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IMPROVED PROCEDURES OF ALKALI-HEMOCHROME AND PYRIDINE HEMOCHROME METHODS FOR THE DETERMINATION OF HEMOGLOBINS (NORMAL AND ABNORMAL) AND THEIR DERIVATIVES

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Abstract

Pyridine hemochrome (PH) and alkali-hemochrome (AH) methods for the determination of blood hemoglobin concentration were improved in precision and accuracy through detailed examination of the original procedures. As to the PH method, an aliquot of 10.0 μl of blood or hemolysate is poured into 5.0 ml of 0.1 N-NaOH solution containing detergent (SDS in concentration of 3 g/dl), followed by addition of 1.0 ml of pyridine and a small amount of hydrosulfite (4-5 mg) to produce hemochrome.

In alkali-hemochrome method, a volume of 10.0 μl blood or hemolysate is diluted with 4.0 ml of water to hemolyze the erythrocytes and subsequently 2.0 ml of 20.0 g/dl NaOH solution and a small amount of hydrosulfite (4-5 mg) are added.

Both of these methods are suitable for the accurate determination of all derivatives and types of hemoglobins including Hb Ms, unstable Hbs, hemochromes, sulf-Hb, etc.

The maximum discrepancy of Hb value of the same blood specimen between the AH and the standard cyanmethemoglobin (CMHb) methods is 1.46%, (Correlation coefficient $r=+0.995$, $n=109$) and that between the PH and the CMHb method is 1.99% ($r=+0{,}990$, $n=77$). Virtually Hb values identical with those by the standard method are obtained.

The major points of improvement are: i) the use of 20.0 g/dl NaOH solution instead of the originally described 3 g/dl solution in the AH method and use of 0.1 N-NaOH solution containing 3 g/dl SDS in substitution of 0.1 N-NaOH solution in the original PH method

井内岩夫，島崎俊一，柴田　進

and ii) specified time of alkaline incubation of blood for 10 minutes to attain strict accuracy of estimation.

Both hemochrome methods are recomended for estimation of blood hemoglobin concentration as the alternate procedures of CMHb method, because they do not cause disastrous water pollution. Heme concentration of blood in hemoglobinopathies is easily determined by these methods.

INTRODUCTION

Determination of hemoglobin (Hb) concentration is mentioned as an important procedure in the studies of hemoglobinopathy as well as in the routine hematological examinations. Cyanmethemoglobin (CMHb)[1] and azide methemoglobin (AMHb) methods[2] have been recommended for this purpose and they are, at present, accepted as standard procedures internationally. However, they occasionally yield inaccurate estimations for some abnormal hemoglobins. The cause of the inaccuracy are thought to be in i) abnormal absorption spectra of CMHb and AMHb of the abnormal hemoglobins, which are exemplified by Hb Ms[3], and ii) partial loss of heme from the hemoglobin molecule, which is seen in some of the unstable hemoglobins, resulting in undue diminution of light absorbance of their CMHb and AMHb[4]. There will be two ways for the resolution of this problem. One is determination of globin by means of spectrophotometry in the ultraviolet region (at 275 nm), and the other is direct estimation of heme content of the relevant hemoglobins. Since ultraviolet spectrophotometry is inconvenient to routine works, direct heme estimation was taken as a candidate of useful measure, and the alkali-hemochrome method of Kajita[5] and the pyridine hemochrome method of Rimington[6] were examined for their accuracy and precision. The result of our reexamination of their methods in comparison with CMHb method disclosed the necessity of some improvement or modification of the original procedures.

This paper aims to present our improved hemochrome methods for Hb determination.

MATERIAL AND METHOD

The blood specimens were obtained from normal subjects and patients with hemoglobinopathies (Hb M Hyde Park[7,8], Hb Köln[9], Hb Hoshida[10] and Hb G Taichung[11]). The blood was prevented from coagulation with Anti-clot-ET and hemolysate was prepared by the conventional procedures[12]. Purified hemoglobin solutions were prepared by cellulose acetate membrane electrophoresis (pH 8.6) of the hemolysates[12] of the carriers of abnormal hemoglobins.

[1] Alkali-hemochrome (AH) method

A] Reagents:

i) 20 g/dl aqueous NaOH solution: 20.0 g of sodium hydroxide (Analytical Grade) is dissolved and made to 100.0 ml with water.

ii) $Na_2S_2O_4$: Commercially available fresh sodium hydrosulfite ($Na_2S_2O_4$, Analytical Grade) is used. It is stored in a desiccator.

iii) Standard Hb solution: Either whole blood or hemolysate is used as the standard Hb solution after their Hb concentration have been determined by CMHb method.

B] Apparatus:

i) Micropipette: Pipettes for taking aliquots of 5.0, 10.0, 15.0 and 20.0 μl.

ii) Photoelectric spectrophotometer for measuring the absorbance at 557 nm.

C] Procedures:

i) To each of the 10 ml test tubes labelled A, B, C, D and S, 4.0 ml of water is introduced. An aliquot of 10.0 μl of the blood sample is added to test tube S and mixed by inversion to get hemolysate. To the other test tubes (A, B, C and D) 0.0, 5.0, 10.0 and 15.0 μl of standard Hb solution are added individually in the order described and mixed to obtain hemoglobin solutions in the same way.

ii) To each test tube 2.0 ml of NaOH solution is added, mixed by inversion and allowed to stand for 10 min at room temperature (25–30°C) in order to release heme from molecule completely.

iii) A small quantity (4–5 mg) of sodium hydrosulfite is added to each tube, dissolved by inversion, and allowed to stand for about 10 minutes to get a maximum coloration of alkali-hemochrome.

iv) Hb concentration of blood sample (test tube S) is determined by collation to the calibration curve which is constructed by the optical densities at 557 nm and the hemoglobin concentrations of test tube B, C and D. Tube A is employed as the blank solution of the colorimetry.

[II] Pyridine hemochrome (PH) method

A] Reagent:

i) 0.1 N-NaOH solution containing 3% SDS*: To 500.0 ml of 6.0 g/dl SDS solution, 20.0 ml of 5.0 N-NaOH solution is added and made to 1,000 ml with water.

ii) Pyridine: Pyridine (Analytical Grade) is refluxed with ninhydrin and distilled in all glass ware vessels before use.

* SDS = Sodium dodecyl sulfate

iii) Sodium hydrosulfite and iv) Standard Hb solution are the same as those used for AH method.

B] Apparatus :

i) Micropipette and ii) photoelectric colorimeter are the same as in AH method.

C] Procedures ;

i) Into each of the ten ml test tubes labelled A, B, C, D and S, 5.0 ml of 0.1 N-NaOH containing SDS is introduced. Aliquot of 10.0 μl of blood sample is added to tube S, while 0.0, 5.0, 10.0 and 15.0 μl of the standard Hb solution are put into the tubes A, B, C and D in the order described.

ii) They are mixed and allowed to stand for about 10 minutes at 25–30°C to be hemolysed.

iii) 1.0 ml pyridine is added to each tube and mixed by inversion.

iv) A small quantity of sodium hydrosulfite (4–5 mg) is added to the individual tubes and dissolved by inversion.

v) The optical density of the colored solution is measured at 554 nm employing the solution in the tube A as the blank. Calibration curve is drawn with the optical densities of solutions A, B, C and D. The concentration of hemochrome of the blood sample (tube S) is read by collation of its optical density to the calibration curve.

RESULTS

The absorption spectrum of the colored solution in the AH method is shown in Figure 1. There sre peaks at wave length 557(α), 527(β), 475.5 and 424 nm, and bottoms are seen at 538(α') and 490(β') nm.

The absorption curves of whole blood, hemolysate, the solutions of hemoglobin derivatives (COHb, met Hb, Sulf Hb, Hb H and hemichromes) and abnormal hemoglobin (Hb Köln, Hb Akita, Hb Ms and so forth) were virtually identical in shape with respect to the height and the wave lengths of α and β peaks.

Thc shape of absorption spectra was affected to a considerable extent by the concentration of alkali (0.5–50.0 g/dl). About 20 g/dl NaOH (but not 3.0 g/dl which is described in original method) was found to be the most appropriate. There was no significant difference in absorption spectra between the individual derivatives. They gave the same α and β peaks and α-β peak separation giving the absorbance ratio of peak/bottom as $\alpha/\alpha'=3.20$, $\beta/\alpha'=1.48$ and $\alpha/\beta=2.16$.

Incubation time in alkaline medium to release heme from hemoglobin (in step 2) exerted influence upon the final coloration. Variation of the absorb-

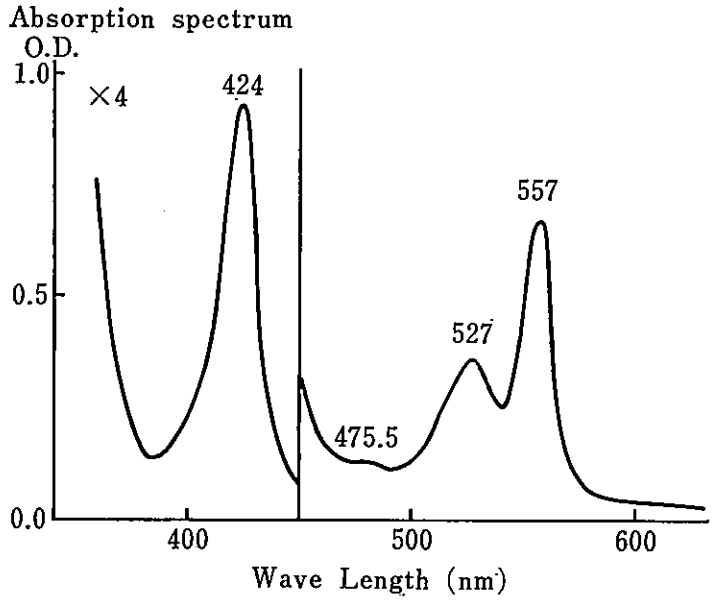

Fig. 1. Absorption curve of the colored solution in the alkali-hemochrome method

ance at 557 nm with time was as follows: at time zero, maximum absorbance, but unstable and tend to decrease; at 10 minutes, 5–10 percent decrease in comparison with the maximum coloration; at 10–60 minutes, the coloration was stabilized at the 10 minute-level, and at 60–240 minutes, the absorbance decreased gradually. The incubation for ten minutes was accordingly chosen.

The quantity (3–20 mg) of hydrosulfite which is added to the solution in step 3 did not affect the coloration.

The average value of the maximum discrepancy of hemoglobin estimation between the present procedure and the standard CMHb method was 1.46%. The coefficient of correlation (r) was quite satisfactory, being $+0.995$ ($n=109$) (Fig. 2).

In pyridine hemochrome method, the maximum coloration was seen immediately after addition of sodium hydrosulfite. This makes a striking contrast with the 10 minute incubation which is necessary in AH method. Coloration was stable for about 50 minutes.

The visual spectrum of the hemolysate by pyridine hemochrome method showed its peaks at 554(α), 522(β), 475(shoulder) and 418 nm and its bottoms at 536(α') and 498(β') nm (Fig. 3). It was quite similar in shape to that of AH, and looked superimposed when its curve was shifted by 4–5 nm toward

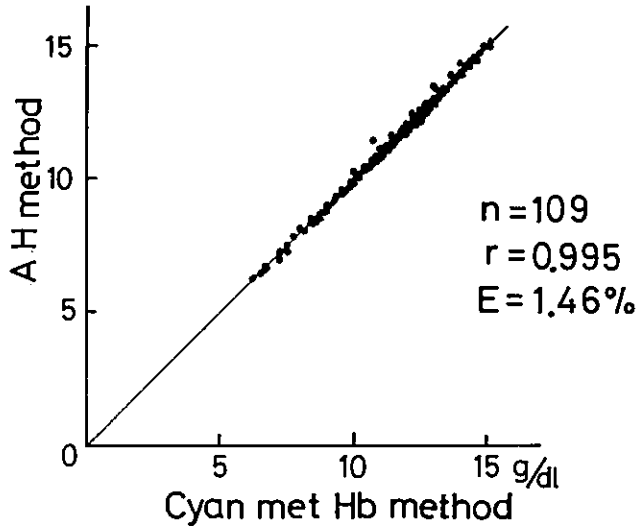

Fig. 2. Correlation of the alkali-hemochrome method with the cyanmethemoglobin method

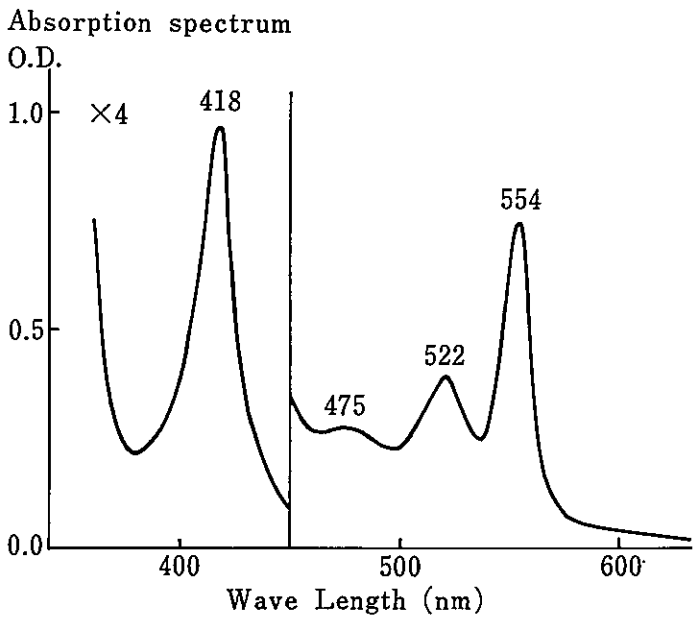

Fig. 3. Absorption curve of the colored solution in the pyridine hemochrome method

the side of longer wave length. There was no difference in the shape of absorption curve between the hemolysate and the Hb solution, but slight deviation was noted with the whole blood. The absorption spectrum with whole blood showed decrease in the α and β peaks, and the appearance of shoulder peak around 590 nm. This deviation was found to be alleviated by addition of the detergent (SDS). The concentration of 2–5% SDS in the alkaline solution was most satisfactory, giving a typical pyridine hemochrome pattern ($\alpha/\beta=1.99$, $\alpha/\alpha'=1.92$, $\beta/\alpha'=1.68$) which is seen in similarly treated hemolysate and hemoglobin solutions.

Similar examination of the AH method was made about the factors exerting influence on the final coloration, and the concentration of alkali, the incubation time and the amount of pyridine and hydrosulfite were specified as described in the methodology of the present procedure.

Maximum discrepancy of hemoglobin estimations between the pyridine hemochrome method and the CMHb method was very small, being 1.99% and the coefficient of correlation was $r=+0.990$ ($n=77$) (Fig. 4). There was no trouble when the pyridine hemochrome method was used for the determination of abnormal hemoglobins and their derivatives.

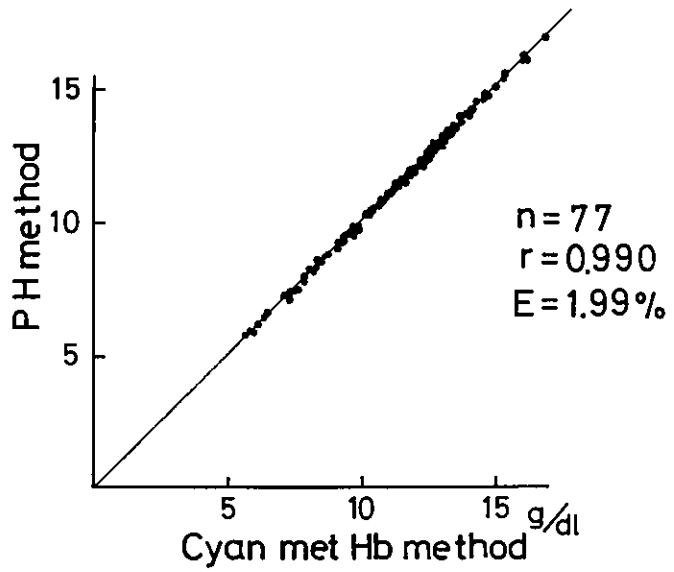

Fig. 4. Correlation of the pyridine hemochrome method with the cyanmethemoglobin method

DISCUSSION

The coloration mechanism of AH and PH methods are essentially the same. Heme is released from the hemoglobin molecule by alkali, and the chemical valency of heme iron is reduced from Fe^{3+} to Fe^{2+}. The fifth and sixth coordinate position of the heme Fe^{2+} are recordinated with either neutral His (Imidazole - N) residue of the nonspecific protein in AH method or uncharged pyridine-N which may have slightly stronger affinity in PH method. Both result in production of hemochrome pigments[13].

The plausibility of this hypothesis is corroborated by for following observation: i) Absorption spectra of the two pigments are quite similar. ii) Pure hemin solution produces hemochromes when it reacts with free histidine (amino acid), nicotinic acid (piridine derivatives). iii) Pure hemin solution forms hemochromes readily when it is mixed with human albumin, globulin or α or β polypeptide chain of hemoglobin.

Therefore, the ligands which have stronger affinity for Fe^{2+} than His-N, such as pyridine will interfere with the hemoglobin estimation by the AH method. However, ordinary ligands such as O_2, CO, H_2S and EDTA do not disturb the AH method.

The major improvements over the original procedures are as follows: i) use of 20 g/dl alkaline solution instead of 3g/dl in the AH method and use of 0.1 N-NaOH containing SDS instead of simple 0.1 N-NaOH solution in the PH method.

These improvements contributed much to solubilization of the pigment, clarification of plasma lipid and non specific protein, and to the typical hemochrome formation. Specification of the time for incubation of hemoglobin with solution of alkali which was not described in the original procedure is also important for accurate hemoglobin estimation.

The advantages of the AH and the PH methods described in this paper consist in i) their applicability to the estimation of concentration of various hemoglobins of men and animals, ii) their utility for the appraisal of partial loss of heme from hemoglobin molecule through comparison of estimation value of hemoglobin by the AH or PH method with that by the ultraviolet spectroscopy at 275 nm, and iii) freedom from the water pollution with toxic substances, such as potassium cyanide and potassium ferricyanide which are used in the CMHb method or NaN_3 in the AMHb method. From the stand point of view of present procedure the AH method will be preferred to the PH method, because the former does not demand pyridine which smells offensively.

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