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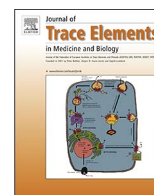

Analytical methodology

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

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A R T I C L E I N F O

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A B S T R A C T

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

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