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A Modified Spectrophotometric Micromethod to Determine Serum Copper*

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Abstract: Most of the available spectrophotometric methods to determine serum copper requires large volume of sample and involves several steps. We modified literature known method to estimate serum copper. Guanidine hydrochloride was used to release ceruloplasmin bound copper and determined using bathocuproine disulphonate disodium salt (BCDS). Our modified micromethod correlated well with the literature known method and shown good recovery and precision. Modified spectrophotometric micromethod is simple, sensitive, rapid, requires very less sample and coloring reagent and can be adopted in any clinical laboratory setups.

Key words: Serum copper, guanidine hydrochloride, bathocuproine disulphonate disodium salt

INTRODUCTION

Over the years, several procedures have been reported to determine copper in biological fluids. Most of the available methods are based on spectrophotometric (Landers and Zak, 1958; Williams *et al*., 1977; Kossman, 1983), Atomic Absorption Spectrophotometry (AAS) (Meret and Henkin, 1971; Makino *et al*., 1981) or neutron activation analysis (Smeyers-Verbeke and Massart, 1973). Although methods based on AAS are most accurate, specific, reproducible and reliable (Meret and Henkin, 1971) but it is costlier and the instrument is not available in all the clinical laboratories.

Spectrophotometrically copper in biological fluids has been determined using various chromogens like diethyl dithiocarbamate, biquinoline, dimethyl diphenyl phenanthroline (Mikac-Devic, 1969) and BCDS (Landers and Zak, 1958; Zak, 1958; Kossman, 1983). All spectrophotometric methods that are in use either contain several steps or require large amount of sample (Landers and Zak, 1958; Mikac-Devic, 1969). Various micromethods are also available to determine copper using small sample volume but involve several steps comprising plasma protein precipitation, centrifugation, extraction into organic solvent, or incubation at low and high temperatures (Kossman, 1983; Smeyers-Verbeke and Massart, 1973; Mikac-Devic, 1969). Measurement of serum copper is required in number of clinical situation like Wilsons disease, chronic renal failure and in neonatal neurological disorders. Due to lack of availability of simple and cost effective methods serum copper estimation is not routinely done in all small laboratory set ups. In this study, we have modified literature known method into few simple steps to determine serum copper, which can be adopted in any clinical laboratory set-ups.

MATERIALS AND METHODS

Pooled serum samples were obtained from Department of Biochemistry, Clinical Laboratory Division, which was sent for routine analysis. BCDS and guanidine hydrochloride were obtained from

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Sigma Chemicals, St. Louis, MO, USA. All others reagents were of analytical grade. Metal free double distilled deionized water was used throughout the study. Glass wares were acid washed and all necessary precautions were taken to avoid trace element contamination.

Reagents

Stock Copper Standard

Copper sulphate ($CuSO_4.5H_2O$) (100 μg mL^{-1}), working copper standard (2 μg mL^{-1}), 0.09 M hydroquinone in 1 N HCl, 0.89 mM BCDS in sodium acetate and 0.89 mM BCDS in 2 M tris solution, 0.1 M sodium acetate/acetic acid buffer pH 4.8, 6 M guanidine hydrochloride and 0.1 M ascorbic acid in 0.1 M acetate buffer pH 4.8.

Procedure

Calibration Curve

Serial copper working standards were prepared with concentration ranging from 40 to 200 μg dL^{-1}. One hundred microliter of each copper working standard was taken in different tubes into which 200 μL of guanidine hydrochloride containing ascorbate was added. After incubating at room temperature for 10 min, 200 μL of BCDS in tris was added and the volume is made up to 500 μL by using tris solution, vortex mixed and incubated at room temperature for 5 min and absorbance was read at 480 nm using Genesys 10 UV spectrophotometer. Calibration curve was prepared after subtracting absorbance of each standard by absorbance of reagent blank.

Sample

Three tubes were labeled as Sample Test (ST), Sample Blank (SB) and Reagent Blank (RB). One hundred microliter of serum was added to each ST and SB and 100 μL of tris solution to RB. Two hundred microliter guanidine hydrochloride containing ascorbate was added to each tubes, vortex mixed and incubated at room temperature for 10 min. 200 μL BCDS in tris was added into each tubes except SB, into which 200 μL of tri sol added. Absorbance was read at 480 nm after 5 min. The corrected absorbance of the sample (ST-{SB + RB}) was used to determine amount of copper by using extinction co-efficient derived from the calibration curve.

We have compared our modified micromethod with that of literature known method (Landers and Zak, 1958) using 22 serum samples. Accuracy of the assay was checked by recovery experiments where serum was enriched with known concentrations of copper and analyzed by the proposed micro method. The reproducibility of the proposed micro method was checked by precision assay where same serum sample was analyzed for within-run, within-day and day-to-day variations.

RESULTS AND DISCUSSION

We found 200 μL of 0.89 mM BCDS was sufficient for chromogen formation (Fig. 1) when compared to other methods requiring 500 μL of BCDS (Landers and Zak, 1958). We have rechecked the absorbance maxima of copper-BCDS chromogen and it was found to be at 480 nm. We have utilized the fact that a guanidine hydrochloride at its concentration of 4 M or greater was sufficient to completely relax and alter the tertiary structure releasing protein bound metal ions (Tanford *et al*., 1966). The copper released from ceruloplasmin will form complex with copper specific chromogen BCDS, which can be estimated spectrophotometrically. The extinction co-efficient calculated from the Cu^{+}-BCDS standard graph was 2.388×10^{3} M^{-1} cm^{-1} (Fig. 2).

The minimum detection limit of our proposed micromethod is 0.4 μg mL^{-1} as that of literature known method (Landers and Zak, 1958). Thus our micromethod is as sensitive as the currently followed spectrophotometric method. Serum copper values of our modified micromethods compared

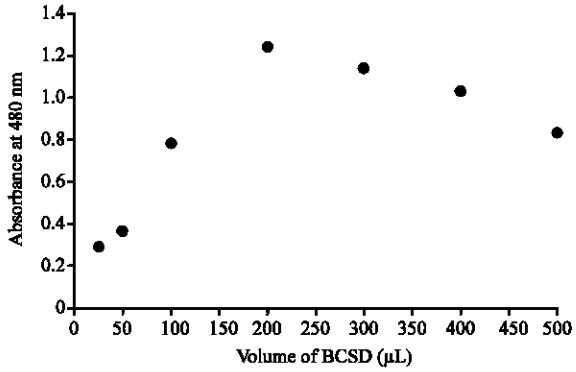

Fig. 1: Optimum volume of BCDS color reagent required for chromogen formation, keeping concentration of copper standard constant (600 µg dL^{-1})

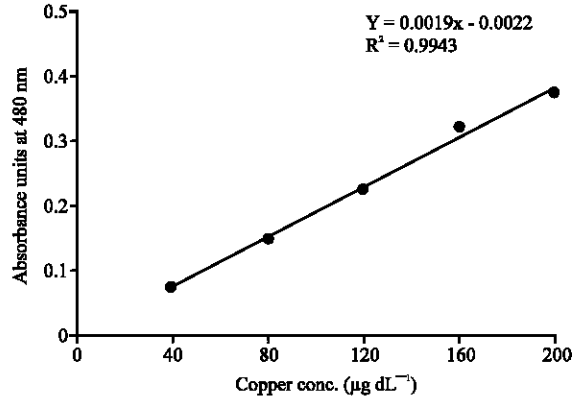

Fig. 2: Calibration curve of copper-BCDS complex using guanidine hydrochloride

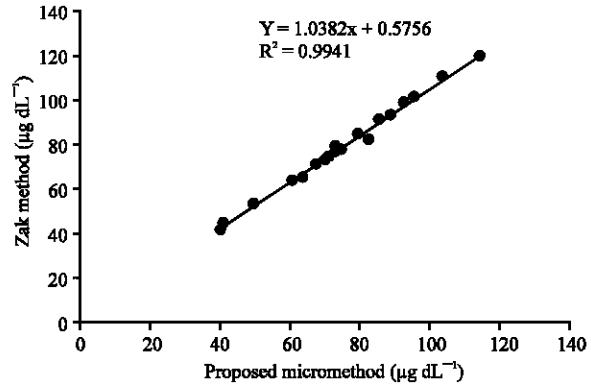

Fig. 3: Comparison between proposed micromethod with literature known method

well with that of literature known method. The resulting correlation coefficient was $r^2 = 0.9941$ (Fig. 3). Recoveries were related linearly to serum copper concentration over wide range with mean value of 92.3% of copper being recovered (Table 1). The precision of the proposed method is statistically significant with coefficient of variance (CV) of about 2% in all the three assays; within-run, within-day and day-to-day variations assay (Table 2). We measured serum copper levels (in μg dL^{-1}) using both our modified micromethod and literature known method in ten serum samples each of healthy individuals (112.40±2.2, 110.60±1.2), chronic renal failure (210.45±4.8, 212.42±2.4) and hyperlipidemia (54.6±4.2, 56.1±3.2), respectively.

The rise in copper level has been reported in various diseases like chronic renal failure (Navarro-Alarcon *et al*., 2006; Panichi *et al*., 2004), Hodgkin's lymphoma (Cunzhi *et al*., 2001), brain tumors (Floriañczyk *et al*., 2003). In these conditions, the amount of sample that is available for determination of copper along with other battery of tests that are done for the patient management will be less specially in neonatal disorders. So it becomes important to minimize the sample volume. To our knowledge the modified micromethod needs less sample (100 μL) when compared to other spectrophotometric methods which requires 1000 μL of sample (Landers and Zak, 1958).

Use of guanidine hydrochloride in our proposed micromethod avoids protein precipation and denaturation steps used in literature known methods and also it avoids the additional steps like centrifugation. This simple modification makes our proposed micromethod simple and avoids possible trace element contamination present in steps like precipitation and centrifugation. The proposed micromethod is simple without plasma protein precipitation, centrifugation, or extraction steps. Our proposed method is as sensitive as the existing spectrophotometric method. Although AAS is more accurate method to estimate copper in biological samples (Meret and Henkin, 1971; Smeyers-Verbeke and Massart, 1973), but the cost factor restricts its use in small laboratories. The difference in copper estimation between AAS and spectrophotometry was studied by Smeyers-Verbeke and Massart (1973) and observed only small difference (2%) and such a small difference can be compromised in situations where the AAS is not available because of its cost and expertise to handle it.

In conclusion, our modified micromethod is simple, sensitive, rapid, cost effective, requires less amount of sample and color reagent. The method can be adopted in any clinical laboratories using simple spectrophotometer.

Table 1: Analytical recovery of the modified micromethod to determine serum copper (μg dL^{-1})

Added	Expected	Found	Recovered (%)
0	-	50.8	87.80
25	75.8	72.3	91.96
50	100.8	96.6	94.58
75	125.8	121.2	94.92
100	150.8	145.0	94.92

Table 2: Precision of the modified micromethod to determine serum copper (μg dL^{-1})

Within-run	Run-to-run	Day-to-day
51.3	51.0	49.8
49.2	48.9	49.3
48.8	47.8	48.1
50.2	50.8	50.3
49.6	49.2	48.4
51.0	50.6	50.3
48.7	50.1	49.2
47.9	48.9	50.8
49.9	51.4	47.4
48.2	49.5	49.6
Mean±SEM	Mean±SEM	Mean±SEM
49.48±0.35	49±0.36	49.32±0.34
CV	CV	CV
2.3	2.3	2.3

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