Determination of Human Hemoglobin in Blood Based on Its Spectral Change Due to the Solvent Effect of Ethanol

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Determining the blood hemoglobin concentration as well as the hematocrit and erythrocyte counting is of great importance for screening and diagnoses related to anemia and erythrocytosis. Many procedures have been reported for determining the blood hemoglobin concentration.¹⁻⁶ Of these methods, the cyanmethemoglobin method^{4,5}, recommended as the preferred technique for routine laboratories, has been widely employed in clinical work. However, there is still a problem that this color reagent contains potassium cyanide, which is extremely harmful to humans, and causes environmental pollution by waste fluids. Recently, a sodium lauryl sulfate method⁶, which requires no cyanide, has been developed by Oshiro *et al.* In this method, since the absorption maximum of hemoglobin is 560 nm, there is only a slight possibility that turbidity originating from plasma proteins or lipids, or coloring substances, interfere with the measurement of blood hemoglobin. The absorption of these interferences decreases as the measuring wavelength is shifted to longer wavelengths. Thus, the author examined a reaction condition which causes a shift of the absorption maximum of hemoglobin to a longer wavelength without using potassium cyanide. For this purpose, the addition of water-soluble organic solvents to hemoglobin lysed by an NaOH-EDTA reagent was effective. Here, the author describes a simple method for the determination of blood hemoglobin based on measuring its absorption spectral change, using a NaOH-EDTA reagent and ethanol.

Materials and Methods

Reagents

All of the reagents were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan, and were of the best quality available.

NaOH-EDTA reagent: 6.7 g of ethylenediaminetetraacetic acid, disodium salt, was dissolved with 1000 cm³ of a 1/3 mol dm⁻³ sodium hydroxide solution.

Standard solution: 3.0 g of hemoglobin from humans was dissolved to 10 cm³ with distilled water.

Other hemoglobin solutions: the oxyhemoglobin was prepared by lysing erythrocytes centrifuged from human blood with distilled water. Reduced hemoglobin was prepared by reducing blood hemoglobin with a 2 g dm⁻³ sodium hydrosulfite solution. Methemoglobin was prepared by treating blood hemoglobin with a 10 g dm⁻³ potassium ferricyanide solution.

Procedure

To 0.02 cm³ of blood 2.0 cm³ of the NaOH-EDTA reagent was added in order to lyse the red blood cells, and mixed well. A few minutes after the reaction at room temperature, 3.0 cm³ of ethanol was added to the reaction mixture, mixed well, and allowed to stand for a few minutes at 25°C. The absorbance was then measured at 600 nm using a Hitachi 7010 Clinical Spectrophotometer or a Hitachi 101 Spectrophotometer against a reagent blank, comprising 0.02 cm³ of distilled water, 2.0 cm³ of the NaOH-EDTA reagent and 3.0 cm³ of ethanol. A calibration curve was prepared for up to 300 g dm⁻³ of hemoglobin concentration.

Results

Absorption spectrum of hemoglobin in the presence of water-soluble organic solvent

In general, the absorption spectrum of a substance is noted to be affected by the coexistence of a solvent; this phenomenon is called the solvent effect.^{7,8} Thus, the effects of water-soluble organic solvents, methanol, ethanol, isopropanol, acetone, ethylene glycol and glycerin, on the absorption spectrum of hemoglobin were examined by adding these solvents at a final concentration of 59.4%(v/v) to blood lysed by the NaOH-EDTA reagent. The color tone of the reaction mixture containing the lysed hemoglobin changed from red to green upon adding the water-soluble organic solvents with the exception of ethylene glycol and glycerin. Its absorption spectrum markedly changed, as shown in Fig.1. Blood hemoglobin, diluted only with distilled water, showed two sharp absorption peaks at about 540 nm and 576 nm, while upon the addition of methanol,

Fig. 1 Absorption spectra of blood hemoglobin in the presence of the water-soluble organic solvents. Each curve indicates the absorption spectrum of hemoglobin lysed by the NaOH-EDTA reagent in the presence of 1, acetone; 2, methanol; 3, ethanol; 4, isopropanol; 5, ethylene glycol; 6, glycerin and 7, distilled water. Curve 8 indicates the absorption spectrum of oxyhemoglobin prepared from patients' blood by diluting it directly with distilled water without adding any organic solvents. All measurements were made against a reagent blank, using a Hitachi 101 Spectrophotometer.

ethanol, isopropanol and acetone, the two maxima came to only one broad peak, and exhibited a large bathochromic shift to 605 nm for methanol, 595 nm for ethanol, 593 nm for isopropanol, and 570 nm for acetone. These spectral changes of hemoglobin occurred very rapidly, and the appearance of the new absorption peak is thought to be related to the values of the dielectric constants of the organic solvents (water, 78.3; glycerin, 42.7; ethylene glycol, 37.7; methanol, 32.6; ethanol, 24.6; acetone, 20.7; isopropanol, 19.9 at 25°C).

Effects of the organic solvents on the color intensity

The relationship between the amounts of the water-soluble organic solvents added and the color intensity was examined. Figure 2 indicates the absorption spectra of hemoglobin when ethanol was added at final concentrations varying up to 59.4% (v/v). The absorption peak was obscure at its low concentration. However, as the concentration of ethanol increased, a broad absorption peak appeared at about 600 nm, and its absorbance increased. Such findings were also observed in the case of methanol, isopropanol and acetone. The molar absorptivities of hemoglobin in the presence of the four organic solvents at a final concentration of 59.4% (v/v) were 2.63×10^7 for methanol, 3.44×10^7 for ethanol, 3.17×10^7 for isopropanol, and $2.85 \times 10^7 \text{ mol}^{-1} \text{ cm}^2$ for acetone.

Spectral changes of three kinds of hemoglobins

The absorption spectra of reduced hemoglobin, oxyhemoglobin, and methemoglobin, which were prepared

Fig. 2 Effects of the water-soluble organic solvents on the color intensity of blood hemoglobin. Each curve indicates the absorption spectrum of blood hemoglobin in the presence of ethanol at different concentrations, measured against a reagent blank using a Hitachi 101 Spectrophotometer. The final ethanol concentrations are 1, 59.4% (v/v); 2, 49.5% (v/v); 3, 39.6% (v/v); 4, 29.7% (v/v); 5, 19.8% (v/v); 6, 9.9% (v/v) and 7, no added.

from the same patients' erythrocytes, were examined by adding organic solvents (methanol, ethanol, isopropanol and acetone). Figure 3 indicates the results in the presence of ethanol at a concentration of 59.4% (v/v). The absorption spectra of these three hemoglobins were markedly different before adding the NaOH-EDTA reagent and ethanol. However, all of the absorption spectra of these hemoglobins after adding these reagents coincided with that of the blood hemoglobin formed when ethanol was directly added to blood lysed by the NaOH-EDTA reagent according to the procedure described above.

Stability of the absorption spectrum newly appearing in the presence of organic solvents

The stability of the absorption spectrum of hemoglobin newly appearing was examined, adding four organic solvents at the final concentration of 59.4% (v/v). The absorption spectrum which newly appeared remained almost unchanged for at least 1 h. Moreover, no precipitation or turbidity due to the coagulation of hemoglobin or plasma proteins was observed, even 24 h after adding the organic solvents. The color intensity was slightly higher at 37°C than at 20°C. Although it was believed that the difference in the color intensity due to the temperature change could be neglected, further measurements were carried out at 25°C.

Application of the spectral change of hemoglobin to the determination of the blood hemoglobin concentration

A linear correlation was observed between the absorbance and the hemoglobin concentration for up to 300 g dm^{-3} in the working curve ($r=0.995$, $p<0.001$). The analytical recoveries of the commercial human

Fig. 3 Changes of the absorption spectra of various kinds of hemoglobin in the presence of ethanol. The upper panel indicates the absorption spectra of 1, oxyhemoglobin; 2, methemoglobin and 3, reduced hemoglobin before adding the NaOH-EDTA reagent and ethanol. The lower panel shows those of these three hemoglobin (1, 2 and 3) after adding the NaOH-EDTA reagent and ethanol. The curve, 4, indicates the absorption spectrum obtained according to the procedure as mentioned in the materials and method. All of the measurements were made against a reagent blank, using a Hitachi 101 Spectrophotometer.

Fig. 4 Comparison of the measurement values of the blood hemoglobin concentrations in 45 patients determined by the present method and the two conventional methods.

hemoglobin added to five patients' bloods varied from 95.5 to 101% (mean, 98.5%). The intra-assay RSD obtained from three levels of hemoglobin concentrations at 92, 145, and 166 g dm⁻³ were less than 1.1% ($n=10$). The inter-assay RSD at 150 g dm⁻³ of the hemoglobin concentration was 2.1% ($n=15$). No interference by sodium chloride (0.1 mol dm⁻³), calcium chloride (0.2 g dm⁻³), albumin (50 g dm⁻³), glucose (10 g dm⁻³), bilirubin (0.1 g dm⁻³), tryptophan (0.1 g dm⁻³), ascorbic acid (1.0 g dm⁻³), creatinine (1.0 g dm⁻³) or urea (1 g dm⁻³) was observed. The concentrations of hemoglobin in patients' blood samples obtained by the present method gave a good correlation with those obtained by the cyanmethemoglobin method⁵ and the sodium lauryl sulfate method⁶, as shown in Table 1 and Fig. 4. The mean value of the present method was slightly higher than those of both conventional methods. Although the reason generating a difference in the measurement value is not clear, it can be considered that there is no effect of this difference on the clinical diagnosis.

Discussion

In determining the constituents in blood, plasma and

Table 1 Correlation of the present method (y) with the two conventional methods (x)

Comparative method (x)	Regression line ($y=ax+b$)	Mean of x /g dm ⁻³	Mean of y /g dm ⁻³	r	n
Cyanmethemoglobin method	$1.007x+3.952$	120.5	125.3	0.977	45
Sodium lauryl sulfate method	$0.955x+10.26$	120.6	125.3	0.995	45

The determination of hemoglobin in blood by the cyanmethemoglobin method and the sodium lauryl sulfate method was carried out, using the kits obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan.

serum by spectrophotometry, the existence of turbidity and coloring substances, such as bilirubin and carotenoid, become a serious problem. Therefore, the measuring wavelength, where the absorption of the coexisting constituents is very small or negligible, is generally selected. The absorption spectrum of hemoglobin treated with the NaOH-EDTA reagent was changed by the addition of water-soluble organic solvents, and its absorption peak shifted to a longer wavelength, about 600 nm, where the absorption of the turbidity and the coloring substances became smaller. Moreover, the oxyhemoglobin, reduced hemoglobin and methemoglobin were also converted to the same substance by these reagents. The absorption maximum newly appeared was very stable, and in spite of the addition of the organic solvents, turbidity and precipitation due to the coagulation of plasma proteins were not occurred for a long time. Thus, the addition of water-soluble organic solvents can shift the absorption maximum of hemoglobin to a longer wavelength, and is thought to become an effective means to design a spectrophotometry which is hard to be affected by the absorption of the coexisting constituents.

With respect to the generation of a measurement error, when the absorption spectrum is sharp-pointed, slight errors in the wavelength setting of the instrument can produce significant error in the measurement. Conversely, when the absorbance of a solution is gradually changing with the wavelength, a slight error in the setting is of little consequence.⁹ In this regard, the spectrophotometry of blood hemoglobin at the broad absorption peak of about 600 nm which newly appeared in the present method, seems to be more precise than that at the two sharp absorption peaks of 540

or 576 nm, which appeared when blood hemoglobin was diluted with water. As can be seen from Table 1, the values obtained by the present method correlate well with those by the conventional methods. In addition, the present method is very simple, and has almost no problem for the human body and environmental pollution. In view of the present results, it is concluded that the present method can be used for the assay of the blood hemoglobin concentration, and will provide a simple, rapid and safe assay for routine blood chemistry. Also, it seems that, since the operation of the present method is simple, an automated method using the NaOH-EDTA reagent and ethanol can be designed.

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