

Speciation Analysis of Serum Copper by Ultrafiltration Combined with Graphite Furnace Atomic Absorption Spectrometry

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Ultrafiltration combined with graphite furnace atomic absorption spectrometry (GFAAS) was used to study protein binding and speciation of copper in human serum. Ultrafiltration was carried out using a cell unit with ultrafiltration membranes having a nominal cut-off of 10,000 Dalton. The effects of various experimental factors including the kind and concentration of electrolyte, sample storage, pH, pressure and the preconditioning of the membranes on the speciation analysis of serum copper by ultrafiltration were examined. It was observed that $4.5 \pm 2.3\%$ of the total copper in serum was ultrafiltrable and this value did not seem to be influenced by the total serum elemental concentration, the pH (6.5–10) and the pressure ($\leq 1.5 \text{ kg/cm}^2$). The preconditioning of the ultrafiltration system with 0.1 mol/L calcium nitrate can overcome the adsorption loss of copper effectively, and the addition of tris-HCl solution (pH 7.4) to serum accelerates the ultrafiltration. The present method was proved to be suitable for speciation analysis for its simplicity, rapidity, small sample requirement and easy control. The results obtained with the method are accurate and reliable.

Keywords Ultrafiltration, speciation analysis, serum copper, graphite furnace atomic absorption spectrometry

Introduction

Copper is an essential element of transport and depot protein for oxygen in blood. Its variation is closely related to the appearance of cancer,¹ viral hepatitis, coronary heart disease,² etc. So, speciation analysis of serum copper is of great importance in further understanding various diseases, but it is a difficult task due to the low concentration and complex organic matrix. Tech-

niques used for this purpose should be of simplicity and not disturb the specific metal protein associations. Metal contamination should be easy to control and less than 10% of the initial total metal concentration in the serum sample.³ Also, the analytical technique chosen for the determination of metal content in the fractions must be sensitive enough and the required sample size should be small. In connection with a program of investigating new sampling methods for speciation analysis of biologically active elements, two-phase aqueous extraction has been proposed in this laboratory.^{4,5}

Ultrafiltration is a useful tool for sample preparation and has already been utilized for the separation of high molecular weight (HMW) species and low molecular weight (LMW) species,⁶⁻¹¹ but there seems no report on the speciation analysis of copper by this method, and little information about the real influence of important parameters such as the kind and concentration of electrolyte, the pH and the pressure on the results of the separation. Furthermore, in the ultrafiltration the membrane filters can adsorb various chemical species that are present in the fluids being filtered.^{6,12} The adsorption loss is often serious and sometimes the species concerned can not be obtained. Obviously, the sample treatment technique that can overcome these problems is desirable for speciation analysis. The aim of the present work was to develop such a technique for serum samples by studying various factors affecting the ultrafiltration and demonstrate the speciation analysis of copper in human serum by ultrafiltration-GFAAS.

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Experimental

Apparatus and operating conditions

Ultrafiltration was carried out using a cell unit fitted with ultrafiltration membranes having a nominal cut-off of 10,000 Dalton (Research Center for Eco-Environmental, CAS, Beijing). A Hitachi 180-70 polarized Zeeman atomic absorption spectrophotometer with an electrothermal atomizer was used for copper measurements and its operating conditions were set as previously reported.⁴

Reagents and samples

A 1 mg/mL stock solution of copper was prepared by dissolving cupric sulfate (spectralpure grade) in water, which was diluted with water gradually to give a working solution. Isotonic tris-HCl solution containing 0.05 mol/L tris and 0.15 mol/L NaCl was prepared by dissolving 6 g tris(hydroxymethyl)aminomethane and 8.7 g sodium chloride in 1 liter of deionised water, and the pH of the eluent was adjusted to pH 7.4 by addition of 0.01 mol/L HCl. All other chemicals used were of analytical grade. Human serum samples were collected from Beijing Zhongguancun Hospital.

Procedures

All components of the ultrafiltering system were soaked for 24 h in 10% V/V nitric acid and rinsed with copious amount of water before use. The membranes were washed twice by ultrafiltering with 1 mL of 0.1 mol/L sodium hydroxide solution and then with water until the washings were free from copper (as established by GFAAS).^{8,9} Before each ultrafiltration experiment, the system was preconditioned by ultrafiltering 1 mL of 0.1 mol/L $\text{Ca}(\text{NO}_3)_2$ first to avoid the subsequent adsorption of copper. And then 0.5 mL of serum sample and 0.5 mL of electrolyte solution were placed in the cell and ultrafiltered under nitrogen pressure (1.5 kg/cm²) for 20 min at room temperature. The liquid that passed through the membrane was collected in a tube, then the system was washed twice by ultrafiltering 1 mL of electrolyte solution and each washing was collected in a separate tube. All the three collections were immediately analyzed for copper without any further treatment, and the amount of copper was determined as ultrafiltrable

species (including free ion species and low molecular weight complexes).

The loosely bound copper was studied by adding 0.2 mL of EDTA (0.4 g/L) to serum (1 mL) before ultrafiltration. The copper in the ultrafiltrate was determined as loosely bound species, which included free ion species, low molecular weight complexes and the protein bound species that can be stripped by EDTA (such as albumin copper).

Another portion of 0.5 mL serum sample was directly diluted to 5 mL with water to determine the total amount of copper by GFAAS.

Total amount = loosely bound species (including free ion species) + tightly bound species

Results and discussion

Ultrafiltration has already been used for the separation of HMW and LMW for its simplicity and small sample size requirements.⁹ However, ultrafiltration has two serious problems, one being the long filtration time and the other being contamination and adsorption loss of species. Again, so far there has been little information on the influence of important parameters such as the kind and concentrations of electrolyte, the pH and the pressure on the results of the separation. To overcome the adsorption loss, in this experiment, an attempt was made on preconditioning the system by ultrafiltering an electrolyte solution (H_2O , 0.1 mol/L of NaOH, 0.1 mol/L of NaNO_3 , 0.1 mol/L of $\text{Ca}(\text{NO}_3)_2$, 0.1 mol/L of CaCl_2 , 0.1 mol/L of NH_4SCN or 1 g/L of EDTA). The results showed that NaNO_3 , $\text{Ca}(\text{NO}_3)_2$ and CaCl_2 all can decrease the adsorption loss of copper from 80% to 12%, 6% and 8%, respectively, which may be attributed to the fact that Ca^{2+} or Na^+ can form surface complexes or is adsorbed on the surface of the cell and filters, and thus occupies the active adsorption sites. If the cell was rinsed with NH_4SCN or EDTA, the ultrafiltrable copper was nearly zero, which can be explained by the complexation of copper with NH_4SCN or EDTA staying in the membrane. So $\text{Ca}(\text{NO}_3)_2$ was used to precondition the ultrafiltration system.

To increase the ultrafiltration rate, an experiment was also made to improve the penetrating properties of samples by adding an electrolyte solution to them, *i. e.*, adding 0.5 mL of an electrolyte solution to 0.5 mL of serum to be ultrafiltered. The tested electrolyte

solutions were isotonic tris-HCl, NH_4HCO_3 (0.05 mol/L) and NH_4Cl (0.05 mol/L). The experimental results were shown in Table 1.

Table 1 Effects of different electrolyte solutions on ultrafiltration time and ultrafiltrable copper of serum

Electrolyte solution	Ultrafiltration time (min)	Ultrafiltrable /total amount (%) ^a
serum alone (0.5 mL)	> 40	1.5 ± 0.6
isotonic tris-HCl (pH 7.4)	20	4.5 ± 2.3 ^b
NH_4Cl (pH 6.5)	20	5.0 ± 2.8
NH_4HCO_3 (pH 3.4)	20	12.0 ± 3.0

^a Mean ± standard deviation ($n = 3$); ^b $n = 5$.

It can be seen that all the electrolyte solutions can largely decrease the filtration time. It is perhaps ascribed to that the addition of electrolyte solutions affected the association of molecules and the coagulation of colloids, as well as the electrostatic forces between the molecules to be filtered and the charged layer at the membrane surface caused by concentration polarization. According to the reported results that the percentage of copper combined with ceruloplasmin in healthy normal serum was $92.7 \pm 7.3\%$,³ which is obtained by gel filtration chromatography (GFC), the ultrafiltrable serum copper (free ions and small molecular species) should be about 7.3%. From Table 1, however, it is seen that ultrafiltrable copper can not be obtained quantitatively when serum itself is ultrafiltered. Only with the help of the electrolyte solutions such as isotonic tris-HCl or NH_4Cl can satisfactory results be achieved. In this experiment, isotonic-HCl (pH 7.4) was chosen and the ultrafiltrable copper obtained was approximately $4.5 \pm 2.3\%$ of the total copper. Incorrect and greater amount of the ultrafiltrable copper (12.0%) was gained with NH_4HCO_3 , because this electrolyte (pH 3.5) changed the pH of the serum sample and the dissociation of copper from proteins occurred. As shown in Fig. 1, the optimal pH range for the ultrafiltration of serum copper is pH 6.5–10. Out of this pH range, the protein is destroyed and the protein-bound copper is released, therefore the ultrafiltrable copper increases.

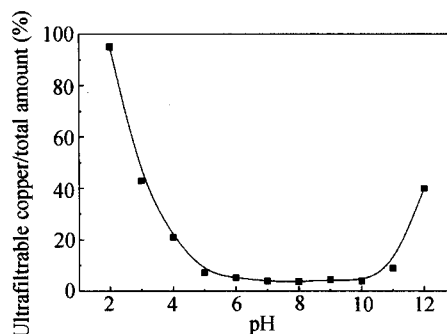


Fig. 1 Effect of pH on the ultrafiltrable serum copper.

Although ultrafiltrable copper varies as a function of serum pH, the effect is not appreciable when pH varies within physiological values, so no special precaution is needed before or during ultrafiltration to make serum pH stable.

We also found that the relative distribution of copper between LMW (ultrafiltrable) and HMW (nonultrafiltrable) serum fractions was constant. This partitioning does not seem to be influenced by the sample aging (at 4°C) and the total serum copper concentration, but if the conditions change greatly, the protein will be denatured and copper can be released to the ultrafiltrable fractions (Table 2). This is agreed with the case of ultrafiltered serum Al.⁹

Table 2 Effects of important parameters on the ultrafiltrable copper of serum

Sample	Ultrafiltrable /total amount (%) [*]
Fresh serum	4.5 ± 2.3
Stored serum (4°C up to 3 months)	6.6 ± 3.2
Fresh serum + 0.1 mL of 0.01 mol/L NaOH	8.0 ± 2.5
Stored serum (25°C for one week)	25.0 ± 4.3

^{*} Mean ± standard deviation ($n = 3$).

Buffle *et al.*¹³ reported that the pressure has no significant effect on ultrafiltration for a given pH and membrane. But our results disagree with him in that when the pressure increased the rate of filtration increased too, and as the pressure increased to a level of 1.8 kg/cm² the rate of filtration decreased considerably due to the strong concentration polarization at high pressure. In this experiment, the pressure of 1.5 kg/cm² was chosen. The analytical results of serum copper and recovery tests in the present study are shown in Table 3.

Table 3 Analytical results of serum copper and recovery tests of free ion state

Sample	Total amount ^a ($\mu\text{g/mL}$)	Ultrafiltrable species ($\mu\text{g/mL}$)	Ultrafiltrable/ Total (%)	Added amount ^b ($\mu\text{g/mL}$)	Recovery (%)
1	1.16	0.05	4.3	0.20	102.0
				0.05	95.6
2	0.78	0.02	2.6	0.20	99.2
				0.05	101.0
3	1.22	0.07	5.7	0.20	96.8
				0.05	103.2

^aTotal amount of serum copper determined directly by GFAAS; ^b Added amount of copper ions.

We tried to separate that portion of copper which loosely binds to protein (such as albumin) by ultrafiltration after a treatment of the serum with EDTA (0.4 g/L—2 g/L), but owing to the low concentration of loosely bound copper in serum, such an attempt did not succeed. However, from Table 2 and Table 3, we can get a result that 94%—97% of serum copper is strongly bound species, which is obtained by subtracting the ultrafiltrable copper (including loosely bound species and free ion species) from the total amount of copper.

Comparison with other methods

There was no paper on the speciation analysis of copper in serum by ultrafiltration in the literature. Besides using starch block electrophoresis,¹⁴ different kinds of proteins were usually separated by chromatography.¹⁰ Gardiner *et al.*³ determined copper and zinc in blood by ETAAS, after the identification of the free ionic species and separation of the proteins being carried out by GFC. Schoeppenthau *et al.*¹⁵ have recently reported the separation of serum for the characterization of metal (including copper and zinc) and non-metal species by using a Nucleogel GFC column and 0.2 mol/L NaCl as the mobile phase. Shum and Houk¹⁶ studied the speciation of various metals (including copper) in human serum by anion exchange and size exclusion chromatography with detection by inductively coupled plasma mass spectrometry (ICP-MS). GFC coupled with AAS or with ICP-MS is very useful for a simultaneous identification and determination of different species in biological samples. However, it is time-consuming and in some cases has a dilution effect for the concerned species.¹⁷ From our results, the two problems mentioned above in ultrafiltration can be effectively solved. Its slow filtration can be overcome by adding a suitable electrolyte solution to the sample;

and the adsorption loss can be greatly decreased by pre-conditioning the system with $\text{Ca}(\text{NO}_3)_2$. By using the present technique, the speciation analysis of serum copper has been successfully demonstrated. It is proved that ultrafiltration is a suitable method for speciation analysis for its simplicity, small sample size requirement and easy control.

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