A Rapid and Simple Colorimetry of Iron in Hemoglobin with Sodium Hypochlorite and Bathophenanthroline

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SUMMARY A rapid and sensitive technique for colorimetric determination of iron in hemoglobin which requires no deproteinization is described. Iron (about 17 nmoles) in hemoglobin of 200 µl of 100 times diluted blood is oxidized to Fe³⁺ and released by adding 200 µl of sodium hypochlorite (active chlorine: 0.28 mol/l). This released Fe³⁺ is reduced to Fe²⁺ by adding 300 µl of 0.17 mol/l ascorbic acid. In order to completely release and reduce iron, after standing for 5 minutes at a room temperature, the released Fe³⁺ is determined by colorimetry with 300 µl of 1.05 mmol/l bathophenanthroline at 535 nm. Simultaneously, denatured protein should be dissolved by adding 500 µl of 0.2 mol/l tris-maleate buffer (pH 7.0). Thus this method (C.V.=2.15%) requires no deproteinization procedure. This method correlates enough with mineralization-bathophenanthroline method (y=1.005x–0.01, r=0.995, n=30).

Introduction

The determination of iron content within hemoglobin has been established especially by atomic absorption method. The specificity and accuracy of the method and moreover, the convenience of continuous and direct analysis of samples, requiring no preparation other than simple dilution makes this technique highly desirable1. However, this method requires such an expensive instrument as an atomic absorption spectrophotometer.

Iron within hemoglobin has been recently analysed by colorimetry. In this method, iron is released from hemoglobin by mineralization and thus released iron (Fe³⁺) is reduced to Fe²⁺ by a reducing agent and then determined by colorimetry under a reaction with bathophenanthroline disulfonic acid disodium salt • 2H₂O (BPT). However, this mineralization technique is rather troublesome and time consuming2.

In our new method, sodium hypochlorite is used as an oxidizing agent of iron to Fe³⁺ state and also as a powerful releasing agent of iron from hemoglobin.
Furthermore, the released Fe$^{3+}$ is reduced to Fe$^{2+}$ by ascorbic acid and led to a colored substance by BPT. But through this procedure, denatured protein is formed that the reaction mixture is turbid. This denatured protein may be dissolved by tris-maleate buffer (pH 7.0). In this paper, the method without deproteinization is briefly described.

**Materials and Methods**

**Sample preparation**

Only venous blood from healthy subject was used. Blood was drawn by Vacutainer or syringe into heparinized tubes.

**Reagents**

All reagents used were of analytical grade. To avoid even a subtle contamination by iron water, water was deionized by MILLI-Q Water Purification System (Millipore Corp., Bedford, MA, U.S.A.). Working iron standard solutions (17.9–179 μmol/l) were prepared from the certified iron standard solution (FeCl$_3$, Fe: 17.9 mmol/l) for atomic absorption measurement (Kishida Chemical Co., Osaka, Japan). NaClO solution (active chlorine: 0.28 mol/l) was prepared from a commercial stock solution (active chlorine: 2.82 mol/l, Katayama Chemical Co., Osaka, Japan). Each of 0.17 mol/l ascorbic acid solution and 1.05 mmol/l BPT chromatogen solution was prepared by dissolving ascorbic acid or BPT (disodium) in deionized water respectively. Tris-maleate buffer (0.2 mol/l, pH 7.0) was prepared by mixing tris(hydroxymethyl) aminomethane, maleic acid with a small amount of 1N NaOH and deionized water.

Deionized water was used throughout the procedure.

**Apparatus and Glasswares**

The absorbance of solution was measured by a spectrophotometer (UVIDEC-510, Japan Spectroscopic Co., Ltd., Tokyo, Japan). The glasswares used were first rinsed with 4N HNO$_3$ and then washed with deionized water.

**Procedure**

Hemoglobin iron was measured as follows: 200 μl of 100 times diluted blood containing about 17 nmoles was put in a test tube into which 200 μl of 0.28 mol/l NaClO and 300 μl of 0.17 mol/l ascorbic acid were added successively and mixed well. At this point, protein was denatured and the reaction mixture became turbid. After standing it for 5 minutes at a room temperature, 300 μl of 1.05 mmol/l BPT solution was added. To dissolve the denatured protein, 500 μl of 0.2 mol/l tris-maleate buffer (pH 7.0) was added. The optical density (A') of the test solution was measured by UVIDEC-510 at 535 nm. For the blank test, deionized water was used instead of diluted blood. The optical density (B') of the blank test was also measured similarly at 535 nm.

**Calculation of iron amount of hemoglobin**

Iron amount of hemoglobin in the test blood is calculated as follows (Eq. 1).

Iron amount (nmol) in 1 ml blood

\[ \text{Iron amount (nmol) in 1 ml blood} = 17.9 \times 500 \times \frac{(A' - B')}{0.256} \]  

(0.256 : optical density of 17.9 nmol iron in the present method)

From the iron amount, hemoglobin amount in the specimen blood was easily calculated from Eq. 2.

Hemoglobin amount (nmol) in 1 ml blood
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\[ = \frac{1}{4} \text{ iron amount (nmol)} \ldots \ldots (2) \]

**Results**

*Calibration curve and sensitivity*

A typical calibration line of blood sample from a healthy subject (1–10 µl) is shown in Fig. 1. An absorbance (O.D. = 0.126, at 535 nm) of normal human blood nearly corresponds to 8.40 nmol of iron.

*Recovery test*

Table I shows the results obtained from tests in which 3.58, 7.17, 10.75, 14.34 and 17.92 nmol of iron were added to a blood sample. The recoveries are in the range of 99.9–102.4%.

<table>
<thead>
<tr>
<th>Table I Recovery of ferric iron added to blood specimen</th>
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<tbody>
<tr>
<td>Fe conc. (nmol)</td>
</tr>
<tr>
<td>----------------------------------</td>
</tr>
<tr>
<td>18.64</td>
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<td>18.64</td>
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<td>18.64</td>
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</table>

Table II Coefficient of variation

<table>
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<th>Sample No</th>
<th>O. D.</th>
<th>Sample No</th>
<th>O. D.</th>
</tr>
</thead>
<tbody>
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<td>11</td>
<td>0.227</td>
</tr>
<tr>
<td>2</td>
<td>0.232</td>
<td>12</td>
<td>0.220</td>
</tr>
<tr>
<td>3</td>
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<td>13</td>
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<tr>
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</tr>
<tr>
<td>5</td>
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</tr>
<tr>
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</tr>
<tr>
<td>10</td>
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<td>20</td>
<td>0.228</td>
</tr>
</tbody>
</table>

\[ m = 0.224 \quad \text{S. D.} = 0.0048 \quad \text{C. V.} = 2.146\% \]

Blood (×100) 200 µl

Comparison of the present method with the BPT method with mineralization

Diluted blood (25–200 times, 200 µl) was ashed together with conc. HNO₃ by heating and the iron in the residue was analysed by colorimetry with BPT. This method and the present method made a good accordance as shown in Fig. 2.
Coefficient of variation

C. V. was determined by measurements of 20 samples of the another diluted blood, and the value was 2.146% as shown in Table II.

Discussion

In 1959, Fischl3) devised a colorimetry of iron in blood with potassium thiocyanate. However, in his method, deproteinization of blood must be done before colorimetry. Connerty et al.4) reported a procedure for colorimetry with deproteinization: NaClO is added to blood to release Fe^{3+}; 5.10 mol/l H_{2}SO_{4} is added to the reaction mixture for deproteinization; and iron in the supernatant is determined by colorimetry with ammonium thiocyanate. In 1967, Zettner et al.1) measured the amount of iron within 100-200 times diluted blood by an atomic absorption spectrophotometer. This method is quite easy but a special and expensive instrument is necessary.

In 1969, Baginski et al.2) devised the micromethod of iron in blood. In this method, 0.1 ml of blood is ashed by mineralization and the released iron is determined by colorimetry with BPT. Its mineralization technique is somewhat troublesome.

The micromethod for hemoglobin iron determination reported here only requires 2 µl of blood and 6 minutes of time. Deproteinization and mineralization techniques are not necessary in the present method because 0.2 mol/l tris-maleate buffer (pH 7.0) added dissolves partially denatured protein. Furthermore, the present method is as precise, sensitive and reproducible as that of Baginski et al.2) with mineralization technique.

As shown in Fig. 1, hemoglobin iron in only 1 µl of normal blood can be determined by our method. This means that our standard method can be applied to the determination of hemoglobin iron in blood of patient with anemia, if 5 µl of the blood is obtained.

On the other hand, as to the use of all blood in the present method, the amount of transferrin is double measured. However, because the average amount of serum iron in a grown man is 0.0269 mmol/l generally, and even considering hemoglobin iron in whole blood is 8.96 mmol/l, the amount of transferrin which may affect the measurement is 1/333. Also, the used blood amount is so small as 2 µl that iron in transferrin can be ignored.

The amount of iron taken from normal human blood heated at 60 ºC for 15 minutes could be determined mined by the present method and the value was the same as that of the unheated blood. Iron amounts of human, hog, rat and bovine blood specimens which had been dried for 30 days in a desiccator and dissolved in saline, were measured and the values turned out to be the same as those of fresh blood specimens. From these observations, it is clear that the present method can be applied not only to fresh blood samples but also to old and partially denatured blood such as blood specimen in legal medicine.

The present method, however, can not be directly applied to the determination of iron in a small amount (a few µl) of serum because the amount of iron contained in serum is minute (approx. 0.3%).

In 1977, Williams et al.5) reported the microdetermination of iron in serum by using 2,4,6-tripyridyl-s-triazine (color reagent) and guanidine (releasing reagent of
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iron from serum), but when this method was applied to the determination of hemoglobin iron, it was discovered that guanidine can not release iron from hemoglobin. In addition to this, other oxidizing agents, K₃Fe(CN)₆ or H₂O₂ are deficient that K₃Fe(CN)₆ takes more than 30 minutes to release iron, and H₂O₂, inspite of its releasing time is 5 minutes, brings about bubbles which likely to cause misjudgement.

Therefore, it became clear that NaClO is the most adequate reagent for releasing iron from hemoglobin.

References