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Spectrophotometric Determination of Copper in Serum Using 6-(2-Naphthyl)-2,3-dihydro-1,2,4-triazine-3-thione

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A simple and accurate spectrophotometric method for determination of copper in serum using 6-(2-naphthyl)-2,3-dihydro-1,2,4-triazine-3-thione (NDTT) has been described. This method is based on the formation of a red coloured complex of copper with a reagent, 6-(2-naphthyl)-2,3-dihydro-1,2,4-triazine-3-thione. The pink coloured Cu-NDTT complex was soluble in chloroform and showed maximum absorbance at 314 nm. In this study, derivative spectrophotometric method is proposed for determination of Cu(II) in serum based on the reaction between copper and new chromogenic compound NDTT. Serum was first treated with trichloroacetic acid for deproteination and ascorbic acid to release Cu from protein. After addition of tartaric acid and NDTT, complex was extracted by chloroform. The optimum reaction conditions and other important analytic parameters have been investigated. Beer's law was obeyed in the copper concentration range of 0.5-6 μg mL^{-1} of serum and the detection limit was 0.106 μg. The linear regression equation was A = 0.04507C–0.0001. The within-day and between-day variations in four selected concentration (0.5, 1, 4, 6 μg/mL) were less than 2.68 and 3.07 %, respectively. The procedure was validated by analyzing control serum samples. The results were in good agreement with the concentration range of copper in the control serum done by atomic absorption. The method was applied to human serum samples with satisfactory results. The proposed method was simple, selective and sensitive for determination of copper in serum.

Key Words: 6-(2-Naphthyl)-2,3-dihydro-1,2,4-triazine-3-thione, Spectrophotometry, Copper.

INTRODUCTION

Copper is both micro-nutrients as well as toxic element for living beings, depending upon the concentration level. The essential role of copper in several biological processes in living organisms is well accepted. It is known that concentration lower than the optimum may lead to a poor performance of the organism; whereas higher

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concentrations may be deleterious or even lethal[1,2]. Therefore, monitoring of the concentration of copper and some other trace elements may be helpful in regulating the proper performance of the body functions, as well as, an important tool in the diagnostic of physical disorders[3]. This explains the increasing interest towards the need for determination of the smaller analyte quantities each time and the search for new, more sensitive analytical methods. Also more developed procedures regarding the extraction and conservation of samples, pretreatments, employment of higher purity reactants should be studied.

Various methods including GC-MS[4], ICP-MS[5], X-ray fluorescence[6], atomic absorption spectrometry[7,8] and voltametry[9] have been used for determination of copper in serum. Many of these methods either are time consuming or required complicated and expensive instruments. Therefore the availability of new, simple, effective and low cost instrumental methods for determination of copper and other metal ions particularly in serum samples is often useful. Spectrophotometric methods generally fall in to the above category of simple and low cost procedures and often utilize a metal complex formation reaction with some chromogenic reagents, for the formation of coloured products[10-12].

The newly synthesized reagent, 6-(2-naphthyl)-2,3-dihydro-1,2,4-triazine-3-thione (NDTT), was used as a chromogenic reagent. 6-(2-Naphthyl)-2,3-dihydro-1,2,4-triazine-3-thione forms a red complex with Cu^{2+}, which is easily extractable with chloroform over a wide range of pH[13]. In the present work, the simple and convenient spectrophotometric method for determination of copper in serum based on the complex formation with the new chromogenic reagent NDTT is reported.

EXPERIMENTAL

Absorption measurements were performed with a spectrophotometer (Shimadzu 160 a, Japan) at 200-800 nm. To prepare serum samples a Hettich-EBA-20 centrifuge was used.

The reagent, 6-(2-naphtyl)-2,3-dihydro-as-triazine-3-thione (NDTT) (Fig. 1) was synthesized by reaction of 2-acetylnaphthalene with amyl nitrite under anhydrous conditions. It produced 2-naphthylglyoxaldoxime which upon reaction with thiosemicarbazide yielded 6-(2-naphthyl)-2,3-dihydro-1,2,4-triazine-3-thione[13]. Its 0.0002 M solution in sodium hydroxide 1 M was employed as complexing agent. Copper, tartaric acid, ascorbic acid, trichloroacetic acid and chloroform were of analytical grade and purchased from Merck (Darmstadt, Germany). Universal control serum was purchased from trulab-N and prepared according to its direction.

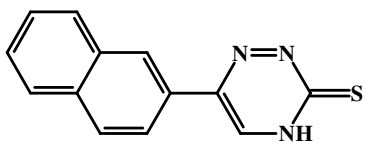

Fig. 1. Chemical structure of 6-(2-naphtyl)-2,3-dihydro-1,2,4-triazine-3-thione (NDTT)

Reagent solutions: 6-(2-Naphthyl)-2,3-dihydro-1,2,4-triazine-3-thione solution (0.0002 M) was prepared by dissolving proper amount of pure reagent in sodium hydroxide (1 M). It should be freshly prepared before use.

Standard solutions: Copper nitrate solution: The standard Cu(II) solution was prepared by digestion of a 100 mg copper metal (99.9 %) in 5 mL concentrated nitric acid. The solution was heated up to dryness. The dried mass was dissolved in 5 mL of nitric acid solution (1:1) and diluted to 100 mL with distilled water. The working standard solutions were prepared by diluting with distilled water.

Ascorbic acid solution: The solution of 1 M ascorbic acid was prepared by dissolving the proper amount of Merck reagent in buffer solution (pH = 4).

Serum samples: All experiments for method optimization were performed using a single lot of a pooled human serum collected at the Children Hospital Medical Center. The standard addition method was used for calibration curve.

Spectrophotometric measurements: One mL of serum sample was treated with 1 mL of trichloroacetic acid (0.6 M) and 1mL of ascorbic acid (0.05 M). After centrifugation (5000 RPM, 5 min), supernatant was transferred to a 100 mL separator funnel and 1 mL of standard Cu^{2+} solution (0.5-6.0 mg/mL), 1 mL of tartaric acid (1 M) and 2 mL of NDTT (0.0002 M) were added together. Then the volume was brought up to 10 mL with deionized water. The resulting complex was extracted with 4, 3, 2 mL chloroform and the total organic phase was made up to the mark with chloroform in 10 mL volumetric flask. The absorbances of the organic phase were recorded in the range of 200-800 nm against blank solution which was prepared in parallel and contained all reagents without Cu^{2+}.

Linearity: The calibration curve of Cu(II) ion was constructed using 5 series of standard copper solutions (0.5, 1, 2, 4, 6 μg/mL) in 2 mL of serum with UV-visible spectrophotometer at 314 nm. The regression equation and the detection limit were determined.

Accuracy and precision: To establish within-run precision 3 series of solutions containing 0.5, 1, 4 and 6 μg/mL of standard copper in 1 mL serum were prepared, respectively and analyzed according to described method. Three series of these samples were assessed in one day using their corresponding calibration curves. Between-run precision was determined by analyzing similar concentrations on three different days within one month. Eight samples of control serum Trulab-N were analyzed according to the procedure. To obtain the accuracy of the method the percentage of error was calculated.

RESULTS AND DISCUSSION

The aim of this work is to develop a spectrophotometric method for determination of copper in serum. In previous study it was shown that Cu(II) reacts with newly synthesized reagent, NDTT, giving a pink complex in basic medium. The spectral characteristic has been discussed before[13].

Absorption spectra: The absorption spectra of the Cu-NDTT complex in chloroform against a reagent blank prepared under similar condition was recorded. It showed a maximum absorption at 314 nm that was used for determination of copper in serum samples.

Calibration curve and detection limit: The calibration curves were constructed according to calibration curve procedure. Beer's law is obeyed over the concentration range 0.5-6 μg/mL of serum at 314 nm with equation of Y = 0.04507X - 0.0001. The correlation coefficient (R^2) was 0.9997 showing good linearity of calibration curve. The limit of detection (LOD) was as low as 0.106 μg/mL based on the blank average signal plus three times the standard deviation of blanks. The results are summarized in Table 1.

TABLE-1
STATISTICAL DATA OF CALIBRATION CURVES OF Cu-NDTT WITH DIFFERENT CONCENTRATIONS

Parameters	Copper
Linearity	0.5-6 μg/mL
Regression equation	Y = 0.04507X – 0.0001
SD of slope	0.00043
RSD of slope	0.95
SD of intercept	0.014
Correlation coefficient	0.9997

Accuracy and Precision: The precision of the proposed method was examined by determining the relative standard deviation (RSD) of three replicate analyses on the same solution containing 0.5, 1, 4 and 6 μg/mL of standard copper in 1 mL of serum. The within-day RSD at copper concentration 0.50, 1, 4 and 6 μg were 2.68, 0.99, 0.33 and 0.12 %, respectively. The low values RSD (less than 2.68 %) in different solutions reflect the high precision of the proposed method.

The accuracy of the proposed method was examined by performing recovery experiments using same aqueous solutions containing known amounts of copper. High values of recovery imply high accuracy of proposed method.

The low values of between-day RSD for the same concentrations of copper (less than 3.07 %) proved the above results. Errors lower than 3.86 % were observed in all cases (Table-2).

TABLE-2
ACCURACY AND PRECISION DATA OF DETERMINATION OF COPPER (0.5-6 μg/mL) OF SERUM

Added (μg/mL)	Within-day (n=3)			Between-day (n=9)		
	Found (μg/mL)	CV (%)	Error (%)	Found (μg/mL)	CV (%)	Error (%)
0.5	0.48 ± 0.013	2.68	-3.86	0.49 ± 0.015	3.07	-2.35
1.0	1.02 ± 0.010	0.99	1.49	1.03 ± 0.020	1.95	3.32
4.0	4.01 ± 0.013	0.33	0.30	3.98 ± 0.041	1.05	-0.55
6.0	5.98 ± 0.007	0.12	-0.25	6.01 ± 0.025	0.42	0.23

Application to biological sample: To ensure the accuracy and reliability of the developed method eight samples of control serum (Trulab N) with a certified amount of Cu analyzed. The relative standard deviation of 8 measurements, performed with 2 mL of control serum was 4.7 %. The results showed that good agreement was obtained between the estimated content by the developed method and certified value (Table-3).

TABLE-3
CONCENTRATION OF COPPER (μg/mL) IN CONTROL SERUM (TRULAB-N)

Control serum (Trulab N)	Cu found (μg/dl)		
	Proposed method ± SD (n = 8)	Reference method	
		Atomic absorption	GC-MS
Range	114-127	102-147	80.9-116
Mean ± SD	122.01 ± 5.11	125	98.7
RSD	4.19 %	–	–
Error	*2.39 %	–	–

*Error is calculated according to the results of atomic absorption method.

Determination of copper in human serum: Copper occurs in serum at the low level between 70-150 μg/dl. Nine people were tested for the Cu level in serum (four women and five men). The samples deproteinized with trichloroacetic acid as reported and were analyzed by the procedure described above (Table-4).

TABLE-4
CONCENTRATION OF COPPER (μg/mL) IN HUMAN SERUM

Individual	Sex	Age	Cu concentration (μg/dl)
1	Female	40	62.61
2	Female	45	69.29
3	Female	25	115.83
4	Female	44	68.18
5	Male	60	100.30
6	Male	55	93.65
7	Male	28	95.85
8	Male	25	102.50
9	Male	39	102.50

Conclusion

In present studies, determination of copper in serum by spectrophotometry with the newly synthesized reagent, 6-(2-naphthyl)-2,3-dihydro-1,2,4-triazine-3-thione (NDTT) has been proposed. The elaborated method was simple, selective and reliable.

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