ANALYTICAL SCIENCES AUGUST 2000, VOL. 16

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Rapid Communications

Speciation of Trace Elements, Binding and Non-binding with Proteins in Human Blood Serum, by Surfactant-Mediated HPLC with Element-Selective Detection by ICP-MS

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(Received June 14, 2000; Accepted June 30, 2000)

The total concentrations of some trace elements in blood serum are generally used as convenient parameters in medical diagnosis because of their extreme importance for diagnosis of health and disease,[1] as well as their easy accessibility. However, most of the trace elements in blood serum are combined with biological substances to have their own specific functions. Therefore, more information about chemical species of the elements, in addition to the total concentrations,[2,3] is required for the investigations on the biological and physiological functions of trace elements as well as for biomedical diagnosis of the physical conditions. Chemical species of trace elements in blood serum are generally divided into two chemical forms.[2] One is protein-associated species, which play important roles in enzymatic activities, and in transportation and storage of trace elements. The other is protein-non-associated species, which are related to membrane transport or excretion of the elements.

In a previous work,[4] the present authors developed a surfactant-mediated chromatographic separation system which allowed simultaneous multi-component separation, based on the mixed-mode separation properties such as size-exclusion, electrostatic and hydrophobic interactions. In this system, large-molecule species (MW 6500–2000000) were eluted within 3 min of elution time, while small-molecule species (MW< 1000) were eluted after 3.5 min. Thus, the surfactant-mediated separation system could be used for direct injection analysis of the serum samples to separate small-molecule drugs rapidly from proteins in human blood serum without any pretreatment,[4] where the UV absorption detector was employed. Since the surfactant-mediated separation column also allows us to couple with inductively coupled plasma mass spectrometry (ICP-MS) for simultaneous multielement detection, the HPLC/ICP-MS combined system using the surfactant-mediated separation column can be applied to speciation of trace elements in blood serum with direct sample injection. In the present paper, we report a preliminary study on speciation of trace elements binding and non-binding with proteins in human blood serum which was carried out by using the surfactant-mediated HPLC/ICP-MS system.

The HPLC system was composed of a pump (Model LC-10AD; Shimadzu, Kyoto), a sample injector (Model V7, Pharmacia-LKB, Uppsala, Sweden) with a 20 μl sample loop, and a UV absorption detector (Model 870-UV; Jasco, Tokyo). The UV detector was used for detection of UV-absorbing species. In the present experiment, an ODS column (L-column; 4.6 mm i. d. × 250 mm long; Chemical Inspection and Testing Institute, Tokyo) dynamically coated with 3-[(3-cholamido-propyl)dimethylammonium]-1-propanesulfonate (CHAPS), zwitterionic bile acid derivative, was employed as the separation column. The CHAPS-coated ODS column was prepared in a manner similar to that used in the previous paper.[4] The mobile phase of 0.2 mM Tris-HCl buffer solution (pH 7.4) was used at a flow rate of 0.7 ml min^{-1}, into which 0.2 mM CHAPS was added to prevent the degradation of the separation column.

For the on-line element-selective detection, an ICP-MS instrument (Model SPQ8000A, Seiko Instruments, Chiba) was used with direct introduction of the eluent from the column into the nebulizer of ICP-MS *via* a Teflon tubing. The ICP-MS instrument was operated using a time-sequential program, which allowed simultaneous multielement chromatogram measurements.

The human blood serum samples were obtained from healthy volunteers. The sampling and pretreatment procedures of the serum samples were performed in a manner similar to those in the previous paper.[5]

The CHAPS-coated ODS column with diverse physico-chemical properties can separate a variety of solutes, such as inorganic anions, amino acids, aromatic compounds, and

Table 1 Elution times of various analytes obtained by the surfactant-mediated HPLC system with UV-absorption detection[a]

Analyte	Elution time/min	Molecular weight/Da
Blue dextran	2.81	2000000
β-Amylase	2.88	200000
Albumin	2.93	66000
Carbonic anhydrase	2.97	29000
Citric acid	3.57	192.1
Acetic acid	4.15	60
Cl^-	4.41	35.5
Br^-	4.60	79.9
I^-	4.73	126.9
Glycine	4.55	75.1
Leucine	5.10	131.2
Phenylalanine	5.86	165
Tryptophan	10.55	204.2
Caffeine	16.47	194.2

a. Detected at 280 nm.

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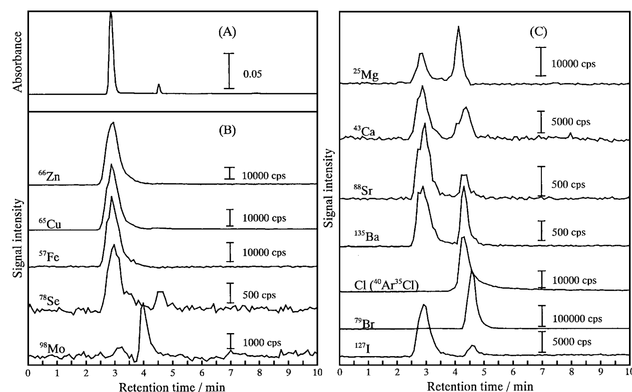

Fig. 1 Element-selective chromatograms of metals and halogens in human serum obtained by the HPLC/ICP-MS combined system. (A) UV absorption-detected chromatograms at 280 nm, (B) ICP-MS detection chromatograms for Zn, Cu, Fe, Se and Mo, (C) ICP-MS detection chromatograms for alkaline earth and halide ions. Column, ODS column dynamically coated with CHAPS; mobile phase, 0.2 mM Tris–HCl buffer + 0.2 mM CHAPS (pH 7.4); flow rate, 0.7 ml min^{-1}; injection volume, 20 μl.

proteins, as reported in our previous paper.[4] In the present experiment, 0.2 mM Tris–HCl buffer solution (pH 7.4) was used as the mobile phase solution, instead of 0.2 mM Na_2HPO_3 solution (pH 7.4).[4] In addition, 0.2 mM CHAPS was added in the mobile phase to prevent the degradation of the separation column. Therefore, the elution behaviors of various representative analytes were re-examined by using the present CHAPS-coated ODS column with the UV absorption detection. The results are shown in Table 1, together with their molecular weights. It can be seen from Table 1 that proteins with the molecular weight larger than 30000 Da provided the elution time shorter than 3.0 min, while small-molecule species such as organic acids, halide ions and amino acids had an elution time longer than 3.5 min. Thus, the elution time ranges shorter than 3.0 min and longer than 3.5 min are hereafter referred to as the large-molecule and small-molecule zones, respectively.

The chromatograms of some trace elements (Zn, Cu, Fe, Se, and Mo) in human blood serum, which were obtained by the ICP-MS detection at each *m*/*z*, are shown in Fig. 1, together with that obtained by the UV absorption detection at 280 nm. In this experiment, the blood serum sample was directly injected into the separation column without any pretreatment. It can be seen in Fig. 1(B) that the peaks of Zn, Cu, and Fe are mainly observed in the large-molecule zone. The elution times of those elements are well consistent with those of serum proteins observed by UV-absorption in Fig. 1(A). Thus, the peaks (around 3.0 min) can be attributed to the elements binding with serum proteins, such as albumin (MW 66000), ceruloplasmin (MW 150000) and transferrin (MW 80000).[6,7] On the contrary, the peak of Mo is observed only in the small-molecule zone (longer than 3.5 min), which may be attributed to the inorganic ion form (MoO_4^{3-}) because MoO_4^{3-} added in serum was observed at the same elution time.

It is seen in Fig. 1(B) that Se provides two peaks in the large-molecule and small-molecule zones. This result suggests the existences of selenoproteins (selenoprotein P and glutathione peroxidase (MW 76000 – 92000)), albumin, and small molecular compounds (probably selenocysteine and selenomethionine). In the previous papers,[8,9] speciation of Se in serum has been mainly focused on selenoproteins as main species,[8,9] and speciation of small molecular species has rarely been carried out. However, selenocysteine and selenomethionine are important species as raw materials in synthesis of selenoproteins.[8,9] It should be noted that significant amounts of small-molecule species of Se exist in blood serum, as is seen in Fig. 1(B). Thus, speciation of small-molecule selenium species may also be necessary to elucidate selenium-cycles in blood serum as well as in various organs.

Furthermore, the element-selective chromatograms of alkaline earth and halide ions are shown in Fig. 1(C). It should be noted here that all alkaline earth elements and iodide provide two peaks in the large- and small-molecule zones. These results indicate that alkaline earth and iodide ions are partly binding with serum proteins. The amounts (%) of protein-binding species of these ions in serum, estimated from the chromatographic peaks in Fig. 1(C), were *ca*. 35% for Mg, *ca*. 70% for Ca, *ca*. 80% for Sr, *ca*. 60% for Ba, and *ca*. 90% for I.

On the other hand, Cl and Br provided the single peaks only in the small-molecule zone. It is noticed that the retention times of Cl, Br and I are slightly different from each other because of their ion-pair formations with alkali and alkaline earth ions, as reported in the previous paper.[10]

In the present surfactant-mediated HPLC/ICP-MS system, valuable information about the bindings of trace elements with serum proteins as well as about the existences of small molecular species could be obtained simultaneously, although separation selectivity for large-molecule species is poor because of the characteristics of the restricted access-type size exclusion of the surfactant-mediated column. However, it should be stressed here that the quantification of the distributions of trace elements existing as large-molecule and small-molecule species often provides important information in the biomedical study on trace elements. Indeed, for example, free iodide ion in human serum was used as the index of iodine-deficiency in a human body.[11] In consequence, it is concluded that the present surfactant-mediated HPLC/ICP-MS system with element-selective detection may be helpful to obtain the information about trace elements binding and non-binding with proteins in blood serum and other biological fluids.

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