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Short Communication

Determination of human serum ceruloplasmin by measurement of its 3,3′,5,5′-tetramethylbenzidine oxidase activity

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Introduction

The copper-containing glycoprotein, ceruloplasmin, in plasma shows oxidase activity towards various substrates [1, 2] such as aromatic polyamines, polyphenols, ascorbic acid, and Fe^{2+}.

The determination of ceruloplasmin by a colorimetric method using *p*-phenylenediamine (PPD) was first reported by Ravin [3]. Thereafter, many methods based on several reactions have been reported; these include oxidative colorimetric methods [4–8] using PPD or its derivatives and ascorbic acid, enzymatic methods [9, 10] using Fe^{2+}, chromatographic methods [11, 12] and immunodiffusion methods [13, 14]. The colorimetric methods are the most widely used but are not as sensitive as the enzymatic methods.

Schosinsky *et al.* [15] devised a sensitive colorimetric method to determine the *o*-dianisidine oxidase activity of ceruloplasmin but such benzidine derivatives are not used now because they are carcinogenic [16, 17]. However, some benzidine derivatives such as 3,3′,5,5′-tetramethylbenzidine (TMB) [18] and dicarboxidine are not carcinogenic [19].

Experiments have been conducted to test whether TMB can be used to determine ceruloplasmin by measurement of its TMB oxidase activity.

Experimental

Chemicals

Human ceruloplasmin solution and human albumin were purchased from Sigma Chemical Co., St. Louis, USA. Anticoagulants sealed in Venoject tubes were used: VT-

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050H (sodium heparin), VT-050NA (sodium EDTA), and VT-050CS (sodium citrate) were obtained from Terumo Corp. (Tokyo, Japan). The haemoglobin solution was prepared as described previously [20]. Other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Preparation of reagents

TMB solution. To 20 mg of TMB dissolved in 2 ml of glacial acetic acid was added 8 ml of water.

0.1 M *Acetate buffer solution* (*pH* 6.0). Twenty millilitres of 0.2 M acetic acid, 580 ml of 0.2 M sodium acetate and 400 ml of water were mixed in a 1-l volumetric flask. The pH was adjusted to 6.0 by addition of sodium acetate or glacial acetic acid, and the solution was diluted to 1 l with water.

Sodium azide solution. Ten milligrams of sodium azide was dissolved in 10 ml of water.

Ceruloplasmin standards. Ten microlitres of the ceruloplasmin solution was diluted to 5 ml with water. Ceruloplasmin standards were prepared by further dilution with water to concentrations of 20, 50, 80, and 100 mg/dl.

Instruments

Absorbance of the solutions was measured by a spectrophotometer (Model 200-20, Hitachi Ltd., Tokyo, Japan).

Procedure

The mixture of 2.0 ml of 0.1 M acetate buffer solution (pH 6.0) and 10 μl of each ceruloplasmin standard or sample was warmed at 37°C for 5 min. After the addition of 0.1 ml of TMB solution warmed at 37°C, the solution was mixed well and kept at 37°C for 30 min. The reaction was terminated by the addition of 0.1 ml of sodium azide solution and the solution was mixed well. The absorbance of this solution was measured at 645 nm.

For comparison, ceruloplasmin was determined by the 2-nitroso-5-(*N*-propyl-*N*-sulfopropylamino)phenol method (the nitroso–PSAP method) [10].

Results

Optimal concentrations and pH

The optimal concentrations of TMB and sodium azide (tested between 8.3 μM–16.6 mM and 0–1.0%, respectively) for the assay of ceruloplasmin in concentrations up to 100 mg/dl were 8.3 mM and 0.1%, respectively. The optimal pH of the 0.1 M acetate buffer solution (tested between 3.6 and 7.0) was 6.0.

Effects of temperature and time

The extent of oxidation of TMB was affected by temperature (25–45°C) and increased with time (Fig. 1). Generally, in the colorimetric methods published previously, the reaction time was set to be within 30 min [7]; it was considered that such a time limit would be suitable for routine use of the proposed method. Within this time limit, the highest absorbance was attained at 37°C for a reaction time of 30 min.

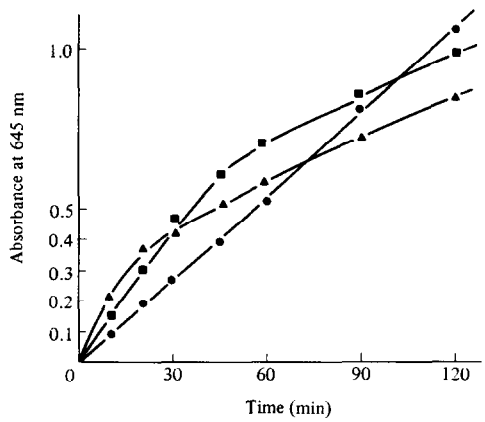

Figure 1
Time-course of the TMB–ceruloplasmin reaction at different temperatures. Ceruloplasmin concentration in sample: 54.83 mg/dl. Reaction temperatures: 25°C (●), 37°C (■), and 45°C (▲).

Effects of anticoagulants

The final concentrations of sodium heparin, sodium EDTA, and sodium citrate used as anticoagulants were 18 IU/ml, 1.5 mg/ml, and 3.8 mg/ml, respectively. These three anticoagulants were added in the determination of ceruloplasmin in concentrations of 21.93, 54.83, and 87.74 mg/dl; the recovery of ceruloplasmin was 85% or less by the proposed method.

Effects of interfering substances

Table 1 shows the recovery of ceruloplasmin by the proposed method when each interfering substance, albumin, bilirubin, lipids, haemoglobin, or chloride, was added to the serum containing ceruloplasmin in concentrations of 21.93, 54.83, and 87.74 mg/dl.

Accuracy

The calibration graph was linear up to 100 mg/dl, with a sensitivity of 2 mg/dl ceruloplasmin per 0.015 absorbance unit at 645 nm. The regression equation of the curve was represented by: $y = 0.0120x - 0.0213$ ($n = 11$, $r = 0.997$); the standard error of the slope was 1.16×10^{-7} ($n = 8$).

Precision

Table 2 gives the intra-assay results of the samples containing 21.93, 54.83, and 87.74 mg/dl of ceruloplasmin in 10 different test tubes, and the inter-assay results for the same concentrations of ceruloplasmin in 20 test tubes. The intra-assay precision or relative standard deviation (RSD) was 2.95%; the inter-assay RSD was 3.02%.

Comparison of methods

Figure 2 shows the ceruloplasmin concentrations in identical samples determined by the proposed method and the nitroso–PSAP method simultaneously. Correlation between these two methods was good ($n = 27$, $r = 0.995$); the regression equation of the graph was represented by $y = 0.983x + 1.373$.

Discussion

It was found that the TMB oxidase activity of ceruloplasmin was inhibited by the anticoagulants, sodium heparin, sodium EDTA and sodium citrate. Accordingly, only

Table 1
Effects of albumin, bilirubin, triglyceride, haemoglobin and chloride on the assay of human serum ceruloplasmin*

Agents	Concentration (g/l)	Range of recovery (%)
Albumin	15, 30, 60, 90, 120	94.4–103.0
Bilirubin	0.0078, 0.0156, 0.0313, 0.0625, 0.125	96.5–105.3
Triglyceride	1.9, 3.8, 7.5, 15.0	97.1–105.5
Haemoglobin	0.125, 0.25, 0.50, 0.75, 1.0	95.1–104.0
Chloride	10†, 25†, 50†, 75†, 100†	95.0–104.6

*Samples were prepared in human serum; concentrations of ceruloplasmin were 21.93, 54.83 and 87.74 mg/dl.
†Units for chloride are expressed in mM.

Table 2
Precision and recovery of human serum ceruloplasmin*

	Added ceruloplasmin (mg/dl)		
	21.93	54.83	87.74
Intra-assay precision (n = 10)			
Mean (mg/dl)	22.81	53.51	89.05
SD (mg/dl)	0.57	1.58	1.79
RSD (%)	2.52	2.95	2.01
Inter-assay precision (n = 20)			
Mean (mg/dl)	22.81	53.95	87.74
SD (mg/dl)	0.62	1.63	2.42
RSD (%)	2.73	3.02	2.76
Recovery (n = 20)			
Mean (%)	104.01	98.39	100.12
SD (%)	2.83	2.97	2.76

*Samples were prepared in human serum.

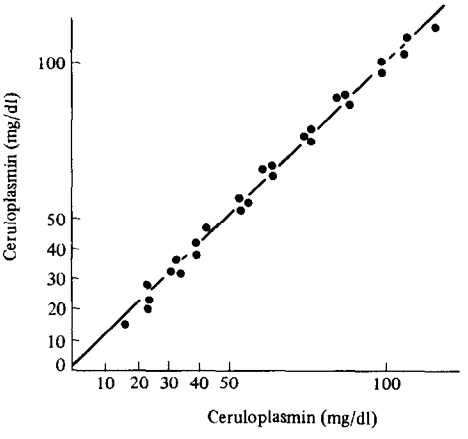

Figure 2
Correlation between the nitroso–PSAP method (y) and the proposed method (x). Samples were prepared by diluting serum from healthy human subjects or patients with low ceruloplasmin concentrated serum.

samples (such as serum) that did not contain anticoagulants, were used in the proposed method.

For a reaction time of 30 min the highest absorbance was attained at 37°C (Fig. 1); these conditions were specified in the proposed method.

The normal range of ceruloplasmin in the human serum of healthy subjects is usually considered to be 27–63 mg/dl [21]. Serum ceruloplasmin levels are influenced by various diseases; for example, they are elevated in anaemia and cholangitis but lowered in Wilson's disease [22–24]. The present method, which has a determination range up to 100 mg/dl, is thus useful clinically for the determination of ceruloplasmin.

Generally, the oxidation reactions of ceruloplasmin toward Fe^{2+} proceed more quickly than do those toward polyphenols and polyamines. Since the sensitivity of TMB itself for colorimetry is generally high, the same results were expected to be obtained using Fe^{2+} and TMB. The method that was developed, however, had a lower sensitivity than that of the method using Fe^{2+} and a higher sensitivity than that using PPD. However, a good correlation was obtained between the proposed method and the nitroso–PSAP method; in addition, results of studies on the interference of serum components were acceptable and a useful determination range was confirmed. Therefore, the present method is expected to be very useful in clinical practice.

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