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Liquid Chromatographic Assay of Plasma Hemoglobin Based on Oxidative Quenching of Serotonin by Pseudoperoxidase Effect of Hemoglobin

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A high-performance liquid chromatographic method for the determination of hemoglobin is described. The method uses the pseudoperoxidase effect of hemoglobin in the presence of hydrogen peroxide and is based on the oxidative quenching of the native fluorescence of serotonin. The determination range for hemoglobin was between 3 and 100 mg/dl. The intra-assay coefficient of variation was 2.04% or less, with a mean recovery of 102.0% at hemoglobin concentrations of 17.28, 43.20, and 69.12 mg/dl. A close correlation was found between the results of this method and the leuco crystal violet method.

Keywords——plasma hemoglobin; pseudoperoxidase effect; fluorometry; serotonin oxidation; HPLC

The concentration of plasma hemoglobin (Hb) has been considered as a useful parameter in various studies, such as in the clinical field[1-4] and blood banking.[5,6] Several methods have been established and are widely used for the determination of plasma Hb, based on the pseudoperoxidase effect in the presence of hydrogen peroxide.

The determination of plasma Hb has been carried out by the colorimetric method, generally with benzidine. However, since the carcinogenicity of benzidine and its derivatives was reported,[7-9] safer research methods have been sought by many investigators. Recent methods for the determination of plasma Hb have employed several chromogens, such as 3,3′,5,5′-tetramethylbenzidine,[10-13] 2,2′-azinodi-(3-ethylbenzthiazoline-6-sulfonic acid),[14] leuco crystal violet,[15] and some others.[16,17]

Recently we reported[18] a method for high-performance liquid chromatographic determination of hydrogen peroxide based on the oxidative quenching of the native fluorescence of serotonin (5-hydroxytryptamine, 5-HT) oxidation. The sensitivity of this method was very high. In this study, we applied this method to plasma Hb determination in various samples.

Experimental

Reagents——5-HT hydrochloride was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 5-Methoxytryptamine (5-MT) hydrochloride was obtained from Fluka AG (Buchs, Switzerland) and 30% hydrogen peroxide solution was from Mitsubishi Gas Chemical Co. (Tokyo, Japan). Hb stock solution was prepared by the method previously described[14] and other chemicals were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Standard solutions for obtaining the calibration equation were prepared by dissolving the Hb stock solution in water to yield Hb standards with concentrations of up to 100 mg/dl.

Chromatographic Conditions——Chromatography was performed by the method previously described[18] with a Tri-rotar VI liquid chromatograph (JASCO, Tokyo, Japan). The system included a manual injector, a DG-3510 degasser, Tri-rotar VI pump, and a 250 × 4.6 mm Finepak SIL C_{18T-5} analytical column (JASCO). The eluate was

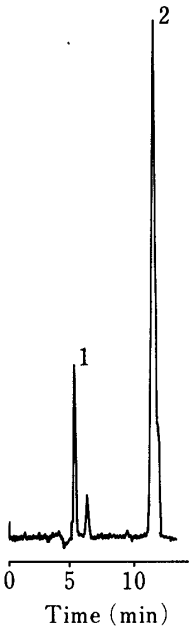

Fig. 1. HPLC Chromatogram Obtained by the Proposed Method

Concentration of hemoglobin in the sample was 93.6 mg/dl. Peaks: 1 = 5-hydroxytryptamine (serotonin); 2 = 5-methoxytryptamine (IS).

continuously monitored with an FP-210 variable-excitation and emission-wavelength spectrophotometer (JASCO), in a flow cell during high performance liquid chromatography (HPLC).

A mixture of 40 mM sodium acetate solution (pH 4.0) and 20% methanol (volume) was used as the mobile phase, at a flow rate of 1.0 ml/min. Fluorescence intensity was measured at excitation and emission wavelengths of 302 and 350 nm, respectively.

Procedure——Pipet 0.1 ml of the substrate solution in 0.1 M phosphate buffer (pH 6.0) containing 37.6 μM 5-HT, 88.2 μM 5-MT (internal standard, IS) and 0.3 ml of 0.0075% hydrogen peroxide solution into each test tube, then pipet 0.3 ml of each sample into the test tube and mix well. Incubate at 37 °C for 30 min, add 0.5 ml of 20% trichloroacetic acid (TCA) solution and mix well. Centrifuge at $1500 \times g$ for 10 min, analyze by HPLC and calculate the concentrations of Hb in the samples from the following equation:

$$\text{Hb (mg/dl)} = -212.8 \frac{\text{sample peak height (cm)}}{\text{IS peak height (cm)}} + 174.0$$

Figure 1 shows the HPLC profile of 5-HT and 5-MT obtained from the Hb assay by the proposed method.

For comparison, Hb was determined by the leuco crystal violet method[15] (the LCV method) as previously described.

Results and Discussion

Effect of Reaction Conditions on the Oxidative Quenching Reaction

5-HT, used as the substrate in the proposed method, is present in platelets, but it scarcely exists in plasma, as determined by our method. 5-HT was oxidized by the pseudoperoxidase effect of Hb in the presence of hydrogen peroxide and lost its fluorescence (302 nm excitation and 350 nm emission).[18] The optimal level of each reagent in the reaction mixture for the determination of plasma Hb at concentrations up to 100 mg/dl was first examined. The concentrations of 5-HT and 5-MT in the substrate solution were set at 37.6 and 88.2 μM respectively, because suitable peak heights were obtained under the proposed conditions. Optimal concentrations of hydrogen peroxide and TCA were 7.5×10^{-3}% and 20% (tested in the ranges of 3×10^{-5} to 3% and 0.02 to 100%, respectively). The optimal pH of 0.1 M phosphate buffer for the substrate solution was 6.0 (tested between 5.0 and 7.6). The oxidation of 5-HT proceeded most effectively at 37 °C (tested at room temperature, 37 and 45 °C). At 37 °C, the oxidation reaction proceeded gradually for 30 min.

Table I. Effect of Anticoagulants, Albumin, Bilirubin, and Triglyceride on Plasma Hemoglobin Assay

Agents	Added		Range of recovery (%)
	Agent (g/l)	Hemoglobin (mg/dl)	
Anticoagulants	9.0,[a] 0.75,[b] 1.9[c]	17.3, 69.1	100.5—102.7
Albumin	15, 30, 45, 60	18.0, 72.0	97.8—102.2
Bilirubin	0.0156, 0.0312, 0.0625, 0.125, 0.250	18.0, 72.0	97.2—102.7
Triglyceride	1.8, 3.7, 7.5	17.3, 69.1	97.6—104.1

a) Sodium heparin, unit: I.U./ml. *b*) Disodium ethylenediaminetetraacetate. *c*) Sodium citrate.

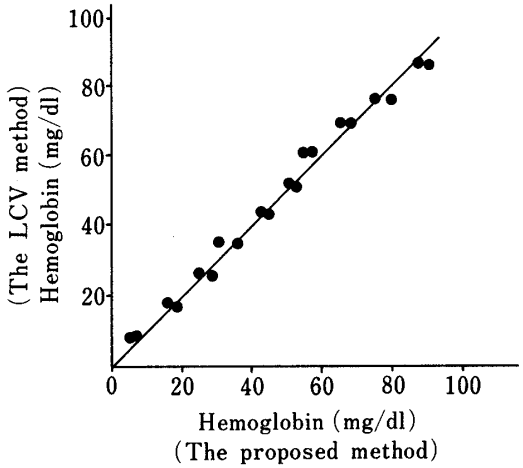

Fig. 2. Correlation between the Results of the Determination of Plasma Hemoglobin by the LCV Method and by the Proposed Method

Effect of Plasma Components and Anticoagulants

The interferences by albumin, bilirubin, and triglyceride as plasma components were examined. When these components, at the final concentrations shown in Table I, were added to pooled human plasma, the Hb concentrations found by this method were between 97.2 and 104.1% of those actually present.

We also studied the effects of anticoagulant such as sodium heparin, disodium ethylenediaminetetraacetate (EDTA-2Na), and sodium citrate, and the concentrations of Hb found under the present conditions were between 100.5 and 102.7% of the added amounts (Table I). Thus, these plasma components and anticoagulants, at the concentrations examined, did not affect the results.

Accuracy

The determination range of Hb was found to be up to 100 mg/dl. The lower limit of concentration detectable was 3 mg/dl (the sample concentration giving a peak height of twice the noise level). The intraassay precision was studied with plasma Hb concentrations of 17.28, 43.20, and 69.12 mg/dl, in 5 repeated tests. The coefficient of variation (C.V.) was 2.04% or less with a mean recovery of 102.0%. Figure 2 shows the comparison of the Hb concentrations in identical samples, prepared by adding the Hb stock solution to pooled human plasma, as measured by the proposed method and by the LCV method. A good correlation ($n=20$, $r=0.995$) was observed, and the regression equation of the curve was $y=1.012x-0.736$ (y, the LCV method; x, the proposed method).

The lower limit of detection with the present method is 3 mg/dl, that is, this method is not as sensitive as other recently reported methods, but it is sufficiently reproducible and it offers a determination range which is appropriate for ordinary use (standard values of human plasma Hb concentrations: stored whole blood, 25 mg/dl or less; concentrated red cells, 50 mg/dl or

less); thus it appears to be a useful alternative method for the determination of Hb in human plasma samples.

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