Analysis of mercury-containing protein fractions in brain cytosol of the maternal and infant rats after exposure to a low-dose of methylmercury by SEC coupled to isotope dilution ICP-MS†

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Received 6th February 2008, Accepted 8th May 2008
First published as an Advance Article on the web 5th June 2008
DOI: 10.1039/b802124d

The mercury-containing protein fractions in the brain cytosol of the maternal and infant rats after exposure to low-dose of methylmercury chloride (0.3 mg Hg kg^{-1} day^{-1}) were analyzed by a SEC-ICP-MS method with the postcolumn isotope dilution analysis. The enriched spiking including ^{34}S, ^{65}Cu, ^{67}Zn and ^{198}Hg was continuously added into the eluate from the HPLC column and then the isotope-diluted fractions were on-line measured by ICP-MS. Therefore, the absolute amounts of sulfur, mercury, zinc and copper in the eluted protein fractions could be attained after calculation of the corresponding peak areas in the mass flow chromatogram. Five mercury-containing protein fractions were monitored in the maternal sample, whereas only one was in the infant sample. The mercury content in the infant sample was about 10 times lower than the one in the maternal sample. In the meantime, two copper- and four zinc-containing fractions were found in both maternal and infant samples. The detection limit for S, Cu, Zn and Hg are 11, 0.1, 1.5 and 0.2 ng, respectively. The results demonstrate that different mercury-containing protein fractions may exist in brain cytosol between maternal and infant rats and the quantitative calculation may be helpful for the toxicological study.

Introduction

Mercury is a hazardous pollutant in the environment. Among mercury species, methylmercury (MeHg) is considered as one of the most toxic forms and a well known neurotoxicant, which may particularly cause adverse effects on the developing brain.[1] Prenatal exposures interfere with the division and migration of neuronal cells and disrupt cytoarchitecture of the developing brain.[2] Recently, concerns have been focused on the potential damage associated with the exposure to low-dose MeHg. Although the toxicity of MeHg has long been studied since the outbreak of Minamata disease, there are still some gaps in our knowledge about mercury toxicity, especially about the exact toxic mechanism of mercury to a fetus under low-dose, *in utero* exposure. For example, the potential harm of human exposure to low-dose MeHg is still disputable, such as the epidemiologic studies in the Faroe Islands[3] and in the Seychelles Islands.[4] More sensitive and reliable analytical methods, especially the quantitative studies are required to further understand the differential toxicity between mothers and their offspring after their exposure to a low-dose of MeHg.

The toxicity of mercury is generally considered to derive from the high affinity of mercury species to sulphydryls or thiols in proteins.[5] In contrast to inorganic mercury, methylmercury is almost totally absorbed in the gastrointestinal tract and readily passes the placenta and blood-brain barrier. Therefore, the MeHg is liable to accumulate in the brain and the fetus. Once MeHg enters the body, it can react with many kinds of proteins to form mercury–protein adducts, which will result in adverse effects on normal physiological process, such as inactivation of enzymes and alteration of cell membrane permeability.[5] On the other hand, some kinds of mercury-containing adducts are considered to have detoxification effects. For example, metallothionein (MT), a small-molecular weight, cysteine-rich, metal binding protein, is liable to bind to mercury and seems to play a protective role of mercury toxicity in mammals.[6] Thus, analysis of mercury-containing protein is important for toxicity study after mercury exposure.

Metal species have been successfully studied by many kinds of hyphenated techniques, such as high performance liquid chromatography[7] or electrophoresis with atomic spectrometry.[7–9] Among the methods, size exclusion chromatography (SEC) hyphenated to inductively coupled plasma-mass spectrometry (ICP-MS) has many unique advantages for the study of metal-containing proteins. One of the advantages is that the proteins can be isolated by the isocratic elution with an aqueous mobile phase containing a low concentration of salts, which is tolerable to ICP-MS. In addition, the relative size of a protein, which is correlative to its molecular weight, can be evaluated using a calibration curve of standard proteins. Therefore, many applications of SEC-ICP-MS for analysis of metal-containing proteins have

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† Presented at the International Symposium on Metallomics 2007, Nagoya, Japan, November 28–December 1, 2007.

been found in the literature,[10–12] such as metal-containing proteins in gibel carp,[10] human brain,[11] and legume seeds.[12] However, it should be mentioned that low chromatographic resolution is usually one of the main disadvantages of SEC.

In spite of the development of hyphenated techniques, the reliable quantification of species in speciation analysis is still difficult. The main obstacle to the accurately quantitative analysis by SEC-ICP-MS is derived from the instabilities of ICP-MS during HPLC process, such as signal shifts and matrix effects. In contrast to other calibration methods, isotope dilution analysis (IDA) is based on the measurement of the isotope ratio and thus the instabilities of the instrument can be overcome.[13,14] When IDA is applied to HPLC-ICP-MS, two spiking modes can be used: the species-specific and species-unspecific mode (also called post-column isotope dilution). The former is used only when the analytical compounds have been known and isotopically enriched species are available. The latter, however, is possible to quantitatively analyze the unknown species of an element.[13,14] Recently, Schaumlöffel *et al.*[15] have successfully developed a method based on pre-column isotope dilution nano-HPLC-ICP-MS for the absolute quantification of sulfur-containing peptides.

In our previous works, a method of enriched stable isotope tracer combined with SEC-ICP-MS was developed to investigate the mercury-containing proteins in maternal and infant rats.[16] Recently, we developed a method of absolute quantification of proteins *via* natural-containing sulfur by a collision cell ICP-MS combined with postcolumn isotope dilution analysis.[17] In this work, a SEC-ICP-MS method with the postcolumn isotope dilution analysis for the determination of mercury-containing protein fractions in the brain cytosol of the maternal and infant rats after exposure to a low-dose of MeHg has been developed. The absolute amounts of sulfur, mercury and some essential elements (zinc and copper) in the protein fractions have been attained after calculation of the chromatographic peaks. The differential distribution of mercury-containing protein fractions in the dam and pup rats were found by means of the established method.

Experimental

Instrumentation

The high performance liquid chromatography system consisted of a Waters metal-free 626 gradient pump, a Rheodyne 9725 injector with a 100 μL loop and a Waters 2487 detector. A TSK-GEL G3000SWxl column (7.8 × 300 mm) and a TSK SWxl guard column (6 × 40 mm) with the mass ranges from 10–500 kDa were used for protein separation.

A Thermo X7 ICP-MS (Thermo Electron Corp., USA) used in the experiment had a hexapole collision cell and Xi interface. High purity oxygen (>99.995%) was used as a reaction gas in order to determine sulfur. The instrumental parameters of ICP-MS and SEC are given in Table 1.

Table 1 The instrumental parameters of ICP-MS and SEC

SEC Chromatographic conditions	
Waters 626 HPLC system	
Analytical column	TSK-GEL G3000SWxl column (7.8 mm × 300 mm)
Mobile phase	0.1 mol L^{-1} Tris-HAC
Flow rate	0.5 mL min^{-1}
Injection volume	100 μL
ICP-MS conditions	
Spray chamber	Quartz impact bead
Nebulizer	Glass concentric
Interface	Xi cones
Chamber temperature	2 °C
Forward power/W	1350
Collision cell gas, oxygen/mL min^{-1}	0.10
Acquired mode	Time resolved analysis
Isotopes monitored	$^{32}S^{16}O$, $^{34}S^{16}O$, ^{63}Cu, ^{65}Cu, ^{66}Zn, ^{67}Zn, ^{198}Hg, ^{202}Hg
Dwell time per points/ms	100
Total analytical time/s	1500

Reagents, proteins and standards

The enriched mercury oxide (^{198}Hg: 39.03%) was purchased from Isotope Production & Distribution, US Department of Energy and the enriched elemental sulfur (^{34}S: 99.90%) was from Isoflex (San Francisco, USA). The enriched ^{65}CuO and ^{67}ZnO were supplied from China Institute of Atomic Energy (Beijing, China). The stock solution of each enriched isotope was prepared by closed-vessel digestion with concentrated nitric acid. The accurate concentration of each enriched isotope was calculated by the reverse isotope dilution. The detail process was described in the previous papers.[17]

The blue dextran, bovine serum albumin (BSA, 66.4 kDa) and the bovine erythrocytes CuZn–superoxide dismutase (SOD, 31.4 kDa) were purchased from Sigma–Aldrich (St. Louis. MO, USA). Rabbit liver metallothionein-II (MT-II, 6.2 kDa) was bought from Lugu Biotechnology Company (Hunan, China). The above proteins were dissolved in the mobile phase for calibrating the SEC column.

Tris (hydroxymethyl) aminomethane was purchased from Roche (Germany); phenylmethylsulfonyl fluoride (PMSF) was bought from Amresco (USA). The analytical grade acetic acid was from Beijing Chemical Reagents Company. Methylmercury chloride was supplied by Riedel-de Haen, Germany, and prepared to 1 mg mL^{-1} as stored solution.

Ultra pure water (18.2 MΩ cm) from a Milli-Q water purification system was used throughout the experiment. The Millex-HV 0.45 μm pore-size filter was purchased from Millipore Corporation (Bedford, USA).

Animal experiment

Healthy adult male and female Sprague–Dawley rats were purchased from the Department of Laboratory Animal Science, Beijing University Health Science Center. After one week adapting feeding, male and female rats were put together at the mating ratio of 1 : 1 in the evening. The gestational Day "0" of female rats was defined as the day when vaginal plug was confirmed. One group of female rats were gastrointestinally administrated by CH_3HgCl (0.3 mg Hg kg^{-1} day^{-1}) from gestational Day 0 to lactation Day 20. All experiments were performed in compliance with the relevant laws and institutional guidelines.

Sample preparation

At the lactation Day 20, all the dam and pup rats were killed. Rat brains were quickly collected and frozen in liquid nitrogen. The

whole brain tissue was weighed and added into 3 times weight of cooled (4 °C), degased Tris buffer (0.1 mol L^{-1}, pH = 7.4) containing 40 μg mL^{-1} PMSF. The suspension was homogenized in a glass homogenizer, centrifuged at 100 000 *g* for 1 h at 4 °C by an ultra speed centrifuge (CP-70 MX, Hitachi, Japan), then the supernatant containing cytosol proteins was obtained.

Postcolumn isotope dilution analysis with SEC-ICP-MS

The brain cytosol (100 μL) was filtered by 0.45 μm membranes (Millex-HV, Millipore) and injected onto the SEC column. The 0.1 mol L^{-1} Tris–HAC buffer (pH = 7.4) was used to separate the proteins in the brain cytosol at the flow rate of 0.5 mL min^{-1}. The enriched spiking mixture solution containing ^{34}S, ^{65}Cu, ^{67}Zn and ^{198}Hg was continuously added by a peristaltic pump into the eluate of the HPLC column *via* a "T"-formed three-way connection, and then the isotope-diluted fractions were introduced into the nebulizer of the ICP-MS. The quantification of sulfur, mercury, zinc and copper in the different protein fractions was performed by postcolumn isotope dilution analysis. In brief, the variation of the isotope ratios, including $^{32}S^{16}O/^{34}S^{16}O$, $^{63}Cu/^{65}Cu$, $^{66}Zn/^{67}Zn$ and $^{198}Hg/^{202}Hg$, was monitored in the total chromatographic process. After corrected by dead time, spectral interference and mass bias of ICP-MS, the isotope ratio chromatogram was converted into the mass flow chart by the isotope ratio equation calculation.[13] The absolute elemental amount in the different protein fractions could be calculated by the integration of the corresponding peaks in the mass flow chart. The details were described in previous literature.[13,16,17]

Results and discussion

Sulfur exists in the amino acids of cysteine and methionine, thus contained in almost all the proteins. Moreover, a sulfur atom in a protein is usually a binding site for metals, especially for mercury. A well known example might be metallothionein (MT). MT is a class of cysteine-rich proteins, which can bind many kinds of metals, such as zinc, copper, cadmium, lead, silver and mercury.[18] The physiological functions of MT include homeostasis of zinc and copper, detoxification of heavy metals (Cd, Pb and Hg), roles as antioxidants and radical scavengers and acute phase.[18] Therefore, simultaneous determination of sulfur, mercury and other essential metals in proteins and further quantification of metal species may be helpful to understand the homeostasis of essential metals and the toxicity of heavy metals.

In order to analyze sulfur by a quadrupole ICP-MS, the oxygen is added as a reactive gas into the hexapole collision cell where S^+ reacted with oxygen to form SO^+; thus, the ratio of $^{32}S^{16}O/^{34}S^{16}O$ representing $^{32}S/^{34}S$ is measured. Under the collisional conditions, Cu, Zn and Hg can be directly determined because of the prohibited oxidation of these elements.

For SEC column calibration, the mixed standard proteins (BSA: 1.8 mg mL^{-1}, SOD: 1.2 mg mL^{-1}, MT-II: 0.6 mg mL^{-1}) are determined *via* sulfur by SEC-ICP-MS. Then the relative molecular mass of protein fractions in the samples can be evaluated by the retention times (t_R). The Tris–HAc buffer is used due to the compatibility with ICP-MS detection. In general, the separation of proteins is performed at the physiological pH range in order to prevent the potential transformations between species. The recoveries of BSA, SOD and MT-II in the SEC-ID-ICP-MS system are 87%, 98% and 93%, respectively,[17] suggesting the mass balance of metalloproteins in the system is satisfactory. The average recovery of mercury-containing proteins in brain cytosol of rats by the similar SEC separation was demonstrated as 97% in our previous papers.[16,19]

Fig. 1 and 2 present the typical chromatograms of the mass flow determined by SEC-ICP-MS in the brain cytosol of the maternal and infant rat, respectively. In the brain cytosol of the maternal rats, five mercury-containing protein fractions are eluted from SEC and monitored by ICP-MS. Their relative molecular weights are about 480, 96, 25, 17 and 10 kDa, respectively. However, the different distribution patterns of mercury-containing protein fractions were found in the brain cytosol between maternal and infant rats. Only one mercury-containing protein fraction, whose molecular weight was about

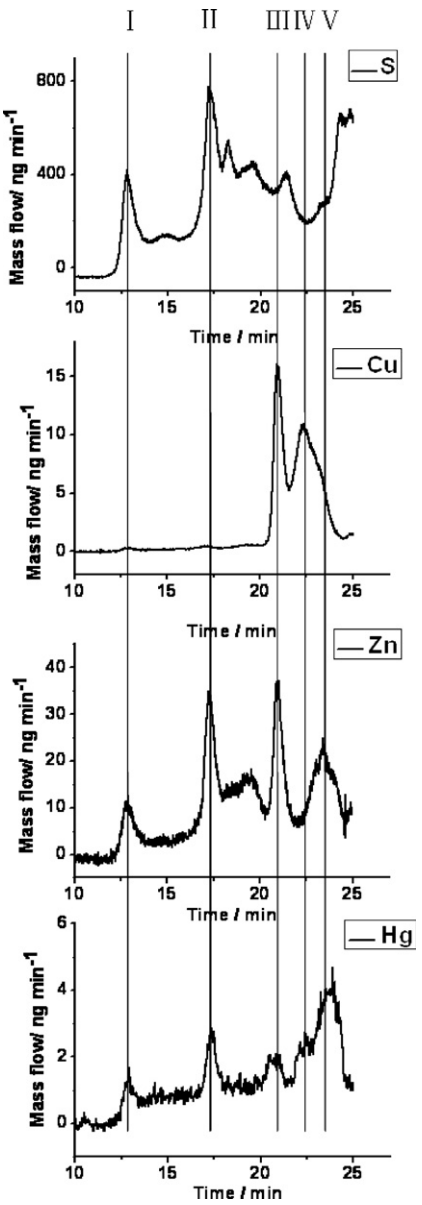

Fig. 1 Chromatograms of the mass flow determined by SEC-ICP-IDMS in the brain cytosol of maternal rats.

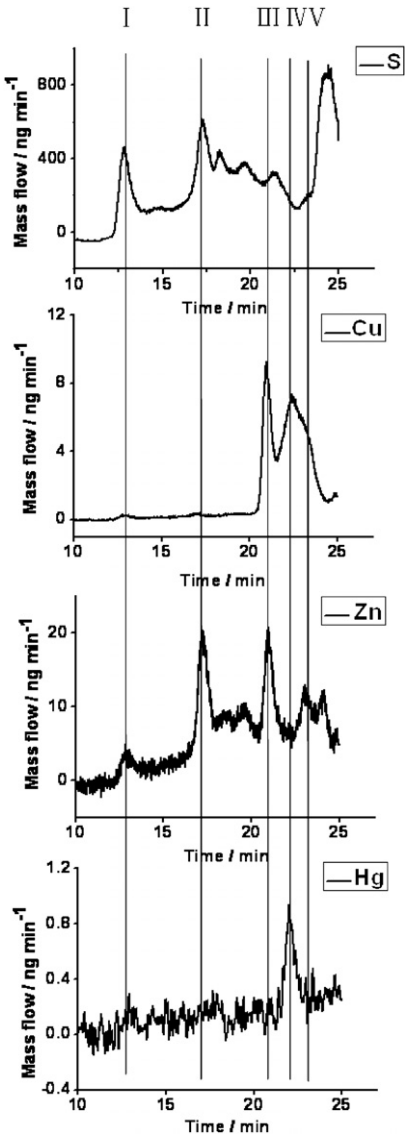

Fig. 2 Chromatograms of the mass flow determined by SEC-ICP-IDMS in the brain cytosol of infant rats.

17 kDa, was found in the brain cytosol of the infant rat. The content of mercury-containing protein in the infant rat was much lower than the maternal rat. We hypothesis that the different patterns of mercury-containing proteins between maternal and infant rats may be attributed to the different ingestion pathway of MeHg, since the maternal rats were administrated mercury *via* the gastrointestinal system, whereas the infant rats were exposed to mercury *via* placenta and the milk of their mother. In the meantime, the Cu- and Zn-containing protein fractions were similar in both the maternal and infant samples. Two copper-containing fractions and four zinc-containing fractions were found in both maternal and infant samples. The quantitative results of sulfur- and metal-containing protein fractions are shown in Table 2. The detection limits for S, Cu, Zn and Hg are 11, 0.1, 1.5 and 0.2 ng, respectively.

The ratios of sulfur to metals in some chromatographic fractions can be obtained if the fractions have been fully separated

Table 2 Quantitative results of metal-containing proteins in brain cytosol of maternal and infant rats by SEC-ICP-IDMS

Peak	I	II	III	IV	V	
Molecular weight /kDa	480	96	25	17	10	Total content[a]
Maternal rat						
Hg content /ng	1.2	3.6	3.0	2.4	6.2	20
Cu content/ng			11	18		31
Zn content/ng	10	35	30		37	140
S content/ng	340					4000
Infant rat						
Hg content/ng				0.8		2.4
Cu content/ng			6.1	13		21
Zn content/ng	4.4	20	19		23	87
S content/ng	420					3800

[a] Total content of an element is obtained by integration of the whole mass flow of the corresponding element.

and the metal and sulfur in the fractions have similar eluted peaks. In this research, the peaks I of sulfur, zinc and mercury are eluted at the same time and their peaks have the same approximate peak width. Therefore, the molar ratios of S/Zn and S/Hg of peak I (480 kDa) in the maternal sample are estimated to be about 34 and 280, respectively; while the molar ratio of S/Zn in the infant sample is about 95. The stoichiometry of a protein in a mixture can be attained after the proteins have been fully isolated by an efficient separation.

Conclusions

A method of SEC-ICP-MS combined with isotope dilution analysis has been established for determination of metalloproteins and sulfur-containing proteins in the brain cytosol of maternal and infant rats after *in utero* exposure to low-doses of MeHg. Five mercury-containing protein fractions were monitored in the maternal sample, whereas only one mercury-containing protein fraction was found in the infant sample. The content of mercury in the infant sample is about 10 times lower than the maternal sample. The different speciation distribution and quantification of mercury in brain cytosol between maternal and infant rats suggest that the hyphenated technique, such as SEC-ICP-MS could be useful for the comparative toxicity study. Further studies should separate proteins by more efficient separation methods (*e.g.* reverse-phase HPLC), and identify all eluted proteins by molecular mass spectrometry. We can expect that the combination of quantification and identification studies of mercury-containing proteins in mothers and their offspring would greatly improve our understanding of differential toxicity between them.

Acknowledgements

This work is supported by the foundations of the National Natural Science Foundation of China (10490181, 20475055 and 10525524), the Chinese Academy of Sciences (KJCX3.SYW.N3), MOST 973 program (2006CB705605) and Key Laboratory of Nuclear Analytical Techniques (K135).

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