, ICP-MS is able to measure isotopes, hence it permits the use of enriched stable isotopes as tracers in metabolic studies. The use of stable isotopes rather than radioisotopes is recommended for human subjects.

Speciation of metals in biological samples provides additional information compared with simple data on concentration. The use of AAS [1,2] and ICP-AES [3–5] for element-specific detection coupled with HPLC has been demonstrated. The use of ICP-MS for detection in HPLC (HPLC–ICP-MS) is expected to be a highly sensitive analytical method for simulta-

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neous and element-specific detection and speciation of metabolites. The HPLC–ICP-MS method has been successfully applied to the speciation of metals in biological samples such as iron [6], cadmium [7], arsenic [8], platinum [9] and selenium [10]. When this method is applied to the metabolic fate of enriched external tracer, its interaction with the endogenous pool can be analysed, in addition to the detection and speciation of the tracer, in a single experiment. Using an ^{82}Se-enriched tracer, we have demonstrated that the application of the HPLC–ICP-MS with enriched stable isotopes is a unique method by which speciation of both endogenous elements and external tracers can be achieved [11].

It has been recommended that the presentation of two isotope profiles for each element is adapted to make it easier to identify polyatomic ions peaks originating from interfering ions [10]. However, it was not very informative to present the result simply by showing the profile of an enriched isotope in reference to the corresponding profile for the other natural abundant isotopes of the element.

The paper deals with the methods of representing HPLC–ICP-MS data for the efficient identification of enriched peaks. The first step was to develop an in-house program to present distribution profiles of multi-elements using a conventional personal computer. Then, several expression methods were examined for representing the HPLC–ICP-MS data using three biologically and nutritionally important elements (i.e., copper, selenium and zinc). Cu constitutes active centres for Cu-enzymes in redox reactions, and two stable isotopes, ^{63}Cu and ^{65}Cu, are present at natural abundances of 69.2 and 30.8%, respectively. The more abundant former isotope was used as a natural abundance reference isotope, while the latter was used as a tracer isotope because enriched tracers of less prevalent isotopes allow one to distinguish externally supplied forms easily from the endogenous forms. Se is present in diverse chemical forms with different valence states. It is a constituent of glutathione peroxidase (GPx) [12] and is present in a variety of selenoproteins identified in plasma and organs [13,14]. Although ^{80}Se is the most abundant isotope (49.6%), it cannot be detected by ICP-MS owing to an interfering mass peak of $^{40}Ar_2^+$. Therefore, the second most abundant isotope, ^{78}Se (23.8% natural abundance), was selected as a reference isotope. Among the third to fifth most abundant isotopes, ^{76}Se (9.4%), ^{82}Se (8.7%) and ^{77}Se (7.6%), ^{82}Se was selected based on the availability of the enriched isotope. Zn constitutes not only active centres for biologically important Zn-enzymes, but also key centres for Zn fingers, Zn twists and metallothionein (MT) [15]. Among the stable isotopes of Zn (^{64}Zn, 48.6%; ^{66}Zn, 27.9%; ^{68}Zn, 18.8%, ^{67}Zn, 4.1%; ^{70}Zn, 0.6%), the most abundant isotope was used as a reference isotope, and ^{68}Zn was selected as the tracer in the present study based on its availability at a reasonable cost.

We used plasma and liver supernatant fractions as biological samples prepared from a control mouse and a mouse injected with a mixture of three enriched isotopes (^{65}Cu, ^{82}Se and ^{68}Zn). Reference and enriched isotope profiles of chromatograms for each element are presented as the common presentation of data, then the other two profiles, one with ratios of enriched to reference mass numbers and the other with percentage of control (relative to enriched tracer in treated vs. control mice), are presented and their characteristics are discussed.

2. Experimental

2.1. Reagents

^{65}Cu-enriched copper(II) chloride was prepared by dissolving enriched metallic copper (99.2% enriched, from Bureau des Isotopes Stables, Gif-sur-Yvette, France) with metal-free concentrated HCl. ^{82}Se-enriched sodium selenite was prepared by oxidation of the enriched metal (97.02% enriched, from Oak Ridge National Laboratory, Oak Ridge, TN, USA). Oxidation of the metal was achieved by dissolving the metal in concentrated metal-free nitric acid and subsequent neutralization with 1 *M* NaOH. ^{68}Zn-enriched zinc chloride was prepared by dissolving enriched zinc oxide (98.0% enriched, from

Bureau des Isotopes Stables) with metal-free concentrated HCl.

Standard solutions of Cu, Fe, Se and Zn (1000 μg/ml) (Wako, Osaka, Japan) were used after appropriate dilution with 0.1 M HNO_3.

2.2. Animals

Male ICR mice were purchased at 4 weeks of age from a breeder (Clea Japan, Tokyo, Japan) and were fed a normal diet (CE-2, Clea Japan) and tap water ad libitum. A mixed solution of the three enriched isotopes (containing $CuCl_2$ as 100 μg Cu/ml, Na_2SeO_3 as 15 μg Se/ml and $ZnCl_2$ as 500 μg Zn/ml in saline) was injected intravenously into an animal at a dose of 1 ml/kg body mass. An animal injected with the same volume of saline served as a control. The animals were killed 12 h after the injection by exsanguination under ether anaesthesia, and their plasma and liver were collected.

2.3. Preparation of biological samples for HPLC–ICP-MS

Blood was centrifuged at 1200 g for 10 min at 2°C to separate plasma. Liver was homogenized in four volumes of extraction buffer (50 mM Tris–HCl buffer, pH 7.4) using a Polytron homogenizer (Kinematica, Lucerne, Switzerland) in a nitrogen atmosphere. The homogenate was centrifuged at 105 000 g for 60 min at 0°C for the resultant supernatant.

2.4. Analytical procedures

A 0.2-ml aliquot of plasma or liver supernatant was applied to a size-exclusion column (Asahipak GS 520, 500 × 7.6 mm I.D., exclusion limit M_r 300 000, particle size 9 ± 0.5 μm) (Showa Denko, Tokyo, Japan) and eluted with 50 mM Tris–HCl buffer (pH 7.4), using an LC-10AD HPLC system (Shimadzu, Kyoto, Japan). The flow-rate was maintained at 1.0 ml/min. The eluent was monitored with an ultraviolet (UV) detector and introduced directly into the nebulizer tube of a PMS 2000 ICP-MS system (Yokogawa Analytical Systems, Musashino, Japan) to detect Cu (m/z 63 and 65), Se (m/z 78 and 82), Zn (m/z 64 and 68), Fe (m/z 54) and S (m/z 34). ICP-MS was performed under the following conditions: forward power, 1300 W; reflected power, <10 W; plasma gas (Ar) flow-rate, 14.5 l/min; auxiliary gas (Ar) flow-rate, 1.1 l/min; nebulizer gas (Ar) flow-rate, 0.9 l/min; sampling cone (Cu) diameter, 1.0 mm; and skimmer cone (Cu) diameter, 0.5 mm. Acquisition of data for each element was performed with the following dwell times: Cu 0.8, Fe 0.2, S 2.0, Se 3.0 and Zn 0.8 ms. The parameters for setting the ion lens were adjusted daily to optimize the detection sensitivity.

The other two elements, Fe and S, were monitored to help in the identification of various macromolecular fractions in HPLC fractions. Fractions from the HPLC column were tentatively identified by their element-specific elution pattern based on retention times.

Results are presented as count rates [counts per second (cps)] from the ICP-MS system, in relation to the retention time from the HPLC effluent. As a sequential analytical system was used, an exact calibration of the sensitivity for all elements studied was not possible. With each set of recordings, the calibration equivalent was denoted by a vertical bar, with the corresponding value for each element indicated on the right hand side of each recording. Profiles of isotope ratios were obtained by a laboratory-developed computer program using the direct output from the ICP-MS system, and are presented here since these allow the best way to resolve the broad peaks from the HPLC column. Results for plasma and tissue supernatants from control animals were used to calculate the percentage change in tracer concentrations and therefore are presented here only for major HPLC fractions.

3. Results

Distribution profiles of the three elements in plasma of the control mouse are presented in Fig. 1, together with those of the other two elements and the UV absorption at 280 nm. The three elements were each detected at two mass:

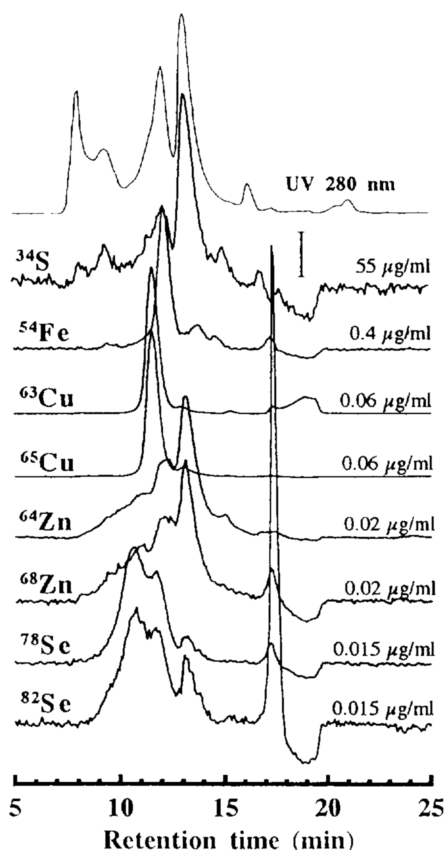

Fig. 1. Distribution profiles of Cu, Se and Zn in plasma of control mouse determined by HPLC–ICP-MS. A 0.2-ml portion of control plasma was applied to an Asahipak GS 520 column and the distribution profiles of each of the three elements at two mass numbers were determined by HPLC–ICP-MS. Profiles of two other elements (Fe and S) and UV absorption at 280 nm were also recorded. The vertical bar indicates the detection level of each element at calibration shown in each profile on the right-hand side, determined using natural abundance standards.

Cu, m/z 63 and 65; Se, m/z 78 and 82; and Zn, m/z 64 and 68. A sequential determination of the other two selected reference elements, was carried out at one mass number: Fe, m/z 54; and S, m/z 34.

3.1. Distributions of Cu, Se and Zn in plasma by HPLC–ICP-MS

Major peaks from HPLC were tentatively assigned according to element-specific distributions [16]. These profiles were obtained by plotting the count rates of each data point against retention time using a laboratory-developed program on a personal computer (Macintosh Classic II; Apple Japan, Tokyo, Japan). The major Cu peak at a retention time of 11.6 min corresponds to ceruloplasmin and the small Cu peak at 13.1 min that co-eluted with Zn, S and UV peaks is assignable to albumin. Other Cu peaks that eluted more slowly than albumin in the ^{63}Cu profile can be considered as polyatomic ion peaks (artifacts due to the presence of interfering ions) because these peaks are not observed in the ^{65}Cu profile (Fig. 1).

The major Zn peaks were consistent between the two Zn profiles, whereas minor peaks slower than 15.0 min were not consistent between the two Zn profiles, suggesting them to be the polyatomic ion peaks.

The two Se profiles are almost identical except for the peak at 17.3 min. Although the reason is not clear, a peak was observed at the same retention time (17.3 min) not only in the ^{82}Se and ^{78}Se profiles but also in the ^{54}Fe, ^{63}Cu and ^{68}Zn profiles.

Two major Se peaks at 10.8 and 11.8 min were observed in plasma profile of some rats [10], the faster peak being eluted at a retention time similar to that of the tentatively assigned selenoprotein P in the rat, while the slower peak being eluted at a retention time similar to that of extracellular glutathione peroxidase (eGPx, molecular mass 92 000 [17]) [10].

The Fe peak at 12.0 min corresponds to transferrin and the S peaks are tentatively assignable as follows; albumin (13.1 min), oxidized form of glutathiones (GSH) (15.3 min) and reduced form of GSH (16.9 min).

Several methods for representing enriched peaks in biological samples were examined for the efficient expression of the HPLC–ICP-MS data. Fig. 2 presents three methods of presentation, obtained by plotting count rates at each data point against retention time after subtracting the mean background, and the ratios vs. percentage change from the control animal. The ^{63}Cu profiles shown by thin lines in Fig. 2A and B are reference profiles for ^{65}Cu in control and

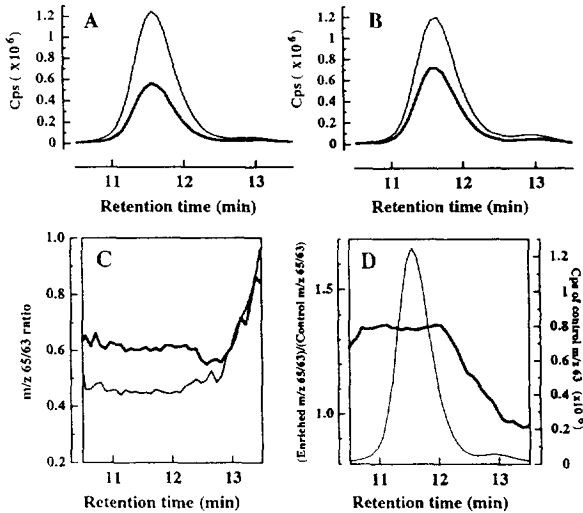

Fig. 2. Distribution profiles of Cu in plasma of mouse injected with a mixture of enriched Cu, Se and Zn. (A) Saline-injected mouse. (B) Mouse injected with the three enriched elements. Counts rates were plotted against retention time as the thin line (^{63}Cu profile) and thick line (^{65}Cu profile) after subtracting the mean background for (A) and (B). (C) Profiles for ^{65}Cu/^{63}Cu ratios. Ratios of ^{65}Cu/^{63}Cu counts at each data point were plotted against retention time. Thin line = profiles of ^{65}Cu/^{63}Cu ratios for control plasma and thick line = profiles of ^{65}Cu/^{63}Cu ratios for plasma from a mouse injected with the three enriched elements. (D) Bold line = profile for ratios of (^{65}Cu/^{63}Cu ratios for plasma from a mouse injected with the three enriched elements)/(^{65}Cu/^{63}Cu ratios for control plasma) against retention time. The thin-line in (A) is redrawn to show the relative peak positions.

treated animals, whereas the profiles of ^{65}Cu are shown by the thick lines for the two animals (Fig. 2A and B), respectively. The two ^{63}Cu profiles are comparable, whereas the ^{65}Cu profile in Fig. 2B suggests that the ceruloplasmin peak at 11.6 min is enriched compared with the profile of the control animal in Fig. 2A and also in relation to the reference ^{63}Cu profile of the treated animal.

The results shown in Fig. 2A and B can be represented better by the presentation shown in Fig. 2C, where the ratio (count rate of ^{65}Cu − mean background count rate)/(count rate of ^{63}Cu − mean background count rate) is plotted against retention time for the control (thin line) and the animal injected with enriched isotope (thick line). The thin line represents the ratio of natural abundance of ^{65}Cu/^{63}Cu = 0.45 for the Cu bound to ceruloplasmin. Ratios for data points without peaks or with low peaks showed a large variance and are not reliable, as seen in the profile later than 12.5 min.

The ratio of each data point shown by thick and thin lines in Fig. 2C is plotted against retention time in Fig. 2D. The thin line corresponds to that in Fig. 2A and indicated as a reference to represent peak positions. The thick line indicates that after injection of the tracer, the Cu bound to ceruloplasmin is enriched approximately 37% more than the control.

Although the amount of Cu bound to albumin was small, it was clearly detectable at 13.1 min. However, the profiles presented in Fig. 2C and D indicated that the Cu bound to albumin was not enriched.

The results for Se distribution in plasma are represented in similar ways to those of Cu, as shown in Fig. 3. The two major Se peaks in plasma were eluted in different patterns even in reference profiles of ^{78}Se between the control (Fig. 3A) and treated animals (Fig. 3B). However, the major peak is enriched more in the treated group (Fig. 3B) than in the control group

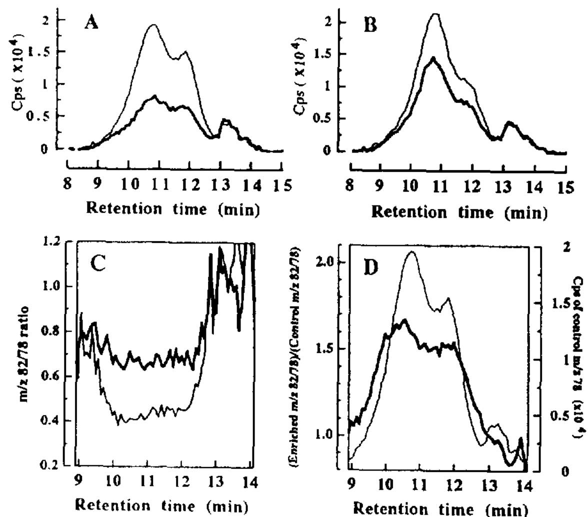

Fig. 3. Distribution profiles of Se in plasma of mouse injected with a mixture of enriched Cu, Se and Zn. (A) and (B) correspond to Fig. 2A and B, with thin line = ^{78}Se profile and thick line = ^{82}Se profile. (C) Profiles for ^{82}Se/^{78}Se ratios, corresponding to Fig. 2C. (D) As for Fig. 2D, for ^{82}Se/^{78}Se ratios.

(Fig. 3A). This is more evident in the profiles shown in Fig. 3C and 3D. The ratios of $^{82}Se/^{78}Se = 0.68$ for the two major Se peaks indicate that the two peaks are enriched more than the control ratio of $^{82}Se/^{78}Se = 0.41$. Further, the thick line in Fig. 3D suggests that the two Se peaks are enriched to different extents.

Fig. 4 shows the results for Zn in plasma. The reference profiles of ^{64}Zn in Fig. 4A and B are similar and the profiles of ^{68}Zn are also not different between the control (Fig. 4A) and the treated animals (Fig. 4B). The two profiles shown in Fig. 4C confirm that the conclusion drawn from Fig. 4A and B is accurate. The ratios were the same between the control and experimental groups in Fig. 4C, showing no enrichment after the tracer dose. The profile (thick line) shown in Fig. 4D also indicates that the major peaks were not enriched.

3.2. Distributions of Cu, Se and Zn in liver cytosol by HPLC–ICP-MS

The control profiles for the three elements Cu, Se and Zn and the two reference elements Fe and S in liver supernatant are shown in Fig. 5. As the liver was not perfused, haemoglobin was detected as a major Fe peak at 13.8 min. The two sharp S peaks can be assigned to the oxidized form of GSH (15.3 min) and reduced form of GSH (16.9 min). The major Cu peak at 12.8 min corresponds to Cu,Zn-superoxide dismutase (SOD) and the slower eluting Cu peak at 13.9 min is that of metallothionein (MT) [4]. The Zn peaks overlap and it is not possible to separate those of Cu,Zn-SOD and alcohol dehydrogenase (ADH). Zn bound to MT is also limited if it was present and it was not detected as a distinct peak. The Se peak at 11.9 min appears to be cGPx [10].

Fig. 6 presents the results for Cu distribution in liver supernatants. The profiles in Fig. 6A and

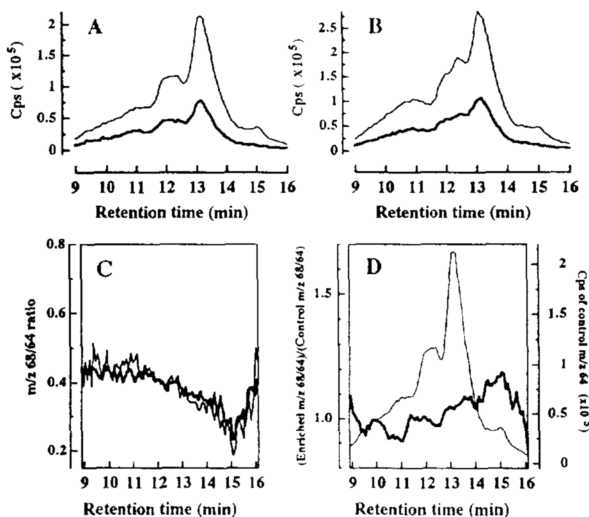

Fig. 4. Distribution profiles of Zn in plasma of mouse injected with a mixture of enriched Cu, Se and Zn. (A) and (B) correspond to Fig. 2A and B, with thin line = ^{64}Zn profile and thick line = ^{68}Zn profile. (C) Profiles for $^{68}Zn/^{64}Zn$ ratios, corresponding to Fig. 2C. (D) As for Fig. 2D, for $^{68}Zn/^{64}Zn$ ratios.

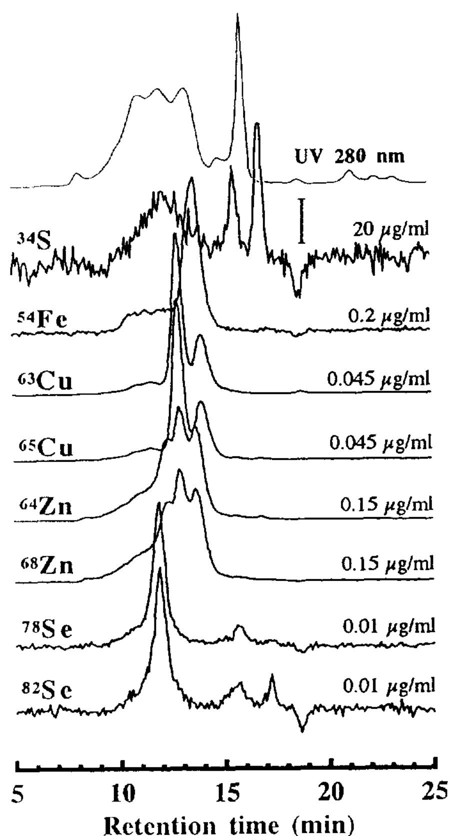

Fig. 5. Distribution profiles of Cu, Se and Zn in liver supernatant of control mouse determined by HPLC–ICP-MS. A 0.2-ml portion of control liver supernatant was applied to an Asahipak GS 520 column. For details, see Fig. 1.

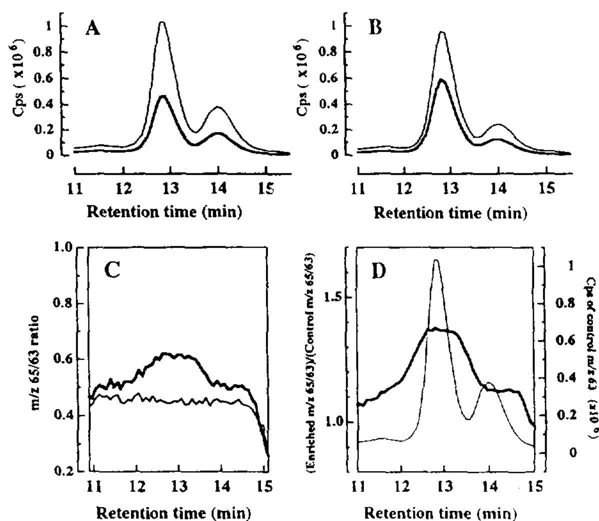

Fig. 6. Distribution profiles of Cu in liver supernatant of a mouse injected with a mixture of enriched Cu, Se and Zn. Details as in Fig. 2, with liver supernatant instead of plasma

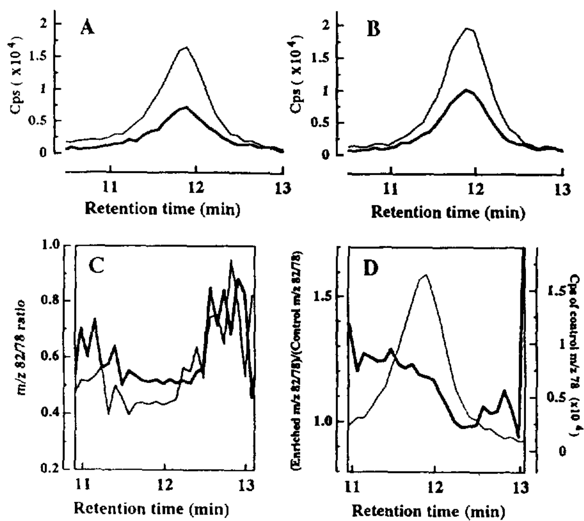

Fig. 7. Distribution profiles of Se in liver supernatant of mouse injected with a mixture of enriched Cu, Se and Zn. Details as in Fig. 3, with liver supernatant instead of plasma.

B indicate that the Cu peak of Cu,Zn-SOD at 12.8 min was certainly enriched by the tracer, whereas that of MT at 13.9 min was not enriched or only marginally enriched. However, the profiles in Fig. 6C and D demonstrate that the Cu peaks of both SOD and MT are enriched by the tracer to different extents, SOD being enriched more than MT.

Fig. 7 demonstrates the results for Se in various fractions of liver supernatant. The Se peak at 11.9 min seems to be slightly enriched by the tracer when Fig. 7A and B are compared. Fig. 7C indicates that the peak is slightly enriched by the tracer and Fig. 7D further suggests that the Se peak contains a shoulder peak around 11.5 min which is enriched more than the major peak (tentatively assigned as cGPx) at 11.9 min.

Fig. 8 shows the Zn profiles for liver supernatants. Fig. 8A and B are similar in shape. However, a close examination suggests that the thick line in Fig. 8B is accompanied by a shoulder peak at the position where MT should be eluted (at 13.9 min). The ratios in Fig. 8C are relatively constant throughout and indicate that the Zn peaks are enriched by tracer almost to the same extent. However, the ratios around 14.0 min suggest that MT is enriched slightly more. The same conclusion is drawn by presentation of the ratio in Fig. 8D. These results suggest that although most of the exogenously provided Zn in liver supernatant was randomized, Zn bound to MT was not equilibrated with the tracer at this time after the injection.

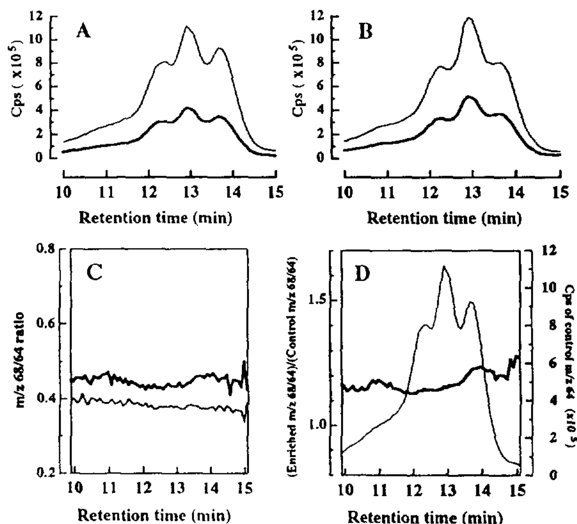

Fig. 8. Distribution profiles of Zn in liver supernatant of mouse injected with a mixture of enriched Cu, Se and Zn. Details as in Fig. 4, with liver supernatant instead of plasma.

4. Discussion

HPLC–ICP-MS is a valuable tool in determining the distribution of stable isotopes in biological systems and is well established [6,11]. This relatively new approach has several advantages over traditional methods for the determination of elements in biological fluids and tissues. Some of these include (i) simultaneous detection of several elements; (ii) use of stable isotopes instead of radioactive isotopes, which reduces the health hazards associated with the radioactive materials and allows the feasibility of using the elements in human studies; (iii) distribution of elements in biological systems, particularly with reference to binding of elements with various proteins or their incorporation in different proteins; and (iv) differentiation of externally provided elements with endogenous pools using isotopes of lower abundance, thereby providing opportunities for investigating the exchange between endogenous and exogenous metals and the turnover of metal-containing macromolecules. The traditional methods of presenting the chromatographic data, however, include a direct output of counts from the mass spectrometer at various m/z levels. The main objective of this work was therefore to examine several other methods of representing the mass spectrometer output which would allow a more complete understanding of the distribution of various elements.

Three methods of presenting the HPLC–ICP-MS data were examined. Presentation of profiles by plotting count rate against retention time is a common and established procedure and provides information about the overall changes, as demonstrated in Figs. 1 and 5. The identification of polyatomic ions peaks can be established by the direct observation of such a presentation. Assignment of peaks is also feasible by comparing the distribution of reference elements if these are associated with established fractions in the tissues or if the tracer element is known to elute with known fractions. However, the quantitative changes can be expressed better by plotting ratios of count rates of the enriched isotope profile/count rates of reference isotope profile after subtraction of the appropriate mean background counts, as indicated in part C of Figs. 2–4 and 6–8. This method allows the differentiation of composite peaks if the level of enrichment in various fractions in that peak is different, and also directly indicates the extent of enrichment by the tracer employed. A tracer of low abundance is therefore desirable in stable isotope studies. This method, however, does not provide information for fractions without appreciable peaks or gives a relatively large scatter if the peaks are small. This method of presentation is therefore effective only for points with appreciable and consistent peaks.

The third method of data presentation is demonstrated by part D in Figs. 2–4 and 6–8. These figures show the distribution of the enriched tracer to indicate the relative locations of the peak(s) and the extent to which the peak was enriched by the tracer. This essentially combines the information included in the two previous methods of presentation.

The three enriched isotopes were selected in this study for their biological or nutritional importance. Interactions are known to occur between elements among themselves, with the HPLC column material and with buffers. This may lead to specific and/or non-specific adsorption and desorption of the ligands on the biological substrates. Under the conditions in this study, Cu was more easily adsorbed than Zn on the column material when eluted with plain buffers. However, Cu binds to biological constituents more firmly than Zn, and therefore eluted more quantitatively than Zn. Although the quantitative aspects were not an objective of this study, it was apparent that Cu was specifically distributed with various plasma or cytosolic fractions and randomization of the label did not appear to be occurring. On the other hand, enriched Zn used as a tracer did not bind rapidly to specific proteins, except for the tiny metallothionein peak in the liver cytosol (Fig. 8). Zn is the most abundant essential heavy metal and interacts with various biological substrates and also with buffers and column materials. It is not clear from these observations whether the even distribution of tracer Zn with plasma proteins and liver cytosolic fractions reflects randomiza-

tion occurring as a biological process or was an artifact. In contrast to Cu and Zn, exchange of ligands is not possible with Se, because it is present in different redox states that are not interconvertible.

The three methods of data presentation given here do not indicate that any one method is superior to another. All methods have their own importance as they reflect different characteristics of tracer distribution. It is therefore inferred that the HPLC–ICP-MS data may be presented in various ways to suit the needs of the information to be conveyed and more than one approach can be employed for this purpose.

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