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A Copper Determination Method Based on the Reaction between 2-(5-Nitro-2-Pyridylazo)-5-(N-Propyl-N-Sulfopropylamino) Phenol (Nitro-PAPS) and Copper for Use in Urine Copper Measurement and Application to Automation

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Aim: To develop a sensitive automated colorimetric urine copper determination method using a water soluble compound, 2-(5-nitro-2-pyridylazo)-5-(N-propyl-N-sulfopropylamino) phenol (nitro-PAPS), as a ligand.

Materials and Methods: The new photometric method using sodium dodecyl sulfate (SDS)-ascorbic acid to dissociate copper from proteins in acetate buffer (pH 3.2) and nitro-PAPS as ligand was also adapted to an automated analyzer. Copper concentrations were determined by atomic absorption spectrophotometric method and by photometric method in 24 h urine samples of patients (n = 100) with various types of diseases including Wilson's disease (n = 12), diabetes mellitus (n = 34), osteoporosis (n = 27), nephrotic syndrome (n = 17), and rheumatoid arthritis (n = 10).

Results: There was a statistically significant correlation ($P < 0.01$) between our new method and the atomic absorption spectrophotometric method ($y = 0.994x + 0.207$, $Sy/x = 1.776$), and the photometric method was linear up to 200 µg/l concentration. Other complexing metals (Zn^{+2} and Fe^{+2}) had no interfering effect on the Cu-nitro-PAPS reaction. The analytical recovery of copper was between 90% and 107% (mean 98%). Within-run and between-run CVs were <5% in normal and high copper containing urine pools.

Conclusions: We have developed a new sensitive and reliable automated colorimetric urine copper determination method.

Key Words: Urine copper, photometric method, nitro-PAPS

2-(5-Nitro-2-Pyridylazo)-5-(N-Propyl-N-Sulfopropylamino) Phenol (Nitro-Paps) ve Bakır Arasındaki Reaksiyonu Temel Alan Metodun İdrar Bakır Ölçümü için Kullanılarak Otomasyona Uyarlanması

Amaç: Bu çalışmada amacımız bağlayıcı olarak suda çözünen 2-(5-Nitro-2-pyridylazo)-5-(N-propyl-N-sulfopropylamino) fenol (Nitro-PAPS) bileşiği kullanarak hassas, otomatize, kolorimetrik bir idrar bakır ölçüm yöntemi geliştirmektir.

Yöntem ve Gereç: Yeni fotometrik metotta kullanılan sodium dodesil sülfat (SDS)-askorbik asit asetat tampon içerisinde (pH 3,2) bakırı proteinlerden ayırır. Bağlayıcı olarak kullanılan Nitro-PAPS aynı zamanda otoanalizöre de adapte edildi. Wilson (n = 12), Diabetes Mellitus (n = 34), Osteoporoz (n = 27), Nefrotik Sendrom (n = 17) ve Romatoid Artrit (n = 10) hastalarını içeren değişik tipteki toplam 100 hastanın 24 saatlik idrar örneklerinde bakır konsantrasyonları atomik absorbsiyon spektrometrisi (AAS) ve fotometrik metot kullanılarak ölçüldü.

Bulgular: Geliştirdiğimiz fotometrik metot ve AAS ($y = 0,994x + 0,207$, $Sy/x = 1,776$) arasında istatistiksel belirgin bir ilişki vardı ($P < 0,01$) ve fotometrik metot 200 mg/l konsantrasyon üzerinde lineerdi. Diğer kompleks metaller (Zn^{+2} ve Fe^{+2}) Cu-Nitro-PAPS reaksiyonun üzerine herhangi bir interferasyon göstermemektedir. Bakırın analitik verimliliği % 90 ile %107 arasında (ortalama: % 98) idi. Normal ve yüksek miktarda bakır içeren idrar havuzunda değerlendirilen çalışma içi ve çalışmalar arası CV <% 5 olarak bulundu.

Sonuç: İdrar bakır ölçümü için hassas, güvenilir ve otomatize kolorimetrik idrar bakır ölçüm yöntemi geliştirdik.

Anahtar Sözcükler: İdrar bakır, fotometrik metot, Nitro-PAPS

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Introduction

Determination of copper in urine is important in the diagnosis of an inborn error of metabolism, Wilson's disease, and various kinds of anemia. Furthermore, abnormal concentrations of copper observed in patients with rheumatoid arthritis (1), abnormal pregnancies (2), or malignancies (3). Copper accumulates in the liver, brain, kidney, and cornea, and urinary excretion of copper is increased. Especially in the diagnosis of Wilson's disease, urine copper determination should be performed because serum copper determination is not sufficient for this purpose in the diagnosis of this disease.

Urine copper is routinely determined by atomic absorption spectrophotometer (AAS) in clinical laboratories. Although AAS is the preferred method, for laboratories without an AAS alternative methods are not available. Due to the extremely low concentrations of urine copper, the colorimetric assays do not have sufficient analytical sensitivity. However, such colorimetric methods are used for serum copper determinations (4-6), in which a sensitive, water-soluble compound, 2-(5-nitro-2-pyridylazo)-5-(N-propyl-N-sulfopropylamino) phenol (nitro-PAPS), is used as a ligand.

We developed a new sensitive colorimetric urine copper determination method using sodium dodecyl sulfate (SDS)-ascorbic acid to dissociate copper from proteins in acetate buffer (pH 3.2) and used nitro-PAPS as ligand. The specific absorbance change near 550 nm wavelength by Cu-Nitro-PAPS complex was directly proportional to the urine copper concentrations.

Materials and Methods

Reagents

All chemicals were reagent grade and commercially available from Sigma Chemical Ltd., IL, USA.

Acetate buffer, 0.4 M; 3.281 g anhydrous of sodium acetate was dissolved in deionized water. Then pH was adjusted to 3.2 with 0.1 N HCl; 3 g of SDS was dissolved in 100 ml of acetate buffer.

Ascorbic acid solution, 20 mg of ascorbic acid was dissolved in 10 ml of acetate buffer.

Nitro-PAPS solution, 7 mg of nitro-PAPS was dissolved in 100 ml of deionized water.

Cu Standards, First, we prepared stock standard (500 µg/l) by adding 50 µl of atomic absorption stock standard (1 g/l) to 100 ml of water. Then we prepared working standards with 10, 20, 30 and 50 µg/l Cu concentrations using appropriate dilutions of stock standard.

Instruments

Atomic absorption spectrophotometric analyses were performed using a Perkin Elmer AAnalyst 800 Atomic Absorption Spectrophotometer (Perkin Elmer Inc., Germany). A Technicon RA-1000 analyzer (Technicon Ltd., Swords, Co. Dublin, Ireland) was used for spectrophotometric determinations. We used a high sample volume (300 µl) to improve the sensitivity of the test. Because the instrument could take a maximum 75 µl volume sample, we put samples in the first reagent position on the reagent tray of the RA-1000 analyzer in order for the instrument to take 300 µl of sample. The first reagent, ascorbic acid solution, was put on the sample tray instead of samples. The second reagent, nitro-PAPS solution, was in the second reagent position of the instrument. Test parameters of urine copper determination in the Technicon RA-1000 are shown in Table 1.

Table 1. Chemistry parameters for urine copper determination on Technicon RA-1000 analyzer.

TYPE	2
%SMP VOL	60
FILTER POS	5 WL 550
DELAY	5 00
%RGT VOL	60
2 RGT VOL	8
A2 DELAY	2 45
RBL LOW	0.000
RBL HI	2.000
CAL FACTOR	2487.84
RGT BLANK	0.0616
STD VAL	500
SLOPE	1.000
INTERCEPT	0.000
EP LIM	0.0800

Samples

Patients (n = 100) with various types of diseases, including Wilson's disease (n = 12), diabetes mellitus (n = 34), osteoporosis (n = 27), nephrotic syndrome (n = 17), and rheumatoid arthritis (n = 10), collected their 24 h urine samples in clean bottles. Samples were taken from the patients immediately after the end of the collection. Thereafter measuring urine volumes, pH of 50 ml of each sample was adjusted to 3.2 with acetic acid prior to colorimetric determination. Then 1 ml of each sample was used for the AAS determination of copper.

The concentration of each specimen was calculated from a daily, freshly prepared calibration with a reagent blank and 500 µg/l of aqueous standard. We checked the calibration with 5 working standards (10, 20, 30, 50 and 200 µg/l) before determining the copper concentrations of urine samples.

Statistical procedures

A linear regression analysis comparing the copper results obtained by our colorimetric method with those obtained by atomic absorption spectrophotometric determinations on 100 specimens was performed using SPSS for Windows (ver. 11.0).

Results and Discussion

Optimization of colorimetric method

pH and buffer selection, Nitro-PAPS complexes with copper but not zinc at pH 3.2 were previously reported (7). Thus, the buffer and samples were adjusted to pH 3.2. Due to the lower logarithmic stability constant of acetate buffer for copper (8) we selected acetate buffer for reaction media.

Effects of interferences, We analyzed the interfering effects of other metal ions (Fe^{+2} and Zn^{+2}) complexing with nitro-PAPS by adding certain concentrations of such metals (500 µg/l of each metal ion) to copper standard and patient samples. Other complexing metals (Zn^{+2} and Fe^{+2}) had no interfering effect on the Cu-nitro-PAPS reaction.

Recovery, Various amounts of copper standards (10-50 µg/l) were added to urine samples (n = 10). Then we determined copper concentrations of standard added and non-added samples. The analytical recovery of copper (Table 2) was between 90% and 107% (mean 98%).

Precision, Urine samples' pools containing normal and high copper concentrations (24 and 171 µg/l, respectively) were used for the precision study. Within-run and between-run (in 1 month period) CVs were estimated in both normal and high copper containing urine pools (Table 3).

Linearity, Standard curves prepared with individual copper standards (10, 20, 30, 50, and 200 µg/l) were straight up to 200 µg/l (Figure 1).

Table 2. Analytical recovery studies of urine copper (recovered copper was the copper concentration difference between copper-added and non-added (endogenous) urine samples).

Cu Concentrations, mg/l			
Endogenous	Added	Recovered	Recovery, %
14	10	10	100
23	20	19	95
30	30	31	103
28	50	49	98
8	100	100	100
44	10	9	90
70	20	19	95
25	30	32	107
33	50	48	96
20	100	97	97
Mean			98

Table 3. Precision studies.

Urine pools	Mean Cu, µg/l	CV, % Within-run	Between-run
Normal	24	4.2	3.8
High	171	2.9	2.5
Number of replicates		20	20

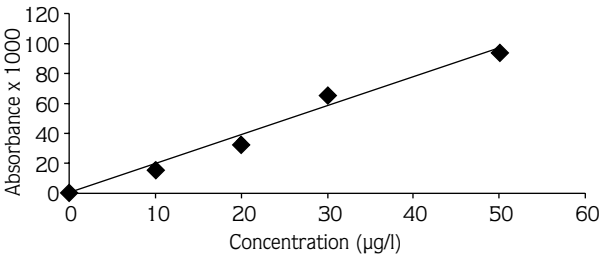

Figure 1. Linearity of the new photometric method.

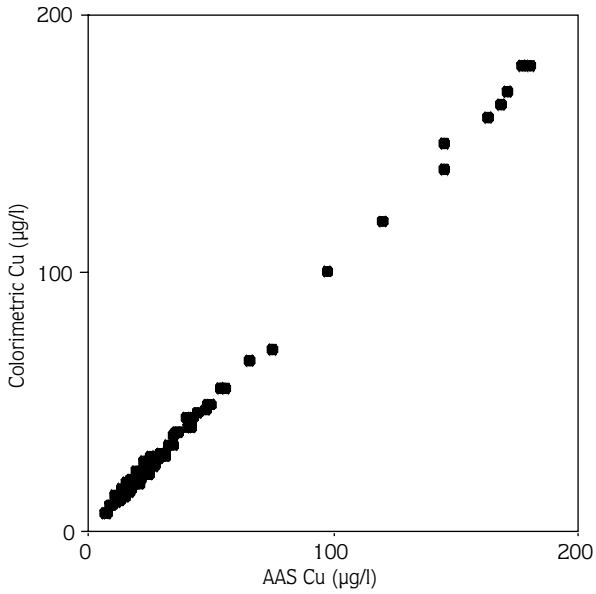

Figure 2. Correlation between AAS and the new photometric methods.

Method Comparison

The mean ± SD values of copper concentrations of urine samples determined by colorimetric and AAS methods were 30.6 ± 21.9 and 32.1 ± 22.1 µg/l, respectively. There was a statistically significant correlation ($P < 0.01$) between our new method and the AAS method ($y = 0.994x + 0.207$, $Sy/x = 1.776$) (Figure 2).

Nitro-PAPS is water soluble and has a sensitivity 4.5 times higher than that of 2-(2-thiozolylazo)-4-methyl-5-sulfomethylaminobenzoic acid for copper assay (6). The standard curves prepared with individual copper standards were straight up to 200 µg/l of copper.

AAS is highly sensitive for copper determination but requires sample pretreatment such as deproteinization and extraction. Our new colorimetric urine copper determination method was as sensitive as the AAS method for the determination of copper in urine. On the other hand, this method is cheaper and faster than the AAS method. The 2 techniques correlated well. Each method was transferable among laboratories.

We conclude that the present method is a simple, sensitive, reproducible, and useful procedure for the assay of copper in urine.

References

1. Youssef AAR, Wood B, Baron DN. Serum copper: a marker of disease activity in rheumatoid arthritis. J Clin Pathol 1983; 36: 14-17.
2. Buamah PK, Russell M, Milford-Ward A, Taylor P, Roberts DF. Serum copper concentration significantly less in abnormal pregnancies. Clin Chem 1984; 30: 1676-1677.
3. Lightman A, Brandes JM, Binur N, Drugan A, Zinder O. Use of the serum copper/zinc ratio in the differential diagnosis of ovarian malignancy. Clin Chem 1986; 32: 101-103.
4. Willims HL, Johnson DJ, Haut MJ. Simultaneous spectrophotometry of Fe^{2+} and Cu^{2+} in serum denaturated with guanidine hydrochloride. Clin Chem 1977; 23: 237-240.
5. Abe A, Yamashita S, Noma A. Sensitive, direct colorimetric assay for copper in serum. Clin Chem 1989; 35: 552-554.
6. Yamashita S, Abe A, Noma A. Sensitive, direct procedures for simultaneous determinations of iron and copper in serum, with use of 2-(5-Nitro-2-pyridylazo)-5-(N-propyl-N-sulfopropylamino) phenol (Nitro-PAPS) as ligand. Clin Chem 1992; 37: 1373-1375.
7. Makino T, Kyonaga M, Kina K. A sensitive, direct colorimetric assay of serum iron using the chromogen, nitro PAPS. Clin Chim Acta 1988; 171: 19-28.
8. Dawson RMC, Elliott DC, Elliott WH, Jones KM. Data for biochemical research 3rd ed. Oxford: Clarendon Press 1986; p 411.