Determination of Ceruloplasmin in Human Serum by Immunoaffinity Chromatography and Size-Exclusion Chromatography-ICP-MS

Application Note

Clinical Research

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Abstract

A new method is described for the analysis of copper (Cu) containing ceruloplasmin (Cp) in human serum. The method uses immunoaffinity plus size-exclusion chromatography (SEC) to separate Cp from other proteins and from inorganic ions, with inductively coupled plasma mass spectrometry (ICP-MS) detection of ^{63}Cu and ^{65}Cu to identify the protein. A reference serum certified for Cp was used to establish method accuracy and precision. The method was applied to 47 human sera samples with one of four different diseases plus a set of normal controls. The Cp concentration was correlated with the total Cu concentration measured by direct ICP-MS analysis. The results demonstrated that SEC-ICP-MS can accurately and reproducibly measure Cp in human serum that is depleted of six most abundant proteins.

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Introduction

Ceruloplasmin (Cp) is a blue alpha-2 glycoprotein with a molecular weight of 132 kDa that binds 90 to 95% of blood plasma copper (Cu) and has 6 to 7 Cu atoms 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]. Data published more recently indicates that albumin and transcuprein are also involved in Cu transport; for example, one report indicates that 65% of Cu is bound to Cp, 15% to albumin, 15% to transcuprein and 5% to low molecular weight complexes [3], and in another report that 71% of Cu is bound to Cp, 19% to albumin, 7% to transcuprein, and 2% to amino acids [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 immunodiffusion (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, which require special precautions (that is, benzidine is a known carcinogen) and purification of the substrate due to light and metal ion-catalyzed oxidation of the substrate [8].

At present there is no standardized reference method for Cp, and the immunologic methods cross-react with apoceruloplasmin (apoCp), which can bias data and deliver higher than expected concentrations for the target protein [9].

This application 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 ICP-MS to detect Cu isotopes at mass-to-charge (*m/z*) ratios of 63 and 65 amu and to identify Cp using 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 antitrypsin using immunoaffinity chromatography prior to SEC. Quantification of the protein in the depleted serum is performed using external calibration with a Cp standard. Method accuracy and precision were established with a reference serum certified for Cp. Forty-seven human sera samples with one of 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 ICP-MS analysis.

Experimental

Materials

Two standards of Cp purified from human plasma were used in the study. One was from EMD Bio-sciences/Calbiochem (La Jolla, CA) in lyophilized form from 133 µL of 50 mM potassium phosphate, pH 6.8, 100 mM potassium chloride, 200 mM e-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) used in the collision/reaction cell (CRC) of the ICP-MS was from Scott Specialty Gases (Plumsteadville, PA) and the liquid argon (purity 99.997%) was from Air Products (Allentown, PA).

Serum samples with one of 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 [10, 11], 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 an Agilent 4.6 mm × 100 mm immunoaffinity column. The recommended column capacity is 40 µL of nondiluted 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 5-fold dilution of serum sample with Buffer A (Agilent) and filtration through a 0.22 µm spin filter (Agilent), 150 µL of the diluted sample was injected onto the 4.6 mm × 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 (Agilent) 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 in a 10 mL round-bottom tube and kept at 4 °C using the thermostatted fraction collector. Bound proteins (Fraction 2) were also collected in a 10 mL round-bottom tube. Buffer A is a phosphate buffer (pH 7.4) and Buffer B is a concentrated urea buffer in water, pH 2.25.

Instrumentation

An Agilent 1100 LC system equipped with a binary pump, degasser, auto sampler (300 µL loop) with thermostat, diode array detector with 6 mm flow cell, and a thermostatted fraction collector was used for the immune affinity work. Protein separation was achieved on a silica TSKGel column SW3000 (30 cm × 4.6 mm id × 4 µm particles × 250 nm pore size) from Tosoh Bioscience (Montgomeryville, PA). All SEC analyses were performed on another Agilent 1100 Series High-Performance Liquid Chromatography (HPLC) system equipped with a binary pump, degasser, autosampler (100 µL loop), and diode array detector (215 nm and 280 nm). The mobile phase used was 0.1 M Tris (pH 7) 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 length), and the nebulizer was fitted to a double-pass Scott spray chamber that was kept at 2 °C. ^{63}Cu and ^{65}Cu detection was performed on an Agilent 7500ce ICP-MS system with a quadrupole mass analyzer and an octopole reaction system (ORS) for matrix-based interference removal. Operating conditions are given in Table 1.

Table 1. 7500ce ICP-MS Operating Conditions.

Parameter	Setting
RF power (W)	1550
Plasma gas flow rate (L/min)	15
Carrier gas (Ar) flow (L/min)	0.8
Make-up gas (Ar) flow (L/min)	0.15
Sampling depth (mm)	8
He gas flow (mL/min)	3.5
Extraction lens 1 (V)	0
Extraction lens 2 (V)	–185
Octopole bias (V)	–18
Quadrupole bias (V)	–16

High levels of Na in the sample can cause the creation of a $^{40}Ar^{23}Na$ polyatomic species that overlaps ^{63}Cu. Similarly, ^{31}P-based molecular species ($^{31}P^{16}O^{16}O$; $^{31}P^{18}O$: ^{16}O) can overlap the Cu isotopes. The ORS was used to eliminate these interfering species and improve signal to noise. Helium was used as the cell gas to pressurize the ORS.

Because He is an inert gas, it removes polyatomic interferences irrespective of their composition or source (matrix or plasma) under a single set of conditions. This universal applicability makes it suitable for the analysis of any isotope in any sample matrix.

Results and Discussion

Determination of Cp by SEC-ICP-MS

Use of SEC coupled with ICP-MS to detect the presence of metals bound to biomolecules has been reviewed by Makarov and Szpunar [12]. More recently, Palacios, et al, [13] reported on the fractionation of Se-containing proteins in serum by multiaffinity liquid chromatography before SEC-ICP-MS. The information presented in Reference 12 was used as a starting point for the selection of the SEC column and mobile phase buffer.

Tris buffer was chosen because it seems to be widely used with ICP-MS detection. A mixture of proteins (identified as the "ladder" in this study) was used to check the SEC column performance regularly. 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 to 300 kDa). IgG elutes at 8.2 minutes and ovalbumin at 9.4 minutes. Cp elutes between albumin and IgG at 8.4 minutes. Although a 1 minute 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 to 40 times higher concentrations in human serum. 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 present in the depleted serum make identification of Cp impossible by UV detection (see Figure 1), but very easy by ICP-MS using the ^{63}Cu and ^{65}Cu isotopes. 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 ions is 13.0 minutes (or scans 740 to 840 in the ICP-MS chromatogram). Figure 1 illustrates SEC-ICP-MS chromatograms for the two standards of Cp that were used in this study. The Sigma standard was found to be more stable than the one from EMD Biosciences/Cal-biochem; however, the purity of the Sigma Cp is only reported as approximate

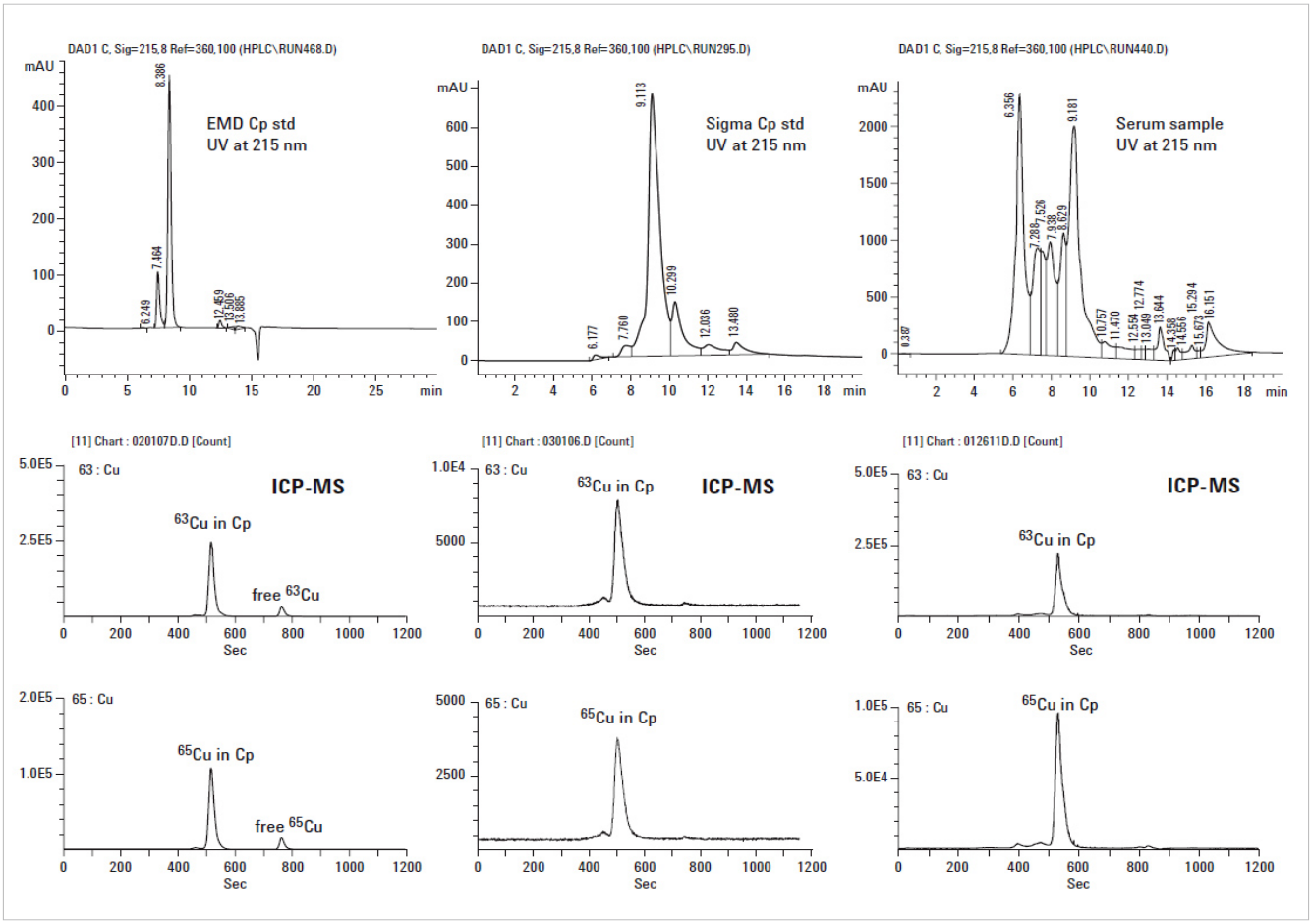

Figure 1. SEC-ICP-MS chromatograms for Cp standards and serum sample. X axis: retention time in minutes for UV detection and in seconds for ICP-MS; Y axis: absorbance at 215 nm for UV chromatograms and total counts for ICP-MS chromatograms.

by the supplier, so we did not pursue a multipoint calibration for this standard. The latter Cp standard is pure but unstable: it is losing the metal, as is evident in the ICP-MS Cu data, and forms a compound that has a retention time near 7.3 minutes. The identity of the Cp standard from EMD Biosciences/Calbiochem was confirmed by electrospray ionization mass spectrometry (ESI-MS) but the peak near 7.3 minutes has not yet been identified. Physicochemical changes in Cp, usually arising from partial loss of Cu, were attributed to aging of solutions, exposure to certain buffers, and ion exchange chromatography [14]. It is well known that a small amount of colorless protein, apoCp, in serum represents 10 to 20% of the total immunologically reactive protein, and that in sera with Wilson's disease, apoCp is present at levels near those found in sera from normal subjects [14].

The loss of Cu from the Cp molecule translates into lower slopes of the calibration curves as shown in Figure 2 (for the EMD Biosciences/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 each day during sample analysis.

Method performance

The performance of this assay was established with the reference human serum ERM DA470, which 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, summarized in Table 2, illustrate that the agreement between the concentration of Cp in the certified serum and the freshly reconstituted serum is excellent. When the serum is kept at room temperature overnight, the concentration of Cp is about 20% lower. The coefficient of variation (CV) for the three replicate measurements of the freshly reconstituted serum and the serum equilibrated overnight are 5.4% and 9.9%, respectively. Because Cp is labile, we recommend that samples be analyzed immediately upon collection.

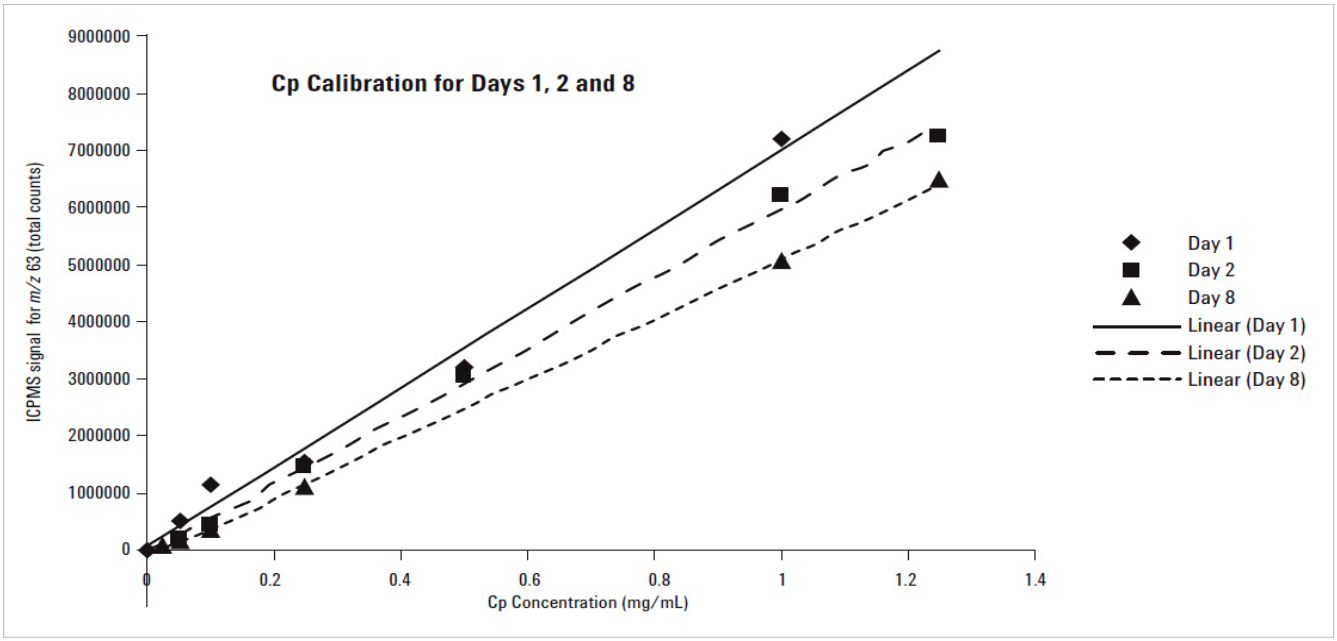

Figure 2. Cp calibration curves for Days 1, 2, and 8 for the EMD standard.

Table 2. Concentration of Cp in the ERM DA470 Reference Serum.

	Certified value (mg/mL)	Concentration measured in this study (mg/mL)	$^{63}Cu/^{65}Cu$
ERM DA470 reference serum (freshly reconstituted)	0.205 (0.011)*	0.208 (5.4%)**	2.1 (3.6%)**
ERM DA470 reference serum (after overnight equilibration at room temperature)	0.205 (0.011)*	0.168 (9.9%)**	2.2 (2.8%)**

*Uncertainty (mg/mL) – 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).

**Average of three determinations; value given in parentheses is the percent coefficient of variation (CV%).

A Cp standard at 5 mg/mL was processed through the immunoaffinity column to verify that Cp is not retained. 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), <0.01% (or 13 ng) was found in the bound fraction that was eluted with Buffer B and analyzed directly by ICP-MS. These data indicate that the recovery of Cp by our method is essentially complete.

Method performance data are included in Table 3. 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 produce even lower method detection limits. The method dynamic range is given as 0.01 to 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 to 0.5 mg/mL range [15]; 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%. Table 3 also includes values at various steps in the analysis. Finally, the identification of Cp is based on the 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 8.39 min with a CV of 0.06%. The natural abundances of the copper isotopes are 69.17% ^{63}Cu and 30.83% ^{65}Cu, 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.

Cp measurements of human sera samples with different diseases

Figure 3 shows the distribution of Cp concentration across four diseases, including MI, PE, RA, and 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 [15] and are shown in the grey area of Figure 3. 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 [16], 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.

Table 3. Determination of Cp by SEC-ICP-MS – Method Performance*

Method indicator	Value
Detection limit (5-µL injection)	0.01 mg/mL
Dynamic range	0.01 to 5.0 mg/mL (tested only to 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

*This method takes approximately 95 min/sample from start to finish (15 min dilution and filtration, 30 min immunoaffinity chromatography, 20 to 30 min concentration, and 20 min SEC-ICP-MS analysis).

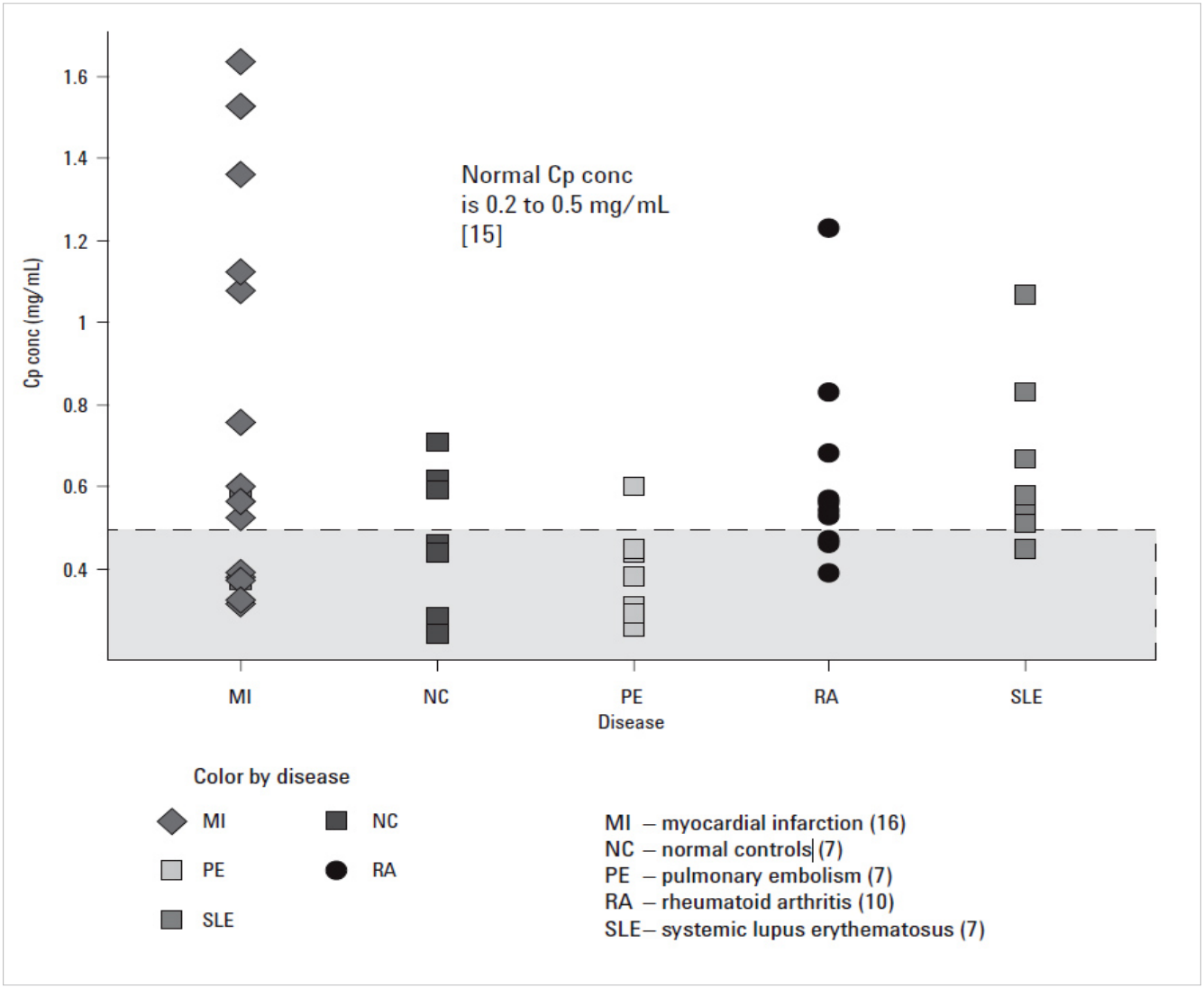

Figure 3. Cp concentration in human sera samples with 4 diseases and normal controls; numbers in parentheses indicate the number of sera analyzed for Cp.

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 sera for which we had enough sample. For total Cu, we analyzed the diluted samples directly using the ICP-MS. We used a 150-fold dilution of the serum with 1% nitric acid. To verify the accuracy of the total Cu measurement, we checked the procedure with two reference sera: Seronorm I and Seronorm II. The total Cu concentration found for Seronorm I is 1,043 ng/mL (certified value is 997 to 1,123 ng/mL) and for Seronorm II is 2,480 ng/mL (certified value is 2,400 to 2,800 ng/mL). Method blanks (that is, 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 illustrated in Figure 4, with an R2 of 0.8, and confirms 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 antitrypsin, 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].

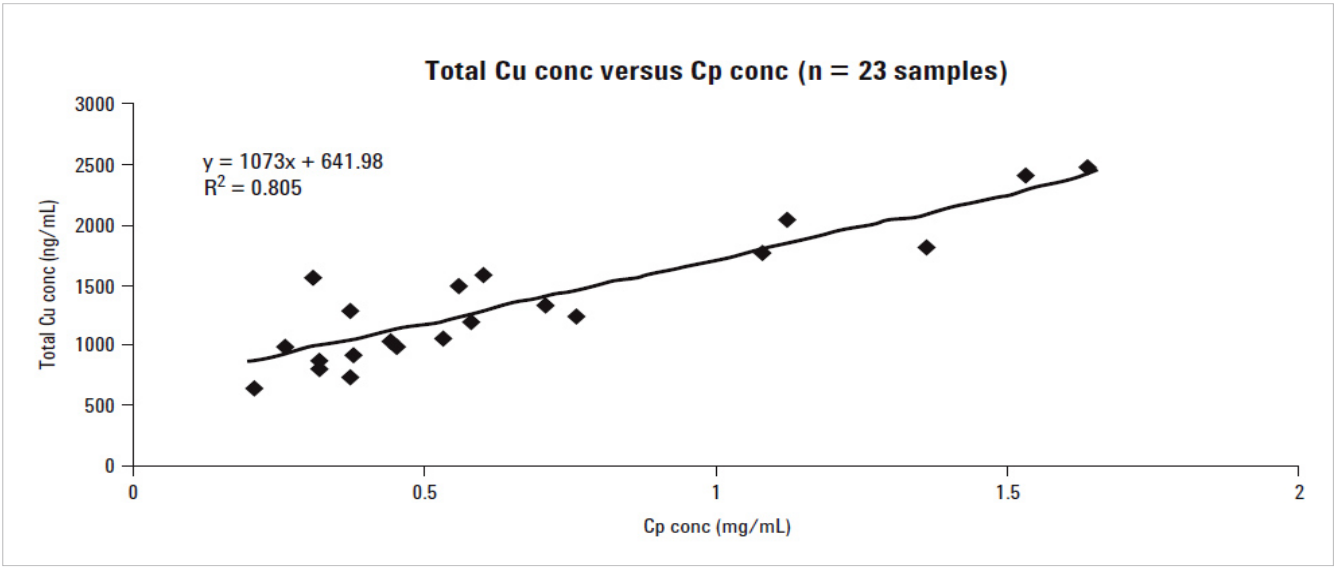

Figure 4. Total Cu versus Cp concentration for 23 serum samples (7 PE, 1 NC, 14 MI, and ERM DA470).

The number of metal atoms in the protein molecule was established using the concentration of Cp that was determined in the ERM DA470 reference serum (by SEC-ICP-MS) and the total Cu measurement of the same sample (618 and 661 ng/mL in duplicate determinations). If Cp contains 6 Cu atoms per molecule, then a Cp concentration of 0.208 mg/mL (measured in this study) would correspond to a total Cu concentration of 596 ng/mL; if Cp contains 7 Cu atoms per molecule, then the total Cu concentration would be 695 ng/mL. Since the Cu concentration was 618 to 661 ng/mL, we can then conclude that Cp in the ERM DA470 reference serum contains between 6 and 7 atoms per molecule, consistent with the published value [1].

Conclusions

Interest in proteins with Cu or Cu binding sites is increasing, and standardized analytical methods are needed to distinguish between Cp and apoCp. The method described in this application uses immunoaffinity chromatography to clean up the sample prior to separation with SEC and detection using ICP-MS. By removing the six most abundant proteins from serum with immunoaffinity chromatography and using SEC to separate Cu bound by Cp from any free Cu in the serum sample, a method has been developed that can accurately and reproducibly measure Cp and has been demonstrated using ERM DA470 reference serum.

The SEC-ICP-MS method was used to analyze 47 human serum samples with one of four different diseases and a set of normal controls for Cp. This is not a diagnostic procedure, but in a research environment, ICP-MS coupled with different forms of chromatography appears to be a desirable analytical tool for the determination of metalloproteins because of its selectivity, especially when dealing with such complex matrices as the human serum.

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Published May 31, 2017
Publication number: 5989-5304EN

Agilent Technologies