SERUM COPPER MEASUREMENT-USING AAS CONTAINING GRAPHITE TUBE

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SUMMARY

The aim of this study was to establish the best method to measure copper (Cu) in serum using the atomic absorption spectrometry (AAS) apparatus (Rank Hilger, H 1550 type, Westwood Margate Kent CT9 4JL-England) with graphite tube. We compared the **"standard curve"** and the **"extrapolation"** methods and found for n=25, X±SD, SE as 78.2±0.07, 0.25 and 82.7±0.11, 0.35 µg/dL, respectively. There was not statistically any significant difference between them. In the end, because the standard curve was linear, recovery values and precision evaluations were better, the accuracy of the standard curve method was accepted to be good. In addition, it is a rapid and easily performed method, and is well suited for routine analysis of Cu in a clinical laboratory.

Key Words: Serum copper, atomic absorption spectrometry, standard curve method, extrapolation method.

INTRODUCTION

In normal human metabolism, copper plays an important role along with other trace elements. It is involved in normal central nervous system function, bone development, in hematopoezis, in the interconversion of catacholamines and as a structural component of certain enzymes in the connective tissue (1,2). Wilson's disease and copper deficiency in adults or infants cause serum Cu concentrations to be subnormal. Other clinical situations such as copper toxicity, Menkes' kinky hair syndrome, leukemias, cirrhosis and infections, cause increased serum Cu levels, as do also the increased concentrations of estrogens in serum in pregnancy or during the use of oral contraceptives (3-8). Diagnosis and confirmation of the above-mentioned abnormalities depends on sensitive, accurate, precise and rapid analytical methods for Cu.

AAS is one of the best methods for measuring Cu in biological samples, with adequate accuracy and precision (9-11). In AAS, the instrumentation required is less expensive, the methods are more sensitive, and the sample preparation in less complicated than that for emmision methods. Either a flame (FAAS) or the more sensitive electrothermal atomic absorption spectrometric (ETAAS) volatilization procedure can be used for measuring Cu. Advantages of flame and/or electrothermal atomization AAS for Cu analysis include high accuracy, high precision, high sensivitiy, minimal chemical and/or matrix effects (interferences), relatively inexpensive instrumentation, and operating costs that are less than most other instrumental methods. In addition , measuring it on micro-scale samples is especially advantageous for pediatric cases (12). The complete analytical method can be guickly taught to relatively inexperienced laboratory workers and FAAS accurate results can be rapidly obtained. The ETAAS when compared with is a more sensitive method which smaller samples can be worked with. In addition extraction is not needed for the analysis and Cu can be measured directly or in the diluted samples.

In this study, we aimed to establish the best method to measure Cu in serum with the AAS apparatus with graphite tube which we had in our laboratory. We planned to measure Cu, by two different methods and compare their accuracy, precision, sensitivity and working conditions with each other.

MATERIALS AND METHODS

To determine the method with highest accuracy, precision and sensitivity for measuring Cu in serum, we planned to work the same samples with two different methods, which were: 1-Standard curve method and 2-Standard addition (extrapolation) method (13).

Apparatus: All the analysis were performed on the type H 1550 atomic absorption instruments with graphite tube (Rank Hilger, Westwood Margate, Kent Ct9 4JL-England). The major units of this apparatus were: 1-Basic unit, Atomspek H 1550, 2-Power supply unit FA 256, 3-Graphite tube atomiser H 1475, 4- Printer AA 6010.

Vacutainer tubes with red-top (lot No. 6430-6P005, Becton-Dickinson, B.P. No. 37-38241 Meylan Cedex-France) were used to draw blood samples. We used a 20 µl adjustable **automatic pipet** (Gilson, Code No. P 20 H 23600-France) with disposable plastic clinipette-tips (Labora Mannheim GmbH für Labor-technik) for the

delivery of the standards, controls and serum samples onto the graphite tube.

All glass tubes, volumetric flasks and pipets were soaked in nitric acid (2 mol/liter) for 24 hours, then rinsed with doubly de-ionized water about ten times. All reagents used were analytical-reagent grade unless otherwise noted.

Nitric Acid: BDH Chemical Ltd-Prod. No. 10168, (England) is used in the preparation of all the standards and reagents. It had d: 1.42 gr/cm^3, HNO_3=% 69-70.5 and its Cu content was % 0.000002.

The nitric acids used in different concentrations were: 2 mol/L (in cleaning and washing), 1 mol/L (in preparation of solutions), 10 mmol/L (in dilution of serum samples), 1 mmol/L (in preparation of standards).

Standards: Cu standards are prepared basically from two substances.
a- Copper Nitrate Standard Solution, BDH Chemicals Ltd. (England) Prod. No. 14139, contained 1 mgCu/1 ml nitric acid 1 N (≅ 15.7 mmol/L.). This is a solution prepared specially for atomic absorption spectroscopy.
b- "Titrisol" Copper Standard Solution, Merck Prod. No. 9987 (W. Germany). It contained 1000 gr±0.002 gr Cu (CuCl2 in water). It is diluted with 1 mmol/L nitric acid to 1000 ml, to give a last concentration of 1 mg Cu/ml.

Stock Cu standard solutions (100 mg/L), was diluted with 1 mmol/L nitric acid to give working standard solutions having Cu concentrations, respectively as 25, 50, 75, 100, 125, 150 µg/dL in a last volume of 2 ml.

After the standards are prepared, 5 µl of each standard was delivered 3 times to graphite tube and absorbances were measured. Then their means are calculated and with these, the standard curves are drawn. The two standard curves obtained with two different standard chemicals are compared with each other. In the end, when we saw that there was no difference between them, in all the following analyses BDH standard is used.

Standard curve analyses are done in diluted serum, serum diluted 10 fold with 10 mmol/L nitric acid and in water to determine the linearity and sensitivity.

Sample Preparation: Consisted of diluting the serum 10-fold with 10 mmol/L nitric acid, to give a final pH of about 3.0 and a final volume of 2000 µl. In dilution procedure we used polypropylene tubes. All the sera analysed in this study were obtained from the children 1-5 years of age.

Control Serum Samples: We prepared a serum pool from the sera of the 1-5 years aged healthy children and it was divided into aliquots. The analysis of one aliquot of the pooled sample was prepared and analysed for Cu just like the other serum samples. Control serum Cu was always measured, along with the serum samples.

PROCEDURE
AAS apparatus was prepared preanalytically as follows:
Wavelength: 325.5 nm, slit width: 6 (100 µm), cathod lamp current: 4 mA, integration time: 10 s, argon gas flow rate: 5L/min, cooling water flow rate: 1.5-2 L/min.

Power and Time Settings: Dry: 35 s-130°C, Ash: 40 s-800°C, Wait: 5s, Atomise: 9 s-2100°C, Cool: 30 s (automatically). After decontaminating the graphite tube, Atomspek is adjusted to zero and then the sample analysis was begun.

Sample Analysis: Each of the prepared standards, control and the serum samples were pippetted 5 µl onto the graphite tube. Each of the samples was measured three times and the averages of their absorbances were calculated and it was accepted as the value of that sample. After giving approximately 10-12 samples onto the graphite tube, it was cleaned by giving argon gas continuously and applying high temperature about 2500°C. Thus the memory effect was prevented.

Method Works:
1- Standard Curve Method: Standard curve was analysed with standard samples prepared in 1 mmol/L nitric acid. Then in each of the 25 serum samples Cu was analysed and the absorbances versus concentration of Cu in µgr/L was read from this curve. Then for n= 25, $\bar{X}$, SD and SE are established, statistically.

2- Extrapolation Method: 10 fold diluted sera with 10 mmol/L nitric acid were individually (n=25) analysed with and without Cu addition. In each serum sample after measuring the basal Cu level, 10 µL Cu standard (100 mgCu/L) was added each time and then Cu level was measured again. Each time when 10 µL standard, was added, Cu content of that serum was increased by 50 µg/dL. We added 4 times totally, and in the end, final concentration of added Cu happened to be 2 mg/L. The absorbances were shown on the y axis, while the added Cu concentrations were marked on the x axis. The cut point of the linear curve on the negative side of the x axis, gave us the Cu concentration of that serum. After establishing concentrations the Cu in each of the 25 serum samples, in the same way, $\bar{X}$, SD and SE values were calculated, statistically.

RESULTS
Serum Cu mean values established in 25 sera by the Standard Curve and the Extrapolation methods are summarized in table I.

Table I. Comparison of the average serum Cu values measured by two methods (In both of the methods, 10 fold diluted serum with 10 mmol/L nitric acid is used).

METHODS										
	Standard Curve				Extrapolation					
Serum	n	$\bar{X}$	SE	SD	n	$\bar{X}$	SE	SD	t	P
Cu (µgr/dL)	25	78.2	0.07	0.23	25	82.7	0.11	0.35	0.114	N.S.*

* N.S. : Non-significant.

Table II. Recovery data for Cu in diluted and undiluted sera.

Added Cu	Diluted Serum		Undiluted Serum	
(µg/dL)	Cu (µg/dL)	Recovery (%)	Cu (µg/dL)	Recovery (%)
-	62	-	143	-
50	109	97	200	104
100	162	100	221	91
150	233	109	289	99
200	348	133	355	104

Recovery Studies:
To further evaluate the accuracy of the standard curve method, and to see the effect of the dilution of serum with 10 mmol/L nitric acid 10 fold, we performed a series of standard addition (recovery) studies (Table II).

In undiluted serum (2 ml) and in serum (0.2 ml) diluted with 10 mmol/L nitric acid to give a final pH 3.0 and final volume of 2 ml, after measuring the basal Cu levels, 10 µL Cu standard (100 mgCu/L) was added. In each adding of 10 µL standard, the content of that serum was increased by 50 µg/dL (0.5 mg/L). The average percent recovery was % 109.8 in the diluted and % 99.5 in the undiluted serum. For all the recovery studies, we used the pooled control sera.

Interferences and Background Effect: To see the background absorption effect we made measurements with deuterium lamp and without it in each of the diluted and undiluted pooled control serum samples. We observed higher absorbances in the undiluted-serum and falsely higher results whether we used deuterium background corrector or not. But in the diluted sera background absorption was minimal and there was no need for the corrector. We thought the contents of serum caused matrix interference. Therefore, we preferred to work in diluted serum without background correction. In addition, recovery results, supported our use of diluted serum also.

Precision Comparisons:
1- Within-run Precision: On a single day, the same sample was analysed 10-20 and 30 times by the standard curve method and then for n=10, n= 20 and n= 30, the mean ($\bar{X}$), the standard deviation (SD) and the coefficient of variation (% CV) were calculated. Using the extrapolation method, on the same day 10 times,

the same sample was analysed, similarly (Table III).

2- Between-run Precision: One aliquot of a single pooled serum sample, once a day for 10 days, was analysed for Cu by two different methods. At the end of the tenth day, the data for n=10 was calculated for each of the methods used, respectively as $\bar{X}$, SD and % CV (Table III).

Sensitivity:
The limit of detection (LOD) and the limit of quantitation (LOQ) values were found to be as 0.008 and 0.08 respectively. A standard curve was analysed with each set of samples to determine the linearity and sensitivity. The "slope" of the standard curve (sensitivity) varied slightly, depending on the instrumental variables from run to run.

Accuracy:
Standard curves were linear when measured in undiluted serum, diluted serum and in standards in water up to concentrations of 250 µg/dL, 350 µg/dL and 600 µg/dL, respectively. Each point on the standard curves was the average of triplacete analysis. The standard curves remained linear from day to day and only the sensitivity changed minimally. As the controls reflected the same change as the standards, thus the results were accepted to be accurate as long as a standard curve is established with each set of samples.

Table III. Within-and between-run precision values obtained by two methods (In all the analyses, serum samples were diluted 10 fold with 10 mmol/L nitric acid).

Methods	Precision					
	Within-run			Between-run		
Standard Curve	$\bar{X}$	SD	% CV	$\bar{X}$	SD	% CV
n=10	50.6	2.45	4.84	62.1	9.76	15.7
n=20	52.3	6.12	11.6			
n=30	52.0	4.81	9.2			
Extrapolation n=10	52.6	3.5	6.65	67.9	7.12	10.4

DISCUSSION

Copper is one of the easiest elements to measure with adequate accuracy and precision if AAS is used because Cu levels are found to be relatively higher than some trace elements in biological materials. In various literature data, serum Cu levels are said to show age-related changes. For example, serum Cu concentrations are found to be 8.9-45.8 in new-borns, 80.3-150 in 1-5 years, 84-136 in 6-9 years, 80.3-121 in 10-14 years and 75-150 µg/dL in adults (over 15 years). These values are regarded to be normal (14,15). Our serum samples were obtained from the children of 1-5 years of age. We measured in their sera Cu levels both with standard curve and extrapolation methods (Table I) and their values were in accordance with the upper-mentioned data in the same age group. When compared statistically, the results that we obtained by two methods, we found no significant difference between them. Thus, we thought that the standard curve method can be accepted as the routine method for determining serum Cu. In most of the literature data also the same method is recommended for serum Cu measurements with AAS (1, 16-18).

In the result of our recovery studies, we have decided that the recovery was better when 10 fold diluted serum with 10 mmol/L nitric acid (pH 2-3) is used (Table II). Although undiluted serum is recommended by some authors (18, 19) the matrix interference is said to be less and there will be no need for background absorption when diluted serum is used (1, 8). We investigated if there is background absorption or not in our method by using deuterium lamp (in corrected mode) and without it. We observed a false increase in both of the conditions if, undiluted serum is used. We could not establish any significant background absorption when diluted serum is used. In the result because the recovery values were fine and there was minimal difference with background corrector, we did not apply it. We thought that the matrix effect was minimal if diluted serum is used.

The between-run precision is a measure of the stability of the sample and the analysis. While the within-run value is the best precision possible and is due to mostly instrumental variation. It is said that between-run values may be as much as double the within-run precision values (8). Our precision findings were in accordance with some literature data (1, 9, 18).

A standard curve was analysed with each set of samples, daily. It helped us to determine the linearity and sensitivity. Although the slope of the standard curve (sensitivity) showed minimal variations from day to day, the Cu value of the serum pool was found to be approximately the same in every analysis, which we used as the control. This has showed us that our measurement was accurate. The most important performance criterion for any analysis is accuracy. Accuracy includes the linearity of the working curve,

freedom from matrix effects that are potential interferences, minimum blank effects, high-yield standard addition studies, analysis of at least a control sample daily. In our work standard curve was linear, the t test values showed us that there was not statistical significance between the results of the standard curve and extrapolation methods. Matrix effect was minimum when the analysis was performed in diluted serum with 10 mmol/L nitric acid, and there was no need for background correction. LOQ and LOD values were found to be small and these showed us that the blank signal effect was minimal and so the sensitivity of our method was high. Recovery results were fine and they were in accordance with the literature data (9).

In conclusion, in the AAS apparatus with graphite tube, for Cu analysis the best method is the standard curve method. In addition, instrumentally the measure of Cu in biological samples can be achieved with high accuracy, high precision, without background correction and with minimum sample pretreatment if care is taken to plan and implement carefully all the critical steps in the analysis prodecure. It can be performed at a reasonable cost. It is an easy method and can be used routinely in the clinical laboratories.

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