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## Note

# Determination of human serum ceruloplasmin by ion chromatography based on its ferroxidase activity

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Ceruloplasmin (Cp) oxidizes various compounds [1, 2], such as polyphenols, polyamines, iron ($Fe^{2+}$) and ascorbic acid, to their corresponding quinonoid compounds, i.e. aldehydes, $Fe^{3+}$ and dehydroascorbic acid.

The oxidase activity of Cp has been considered a useful parameter in various clinical studies [3–5], such as those on anaemia, cholangitis and Wilson diseases. The enzymatic activities of Cp for these compounds were first reported by Holmberg and Laurell [1, 2]. Generally, the determination of the enzymatic activity of Cp was followed by the colorimetric method with *p*-phenylenediamine (PPD), as reported by Ravin in 1956 [6]. Many methods have since been reported: for example, methods using PPD or its derivatives [7—10], ascorbic acid [11, 12], immunodiffusion [13, 14] and enzymatic activity for $Fe^{2+}$ [15–18]. The activity of Cp on $Fe^{2+}$ as a substrate is called ferroxidase activity. The methods for determining this activity have the advantage of being rapid and highly sensitive due to the high molecular activity [15].

Recent methods for the determination of ferroxidase activity generally use apotransferrin as a chromogen [16]. However, this method is time-consuming and is influenced by serum turbidity.

Recently, we reported [19] a method for determining Cp based on ferroxi-

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dase activity using 2-nitroso-5-(N-propyl-N-sulphopropylamino)phenol (nitroso-PSAP) as a specific chelating reagent for $Fe^{2+}$. Here, we report our findings on the application of this nitroso-PSAP method.

## EXPERIMENTAL

### *Materials*

Human Cp solution was purchased from Sigma (St. Louis, MO, U.S.A.). Substrate solution was prepared by dissolving 137 mg of ammonium ferrous sulphate 6-hydrate (Wako, Osaka, Japan) in water to 1000 ml. Human serum samples were obtained from normal healthy adults and were not treated with anticoagulants.

### *Chromatographic conditions*

The ion chromatographic unit consisted of a Model AMP-1 analytical pump, a Model PCR-1 post-column reactor and a Model UVP-2 UV– VIS detector (Dionex, U.S.A.). Analyses were performed by separation using an HPIC-CG5 guard column and an HPIC-CS5 separator column (Dionex), by colorimetry using a PCR-1 with the following reactant and by detection using UVM-2.

Pyridine-2,6-dicarboxylic acid (PDCA) solution (6.0 m*M*, pH 4.8) with 8.6 m*M* lithium hydroxide was used as the eluent at a flow-rate of 0.7 ml/min. 4-(2-Pyridylazo)resorcinol (PAR) (0.4 m*M*) in 3 *M* ammonium hydroxide solution was used as the reactant at a flow-rate of 0.1 ml/min. Absorbance was measured at 520 nm.

### *Calibration curve*

In 1 ml of 0.1 *M* acetate buffer at pH 6.5, various amounts of ceruloplasmin (between 10 and 100 mg/dl) enzymatically oxidized $Fe^{2+}$ to $Fe^{3+}$ at room temperature in 10 min. The reaction was stopped by the addition of 10% trichloroacetic acid solution. The precipitated proteins present with Cp were removed from the reaction solution by centrifugation at 1500 *g* for 10 min. The remaining $Fe^{2+}$ was analysed by ion chromatography and the calibration curve was made by plotting peak height against concentration of Cp.

### *Ceruloplasmin assay*

Pipette 1 ml of 0.1 *M* acetate buffer (pH 6.5) into test tubes, pipette 0.2 ml of each Cp standard and sample into the respective test tube and mix well. Incubate at room temperature for 5 min, add 0.5 ml of substrate solution and mix well. Incubate at room temperature for another 10 min, add 0.5 ml of 10% trichloroacetic acid solution and mix well. Centrifuge at 1500 *g* for 10 min, analyse by ion chromatography and obtain the concentrations of Cp in the samples from the calibration curve.

For comparison, Cp was determined by the PPD method [10] as previously described.

## RESULTS

Fig. 1 shows the ion chromatographic profile of $Fe^{3+}$ and $Fe^{2+}$ obtained

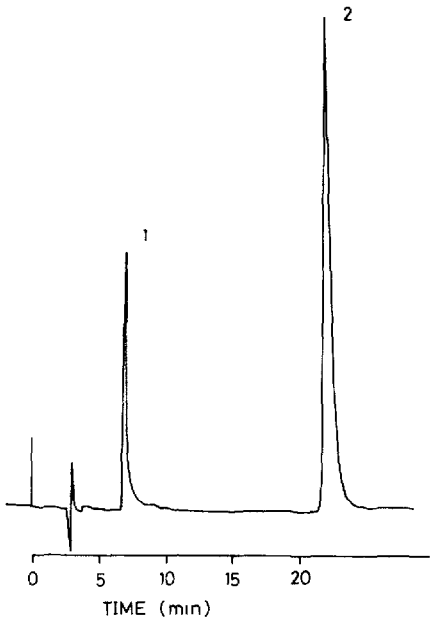


Fig. 1. Ion chromatogram of a reagent blank with $Fe^{3+}$ by the proposed method. Concentrations were: $Fe^{3+}$, 50.6 $\mu M$; $Fe^{2+}$, 795 $\mu M$. Peaks: 1 = $Fe^{3+}$; 2 = $Fe^{2+}$.

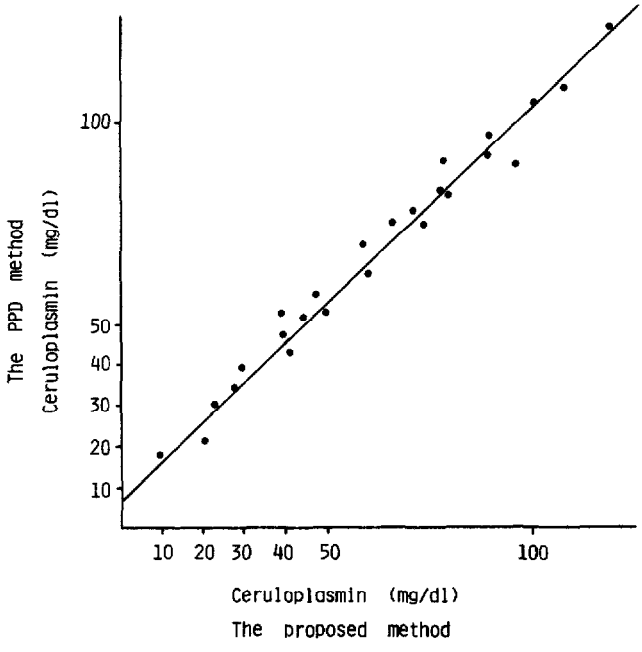


Fig. 2. Correlation between results of determination of human serum ceruloplasmin by the proposed method and the PPD method.

from the ferroxidase assay of Cp by the proposed method. Under the conditions described, the retention times of $Fe^{3+}$ and $Fe^{2+}$ were 7.1 and 21.4 min, respectively.

Under the proposed conditions, we were able to obtain the calibration curves from $Fe^{3+}$ and $Fe^{2+}$. But in the reaction mixture, $Fe^{2+}$ was gradually oxidized to $Fe^{3+}$ with existing oxygen. Under the proposed chromatographic conditions, the sensitivity of $Fe^{3+}$ was ca. six times more than $Fe^{2+}$, i.e. the determination errors with an $Fe^{3+}$ calibration curve were more than the $Fe^{2+}$ curve. Therefore, the $Fe^{2+}$ calibration curve was adopted. A linear relationship between the peak height (cm) of $Fe^{2+}$ and the concentration (mg/dl) of Cp was found with concentrations up to 100 mg/dl ($n = 6$, $r = -0.987$, $y = -0.133x + 19.84$).

The reproducibility of the method was confirmed by repeated analyses of the same Cp concentration. The concentration of Cp was found to be 53.74 ± 1.47 mg/dl (mean ± S.D., $n = 5$).

The correlation between the proposed method and the PPD method was studied by measuring Cp concentrations in identical samples, simultaneously.

Fig. 2 shows a good correlation between these two methods ($n = 25$, $r = 0.986$), with the regression equation of the curve defined by $y = 0.967x + 6.532$.

## DISCUSSION

In the present method, separated $Fe^{2+}$ was chelated and coloured with PAR, which yielded several chelating compounds with metals such as $Fe^{2+}$, $Fe^{3+}$, $Cu^{2+}$ and $Zn^{2+}$; these were coloured, with peak absorbances at ca. 520 nm. As a representative case, the mechanism for the formation of chelating compounds between $Fe^{2+}$ or $Fe^{3+}$ and PAR is shown in Fig. 3.

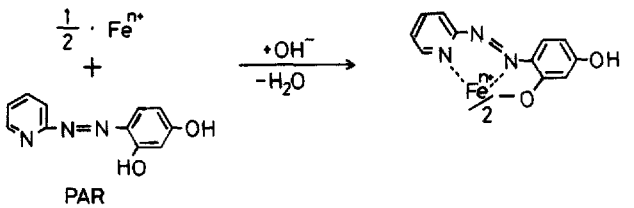


Fig. 3. Scheme of chelation between $Fe^{n+}$ and PAR. $n = 2$ or 3.

In the determination of serum components, colorimetric methods are readily influenced by other serum components because they are usually coloured. However, chromatographic methods, such as the present method, are not readily affected by these components because they are separated before measurement. This accounts for the reliability of the chromatographic methods.

Generally, Cp oxidizes various serum components [20], such as adrenalin, serotinin and catechol, but the oxidation reactions toward them proceed more slowly than toward $Fe^{2+}$ [16]. Curzon and O'Reilly [15] reported that the molecular activity of Cp toward $Fe^{2+}$ was many times greater than any known substrates. Thus, the proposed method does not appear to be affected by the results from human serum components and appears to have a good correlation with the PPD method.

The proposed method is not as sensitive as the recently reported nitroso-PSAP method [19] or high-performance liquid chromatographic method [17,

18], but because it is highly reproducible and has a suitable determination range in ordinary use, it appears to be a useful alternative for the determination of ferroxidase activity of Cp in human serum samples.

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