Journal of Pharmaceutical & Biomedical Analysis
Vol. 6, Nos 6–8, pp. 781–786, 1988
Printed in Great Britain

0731–7085/88 $3.00 + 0.00

Kinetic-spectrophotometric method for the assay of copper(II) in human serum by catalytic oxidation of salicylic acid*

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Abstract: A very sensitive kinetic spectrophotometric method for the determination of copper(II) concentrations as low as 0.07 ng ml^{-1} is described. This method is based on the oxidation of salicylic acid by hydrogen peroxide in ammoniacal medium, catalysed by copper(II) ion. The figures of merit of the procedure and the results of a study of interferences are given. The method is applied to the assay of copper in human blood serum.

Keywords: *Copper(II); analytical kinetic method; human serum; catalytic oxidation; salicylate ion.*

Introduction

The role of copper in living systems has not yet been fully explained. It is known that it has two main functions, namely, electron transport and oxygen transport. It also acts as an essential part of, or as an activator for a number of enzymes, among others probably those involved in the production of haemoglobin [1]. The concentration of copper in body fluids has an important diagnostic value in several diseases, as for instance in Wilson's disease. Several classical procedures for copper determination in serum based on spectrophotometric measurements after a chromogenic reaction [2–4] (with or without a previous precipitation step for splitting off the copper(II) from the proteins) are available; the direct determination by atomic absorption spectrophotometry (AAS) has also been recommended [5, 6].

In this work a new fixed-time kinetic method for copper(II) determination in blood serum after protein precipitation is developed. This kinetic procedure is derived from the equilibrium method proposed by Minzl and Hainberger [7], based on the measurement of the absorbance brought about in the test solution (after 30 min heating at 80°C) by the copper(II)-catalysed oxidation of salicylate ion by hydrogen peroxide in an ammonia medium. The role of copper(II) in the reaction between *o*-hydroxy aromatic carboxylic acids and hydrogen peroxide in an ammonia medium was studied in previous work [8].

*Presented at the "International Symposium on Pharmaceutical and Biomedical Analysis", September 1987, Barcelona, Spain.

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This showed that the yellow-to-reddish colour developed in the reaction is due to a new compound obtained by oxidation of the reagent through an intermediate copper(III) complex.

The proposed method has all the general advantages of kinetic methods and it is easily performed in a relatively short time. It must be noted that the very low limit of detection of the new procedure allows copper(II) determination in very small samples of serum.

Experimental

Apparatus

A Beckman (Acta M VII) double-beam spectrophotometer, with 1.0-cm fused-silica cells, equipped with a Perkin–Elmer Temperature Digital Controller C 570–0701 and cell holders with thermoelectric Peltier modules. A Radiometer PHM 64 pH meter, with a combined glass–calomel electrode GK 2401 B. Atomic Absorption Spectrometer Perkin–Elmer 4000.

Reagents

All reagents (analytical grade) used were from E. Merck. Ultrapure, doubly deionised water (from a Culligan System, 0.08 ohm^{-1} cm^{-1}) was used throughout. Blood serum samples were obtained from Vall d'Hebron Hospital (Barcelona).

Reagent solutions. A. 0.300 mol l^{-1} salicylic acid and 3.0 mol l^{-1} aqueous ammonia solution, pH adjusted with 3 M hydrochloric acid to 9.40; B. 4% (1.18 mol l^{-1}) aqueous hydrogen peroxide solution.

Procedure

A 0.5-ml volume of blood serum is mixed with a 0.5-ml volume of the precipitating reagent (0.6 mol l^{-1} trichloroacetic acid plus 1.5 mol l^{-1} hydrochloric acid). The mixture is heated to 56°C for 15 min and centrifuged. From the supernatant solution a 20-μl volume (equivalent to 10 μl of the original sample) is taken and transferred into the spectrophotometric cell. A 1.0-ml volume of reagent solution A and a 1.0-ml volume of reagent solution B are added. The cell is introduced into the Peltier Cell Holder, homogenised by magnetic stirring and heated up to 65°C. The whole operation takes about 15 s, after which the initial absorbance at 376 nm is read; 5.0 min after cell introduction the increment of absorbance of the solution at the same wavelength is measured. Quantitation is achieved through the use of a calibration curve or by the standard additions method involving 10-μl additions of standard copper(II) solutions [containing 2.00, 4.00 and 6.00 μg ml^{-1} of copper(II)] to the test solution obtained from 20 μl of the precipitated serum sample. Absorbance of a blank solution at 376 nm must be subtracted from all absorbance readings. (For routine work the required accuracy is attained if one single addition of the most diluted copper standard is made to one out of a number of test solutions.)

Results and Discussion

Catalytic effect of copper(II) ion

Salicylic acid in an ammonia medium is only slowly oxidised by hydrogen peroxide. In the presence of traces of copper(II) the oxidation rate is increased and this change in rate

can be measured absorptiometrically at 376 nm. Figure 1 shows the effect of copper(II) ion [added as aqueous copper(II) nitrate solution] on the absorbance versus time plots for this reaction. The influence of temperature on this catalytic effect was studied at 10°C intervals within the range 20–80°C by a fixed-time kinetic method. For a constant copper(II) ion concentration the absorbance increments measured after 5 min reaction time at the different temperatures (Fig. 2) show a steady increase in the reaction rate without levelling out to any plateau. A temperature of 65°C was selected as the working temperature (the equilibrium method [7] is performed at 80°C) because at higher temperatures blank readings are too high and bubble formation is observed. The use of the Peltier heating modules allows a very stable and reproducible setting of temperature, as shown by the precision attained in the results.

Optimisation of reaction parameters

The effect of the concentrations of the reactants (salicylic acid, hydrogen peroxide, ammonia, hydrogen ion) was studied through a multivariate procedure using an aqueous standard solution of copper(II) nitrate containing 1.2 μg ml^{-1} copper(II) ion as a test solution. The Simplex modified method [9] was used to optimise the concentrations of the four reactants. In Table 1 the different regions of the variable domains, taken as input, are shown together with the final output from the modified Simplex. Since the response surface shows a rounded maximum, the Simplex procedure was stopped when

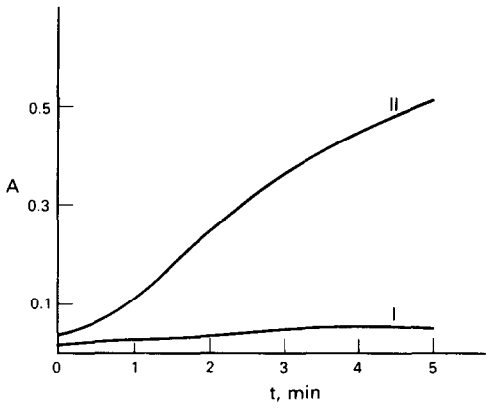

Figure 1
Absorbance at 376 nm vs time curve obtained at 65°C from an aqueous solution containing 0.150 mol l^{-1} salicylate ion, 1.50 mol l^{-1} ammonia and 0.60 mol l^{-1} hydrogen peroxide, pH = 9.40. Curve I = without copper(II) ion; curve II = in the presence of 56 ng ml^{-1} of copper(II) ion.

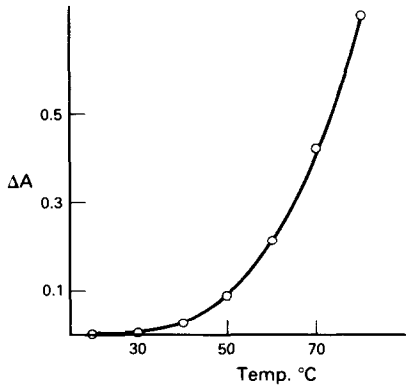

Figure 2
Influence of temperature on the increase of absorbance at 376 nm after 5.0 min reaction time, for a solution containing 0.150 mol l^{-1} salicylate ion, 1.50 mol l^{-1} ammonia, 0.60 mol l^{-1} hydrogen peroxide, pH = 9.40, and 40 ng ml^{-1} copper(II) ion.

Table 1
Simplex optimisation of the procedure, for a test solution containing 12.0 μg ml^{-1} of copper(II) ion

Variable	Initial variable domains	Restricted optimum domains
Salicylate ion (mol l^{-1})	1×10^{-3}–0.30	0.11–0.18
Ammonia (mol l^{-1})	0.10–2.00	0.90–2.00
Hydrogen peroxide (%)	0.00–5.00	1.82–2.82
pH	7.00–10.50	9.04–9.76
Response (*)	—	0.438 ± 0.013

*Absorbance increment at 376 nm after 5.0 min reaction time.

the absorbance response showed changes within a ±3.0% range around the mean value of successive readings. The final output, therefore, is not single values but restricted domains of the variables. Reagent concentrations given in the experimental part lie within these restricted domains.

Figures of merit

Figures of merit for the analytical procedure as described in the Experimental section were determined using the aqueous standard containing 1.20 μg ml^{-1} copper(II) ion instead of the precipitated serum sample.

The linearity range of the procedure is from 0.20 to 20 μg ml^{-1} in the sample solution; this range is very much wider than the normal and pathological ranges of copper concentrations in body fluids. The linear correlation between absorbance (A), and copper concentration in the final cell solution (C), (in ng ml^{-1}), within the linear range is given by the expression:

$$A = 6.85 \times 10^{-3} C + 3.73 \times 10^{-3}, r = 0.9997.$$

Sensitivity of the procedure is therefore, 6.85×10^{-3} absorbance units ng^{-1} ml, indicating higher sensitivity than that of the equilibrium method (and those of most absorptiometric methods).

The limit of detection, determined according to IUPAC recommendations [10] from 12 blank readings and their standard deviation, is 0.07 ng ml^{-1} (whereas the limit of detection for the corresponding equilibrium method [7] is 5 ng ml^{-1}), and the limit of quantification is 1.51 ng ml^{-1} (in the final solutions). The precision of single results, expressed by their relative standard deviation, R.S.D., was evaluated from sets of 12 independent replicates. At the 1.5 μg ml^{-1} copper level in the serum sample, R.S.D. = 8.1%, and at the 13.2 μg ml^{-1} level R.S.D. = 6.0%.

Effect of proteins

In the presence of proteins in the test solutions, systematically low results are obtained. Thus, three blood serum samples analysed by the proposed method yielded the following results for their copper(II) content: (a) after protein removal, 1.32, 1.67 and

Table 2
Copper(II) ion concentration (in μg ml^{-1}) in samples of human blood serum

Sample number	Kinetic method	AAS method	Difference (%)
1	1.32	1.28	+3.1
2	1.67	1.62	+3.1
3	1.03	1.05	−1.9
4	1.15	1.13	+1.7
5	1.34	1.30	+3.0
6	1.41	1.40	+0.7
7	1.23	1.18	+4.1
8	1.25	1.23	+1.6

1.03 μg ml^{-1} (in good agreement with the AAS results shown in Table 2); (b) without protein precipitation: 0.78, 0.84 and 0.42 μg ml^{-1}, respectively. These results show that copper(II) ion bound to the protein is not an effective catalyst for salicylate oxidation by hydrogen peroxide, probably because the lifetime of the copper(III) intermediate is increased by the competing formation of more stable polypeptide complexes [11].

Interfering ions

The effect of several ions in blood serum on the reaction rate was investigated. The ions studied were: Ca(II); Mg(II); Fe(II) and (III); Zn(II); Na(I); K(I); chloride and phosphate. They were added at several increasing concentrations to a copper(II) ion solution containing 16 ng ml^{-1} copper(II), up to concentration ratios (added ion/copper(II) ion) from 2 to 100 times higher than those found in normal human blood serum [12]. The minimum concentration ratio that gives absorbance readings in the Cu(II)-catalysed salicylate oxidation differing by more than ±5% of the reading obtained from a pure copper(II) ion solution was taken as the maximum allowable ratio for the specific ion involved. The maximum allowable concentration ratios of interfering ions are: Ca(II), (500:1); Mg(II), (100:1); Fe(II) and (III), (80:1); Zn(II), (200:1); Na(I), (20,000:1); K(I), (5000:1); chloride, (10,000:1); phosphate, (250:1).

Application

The analytical kinetic procedure proposed in the present work was applied to eight human blood serum samples after protein removal as described earlier. The copper(II) ion concentrations of these samples were also determined by the standard AAS method [5, 6]. It can be seen in Table 2, where the results obtained by both procedures are given, that no significant difference is obtained.

Conclusion

The new kinetic procedure shows a detection limit lower than those of most of the existing kinetic methods for copper(II) determination in blood serum, which range between 1 and 5 ng ml^{-1} [13, 14]. The detection limit of 0.004 ng ml^{-1} claimed by Dolmanova *et al.* [15] for their kinetic method was not calculated according to the IUPAC convention. The precision of the proposed procedure is similar to those of other kinetic methods. The proposed procedure is less time-consuming than the methods mentioned earlier. The new kinetic procedure can be recommended for copper(II) ion determination in blood serum (and in urine) since it gives comparable results to those of

the AAS method, but uses a much lower sample volume. The new kinetic procedure, as all procedures of its kind, can be very easily automated.

Acknowledgement — Dr Lydia García-Beltrán is acknowledged for kindly providing the blood serum samples.

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[*Received for review 23 September 1987; revised manuscript received 18 January 1988*]