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A Simple and Accurate Method for Measurement of the Hemoglobin Content in Blood by Colorimetric Iron Determination

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Summary. A simple and accurate determination of iron in blood involving acid digestion in a heating block is described. The digestion requires little attention and permits the handling of large numbers of samples. The diluted digest is used for the colorimetric determination of iron using o-phenanthroline, and the equivalent hemoglobin in blood is calculated. There was a good correlation between the results of the present method and the cyanmethemoglobin method with 21 standard blood samples containing 4.9–26.8 g/dl of hemoglobin.

Key words: Colorimetric iron determination – Hemoglobin content in blood, colorimetric iron determination

Zusammenfassung. Es wird eine einfache und genaue Methode zur Bestimmung von Eisen im Blut unter Verwendung von Säureaufschluß im Heizblock beschrieben. Der Aufschluß benötigt wenig Mühe und erlaubt die Verarbeitung einer großen Zahl von Proben. Der verdünnte Aufschluß wird zur kolorimetrischen Eisenbestimmung mittels o-Phenanthrolin benutzt und die entsprechende Hämoglobinkonzentration im Blut berechnet. Es zeigte sich eine gute Korrelation der Ergebnisse mit der beschriebenen und der Cyanmethämoglobin-Methode anhand von 21 Standardproben, die 4,9 bis 26,8 g/dl Hämoglobin enthielten.

Schlüsselwörter: Eisenbestimmung, kolorimetrisch – Hämoglobingehalt im Blut, kolorimetrische Eisenbestimmung

The cyanmethemoglobin method [1, 2] is now widely used for the determination of the hemoglobin content in fresh blood. In forensic practice, total hemoglobin is often determined to calculate the saturation percentage of carboxyhemoglobin (HbCO) for diagnosis of carbon monoxide poisoning [3–5]. Blood samples obtained from cadavers sometimes contain various denatured hemoglobin deriv-

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atives which cannot be determined by the cyanmethemoglobin method. Iron determination either by colorimetry [4] or by atomic absorption spectroscopy [6, 7] has been recommended for hemoglobinometry in decayed blood. The colorimetric method for iron determination requires laborious procedures involving acid digestion of blood samples. Atomic absorption spectrophotometric iron determination can be made without acid digestion. However, the results varied, depending upon the presence of inorganic substances or handling of blood samples [8].

In the present study, a simple and accurate colorimetric method for determination of iron involving sulfuric acid digestion in a heating block was described.

Materials and Methods

Instruments

Acid digestion was made in an aluminium block electrically heated and thermostatically controlled, with 40 holes to accomodate Pyrex test tubes (Taiyo dry thermo unit TA-2H, Taiyo Service Center Co. Ltd. Tokyo, Japan). Colorimetry was made with Hitachi 557 spectrophotometer (Hitachi, Ltd. Tokyo, Japan).

Materials

Venous blood from healthy adults was collected into heparinized test tubes. Erythrocytes and plasma of the blood were separated by centrifugation, and the standards containing various concentrations of hemoglobin were prepared by mixing the cells and the plasma. The standards (about 1 ml each) were stored at $-30°$ C and thawed just before the analyses.

The glassware used had been cleaned with chromic or sulfuric acid and subsequently rinsed repeatedly with distilled and deionized water. All reagents used were of analytic grade. Only distilled and deionized water was used for diluting and rinsing purposes.

Iron Determination by Colorimetry

Reagents: (1) Conc. sulfuric acid, (2) hydrogen peroxide, 30%, (3) ammonia water, 28%, (4) p-nitrophenol, 50 mg per 10 ml of ethanol, (5) sulfuric acid, 2.5 N: dissolve 6.9 ml conc. H_2SO_4 in water and dilute to 100 ml, (6) ascorbic acid, 0.24 g/dl: dissolve 48 mg ascorbic acid in water and dilute to 10 ml, prepare freshly, (7) color reagent: dissolve 90 mg o-phenanthroline and 30 g sodium acetate in water, and dilute to 100 ml, and (8) Iron stock solution (50 µg Fe per ml): dissolve 430.5 mg $Fe(NH_4)(SO_4)_2 \cdot 12\,H_2O$ in 1 liter of 0.1 N HCl.

Total Hemoglobin Determination as Cyanmethemoglobin

Total hemoglobin was determined using a commercial kit (Hemoglobin test-Wako, Wako Pure Chemical Industries, Ltd. Osaka, Japan) based on the method of Van Kampen and Zijlstra [1]. The extinction coefficient of cyanmethemoglobin at 540 nm was obtained using a standard cyanmethemoglobin commercially available (18 g/dl, Wako Pure Chemical Industries, Ltd. Osaka, Japan).

Procedure

Digestion. Pipette 0.4 ml of diluted blood containing 10–30 µg Fe into a test tube (Pyrex, 20×200 mm). Pipette 0.4 ml of water into another test tube, which is carried through as a blank. Add 0.4 ml conc. H_2SO_4. Place the tubes in a heating block and heat the block at 200° C for 40 min. After cooling of the digest, add 0.1 ml H_2O_2 solution and heat for an additional 5 min at 200° C. Add 0.1 ml H_2O_2 solution again and heat in the same way. A water-clear or slightly colored residue is then obtained.

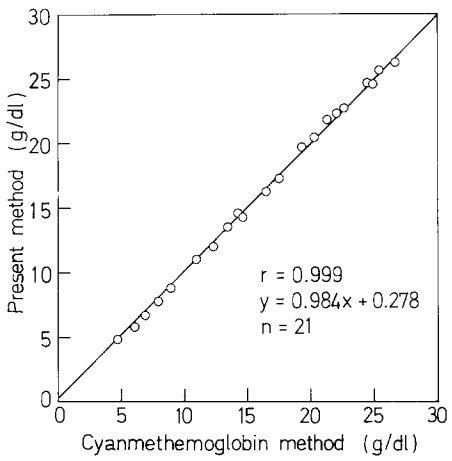

Fig. 1. Comparison of the cyanmethemoglobin method with the present method determining iron in blood by colorimetry

Color Development. Transfer the digest to a 10 ml volumetric flask, using about 6 ml of water, and add 0.9 ml ammonia water to the flask. Cool the flask with tap water and add 1 drop p-nitrophenol. Add ammonia water until the solution becomes yellow. Add 2.5 N H_2SO_4 until the color of the solution disappears and then add 0.4 ml 2.5 N H_2SO_4. Add 0.2 ml ascorbic acid solution and then 1 ml of the color reagent. Dilute the solution to 10 ml with water. Read absorbance at 510 nm. The color appears just after the addition of the color reagent and is stable for at least 24 h.

Standards. For standards, pipette 0–0.8 ml iron stock solution into a 10 ml volumetric flask. Add 0.4 ml 2.5 N H_2SO_4, 0.2 ml ascorbic acid and 1 ml of the color reagent in that order. Dilute the solution to 10 ml with water. Read absorbance at 510 nm.

Calculation. Calculate hemoglobin content in blood from the iron values by the following equation,

$$\text{Hb (g/dl)} = \frac{\text{Fe (}\mu\text{g)}}{\text{x (}\mu\text{l)}} \times \frac{1}{55.85} \times \frac{64500}{4} \times \frac{1}{10}$$

where, Fe represents iron content in a test tube as μg and x original blood volume added in the tube as μl.

Results

The extinction coefficient of four standards of cyanmethemoglobin at 540 nm was 11.01 ± 0.03 for 25 mM which excellently coincides with the value of 11.0 adopted during the 12th Congress of the International Society of Hematology in Sidney, Australia [2]. The value of 11.01 was used in the present study. Two methods for determination of total hemoglobin, the cyanmethemoglobin method and the present colorimetric method for iron determination in blood, were compared with 21 standard blood samples containing 4.9–26.8 g/dl of hemoglobin (Fig. 1). There was an excellent agreement between the results of the two methods.

Discussion

Van Assendelft et al. [8] reported that determination of iron in blood by atomic absorption spectroscopy was influenced by the presence of inorganic substances

and handling of blood samples. Furuno et al. [9] reported that blood samples diluted with water and directly determined by atomic absorption spectroscopy showed higher hemoglobin values than those determined by the cyanmethemoglobin method. On the other hand, Lötterle [7] reported that blood samples diluted with 0.5% Triton X-100 and determined directly by atomic absorption spectroscopy resulted in 4.35% lower hemoglobin values than those determined by the cyanmethemoglobin method. Consequently, the technique is liable to systematic errors and unsuitable for the absolute measurement of hemoglobin iron.

The colorimetric iron determination with acid digested blood has two problems; first, acid digestion requires laborious procedures, and second, the formation of iron hydroxide by alkali preventing the ionization of iron may occur during color development [10]. A simple sulfuric acid digestion technique using an aluminium heating block described in the present study requires little attention and permits the handling of large numbers of samples. The present method for color development is basically according to the method of Matsubara which can avoid the formation of iron hydroxide by using sodium acetate instead of an alkali reagent to get the optimum pH for color development [10]. Thus, the present method could settle both problems with the colorimetric iron determination. Accuracy of the present method was well demonstrated in the comparative study with the cyanmethemoglobin method shown in Fig. 1. The present hemoglobinometry by iron determination can be applied to decayed blood and is suitable for forensic practice.

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