A bis-azo dye as a chromogenic reagent for determining traces of copper in foodstuffs, blood sera and body tissues

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Manuscript received 10 February 2003, revised 6 February 2004, accepted 24 August 2005

Abstract : A newly synthesized bis-azo dye, 2,6-bis(1-hydroxy-2-naphthylazo)pyridine has been found to be a very sensitive and highly selective reagent for determining copper. Beer's law is followed up to 1.6 ppm with an optimum concentration range of 0.25-1.0 ppm of Cu$^{II}$. Sandell's sensitivity was calculated to be 0.0012 µg cm$^{-2}$ with molar absorptivity of 5.2 x 10$^4$ dm mol$^{-1}$ cm$^{-1}$ at 570 nm. Copper has been determined with the reagent in milk, food-grains, tea-leaves, human blood sera and body tissues.

Keywords : Chromogenic reagent, copper determination, spectrophotometry, bis-azo dye, blood sera, body tissues, food stuffs.

Heterocyclic azo dyes$^1$ (PAN and PAR) have been found to be the excellent chromogens for use in spectrophotometric determination of metal ions. In this laboratory the chromogens such as 2-(4,6-dimethyl-2-pyrimidylazo)-1-naphthol-4-sulfonic acid, 4-(2,6-diamino-4-pyrimidylazo) phenol and 2,6-bis(7-hydroxyacenaphthyl-8-azo)pyridine have been synthesized and utilized$^2$. This paper describes the results of synthesis of 2,6-bis(1-hydroxy-2-naphthylazo)pyridine (PBN) and its utility in determining traces of copper in foodstuffs, blood sera and body tissues by spectrophotometry.

Results and discussion

The reagent and its colour reactions:

It is evident from the literature that an azo dye of α-naphthol can be obtained by reacting 1,2-naphthoquinone with an aromatic hydrazine$^3,4$. Thus a bis-azo dye was obtained by reacting 2 moles of 1,2-naphthoquinone with 1 mol of 2,6-dihydrazinopyridine. Its infra-red spectrum confirmed that the compound obtained has an enol-form. The dye showed a light orange colour up to pH 9.0. Below pH 1.5, a hypsochromic shift of 5 nm was observed but a bathochromic shift of 43 nm was seen above pH 9.0. The molar extinction coefficients (ε, dm mol$^{-1}$ cm$^{-1}$) of the dye at different pH values are 1.8 x 10$^4$ (480 nm, pH 0.9), 1.9 x 10$^4$ (485 nm, pH 6.0) and 2.7 x 10$^4$ (528 nm, pH 11.0) The pK$_{NH}$ and pK$_{OH}$ values are 2.2 ± 0.2 and 19.16 as determined spectrophotometrically.

The ethanolic solution of PBN gave very deep colour with a number of metal ions at different pH values i.e. deep blue to violet colour with zinc(II), cadmium(II), mercury(II), copper(II), silver(I), cobalt(II), nickel(II), manganese(II) in neutral to alkaline media; violet colour with iron(II), vanadium(V) and thalium(I) in alkaline media; green colour with palladium(II) in neutral to alkaline media and a pink colour with chromium(III) in alkaline media. In all these colour reactions the ethanol concentration was kept above 50% to avoid precipitation.

Preliminary studies on colour reactions of PBN with metal ions also showed that in a phosphate buffered medium, only copper(II), iron(II), cobalt(II), nickel(II) and mercury(II) gave coloured complexes. The composition of the complex formed was 1:1 as determined by Job's method of continuous variations. The following tentative structure of the complex of bivalent metals with the bis-azo dye may be proposed as:

Further investigations revealed that addition of 2% solution of sodium citrate in phosphate buffered medium
Table 1. Contents of copper in various food-stuffs and biological samples

| Sample                  | No. of analyses | Sample ashed (ml) or (g) | Cu found in whole sample using PBN (µg) | Cu found in whole sample using AAS (µg) | Range of Cu levels (mg/100 ml) or (mg/100 g) |
|-------------------------|-----------------|--------------------------|----------------------------------------|----------------------------------------|-----------------------------------------------|
| **(a) Milk samples:**  |                 |                          |                                        |                                        |                                               |
| Cow                     | 4               | 100                      | 25.5, 20.8,                            | 22.9, 20.7,                            | 0.0208-0.0253                                 |
|                         |                 |                          | 21.7, 25.3                            | 22.1, 25.5                            |                                               |
| Buffalo                 | 4               | 100                      | 24.1, 24.9,                            | 24.1, 24.7,                            | 0.0225-0.0303                                 |
|                         |                 |                          | 30.3, 22.5                            | 29.7, 22.6                            |                                               |
| Goat                    | 4               | 100                      | 39.5, 43.0,                            | 39.5, 42.9,                            | 0.0375-0.0430                                 |
|                         |                 |                          | 42.5, 37.5                            | 42.0, 38.5                            |                                               |
| **(b) Food samples:**  |                 |                          |                                        |                                        |                                               |
| Phaseolus aureus        | 4               | 2                        | 8.94, 7.71,                            | 8.75, 8.15,                            | 0.385-0.478                                   |
| (Mung)                  |                 |                          | 9.56, 8.34                            | 8.90, 8.15                            |                                               |
| Oryza sativa           | 3               | 2                        | 9.86, 10.48,                           | 10.25, 10.30,                          | 0.493-0.478                                   |
| (Bran-rice)             |                 |                          | 10.17                                  | 10.45                                 |                                               |
| Pennisetum typhoidum   | 4               | 5                        | 51.4, 53.76,                           | 51.2, 52.9,                            | 1.028-1.093                                   |
| (Bajara)                |                 |                          | 54.66, 52.25                           | 54.05, 53.1                           |                                               |
| Zea mays                | 4               | 4                        | 61.89, 60.28,                          | 62.7, 61.9,                            | 1.507-1.568                                   |
| (Maize)                 |                 |                          | 62.7, 61.9                            | 62.3, 62.5                            |                                               |
| Lens culinaris          | 4               | 4                        | 22.81, 23.12,                          | 23.3, 23.48,                           | 0.570-0.578                                   |
| (Masur)                 |                 |                          | 22.5, 22.9                            | 22.35, 22.85                          |                                               |
| Triticum aestivum       | 4               | 5                        | 17.0, 17.88,                           | 17.2, 17.35,                           | 0.340-0.358                                   |
| (Wheat flour)           |                 |                          | 17.58, 17.9                           | 17.9, 17.9                           |                                               |
| Milk powder             | 4               | 5                        | 15.4, 16.03,                           | 15.65, 16.2,                           | 0.292-0.321                                   |
|                         |                 |                          | 15.8, 14.6                            | 15.3, 15.2                            |                                               |
| **(c) Tea samples:**    |                 |                          |                                        |                                        |                                               |
| Lipton                  | 4               | 2                        | 10.79, 12.03,                          | 10.98, 12.37,                          | 0.54-0.60                                     |
|                         |                 |                          | 11.71, 11.20                          | 11.82, 11.29                          |                                               |
| Brooke-Bond             | 4               | 2                        | 9.55, 10.79,                           | 9.77, 10.65,                           | 0.478-0.540                                   |
|                         |                 |                          | 10.48, 10.12                          | 10.93, 10.52                          |                                               |
| Taj                     | 3               | 2                        | 15.55, 15.24,                          | 15.85, 15.23,                          | 0.695-0.777                                   |
|                         |                 |                          | 13.90                                  | 14.57                                 |                                               |
| **(d) Human blood sera:** |               |                          |                                        |                                        |                                               |
| Normal                  | 6               | 2.051                     | 2.10, 2.42, 1.98,                      | 2.02, 2.31, 2.08,                      | 0.099-0.121                                   |
|                         |                 |                           | 1.58, 2.20, 2.07                      | 1.58, 2.0, 2.07                       |                                               |
| Diseased                | 6               | 1.995                     | 3.85, 3.50, 3.75,                      | 3.71, 3.65, 3.89,                      | 0.160-0.193                                   |
| (Malignant breast tumor)|                 |                           | 3.35, 3.20, 3.83                      | 3.55, 3.41, 3.67                       |                                               |
| Diseased                | 5               | 1.989                     | 2.16, 1.83, 1.93, 2.08, 2.09          | 2.82, 2.05, 2.01, 2.18, 2.19          | 0.092-0.108                                   |
| (Benign breast tumor)   |                 |                           |                                        |                                        |                                               |
| **(e) Body tissues:**   |                 |                          |                                        |                                        |                                               |
| Eye-lens                | 4               | 1.65                      | 1.02, 0.86,                            | 1.11, 1.03,                            | 0.052-0.073                                   |
| (pooled normal)         |                 |                           | 0.78, 1.09                            | 1.27, 1.25                            |                                               |
Note

| Eye-lens (pooled mature senile cataract) | Prostatic tissue | Carcinoma (prostatic tissue) |
|------------------------------------------|-----------------|-------------------------------|
| 4                                        | 3               | 3                             |
| 1.48                                     | 4.92            | 4.35                          |
| 2.75, 2.03, 2.18, 2.16, 25.78, 26.08, 26.3, 26.7, 55.25, 62.20, 54.1, 60.2, 53.07, 54.5 |
| 2.31, 2.31, 2.25, 2.30, 29.5, 60.2, 54.5 |
| 0.137-0.186, 0.524-0.614, 1.22-1.43      |

*Wet weight was taken.

decomposed all the complexes except that of copper(II), thus making the method highly selective for copper.

**Spectrophotometric studies on copper(II)-PBN complex:**

Ethanolic solution of PBN gave a dark blue complex with copper(II) in neutral to alkaline medium (containing not less than 50% ethanol to avoid precipitation). The maximum and stable absorbance was found at pH 7.0 to 8.5 and phosphate buffer (2 ml, pH 7.6) was found to be suitable to determine copper selectively. The maximum colour intensity was found for Cu^{II} : reagent :: 1 : 5 in molar concentration.

**Effect of diverse ions:** In the procedure under study 1000-fold of chloride, bromide, nitrate, acetate, sulfate, sulfite, citrate, tartrate, phosphate, borate, and 100-fold of Ca^{II}, Sr^{II}, Ba^{II}, Nb^{V}, Ta^{V}, Al^{III} and lanthanides did not interfere, while EDTA, thiosemicarbazide and sulfide interfered seriously. The following anions and cations did not cause a deviation more than ±2%:

- **Anions:** Nitrite, oxalate (500-fold); iodide, thiocyanate (100-fold); thiosulfate (50-fold) and fluoride (25-fold). Cyanide (200-fold) however did not interfere, but a hypsochromic shift of 20 nm was observed producing a bright red colour.

- **Cations:** Hg^{II}, Mg^{II}, Sn^{III}, Bi^{III}, Th^{IV} (50-fold); Pb^{II}, Au^{III} (25-fold); Mn^{II}, Ag^{I}, Mo^{VI}, W^{VI} (20-fold); In^{III}, Pt-metals except Pd^{II} (15-fold); Ti^{IV}, Cr^{III}, U^{VI} (10-fold); Cd^{II}, V^{V} (5-fold); Co^{II}, Ni^{II}, Zn^{II} (3-fold) and Pd^{II} (4-fold masked by thiocyanate).

**Experimental**

2,6-Dihydrazinopyridine (0.01 mol) was dissolved in minimum amount of acetic acid and 1,2-naphthoquinone (0.02 mol) was dissolved in ethanol. These solutions were mixed and kept for sometime and then neutralized with ammonium hydroxide to get a red precipitate. The precipitate was washed with 50% ethanol and dried at 60–70°. Its purity was checked by TLC and elemental analysis. The infra-red spectrum of the compound showed the absence of the v_{C=O} (1670 cm^{-1}, for 1,2-naphthoquinone) and v_{C=O} (1630–1680 cm^{-1}, for hydrazones) and the appearance of a new strong frequency v_{OH} (phenolic) at 3200–3500 cm^{-1}, confirming thereby the enol-form of the compound rather than the keto-form. A 5 x 10^{-4} M reagent solution was prepared by dissolving 0.2092 g of the dye in 1 L ethanol.

A stock solution of copper(II) (0.01 M) was prepared by dissolving copper sulfate pentahydrate in double distilled water and standardized by versenate (EDTA) method. Phosphate buffer (pH 7.6) was prepared as usual. Trisodium citrate solution (2%) was prepared in doubly distilled water.

All the reagents used were of A.R. grade. A Beckman 26 spectrophotometer, Beckman pH-meter and ECIL-4129 atomic absorption spectrophotometer were used.

Samples of milk, food-grains, tea leaves, body tissues, eye lenses and blood sera were prepared by using the reported procedure^{1-3}. Milk was procured from the local dairies and biological samples were procured from the Medical College & Hospital, Rohtak.

**Procedure:**

An aliquot containing 6–25 μg of copper(II) was taken in a 25 ml standard flask and 2 ml of 5 x 10^{-4} M PBN solution, 2 ml of phosphate buffer solution (pH 7.6) and 1 ml of 2% trisodium citrate solution were added to it. The total volume was made up to 25 ml keeping 50% (v/v) ethanol. The absorbance was recorded after 2 min at 570 nm against a reagent blank.

**Copper(II) in foodstuffs, blood sera and body tissues etc.:** The procured samples were analyzed for copper following the procedure under study. The samples were
also analyzed for copper using an atomic absorption spectrophotometer. The results (Table 1) reveal that PBN can successfully be used to determine copper(II) ions in diverse samples.

**Acknowledgement**

The authors are thankful to the M. D. University, Rohtak for facilities.

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