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Analytical methodology

Free copper in serum: An analytical challenge and its possible applications

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ABSTRACT

Copper (Cu), as an essential metal, plays a crucial role in biochemical reactions and in physiological regulations.

Cu in plasma is mostly bound to proteins; about 65–90% of Cu is tightly binds with caeruloplasmin and the rest of Cu is loosely binds with albumin and transcuprein. A small but significant relatively "free" fraction, probably complexed with amino acids, is present at around 5% of the total concentration.

We developed and validated a new method for direct measurement of free Cu in serum by ultrafiltration with AMICON®Ultra 100K device and determination with AAS.

Also, we checked that there is no trace of albumin in the ultrafiltrates and we demonstrated the ultrafiltration of a known concentration of Cu added in artificial serum without albumin and, on the contrary, the retention of the Cu in artificial serum with albumin.

The ultrafiltration procedure and the instrumental determination showed a good repeatability and a very low limit of detection (1μg/L).

The method was applied to 30 healthy subjects, the mean value of the total Cu (994.8μg/L) is included in the normal range for healthy people and the values of free Cu (23.6μg/L) corresponding to 2.37% of the Cu total.

The determination of free Cu by this simple and cheap method may be useful to measure the most bioavailable Cu fraction possibly implicated in neuro-degenerative and oxidative-stress related diseases.

1. Introduction

Copper (Cu) is an essential trace element and acts as a critical cofactor into specific cuproenzymes that catalyze electron transfer reactions required for cellular respiration, iron oxidation, pigment formation, neurotransmitter biosynthesis, antioxidant, defensins, peptide amylation and connective tissue formation. Excessive Cu intake has been associated with toxic effects in animal experiments; known toxic effects in humans include metal fume fever, an influenza-like syndrome occurring after acute exposure to Cu and other metal fumes; hemolysis and kidney failure due to Cu sulfate intake have also been reported [1].

Cu exists in blood plasma in different species; the most (65–71%) of plasma Cu is composed primarily of Cu bound to caeruloplasmin, a 150 Kd glycoprotein enzyme involved in iron metabolism characterized by a Cu-dependent oxidase activity. Caeruloplasmin catalyzes the oxidation of iron Fe^{2+} into Fe^{3+}, therefore allowing its bond to transferrin, which can carry iron only in the ferric state [2].

The fraction of exchangeable serum Cu is composed of Cu-albumin (Cu-ALB) (15–19% of the Cu bound to the N-terminal end of albumin via several amino acids) and Cu bound to transcuprein (7–15%) a high affinity Cu carrier.

Less than 2–5% of Cu that remains free and/or bound to amino acids is also defined as free or ultrafiltrable Cu which is readily available for uptake by cells [3–5].

The concentration of protein-free Cu is very low relative to the total serum Cu concentration, however, some evidences reported that an excess of free Cu fraction, might lead to tissue injury due to pro-oxidant effects and depletion of antioxidant reserves [6].

As a free metal ion, Cu also participates in angiogenesis, nerve myelination and endorphin action [7]. From the toxicological point of view, free Cu ions can interact readily with oxygen to initiate a cascade of biochemical events leading to the production of the highly damaging hydroxyl radical.

The quantitative variations between different Cu-containing compartments in plasma reflect pathological disorders. Much attention has been paid to diseases related to the anomalies in the caeruloplasmin

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level, e.g., Wilson's disease and Menke's syndrome [8]. Variations in the loosely bound Cu fractions may also indicate disease states [9].

A peculiar role of the free Cu fraction in the development of some chronic degenerative diseases, whose pathophysiology is largely attributed to oxidative stress, has been reasonably hypothesized [10].

A key difference between bound and free Cu lies in the fact that the limited size of the low-molecular-weight compounds allow free Cu to easily cross the blood-brain barrier [11].

The hypothesis of a contribution of protein-free Cu to the development of neurodegenerative diseases (e.g. Alzheimer disease) has been studied in its toxicokinetics aspects: it has been demonstrated that Cu uptake through the blood-brain barrier is about 50 and 1000 times higher than Cu-albumin and Cu-caeruloplasmin uptake, respectively [12 Choi].

Furthermore, Cu^{2+} enhances the effect of amyloid-β (Aβ) on microglial activation, important pathological component in the brains of Alzheimer's patients, and the subsequent neurotoxicity [13].

Clinically significant changes in the free Cu concentration may not be detected through measuring total Cu concentration alone because more than two species are present and no easily applicable formula can really help in determining the real amount of each species.

Serum free Cu has historically been defined and calculated as the whole non–caeruloplasminic Cu. The measurement of this so called “free Cu” can be indirectly obtained: the calculation is based on the total serum Cu concentration less the product of the serum caeruloplasmin concentration and a factor correlating to the amount of Cu bound per milligram of caeruloplasmin. This fraction includes both albumin/aminoacid-bound Cu and protein free Cu.

Some inherent weaknesses on this method of indirect calculation are related to the assumptions that caeruloplasmin is saturated and that the proportion of Cu bound to proteins other than caeruloplasmin is not considered in the calculation.

Non protein bound Cu direct measurement can be a precious tool to evaluate the actual contribution of the active and bioavailable fraction of this metallic element into the pathophysiology of oxidative-stress related diseases.

In literature indeed albumin-bound Cu is defined “easily exchangeable” as the albumin-Cu bond is considered weak; free Cu is by definition the actual exchangeable fraction of the whole amount of total serum Cu; though, it seems to be the first and most important species to measure in order to evaluate any clinical or pre-clinical variation of Cu metabolism or intake.

Among the available methods described in literature for the separation and measurement of serum non protein bound metallic species, ultrafiltration and solid phase extraction have already been experimented for the determination of free Cu by some authors [14–19].

The aim of our work was to develop a very simple, cheap and easily reproducible method to assess free Cu concentration in serum and evaluate its potential clinical importance.

2. Material and methods

2.1. Serum samples and ultrafiltration

Blood was collected in serum Monovette® (Sarstedt AG & Co, Germany) and was centrifuged at 3000rcf for 10 min. All material had been previously tested and found neither to release nor bind Cu. Further, to guarantee the absence of environmental dust contamination, all filters, tubes and tips were pre-rinsed with ultra pure water before use; the analysis of the water does not show Cu presence.

After centrifugation, the serum was ready for ultrafiltration.

Serum was ultrafiltered on Amicon® Ultra-4®, 100.000 50.000 and 30.000 NMWL (Millipore, Molsheim, France) to determine Cu free.

2 mL of serum was transferred to the filter reservoir and the filters were centrifuged for different spin conditions (40 min at 1800rcf and 10 min at 3000rcf, room temperature).

For the normal specimens, serum was obtained from 30 healthy elderly subjects (13 female and 17 male) 75.6 mean years (57–88 years).

This study was approved by the committee on research ethics at the relevant institutions in accordance with the Declaration of Helsinki of the World Medical Association. All participants signed an informed consent form agreeing to provide detailed information on their dietary and life-style habits at recruitment and to provide blood samples for use in future research.

2.2. Albumin determination

Albumin were measured in serum, supernatant and ultrafiltrates with a Bromocresol Green Albumin Assay (BCG) (Bromocresol Green Albumin Assay Kit, Sigma Aldrich, Saint Louis, Missouri, USA). The BCG albumin assay kit is designed to measure albumin directly without any pretreatment of samples. The kit utilizes bromocresol green, which forms a colored complex specifically with albumin. The intensity of the color, measured at 620 nm, is directly proportional to the albumin concentration in the sample. The detection range of albumin was between 0.01 g/dL and 5 g/dL. The albumin content (g/dL) was 4.8–5.4 in human serum.

2.3. Reagents

Cu stock standard solutions was prepared from 1000 mg/l in 2% di HNO_3 (Cu metal) (O_2si smart solution, Charleston, USA).

Working solutions were prepared by appropriate dilution of the stock solution. The water used was bi-distilled water, for inorganic trace analysis (Merck KgaA, Darmstadt, Germany).

2.4. Copper determination

Total and free Cu were performed by an Atomic Absorption Spectrometry (AAS Spectra 400 Varian, Medical Systems, Inc. Palo Alto, CA) equipped with a longitudinal Zeeman-effect background correction system and an autosampler was used for all measurements. The Instrumental operating parameters and the temperature ramp of the AAS apparatus was reported in Tables 1 and 2.

For measure of total Cu in the serum and in solution on top of the filter (supernatant), the solutions were diluted 1:40 with bi-distilled water for inorganic trace analysis. Additional calibration was used in serum between 500 µg/L and 2000 µg/L for total Cu and external (standard) calibration was used between 5 µg/L and 50 µg/L for free Cu (Curve of calibration in Appendix A- Supplementary Material, Fig. a, b).

Table 1
Instrument parameters of the AAS method for determination of Copper.

Operating conditions	
Primary source	Copper Hollow Cathode lamp (Agilent Technologies)
Lamp current	4 mA
Analytical wavelength	324.8 nm
Background correction system	Zeeman effect based (Longitudinal)
Slit width	0.5 nm
Mode	Absorbance (high)
Graphite furnace operation	
Atomization tube	Partition tubes (coated)-GTA (Agilent Technologies)
Sheath/Purge gas	Argon (Ar) of 99.999% purity
Injection volumes (sample, µL)	5

Table 2
Temperature ramp of the AAS method for determination of Copper.

Step N°	Temperature °C	Time (s)	Flow (L/min)	Type of gas
1	40	5.0	3.0	Argon
2	180	15.0	3.0	Argon
3	180	20.0	3.0	Argon
4	480	5.0	3.0	Argon
5	480	15.0	3.0	Argon
6	900	5.0	3.0	Argon
7	900	20.0	3.0	Argon
8	900	2.0	0.0	Argon
9	2600	1.0	0.0	Argon
10	2600	2.0	0.0	Argon
11	2800	1.0	3.0	Argon
12	2800	3.0	3.0	Argon
13	40	15.0	3.0	Argon

3. Results

3.1. Ultrafiltration

Different batches of AMICON® Ultra-4 had been previously tested and found neither to release nor to bind Cu.

Ultrafiltration test has been made on 15 samples with three different devices and different spin conditions (300rcf 10 min and 1800rcf 40 min) (Table 3).

Three different cutoffs (molecular weight cutoff (MWCO)) were:
AMICON® Ultra 30k device-3000 MWCO
AMICON® Ultra 50K device-50,000 MWCO
AMICON® Ultra 100K device-100,00 MWCO

The Cu measured in the ultrafiltrates of the device 30k and 50k were below the detection limits; the spin condition increased does not affect the results.

Ultrafiltration with AMICON® Ultra 100K device allowed a recovery of 500 µL and levels of Cu above the detection limit.

3.2. Stability

The stability of Cu in the ultrafiltrates was been tested with repeated measure of Cu in serum and in the ultrafiltered solution.

Cu total and Cu free variation were about 3.5% and 2.8%, respectively, of the initial values when serum was frozen at −20 °C for 1 month.

3.3. Repeatability

A pool of serum (total Cu of 1015 µg/L) was ultrafiltered six times on six different filters. Free Cu was measured to assess the repeatability of the method including the ultrafiltration process. The coefficient of variation obtained was 5.1%.

3.4. Recovery

We added a known amount of 100 µg/L of Cu in 10 samples of serum. The recovery of Cu was 92%.

3.5. Albumin determination

The AMICON® Ultra 100K device should theoretically retain macromolecules with weight greater of 100 kDa and the Cu bound with albumin (67KDa) should be located in the ultrafiltrates.

For this reason, measure of albumin was been conducted in serum, supernatant and in the ultrafiltrates.

No trace of albumin were revealed in ultrafiltrates (Bromocresol Green Albumin Assay Kit, Sigma Aldrich) confirming that the Cu measured was free.

The albumin in the serum was 2.52–4.44 g/dL (reference values 3.5–5.5 g/dL). In the supernatant the albumin were comprised between 2.43–5.15 g/dL.

In addition, further tests were done to confirm the fraction of only free Cu in the ultrafiltrates.

We added 100 µg/L of Cu standard in a solution of artificial serum with and without albumin (NaCl 0.9% + 0.2% NaN3 and NaCl 0.9% + 0.2% NaN3 + 10% fetal bovine serum, biowest, Nuaillé – France).

In the serum without albumin 83% of Cu added was in the ultrafiltrates, while in the artificial serum, the Cu added was bind with albumin and do not pass through the filter (Fig. 1).

3.6. Validation of the method and analytical performances

Instrumental limit of detection of the total and free Cu calculated, as three standard deviations of the background signal obtained on 10 white samples, were 50 µg/L and 1 µg/L respectively.

The coefficients of variation (CVs) of measurements of Cu solutions were 5.1% (tot Cu) and 3% (free Cu).

The linearity of the methods ranged between 50–3000 µg/L.

The quality control of the analysis was reported in a Levey-Jennings chart (Appendix A- Supplementary material, Fig. c).

The accuracy of the method was determined on the mean values obtained by certified reference materials (Environmental and Occupational, G-EQUAS for serum and supernatant, NIST 1643e-1643d trace elements in water for ultrafiltrate).

The laboratory participates in the intercomparison programme for toxicological analysis in biological materials for the determination of Cu in the serum (Institute and Outpatient Clinic for Occupational, Social and Environmental Medicine of the University of Erlangen-Nuremberg), (Appendix A-Supplementary material, Fig. d).

The ultrafiltration with AMICON® Ultra 100K device (3000rcf, 10 min) has been conduct in blood samples of 30 healthy subjects.

For the normal specimens, plasma was obtained from 30 healthy elderly subjects (13 female and 17 male), mean age of volunteers was 75.6 years, range 57–88 years.

The Cu was measured in the serum, in the supernatant and in ultrafiltrates.

The mean value of the total Cu (994.8 µg/L) is included in the normal range for healthy people, the values of free Cu (23.6 µg/L) corresponding to 2.37% of the total (Table 4, Fig. 2).

The albumin test was also repeated in these samples; the concentration of albumin in serum was 4.03 g/dL, while in the ultrafiltrates the albumin was not detectable. Every run was validated with the measurement of internal quality controls (Environmental and

Table 3
Determination of Cu free, total Cu and Cu in the supernatant of three different devices and with different spin conditions (N°15 samples).

	AMICON® Ultra 30K		AMICON® Ultra 50K		AMICON® Ultra 100K	
	1800rcf, 40 min	3000rcf, 10 min	1800rcf, 40 min	3000rcf, 10 min	1800rcf, 40 min	3000rcf, 10 min
Concentrate volume (µl)	100	100	100	100	400	500
Cu free (µg/l)	89% < LOD	89% < LOD	93% < LOD	93% < LOD	25.3 ± 10.6	26.8 ± 11.5
Cu tot(µg/l)	1051 ± 173		1026 ± 171		1143 ± 212	
Cu in the supernatant (µg/l)	1050 ± 176	1081 ± 173	1055 ± 200	1062 ± 192	1130 ± 236	1127 ± 248

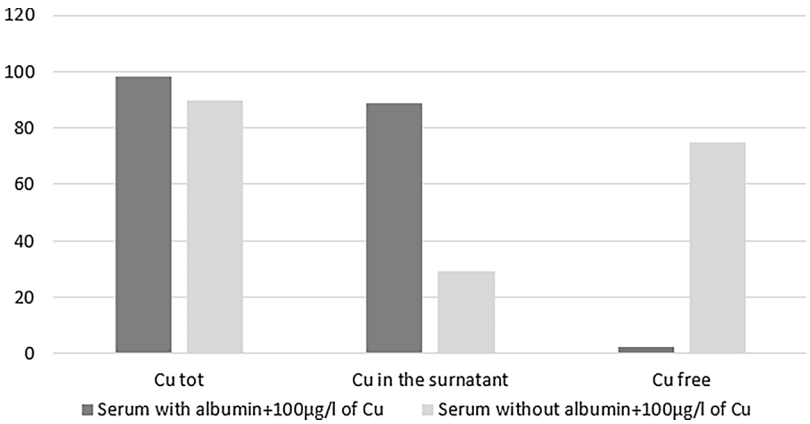

Fig. 1. Average values of three determination (µg/l) (AMICON® Ultra 100K device).

Table 4
Concentration of different fraction of Cu measured in 30 healthy people.

	Cu Tot	Cu in the supernatant	Cu free	Cu free % of total Cu
Mean	994.8	1046.9	23.6	2.37
Median	991.0	1054	22.25	2.24
5°–95°	709.7–1285.7	791–1384	8.9–47.5	1.3–3.7
Range	639–1495	724–1445	7.2–55.2	1.1–3.7

Occupational, G-EQUAS for serum and supernatant, NIST 1643e-1643d trace elements in water for ultrafiltrates).

4. Discussion

Copper serum free fraction represents the active, bioavailable and potentially toxic fraction of this metallic element in the bloodstream.

As with other metals, the bond of Cu with plasmatic protein or macromolecules represent a sort of deposit and sequestration of the free metallic fraction.

Most published studies, including the most of the ones regarding the so-called free-Cu concentration, regard indeed free Cu meant as non-caeruloplasminic Cu, while only few studies evaluate free Cu defining it as the non-protein bound fraction.

Our opinion is that describing the whole non caeruloplasminic Cu as "free" appears to be an equivocal and misleading definition for some reasons: as clinicians, laboratory physicians and technicians we are used to define the free fraction of a substance as the fraction of its whole circulating amount presenting two fundamental characteristics: bioavailability and no binds to proteins.

There are evidences that free Cu is a fraction biologically available and involved in carcinogenesis due to the induction of oxidative damage but overall there is now no conclusive evidence of Cu being a human carcinogen [20].

Free Cu has been shown to involve oxidative modification of low-density lipoprotein (LDL) and promote atherogenesis by enhancing the transformation of macrophages into foam cells and by developing vasoconstrictor and prothrombotic properties [21].

Some authors have related the oxidative stress induced by Cu to the development of degenerative diseases, including, diabetes, neurological disorders and chronic inflammation [22].

Direct measurement of serum free Cu is the only way to evaluate its potential role in the development of the above mentioned chronic diseases, and an easily applicable separation and measurement technique might provide the basis for further research such as case control-studies.

The ultrafiltration was carried out with "AMICON® Ultra" filter devices; as reported by their instructions leaflet, these membranes were developed for concentration of biological samples, purification, desalting, buffer exchange and diafiltration.

We further decided to verify the appropriateness of their use for the ultrafiltration procedure directly with the Technical Service of Millipore.

After many laboratory test, we experimentally demonstrated the best cut off filter to be the AMICON® Ultra 100k device proving a pores size to eliminate the caeruloplasmin, albumin and other proteins expected to bind Cu.

As such, the ultrafiltrates should contain only "free" Cu that is easily measured using conventional AAS.

To reach an effective separation, we started from AMICON® Ultra 30k device that should, theoretically, retaining albumin allowed the passage of free Cu in the ultrafiltrate.

However, no free Cu was measurable in the ultrafiltrate after filtration with AMICON® Ultra 30k or 50k devices, probably because the generation of protein clots within the membrane.

We further dimostrated the efficacy of the separation of our method through the determination of albumin in the ultrafiltrates and some experimental assays with the use of a known amount of Cu in artificial serum with and without albumin.

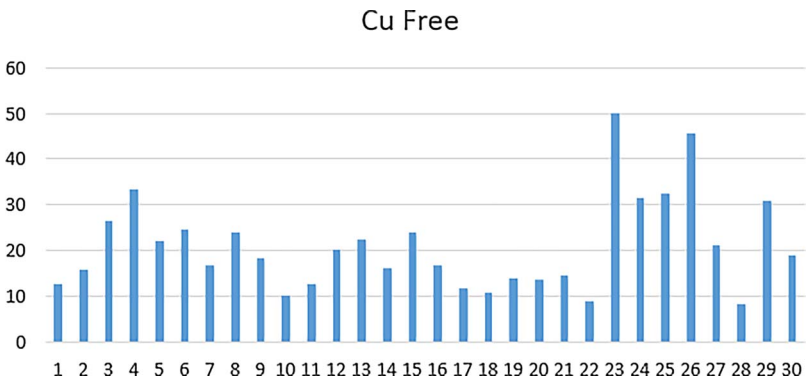

Fig. 2. Distribution of free Cu in a population of healthy people (µg/L).

Table 5
Free Copper determinated by ultrafiltration in healthy subjects in other studies..

	Cu free µg/L	Cu free % of total Cu	method	Filter device and cut off (KDa)
Favier et al. [14]	14.5	1.1	ultrafiltration/AAS	Centrifree® MPS-1 25
Noubah et al. [15]	15.8	1.4	ultrafiltration/AAS	Centrifree® MPS-1 10
Chan et al. [16]	10.7	1.2	ultrafiltration/AAS	Centrifree® MPS-1 30
Bohrer et al. [17]	33.6	2.3	ultrafiltration and solid phase extraction/AAS	Ultrasart® 20
ElBalkhi et al. [18]	6.3	0.6	ultrafiltration/ETAAS	Ultrafree-4® 30
McMillin et al. [19]	50.4	1.0	ultrafiltration/ICP/MS	Centrifree® 30
Present Study	23.6	2.4	ultrafiltration/AAS	Ultra-4 100

Only a few studies investigate the distribution of the non-protein bound Cu fractions in physiological or pathological conditions.

The basic technique, successfully applied to the measurement of numerous non-protein bound analytes in serum is apparently just as useful for quantifying the ultrafiltrable Cu fraction in human serum.

We applied our validated method for quantifying the non-protein-bound Cu fraction in serum samples of a group of healthy subjects.

The mean of free Cu measured in our specimens was 2.37% of the total serum Cu, values comparable with literature data.

Other studies that applied ultrafiltration in serum samples reported lower percentages of free Cu (0.6–1.1%), except the study by Bohrer et al. [17] in which the free Cu was 2.3% of the total Cu (result of 7 determinations) (Table 5).

We hypothesize that some studies may have underestimated the free Cu fraction because did not have considered the protein clots as a potentially source of systematic errors.

A role of Cu in human neurological disorders is known [23], and recent studies report a higher serum concentration of non caeruloplasminic Cu in Alzheimer disease patients [24,25]; further research is needed to clarify the role of really free Cu in these patients in order to better evaluate the biological plausibility of this association.

Conflicts of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jtemb.2017.11.006.

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