Anal Bioanal Chem (2006) 386: 180–187
DOI 10.1007/s00216-006-0528-9

SPECIAL ISSUE PAPER

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Determination of ceruloplasmin in human serum by SEC-ICPMS

Received: 6 March 2006 / Revised: 19 April 2006 / Accepted: 4 May 2006 / Published online: 23 June 2006

Abstract This paper describes an analytical method for the determination of ceruloplasmin (Cp) in human serum. The method uses immunoaffinity chromatography and size-exclusion chromatography (SEC) to "purify" the serum sample prior to analysis of ^{63}Cu and ^{65}Cu by inductively-coupled plasma mass spectrometry (ICPMS). By removing the six most abundant proteins from serum with immunoaffinity chromatography and by using SEC to separate Cu bound by Cp from any free Cu that might be present in the serum sample, we demonstrated that SEC-ICPMS can accurately and reproducibly measure Cp in the ERM DA470 reference serum. Cp identification is based on retention time match of the unknown in the serum sample with the Cp external standard and the presence of ^{63}Cu and ^{65}Cu at a ratio of 2.2±0.1. This method was used to analyze a reference serum certified for Cp, 47 serum samples from four different diseases and a set of normal controls. The reference serum and a serum sample from a patient with myocardial infarction, as well as a Cp standard, were also analyzed by electrospray mass spectrometry to confirm the presence of Cp in the SEC fraction known to contain ^{63}Cu.

Keywords Metalloproteins · Ceruloplasmin · Size-exclusion chromatography · Inductively coupled plasma mass spectrometry

Introduction

Ceruloplasmin (Cp) is a blue alpha-2 glycoprotein with a molecular weight of 132 kDa that binds 90–95% of blood plasma copper (Cu) and has 6–7 Cu ions per molecule [1]. The complete amino-acid sequence was reported in 1984 [1] and the various functions of this protein, although not fully understood, include: ferroxidase activity, amine oxidase activity, superoxidase activity [1] and involvement in Cu transport and homeostasis [2]. More recent published data indicate that albumin and transcuprein are also involved in Cu transport; for example, one report indicates 65% of Cu is bound to Cp, 15% to albumin, 15% to transcuprein, and 5% to low molecular weight complexes [3], and in another 71% of Cu bound to Cp, 19% to albumin, 7% to transcuprein, and 2% to aminoacids [4].

Current analytical procedures for the determination of Cp include immunoturbidimetry and nephelometry assay [5], in which Cp is reacted with anti-Cp antibodies to give insoluble aggregates whose absorbance is proportional to the concentration of Cp in the sample [5], radial immunodifussion (RID) test [6], and bichromatic assay [7]. Comparison of measurements done by radial immunodiffusion with those by immunonephelometry showed a significant bias between the two methods that was in part related to the variation in the antisera sources used in the two methods [6]. In the case of the bichromatic assay, the most commonly used procedures are based on the oxidase activity of Cp on diamines such as benzidine, p-phenylene diamine, and N,N-dimethyl-p-phenylene diamine, that require special precautions (i.e., benzidine is a known carcinogen) and purification of the substrate due to light and metal ion-catalyzed oxidation of the substrate [8]. There is no standardardized reference method for Cp and the immunologic methods cross-react with apoceruloplasmin (apoCp) thus giving higher concentrations for Cp [9].

The most important clinical application of the Cp assay is in detecting Wilson disease where the Cp concentration is reduced and the inorganic Cu-ion is increased, because Cu ion fails to reenter the circulatory system as Cp and, thus, gets deposited in the liver, cornea, kidney, bones, and parathyroids. This disease is fatal if the patient is not treated immediately with Cu chelators, and treatment takes usually 3–6 months, during which the disease progresses.

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The relationship between serum Cp concentration and the incidence of myocardial infarction (MI) was studied by Reunanen et al. [10] using serum from 104 patients with MI or stroke and 104 matched controls. The researchers concluded that high Cp concentration in serum was significantly associated with higher incidents of MI but not of stroke. Besides MI, Cp concentration was also high in a variety of neoplastic and inflammatory conditions like carcinomas, leukemia, Hodgkin disease, primary biliary cirrhosis, systemic lupus erythematosus, and rheumatoid arthritis [11].

This paper describes a method for the determination of Cp in human serum at biologically relevant concentrations >0.01 mg/ml. The method uses SEC to separate Cp from other proteins and from inorganic ions, and ICPMS to detect Cu isotopes at mass-to-charge (m/z) ratios of 63 and 65 amu, and to identify Cp from the ^{63}Cu /^{65}Cu signals. To eliminate possible interference from highly abundant proteins, some of which may bind Cu to form protein-Cu complexes, the serum sample is depleted of albumin, IgG, IgA, transferrin, haptoglobin, and anti-trypsin using immunoaffinity chromatography prior to SEC. Quantitation of Cp in the depleted serum is performed by external standard calibration with a Cp standard. Method accuracy and precision were established with a reference serum certified for Cp. Forty-seven human sera from four different diseases and a set of normal controls were analyzed by this method and the Cp concentration was correlated with the total Cu concentration measured by direct ICPMS analysis. To confirm the presence of Cp, two serum samples and the Cp standard were immunodepleted of the six most abundant proteins, fractionated by SEC, and the Cp fraction was collected, concentrated, and subjected to one-dimensional gel electrophoresis to separate Cp from any coeluting proteins. After tryptic digestion, the digests were analyzed by HPLC with electrospray MS.

Experimental

Materials

Two standards of Cp purified from human plasma were used in the study. One was from EMD Biosciences/Calbiochem (La Jolla, CA) in lyophilized form from 133 µl of 50 mM potassium phosphate, pH 6.8, 100 mM potassium chloride, 200 mM ε-amino-n-caproic acid and 5 mM EDTA, with a purity of >95%. The other standard was from Sigma (Saint Louis, MO), in the form of lyophilized powder containing about 30% protein with the balance of sodium chloride and sodium acetate (6.7 mg solid, 11.2 units/mg solid). Water (18.2 mΩ) was purified with a Milli-Q Gradient A10 system (Millipore Corporation, Billerica, MA). The helium collision gas (99.999% purity) was from Scott Specialty Gases (Plumsteadville, PA) and liquid argon (purity 99.997%) was from Air Products (Allentown, PA).

Serum samples from patients with four different diseases including myocardial infarction (MI), rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and pulmonary embolism (PE) and normal controls (NC) were obtained from Stanford University (Stanford, CA). All samples were kept frozen at −20 °C until analysis.

ERM DA470 is a human serum certified for 15 proteins including Cp [12, 13] and was purchased from RTC (Laramie, WY). Seronorm Level I and Level II sera, certified for trace elements including Cu, were purchased from Accurate Chemical & Scientific Corporation (Westbury, NY). All certified samples were in lyophilized form and were reconstituted (per manufacturer instructions) immediately prior to use.

Immunoaffinity chromatography

High-abundant protein removal from human serum was performed on a 4.6×100 mm immunoaffinity column (Agilent Technologies). The recommended column capacity is 40 µl of non-diluted human serum (capacity is defined as the amount of original serum that can be loaded onto the column such that 99% of the targeted high-abundant proteins are removed for at least 200 injections on a particular column). After a fivefold dilution of serum sample with buffer A and filtration through a 0.22 µm spin filter (Agilent Technologies), 150 µl of the diluted sample was injected onto the 4.6×100 mm column in 100% Buffer A at a flow rate of 0.5 ml/min for 10.0 min. After collection of the flow-through fraction (2 ml), the column was washed and the bound proteins were eluted with 100% Buffer B at a flow rate of 1.0 ml/min (volume of bound protein fraction 3 ml). The immunoaffinity column was then regenerated by equilibrating it with Buffer A for 13 min bringing the total run cycle to 30.0 min. Fraction collection of flow-through proteins was time-controlled and corresponded to the UV 280 nm absorbance of the eluting proteins. The flow-through fraction (fraction 1) was collected into a 10-ml round-bottom tube, and kept at 4 °C using the thermostated fraction collector. Bound proteins (fraction 2) were also collected in a 10 ml round- bottom tube. Buffer A (Agilent Technologies) is a phosphate buffer (pH 7.4) and buffer B (Agilent Technologies) is a concentrated urea buffer in water, pH 2.25.

1-D gel electrophoresis and in-gel digestion

The SEC fractions in which Cp was expected to elute were concentrated to 20 µl using spin concentrators and were diluted twofold with reducing agent (Bio-Rad Laboratories, Hercules, CA) prior to electrophoresis on a 4–20% acrylamide precast-gel (Pierce Biotechnology, Rockford, IL) at 60 V for 1 h. Gels were then stained with gel blue (Pierce Biotechnology) for 18 h. The stained protein bands were cut with a clean razor blade and were immersed in 10 mM ammonium bicarbonate containing 10 mM dithiothreitol and 100 mM iodoacetamide. The gel pieces were treated with 50% acetonitrile in 10 mM ammonium bicarbonate to remove the stain and then digested overnight

at 37 °C with 0.1 mg trypsin in 10 mM ammonium acetate containing 10% acetonitrile.

Instrumentation

An Agilent 1100 LC system (Agilent Technologies) equipped with a binary pump, degasser, autosampler (300 μl loop) with thermostat, diode array detector with 6-mm flow cell, and a thermostated fraction collector was used for the immunoaffinity work. Protein separation was achieved on a silica TSKGel column SW3000 (30×4.6 mm id×4 μm particles ×250 nm pore size) from Tosoh Bioscience (Montgomerryville, PA). All SEC analyses were performed on another Agilent Technologies 1100 Series High Performance Liquid Chromatography system equipped with a binary pump, degasser, autosampler (100 μl loop) and diode array detector (215 and 280 nm). 0.1 M Tris (pH 7) was used as mobile phase at a flow rate of 0.3 ml/min. The exit from the diode array detector was connected to a Micromist nebulizer with PEEK tubing (60 cm in length) and the nebulizer was fitted to a double-pass Scott spray chamber which was kept at 2 °C. ^{63}Cu and ^{65}Cu scan was performed on an Agilent 7500ce ICPMS system with a quadrupole mass analyzer and an Octapole Reaction System for matrix-based interference removal. ICP conditions: outer gas (Ar) flow rate 15 l/min; carrier gas (Ar) flow rate 0.8 l/min; forward power 1.55 kW, sampling depth 8 mm. He was used as collision gas because serum samples were also analyzed for other elements, including Fe, and ^{40}Ar^{16}O was being removed by He in the octapole reaction cell. The flow rate was set at 3.5 ml/min which seemed to be the optimum value to meet the instrument tuning criteria on a daily basis. The voltages for ion lenses were: 0 V for extraction lens 1 and −185 V for extraction lens 2. The octapole bias was set at −18 V and the quadrupole bias was set at −16 V.

MS identification of the tryptic digests was performed by HPLC using a Zorbax 300SB-C18 nanocolumn 75 μm×150 mm, 3.5 μm particles (Agilent Technologies). The peptides were eluted at 300 nl/min with a 60-min linear gradient from 0 to 95% acetonitrile containing 0.1% formic acid in water. The effluent from the HPLC column was electrosprayed into an ion trap MS (Model XCT Plus, Agilent Technologies). The MS parameters were as follows: m/z range 50–2,200 Da; skimmer 40 V; cap exit −200 V; multiplier 830 V; nitrogen as curtain gas and collision gas; source temperature 300 °C. The MS detection was done in the positive mode using repetitively full MS scan (for 0.5 min) followed by collision induced dissociation (CID) of four most intense ions selected from the MS scan. The MS data was compared to simulated proteolysis and CID spectra of proteins in the SwissProt database using Spectrum Mill software (Agilent Technologies).

Results and discussion

Determination of Cp by SEC-ICPMS

Use of SEC coupled with ICPMS to detect the presence of metals bound to biomolecules has been reviewed by Makarov and Szpunar [14] and very recently Palacios et al. [15] reported on the fractionation of Se-containing proteins in serum by multiaffinity liquid chromatography before SEC-ICPMS. We used the information presented in Ref. [14] as a starting point for the selection of the SEC column and the mobile phase buffer. Tris buffer was chosen because it seems to be widely used with ICPMS detection. Addition of NaCl to maximize the molecular sieving and minimize any hydrophobic interactions between the proteins and the column packing material did not improve the chromatographic separation of our test proteins, and caused plugging of the nebulizer; therefore, it was not considered. A mixture of proteins (identified as the "ladder" in this study) was used to check the SEC column performance or a regular basis. With 0.1 M Tris at pH 7, thyroglobulin (669 kDa) elutes in the exclusion volume of the SEC column (TSK Gel SW3000 is suited for separation of proteins with MW of 70 kDa–300 kDa). IgG elutes at 8.2 min and ovalbumin at 9.4 min. Cp elutes between albumin and IgG at 8.4 min. Although a 1-min difference in retention time between Cp and albumin may seem sufficient to resolve these two proteins when present in a mixture at equal concentrations, this is no longer the case when albumin and IgG are present at 10–40 times higher concentrations in human serum than Cp. Therefore, albumin and IgG as well as transferrin, IgA, haptoglobin and anti-trypsin (known to be present at mg/ml concentrations) had to be removed from the human serum prior to SEC. Despite the fact that these six abundant proteins were removed, other proteins are present in the depleted serum that make the identification of Cp impossible by UV detection (see Fig. 1), but very easy to identify by ICPMS signal when monitoring ^{63}Cu and ^{65}Cu. Under these experimental conditions the Cu bound in Cp can be easily distinguished from the free Cu ions since the retention time of free Cu ion is 13.0 min (or scans 740–840 in the ICPMS chromatogram).

The ladder was analyzed daily at the beginning of the day to assess column performance in terms of peak shape and resolution. If peaks tail, there is an indication that the column needs to be regenerated per manufacturer's instructions.

Figure 1 also shows SEC-ICPMS chromatograms for the two standards of Cp that were used to in this study. Sigma standard was found to be more stable than the EMD-Calbiochem standard; however, the purity of the Sigma Cp is only reported as approximate by the supplier, so we did not pursue a multipoint calibration for the Sigma standard. The latter Cp standard is pure but it is losing Cu as evident in the Cu scans by ICPMS and forms a compound that has a retention time of about 7.3 min. The identity of the Cp standard from EMD Calbiochem was confirmed by ESI-MS but the peak at 7.3 min has not been identified yet.

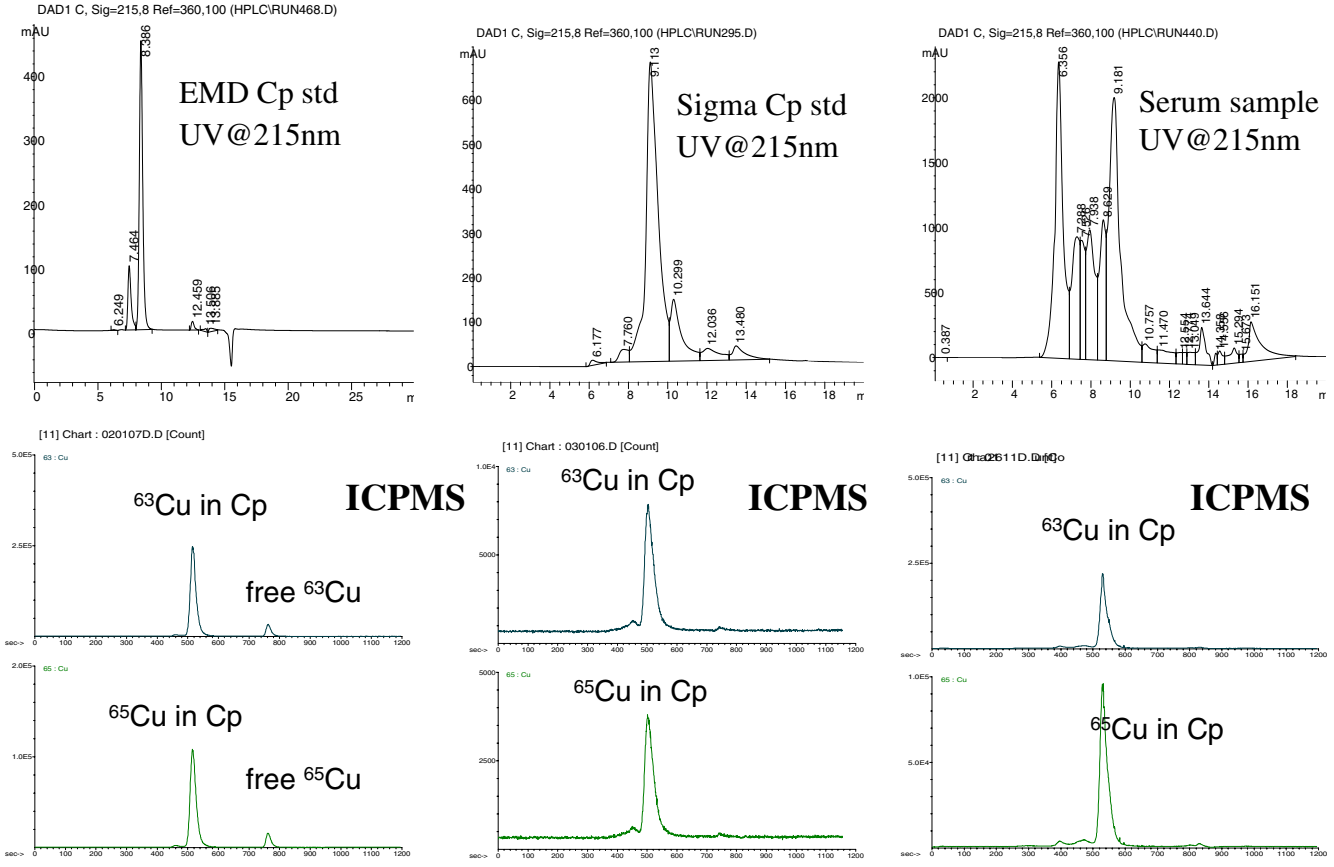

Fig. 1 SEC-ICPMS chromatograms for Cp standards and serum sample; X axis: retention time in min for SEC and sec for ICPMS; Y axis: absorbance at 215 nm for UV chromatograms and total counts for ICPMS chromatograms

Physico–chemical changes in Cp, usually arising from partial loss of Cu, were attributed to aging of solutions, exposure to certain buffers, and ion exchange chromatography [16]. It is well known that a small amount of colorless protein, apoCp, in serum represents 10–20% of the total immunologically reactive protein and that in sera from patients with Wilson disease, apoCp is present at levels near those found in sera from normal subjects [16].

The peak area ratio of the peak at 7.3 min to the Cp peak was plotted in Fig. 2 at three different time events (day 1 when the standards were prepared fresh from the lyophilized material; day 2,a day later; and day 8–7 days later since the preparation of the calibration standards). At day 1, the peak area ratio was about the same (0.22–0.27) for the five standards at concentrations of 0.05, 0.1. 0.25, 0.5, and 1 mg/ml, and kept increasing as a function of time (at day 2 the ratios were 0.28 to 0.42 and at day 8 were 0.47 to 0.86), indicating that Cp is unstable. Five separate injections of the 1 mg/ml Cp standard gave a coefficient of variation (CV) of 5.3%, indicating the injection is reproducible and

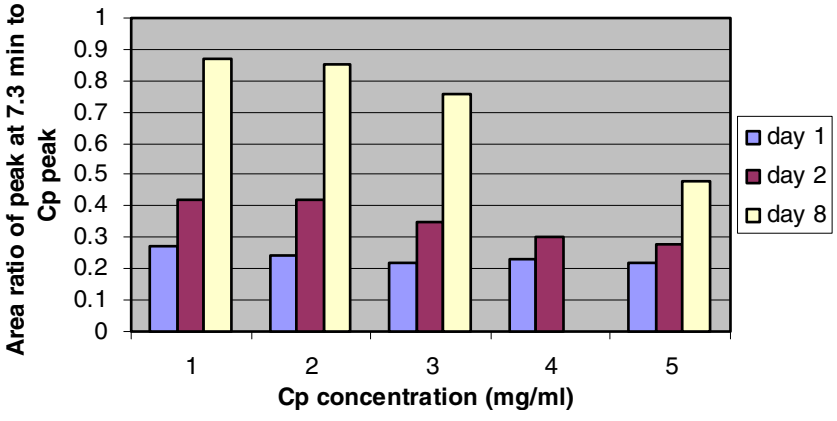

Fig. 2 Area ratio of peak at 7.3 min to Cp peak as a function of Cp concentration and time (X axis: 1 is for Cp at 0. 05 mg/ml, 2 for 0. 1 mg/ml, 3 for 0. 25 mg/ml, 4 for 0. 5 mg/ml, and 5 for 1 mg/ml)

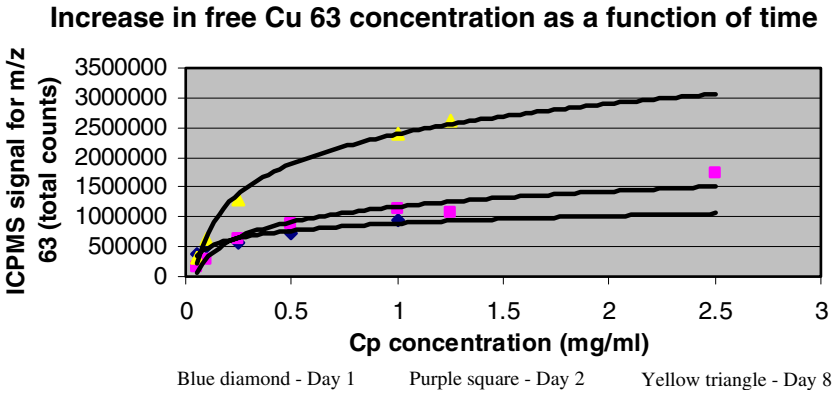

Fig. 3 Increase in free Cu ion concentration as a function of Cp concentration and time

what was observed was not an artifact during the injection of the calibration standards into the HPLC system.

The increase in the concentration of the peak at 7.3 min seems to correlate with the increase in the concentration of free Cu ions in solution (Fig. 3), which substantiates the fact that Cp is losing Cu. The decrease in the concentration of Cp is consistent across various concentrations as shown in the calibration plots obtained at three time intervals (Fig. 4). The loss of Cu from the Cp molecule translates into lower slopes of the calibration curves (for the EMD Calbiochem standard on days 1, 2, and 8; the slopes are 7,000,000, 6,000,000, and 5,000,000 respectively) and therefore calibration standards need to be prepared fresh, on a daily basis, during sample analysis.

Method performance

The performance of this assay was established with the reference human serum ERM DA470 that is certified for Cp at 0.205 mg/ml. This serum was reconstituted with high purity water and analyzed in triplicate. Since the instructions provided with the serum state that the reconstituted serum should be equilibrated overnight at room temperature, we chose to carry out two sets of triplicate measurements. One set was processed immediately on the immunoaffinity column and the other set was processed after overnight equilibration at room temperature (22 °C). The results are summarized in Table 1. The agreement between the concentration of Cp in the certified serum and the freshly reconstituted serum is excellent. The concentration of Cp in the serum kept at room temperature for overnight is about 20% lower. The coefficient of variation for the three replicate measurements of the freshly reconstituted serum and the serum equilibrated overnight are 5.4 and 9.9%, respectively. Given the fact that Cp is labile, we recommend that samples be analyzed immediately upon collection.

A Cp standard at 5 mg/ml was processed through the immunoaffinity column to verify that Cp is not retained by the column. For a 150 μg of Cp loaded to the immunoaffinity column (30 μl of a 5 mg/ml Cp standard diluted to 150 μl with buffer A,) less than 0.01% (or 13 ng) was found in the bound fraction that was eluted with buffer B

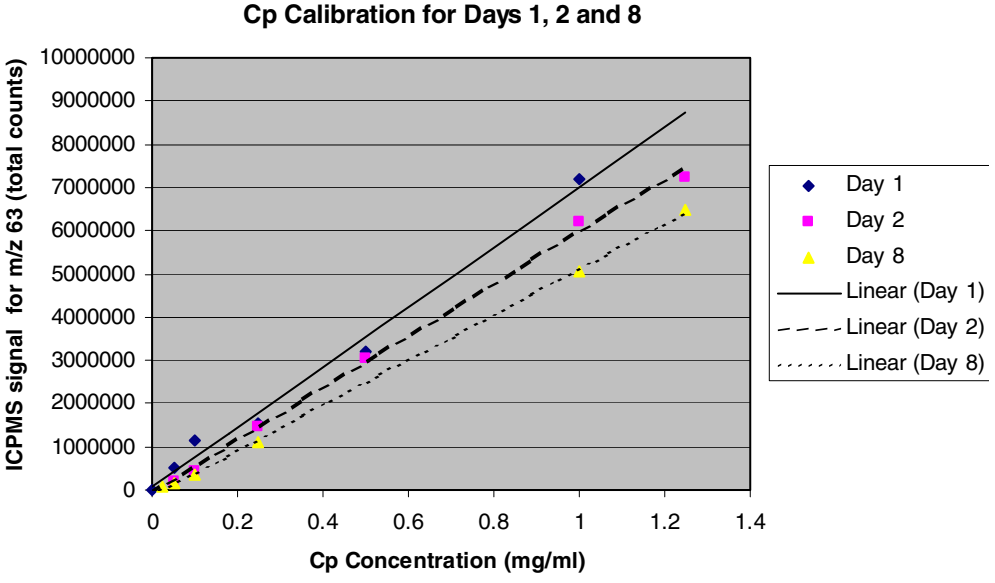

Fig. 4 Cp calibration curves for days 1, 2 and 8

Table 1 Concentration of Cp in the ERM DA 470 reference serum

	Certified value (mg/ml)	Conc measured in this study (mg/ml)	$^{63}Cu/^{65}Cu$
ERM DA 470 reference serum (freshly reconstituted)	0.205 (0.011)[a]	0.208 (5.4%)[b]	2.1 (3.6%)[b]
ERM DA 470 reference serum (after overnight equilibration at room temperature)	0.205 (0.011)[a]	0.168 (9.9%)[b]	2.2 (2.8%)[b]

[a]Uncertainty (mg/ml) is defined as half-width of the 95% confidence interval of the mean value (K factors were chosen according to the *t*-distribution depending on the number of labs)
[b]Average of 3 determinations; value given in parentheses is the coefficient of variation

and analyzed directly by ICPMS. This data indicate that the recovery of Cp by our method is 100%.

Method performance data are included in Table 2. The method detection limit was established from the instrument detection limit and applies only to sample injection volumes of 5 μl; larger injection volumes would allow a lower method detection limit, but such experiments were not pursued here. The method dynamic range is given as 0.01–5 mg/ml since this is the range of concentrations that were tested here. Typical concentrations of Cp in human sera from normal subjects are in the 0.2–0.5-mg/ml range [17]; therefore, a 30-μl volume of the original serum is processed through the analytical method, and the final volume of the depleted serum that is recovered from the spin concentrator is adjusted to 30 μl. The overall CV for method reproducibility is <10% and it is shown in Table 2 for various steps in the analysis. Finally, the identification of Cp is based on retention time match of the unknown peak in the sample to the Cp standard and the ratio of $^{63}Cu/^{65}Cu$. The average retention time for eight consecutive injections of the Cp standards is 8.389 min with a CV of 0.059%. The relative abundance of the ^{63}Cu is 69.17% and ^{65}Cu is 30.83%, thus the theoretical ratio for $^{63}Cu/^{65}Cu$ is 2.24; based on our experimental data we set the acceptance limits for $^{63}Cu/^{65}Cu$ to 2.2±0.1.

Table 2 Cp determination by SEC-ICPMS method performance[a]

Method indicator	Value
Detection limit	0.01 mg/ml
Dynamic range	0.01–5.0 mg/ml (tested only until 5 mg/ml)
Reproducibility	CV for immunodepletion: 0.07% to 2.2%
	CV for injection into HPLC: 5.3% (Cp standard at 1 mg/ml)
	Overall CV:<10%
Accuracy	101% (ERM DA 470)
Cp identification	From retention time match of the unknown peak in the sample to the Cp standard and the presence of ^{63}Cu and ^{65}Cu at a ratio of 2.2±0.1

[a]This method takes approximately 95 min/sample from start to finish (15 min dilution and filtration, 30 min immunoaffinity chromatography, 20–30 min concentration, and 20 min SEC-ICPMS analysis)

Cp measurements in sera from different diseases

Figure 5 shows the distribution of Cp concentration across four diseases, including MI, PE, RA, SLE and normal control sera (total of 47 samples). The Cp concentrations reported for normal subjects range from 0.2 to 0.5 mg/ml [17] and are shown in the dashed area of Fig. 5. Although this is a very limited sample set, the MI samples exhibited some of the highest concentrations of Cp. This seems to be in agreement with published data by Reunanen et al. [10] using serum from 104 patients with MI or stroke and 104 matched controls, who concluded that high Cp concentration in serum was significantly associated with higher incidents of MI but not of stroke.

To find out if there is a correlation between the Cp concentration in the depleted serum and the total Cu concentration in the original serum, we analyzed some of sera, for which we had enough sample, for total Cu by spraying the diluted sample directly into the ICPMS system. We used a 150-fold dilution of the serum with 1% nitric acid. To verify the accuracy of the total Cu measurement by direct ICPMS, we checked the procedure with two reference sera, Seronorm I and Seronorm II, and found that total Cu concentration for Seronorm I is 1,043 ng/ml (certified value is 997–1123 ng/ml) and for Seronorm II is 2,480 ng/ml (certified value is 2,400–2,800 ng/ml). Furthermore, method blanks (i.e., 1% nitric acid) and calibration standards were analyzed in between samples to ensure that there was no cross-contamination between samples and that system calibration was within acceptable limits. The correlation between the total Cu in the original serum and the Cp concentration in the depleted serum for 23 samples is high as indicated by the R^2 of 0.8, which would confirm literature reports that most Cu in serum is bound by Cp [1]. Since in our study all samples have been depleted of albumin, transferrin, IgG, IgA, haptoglobin and anti-trypsin, we are certain that what we measured was Cu bound only to Cp and not to transferrin or albumin, both of which are known to bind Cu to some extent [3, 4].

To establish the number of Cu atoms in the Cp molecule, we used the concentration of Cp that was determined in the ERM DA470 reference serum by SEC-ICPMS and the total Cu measurement of that serum sample, which we determined in duplicate by direct ICPMS and found to be 618 and 661 ng/ml. If Cp would contain 6 Cu atoms per molecule, then a Cp concentration of 0.208 mg/ml

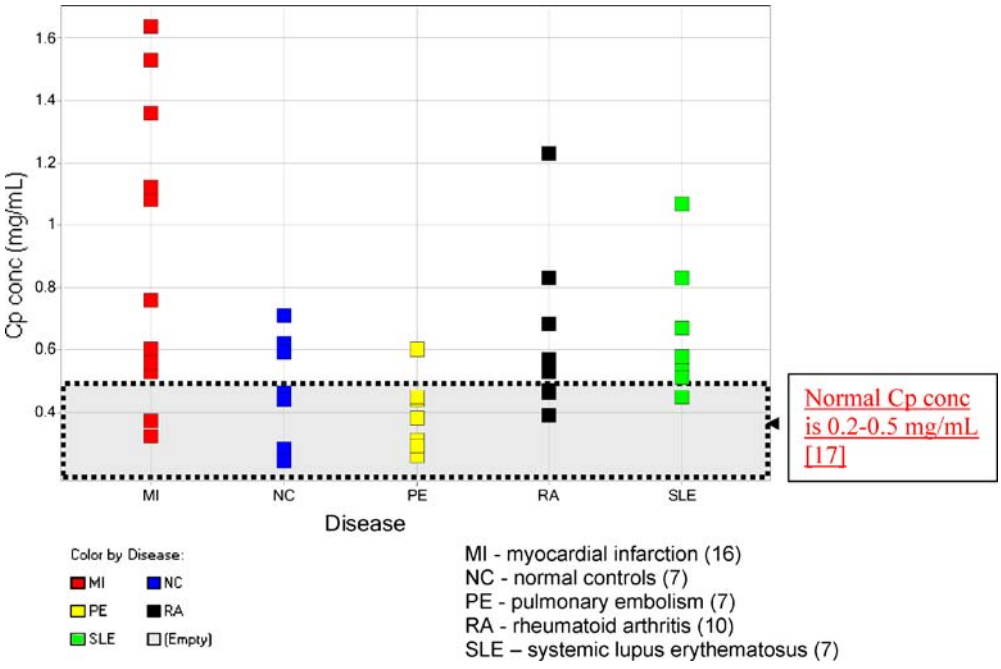

Fig. 5 Cp concentration in human sera from four diseases and normal controls

(measured in this study) would correspond to a total Cu concentration of 596 ng/ml; if Cp would contain 7 Cu atoms per molecule, then the total Cu concentration would be 695 ng/ml. Since the Cu concentration was 618–661 ng/ml, we can then conclude that Cp in the ERM DA470 reference serum contains between 6–7 atoms per molecule, consistent with the published value [1].

The ESI-MS data for the Cp standard and the two serum samples are presented in Table 3 as the protein score, number of peptides found, and the % sequence coverage. The Cp standard was processed by one-dimensional gel electrophoresis only, by SEC and one-dimensional gel electrophoresis, and through all three steps. In all cases involving the Cp standard, the database searches confirm the identity of Cp with different degrees of sequence coverage. A protein score above 20 indicates identity or extended homology with a p value <0.05. The data for the two serum samples (processed through immunoaffinity chromatography, SEC) and from which we collected the peak corresponding to the Cu protein, also resulted in the correct identification of Cp. When the two serum samples were not depleted of the high abundant proteins and were not fractionated by SEC, the identification of Cp separated from the serum only by one-dimensional gel electrophoresis was not possible in the ERM-DA470 serum, but it was successful in the MI-1 serum.

Conclusions

Interest in proteins with Cu or Cu binding sites has increased in the last several years and standardized analytical methods are needed to distinguish between Cp and apoCp. The method described here uses immunoaffinity chromatography and SEC to "purify" the sample prior to analysis of Cp by ICPMS. By removing the six most abundant proteins from serum with immunoaffinity chromatography and by using SEC to separate Cu bound by Cp from any free Cu in the serum sample, we demonstrated that SEC-ICPMS can accurately and reproducibly measure Cp in the ERM DA470 reference serum. The SEC-ICPMS method was used to analyze 47 serum samples from four diseases and a set of normal controls for Cp. The presence of Cp in two of the serum samples, depleted of the six most abundant proteins, was verified by SEC fraction collection, followed by one-dimensional gel

Table 3 ESI-MS data for Cp standard, ERM-DA470 reference serum and MI-1 serum sample

Sample name	Immunoaffinity	SEC	1D GE	Score	Peptides found	% coverage	Identification
Cp std				393	26	28	Cp
Cp std		x	x	45	3	4	Cp
Cp std	x	x	x	162	11	12	Cp
ERM DA470			x	469	28	55	Albumin
ERM DA470	x	x	x	43	3	4	Cp
MI-1			x	45	3	4	Cp
MI-1	x	x	x	85	6	8	Cp

electrophoresis, removal of the protein band of about 130 kDa, in-gel digestion with trypsin, and analysis of the tryptic digest by electrospray MS. ICPMS coupled with different forms of chromatography appears to be a desirable analytical tool for determination of metalloproteins because of its selectivity, especially when dealing with such complex matrices as the human serum.

Acknowledgements The authors thank Toshiaki Matsuda of Agilent Tokyo Analytical Division, Tokyo, Japan, for making available an ICPMS system for performing this research; Alex Apffel of Agilent Labs, Palo Alto, CA for making available an HPLC system with fraction collection and for assistance with the immunoaffinity chromatography process; and to Darlene Solomon and Brian Peter, also of Agilent Labs, for reviewing the manuscript prior to publication.

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