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VARIA

Copper and its role in the human body – the importance of establishing copper concentrations in the body

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Abstract: *Copper is essential to all living beings, as a food mineral, being an essential component of the cytochromoxidase enzymes complex. It is also an essential component of several systems involved in the production of hemoglobin, of sugar metabolism, of catecholamines' synthesis and in the formation of cross-links between collagen, elastin and keratin fibers within the hair. The dysregulation of copper through impaired absorption or excretion, either genetically based or in acute or chronic poisoning, results in deficiency or toxicity. For this reason, the measurement of copper levels in various biological samples: urine, blood, or hair, provides the physician with information regarding contamination, poisoning, or a metabolic disease. Due to low concentrations in most heavy metals' and metalloids' cases, finding traces in biological samples is a difficult problem. Classical methods (staining, flame photometry, reagent kits, electrochemical methods) can only be used for a limited number of elements, such as sodium, potassium, calcium, magnesium, copper, iron and zinc. The analysis of biological samples is the most important use of graphite furnace atomization. The advantages of using this technique are its great sensitivity and the very low quantity of sample necessary for performing the measurement.*

In modern medicine, determining microelements is very important in diagnosing diseases and/or in treating them. The GF-AAS method is quick, reproductible and has very good sensitivity.

Keywords: *copper, toxicity, metabolism disorders, Wilson disease, determination methods, spectrophotometry, GF-AAS*

INTRODUCTION

A great number of chemical elements in nature are present in the body, either as standalone components, or bound one to another, forming organic and inorganic substances that facilitate or create various disorders in the human body. A part of them (Co, Cr, Cu, Fe, Mg, Mn, Mo, Ni, Se, Sr and V) are essential to life.

Other elements (As, Ba, Be, Cd, Li, Hg, Pb, Sb, Sn) are considered toxic, and their concentrations in any given biological product is preferred to be close to nil. When such concentration is different from zero, the value is considered normal if it is lower than the limit established by toxicology authorities around the world.

Copper is a chemical element that has been used for thousands of years in alloyes. Its compounds are frequently found as copper (II) salts, which usually yield the blue or green colours of minerals, such as azurite or turquoiuse, and have been largely used as pigments. Copper sulfate forms are bright blue crystals containing five molecules of water [$CuSO_4 \cdot 5H_2O$]. It is commonly known as the "Blue Vitriol" or "Blue Stone" [1] and it is used mainly for agricultural purposes as a pesticide, and in the leather industry [2, 3].

Biological Roles of Copper

Copper is essential to all living beings, as a food mineral, as

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it is an essential component of the cytochromoxidase enzymes' complex. It is found in the liver in vertebrates. In high concentrations, copper compounds are toxic for evolved organisms, and are used as bacteriostatics and fungicides.

Copper alloys have intrinsical natural properties that kill a large number of microorganisms, such as E. coli O157: H7, methicillin-resistant Staphylococcus aureus (MRSA), Clostridium difficile, influenza virus A, adenovirus, fungi [4, 5]. Some copper 355 alloys have been shown to kill more than 99.9% of germs within two hours, when properly cleaned [4]. The United States Environment Protection Agency (EPA) has authorized these copper alloys as „antimicrobial materials with public health benefits" [4]. Recent biotechnological researches use copper ions coupled with indocyanine and proteins in targeted cancer treatments [6].

Proteins which contain copper have several biological roles in electron and oxygen transport, processes in which interconversion of Cu (I) and Cu (II) easily takes place [7]. The biological role of copper began the moment oxygen appeared in our planet's atmosphere [8].

In the cytochrome-c oxidase, which is necessary for aerobic respiration, copper and iron work together to reduce oxigen. Copper is found in superoxide-dismutase, which catalyzes the breakdown of superoxides, through conversion to oxygen peroxide and hydrogen:

$$2\ HO_2 \rightarrow H_2O_2 + O_2$$

Copper is an essential component of several systems involved in the production of hemoglobin, in sugar metabolism, in catecholamine biosynthesis, and in forming cross-links between collagen, elastin and keratin fibers within hair.

Copper deficits leads to growth and behavioural disorders, especially in children [9]. Copper deficiency is an etiology of anemia, neutropenia, and bone marrow dysplasia that may be under-recognized, being a possible cause of medical errors in clinical practice [10, 11]. The mechanism of anemia in copper deficiency may be related to the role of copper-dependent enzymes, such as ceruloplasmin and cytochrome-c oxidase, in iron metabolism and transportation [12]. Bone marrow findings of marked vacuolization of both erythroid and myeloid precursors have been consistently reported, and ring sideroblasts are also occasionally reported [13].

Kinetics of Copper

Copper intake is mainly achieved through diet : it is easily absorbed, but its absorbtion is limited by homeostatic mechanisms, after requirements have been met. The human body contains aproximately 1.4-2.1 mg/kg of copper [14]. Copper is absorbed in the gut, then transported to the liver bound to albumin [15]. After being processed in the liver, it is then distributed to other tissues. Copper transportation involves a protein called ceruloplasmin, which also transports copper excreted in milk [16]. The body is able to excrete excessive levels of copper, if necessary, through the bile. The excreted excess is not reabsorbed by the intestine [17, 18]. In catalytic oxidation processes, metalloproteins containing copper are also necessary, as they intervene in oxygen metabolism. Also, copper also acts in oxidative phosphorylation reactions. Copper intake from food can also prevent certain diseases or deficiencies, such as allergies, hair loss, AIDS, leukemie, osteoporosis and gastric ulcer. Together with iron, copper helps in red blood cells' synthesis.

Copper metabolism disorders

The dysregulation of copper through impaired absorption or excretion results in deficiency or toxicity, as illustrated by two rare genetically based diseases.

Wilson's disease was first described in 1912 by Kinnear Wilson as "progressive lenticular degeneration". Wilson's disease can manifest itself at birth, but signs and symptoms only appear when copper accumulates in the brain, liver, or another organ. The signs and symptoms vary with the body part that is affected by the disease and can include fatigue, loss of appetite or abdominal pain, jaundice, brown-golden Kayser-Fleischer rings, muscular stiffness, lack of motor coordination, speech impairment and others.

Menkes' disease, when the body cannot stock copper, leading to its deficiency; it is also known and the uncombable hair syndrome (hair lacks in color, is sparse, wiry and rough, with occasional nodosities) or Menkes' kynky hair disease. It is a rare hereditary X-linked transmitted disease, described by Menkes in 1962 as a severe degenerative disease of the nervous system. Biologically, serum copper and ceruloplasmin levels markedly decrease. Some of the clinical manifestations pertaining to this disease are facial dysmorphia, signs of neuropsychic involvement, hypothermia, skeletal manifestations similar to those in scurvy, and the irregular lumen of blood vessels.

Clinical signs in copper intoxication

In acute intoxication, serum copper reaches very high values, ceruloplasmin and hepatic copper are within normal limits, and urine copper is increased.

In chronic intoxication, serum, urinary copper and

ceruloplasmin are increased, and hepatic copper reaches very high values. The acute toxicity of copper in humans is likely due to the generation of reactive oxygen species, which deteriorate DNA [10]. A minimal dietary value of at least 3 ppm was reported for healthy growth in rabbits [20]. However, high concentrations of copper (100 ppm, 200 ppm, sau 500 ppm) in their diets can favorably alter their growth rates [21].

Copper toxicity is determined by the balance between copper intake and excretion. Voluntary copper salts intake, copper intake from food and beverages, inhaling dust which contains copper or aerosols which contain copper salts, through manual spraying of insecticides or fungicides, represent the main risks of exposing the human body to copper contamination.

The main actions of copper on the human body are gastrointestinal irritation, liver and renal disorders, intravascular hemolysis and shock. Acute intoxication following ingestion of copper salts targets the GI tract, cardiovascular and circulatory systems, hematopoietic system, liver, kidneys and nervous system.

The respiratory effects following inhalation are: acute exposure to copper vapors targets the respiratory pathways and chronic exposure to copper salts, seen in those who spray plants, targets the lungs.

The local, irritating effects, through cutaneous or eye contact with copper salts, are seen at skin and eye level.

Other diseases in which abnormalities in copper metabolism appear to be involved include Indian childhood cirrhosis (ICC), endemic Tyrolean copper toxicosis (ETIC), and idiopathic copper toxicosis (ICT), also known as non-Indian childhood cirrhosis. ICT is a genetic disease recognized in the early twentieth century primarily in the Tyrolean region of Austria and in the Pune region of India [22].

DETERMINING COPPER CONCENTRATIONS IN BIOLOGICAL PRODUCTS

Due to low concentrations in most heavy metals' and metalloids' cases, finding traces in biological samples is a difficult problem. Classical methods (staining, flame photometry, reagent kits, electrochemical methods) can only be used for a limited number of elements, such as sodium, potassium, calcium, magnesium, copper, iron and zinc.

Modern methods replace these types of tests. Elements can be determined through atomic absorbtion spectrometry AAS with flame atomization FAAS or graphite furnace atomization GTAAS, inductively coupled plasma and atomic emission spectrometry ICP-AES or inductively coupled plasma and mass spectrometry ICP-MS. ICP-MS is the most sensitive method for determining metals and metalloids, but it also has the highest cost. For determining metals and metalloids in biological samples, the GTAAS method is recommended. For Na+, K+, Cl-, Ca2+ it is much more cost effective to use machines which only measure blood or urine concentrations of these ions, through electrochemical methods.

The analytical method used to determine the concentration of studied elements, from the analyzed matrices, is the atomic absorbtion spectrometry coupled with graphite furnace atomization GF-AAS (Graphite Furnace Atomiser – Atomic Absorbtion Spectrometry). Atomic absorbtion spectrometry is an analytical method used to quantitatively measure metals or metalloids in a sample, based on the quantity of light that is absorbed or emitted by atoms, from a line that is characteristic for the specter of light the atom emits in an excited state.

The first method that was used to bring atoms to an excited state was heat emanated by a flame. The temperature of this flame is approximately 1200 – 13000C. Due to the low temperatures that can be achieved with this method, the sensitivity of this type of atomization is not useful in determining the majority of elements in biological samples.

Graphite furnace atomization has become very useful in analytical chemistry, as a technique which is very good for determining trace elements in a wide variety of sample types. The principle of this method, of achieving a controlled temperature, using electrical current, makes it perfectly reproductible, compared to flame atomization. The greatest advantage, however, consists in the high atomization temperatures, which can never be attained with a flame, which makes analyzing trace elements in biological samples possible.

The number of replicates (repeatedly performing the same determination) is very important for obtaining quality results. Thus, the working program allow real-time quality control of obtained results, by verifying the standard deviation of replicates. If the value of the square mean deviation (standard deviation) of values measured in the same sample exceeds the pre-established value, the system performs the determination once more. The usually admitted standard deviation is 4-5%. The increase of replicates' number increases the duration of a given determination, wears down the graphite furnace, but improves the accuracy of the results. Due to the marked stability of the machine, it is possible to use the minimum number of two replicates.

The absorbance signal has its own noise due to the variations in emitted light, due to its transmission through the optical chain, due to variations caused by the photomultiplier and the electronic system which amplifies the electric signal. This type of noise can be removed by mathematical methods. The methods which may be applied are either in 3, 5, or 9 points. The calibration method is extremely important for the accuracy of the results. For this reason, choosing an optimal calibration method is one of the most important decisions in establishing the method. For standard calibration, the software automatically chooses the liniar calibration. The calibration curbe may or may not pass through point zero. When measured levels are very low, this curve must pass through point zero. Quality control is mandatory to establish the accuracy of the obtained results. Quality control also involves checking: the standard deviation of replicates; the correlation coefficient of the calibration curve; the detection limit of the method and the apparatus.

Table 1: Concentrations of some essential trace elements in blood and urine (mg/l).

Element	Serum (µg/L)	Blood (µg/L)	Urine (µg/L)
Chromium	0.5	0.5	10
Cobalt	0.2	2.0	100
Copper	1100	1200	60
Iron	1200	-	180
Magnesium	0.5	5	300
Molibdenum	1.0	100	100
Nickel	5.0	5.0	85
Selenium	100	100	30
Strontium	50	-	150
Vanadium	0.5	4.0	16

In urine spot samples, there was a great variation in copper content, which is why collecting 24h urine and sampling from the total volume is recommended. The conventional level taken as diagnostic of Wilson's Disease is >100 mg/24 hours in symptomatic patients [23].

A method to determine the concentration of an element in the matrix involves establishing the working conditions of the spectrometer, of the graphite furnace atomizer, of the sample dispenser and the conditions for the automatic quality control of the test.

The calibration curves of the elements, absorbance as a function of concentration, are calculated with the following equation:

$$A/C = a + b * A + cA^2$$

where: A – is the sample absorbance, C – is the sample concentration, A, b, c, d – are coefficients of the calibration curve.

The determination of copper in biological samples is usually carried out for diagnosing Menkes or Wilson disease or for monitoring the distribution and the amount of copper removed during dialysis procedures of patients with chronic renal disease [24, 25].

In order to develop a method for determining direct GF-AAS copper in biological samples (blood and urine) the establishment of operating parameters for atomic absorption spectrometer system – AAS 880 Spectra Varian and for graphite tube atomizer – GTA 100 is of major importance.

The utilized wavelength must be a part of the absorbtion specter of copper and depends on the type of sample. It may be 327.4 nm or as alternative wavelengths, 324.8 nm or 217.9 nm or 218.2 nm. The correct choice is made with the purpose of eliminating the non-atomic absorbtion which may appear during sample analysis. The width of the used diaphragm is of 0.2 or 0.5 nm and its size depends on the type of analyzed sample.

Method for determining the level of copper in whole blood samples

The samples were analyzed with a system formed from the following components:

- Atomic absorbtion spectrometer AAS-880.
- Graphite tube atomizor GTA-100.
- Programmable sample dispenser – PSD.
- Water cooler – Neslab CFT 22.
- Nitrogen generator - Dominick Hunter.
- Gas cylinder (Argon) 99.9999% purity

Optimized working parameters for determining copper levels in whole blood

In order to determine the Cu concentration in the blood samples taken from patients (2 ml of venous blood, taken on anticoagulants), the following method was used:

- Pipetting method – automixing;
- Quantity of injected sample – 10 µl;
- Measurement method – peak height;
- Concentration calibration;
- Smoothing – 9 points;
- Number of replicates - 2 for standard and for sample;
- Cathode tube current – 4 mĂ;

- Wavelength – 327.4 nm;
- Slit width - 0.5 nm;
- Background correction – deuterium lamp;
- Drying – 1200C;
- Calcination - 8000C;
- Atomization - 23000C;
- Cleaning - 25000C;
- Three-point calibration curve – 0, 50 and 100 µg/l;
- Recalibration rate – 15;
- Method detection limit <1.0 µg/l.
- Apparatus detection limit <0.04 µg/l.

Automatic quality control – dispersion <10 %; correlation coefficient of calibration curve >0.998.

The optimization of the temperature program must provide optimal conditions for the measurement of Cu signals by GF-AAS, free of interferences from the background which is a very common problem in the analysis of biological samples (26). The application of the optimized temperature program made possible to eliminate the whole matrix of the sample before the atomization step, as confirmed by the low background signals observed in the measurement of Cu. No chemical modifiers were added. However, biological samples must undergo a deproteinization process, which is meant to eliminate non-atomic absorbance.

Table 2: Established and optimized steps for GTA 100

Step	Temp C	Time s	Flow L/min	Gas Type	Read	Signal Storage
1	40	5	3	N_2	No	No
2	80	10	3	N_2	No	No
3	105	5	3	Ar	No	No
4	105	10	3	Ar	No	No
5	800	20	3	Ar	No	No
6	800	5	3	Ar	No	No
7	800	2	0	Ar	No	Yes
8	2650	0.9	0	Ar	Yes	Yes
9	2650	2	0	Ar	Yes	Yes
10	2650	3	3	Ar	No	Yes
11	40	30	3	N_2	No	No
12	40	5	0	N_2	No	No

Copper stock standard solution containing 1000 mg/L copper was diluted with a premixed solution of distilled water and analytical grade concentrated nitric acid to provide working standards of various concentrations in 0,1% (w/v) HNO3. The calibration blank solution used throughout was a 0.1% w/v HNO3 solution. For copper, the working standards were 100 and 50 µg/L.

Table 3: Performance parameters of linear regression analysis

Conc. ug/L	Abs	Abs. Average	STDEV	RSD %
0	0.0193	0.0183	0.0014	7.55
0	0.0167			
0	0.0188			
50	0.3996	0.4001	0.0009	0.23
50	0.4012			
50	0.3996			
100	0.7214	0.7178	0.0034	0.47
100	0.7147			
100	0.7174			

The whole blood sample (200 µL) was treated with 800 µL solution 5% antifoam B (Sigma) and with 1000 µL of 1.6 M HNO3 solution. The mixture was maintained for 20 minutes and centrifuged with 5000 rpm for 5 min. The supernatant was analyzed by GF-AAS. The results are obtained in µg/dL. A linear relationship was found between the absorbance at 327.4 nm and the concentration of cooper in the range of 0.0 to 100 µg/L. The representative linear equation was y = 0,007x + 0,029 where: y is the absorbance, x is lead concentration (µg/L), calculated by the least squares method. The regression coefficient (r) standard curve was 0.9972, indicating good linearity (r > 0.99).

The performance parameters of linear regression equation are presented in Table 3. The parameters of the GF-AAS analysis method of lead are presented in Table 4. LOD and LOQ were calculated based on the standard deviation and the slope of the regression line.

Table 4: Validation parameters

Standard error	0.0173
R Square	0.9972
Intercept	0.0290
Slope	0.0070
SE of intercept	0.0091
LOD (µg/L)	***4.29***
LOQ (µg/L)	***13.01***

LOD is the lowest concentration of an analyte that can be detected while LOQ is defined as the lowest concentration

of an analyte that can be determined at the acceptable level of precision and accuracy and were calculated according to the formula below: LOD = 3,3*(SD of intercept/Slope) and LOQ = 10*(SD of intercept/Slope)

The established method can be easily used to perform copper measurements in the laboratory, both in urine and blood samples taken from patients suspected for copper intoxication. Copper deficiency and toxicity can be either of genetic or non-genetic origin. The study of copper's genetic diseases, which are the focus of intense international research activity, has shed insight into how human bodies use copper, and why it is important as an essential micronutrient.

The evolution of the copper concentration in urine or whole blood provides very useful information for poisoning diagnostic when the history is not clear [27, 28].

CONCLUSIONS

Microelements play an important role in the normal course of life. The variation of their blood levels within the body can lead to metabolic disturbances. For example, copper deficiency is found in Menkes syndrome or ,,uncombable hair disease", and copper excess leads to Wilson's disease. The rise in blood levels of copper can also be due to acute intoxication, for example, as a result of spraying plants with copper sulphate.

For these reasons, measuring Cu concentrations in various biological samples, urine, blood, or hair, provides the physician with information regarding contamination, intoxication or the existence of a metabolic disease.

The analysis of biological samples is the most important use of graphite furnace atomization. The advantages of this technique are its high sensitivity and the very low quantity of sample necessary for the measurement.

In modern medicine, determining trace elements is very important in diagnosing various diseases and/or in treating them. The GF-AAS is a quick, reproductible and highly sensitive method.

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