A RAPID SPECTROPHOTOMETRIC DETERMINATION OF CARBOXYHEMOGLOBIN IN BLOOD

BY

Hideyuki Muroya and Tamenobu Kubota*1

ABSTRACT

A method is described whereby carboxyhemoglobin in the blood is rapidly and accurately determined by spectrophotometric analysis. The $\gamma$-wave ranging from 400 to 500 nm was used for the determination, and to increase the stability of the absorption value the isobestic point was utilized.

This method is simple; a heparinized whole blood sample, diluted to about 1:100 to 1:200, is placed in a cuvette and the absorbance is measured at the wavelengths of 413.2 nm, 421 nm and 431.3 nm against water as the zero reference standard. The absorbances of the standard solutions of 0% of CO-Hb and 100% of CO-Hb are also measured, and by these absorbance values the concentrations of CO-Hb are calculated.

To make the procedure simpler, it is convenient to use a factor composed by personal and instrumental ones, which is obtained during the calibration by the spectrophotometer. Using this factor, the determinations of the absorbances of standard solutions of 0% and 100% of CO-Hb can be omitted.

INTRODUCTION

A series of methods for estimating the carbon monoxide in the blood have been reported; these are classified as gasometric1, infrared analytical2-3, gas chromatographic4 and spectrophotometric ones5-9. And each of these has its own problems such as time consuming, technical difficulty, accuracy, need of trained personnel and availability of equipments. The spectrophotometric method has long been studied9, because of its technica! simplicity and availability of equipment, but also it has been reported that this method is not sensitive for the relatively low concentration of carboxyhemoglobin.

The methods of Kampen7 and Commins and Lawther8,9 were recently reported and used as the standard methods, as they are sensitive and stable enough for the measurement of CO-Hb of low concentration. However, the authors were not content with these methods and tried to find

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*1 墨屋 博顕, 久田 敦: Department of Hygiene (Chief: Prof. H. Kita), School of Medicine, Tokyo Medical and Dental University (Tokyo Ika Shika Daigaku). Received for publication, December 1, 1973.
a new method which is especially more rapid.

Exposure of a large population to CO in the urban environments has been a subject of increasing interest, and it is necessary to have a rapid and sensitive method for the measurement of the low value of CO-Hb in the blood to know the effects of air-pollution on the human body.

In this study, an accurate method based on spectrophotometric analysis is reported and this permitted us to achieve good results in the measurement of the carboxyhemoglobin in the blood of a large number of samples.

**Principle of Method**

The concentration of CO-Hb in the blood is expressed in two ways, the one is the concentration of CO-Hb in the total hemoglobin

\[
\text{CO-Hb} \left( \frac{\text{act. Hb} + \text{inact. Hb}}{\text{Hb}} \right)
\]

and the other, the concentration of CO-Hb in the active hemoglobin which combines with oxygen (CO-Hb/act. Hb). In this study, the CO-Hb\% was shown in the latter.

Hemoglobin and its derivatives have specific absorbances respectively between the wavelengths of 400 to 500 nm. CO-Hb has an absorbance maximum at the wavelength of near 420 nm and O₂Hb near 414 nm.
Also it is reported that the isosbestic points* of the spectra of CO-Hb and O₂-Hb are at the wavelengths of 413.2 nm and 431.3 nm (Fig. 1). The absorbances at the three wavelengths (413.2, 421 and 431.3 nm) of the unknown solution of CO-Hb (x%) and the solution of CO-Hb (100%) and CO-Hb (0%) against water as the zero reference are as follows:

$$E_{413.2}(x\%) = E_{421}(x\%) = E_{431.3}(x\%)$$
$$E_{413.2}(100\%) = E_{421}(100\%) = E_{431.3}(100\%)$$
$$E_{413.2}(0\%) = E_{421}(0\%) = E_{431.3}(0\%)$$

E: absorbance of a solution of a certain concentration (%) of CO-Hb at a certain wavelength.

These three absorbances at the 413.2 nm and 431.3 nm are equal, respectively, that is: (Fig. 2).

$$E_{413.2}(x\%) = E_{421}(100\%) = E_{431.3}(0\%) \quad \cdots \quad (1)$$

Also, the absorbances of $E_{421}(100\%)$ and $E_{431.3}(0\%)$ are dependent upon the Hb concentration of the diluted blood solution, but the absorbance of $E_{421}(x\%)$ changes parallelly to that of $E_{421}(100\%)$ and $E_{431.3}(0\%)$, therefore the concentration of the unknown CO-Hb% in the blood (CO-Hb [%]) is obtained theoretically by the following equation:

$$C_{CO-Hb(\text{X}\%)} = \frac{E_{421}(x\%) - E_{421}(0\%)}{E_{421}(100\%) - E_{421}(0\%)} \times 100 \quad \cdots \quad (2)$$

* isosbestic = absorption curves of solutions of the same concentration at different states.
But to determine the blood CO-Hb % accurately, the absorbances of \( E_{421}(x\%) \), \( E_{421}(100\%) \) and \( E_{421}(0\%) \) must be stable during the time of repeated determinations. According to our experiments, these absorbances show slight changes with each determination, being dependent upon the diluting procedure of the solutions. And because of that, the CO-Hb % in the blood obtained by equation (2) cannot be accurate enough for the determination.

Therefore, to level up the accuracy of such a spectrophotometric method, the authors applied the isosbestic phenomena mentioned above and introduced the value \( r \) to neglect the slight instability of the absorbances at the wavelengths of 413.2 nm, 421 nm and 431.3 nm. The ratios \( r \) are calculated as follows:

\[
\begin{align*}
    r(x\%) &= E_{421}(x\%) / \sqrt{\left( E_{413.2}(x\%) + E_{431.3}(x\%) \right)} \\
    r(100\%) &= E_{421}(100\%) / \sqrt{\left( E_{413.2}(100\%) + E_{431.3}(100\%) \right)} \\
    r(0\%) &= E_{421}(0\%) / \sqrt{\left( E_{413.2}(0\%) + E_{431.3}(0\%) \right)}
\end{align*}
\]

Then the following equation (4) was obtained instead of the equation (2), and the sensitivity and stability increased much in determining the concentration of the CO-Hb % in the blood.

\[
C_{CO\text{-Hb}(x\%)} = \frac{r(x\%) - r(0\%)}{r(100\%) - r(0\%)} \times 100
\]

But, to determine the \( C_{CO\text{-Hb}(x\%)} \) using the equation (4), \( r(0\%) \) and \( r(100\%) \) must be determined for each sample. It requires a complicated procedure of preparing oxygen or CO gas bubbling into the blood to make a solution of 0\% or 100\% concentration of CO-Hb for the determination of \( r(0\%) \) and \( r(100\%) \).

If the values of \( r(100\%) \) and \( r(0\%) \) become a constant \( R(100\%) \) and \( R(0\%) \) after repeated examinations, i.e. characteristic to the analyst according to his technique, this procedure can be neglected. The absorbances of \( E_{421}(100\%) \) and \( E_{421}(0\%) \) vary in each blood sample as described above. The values \( r(100\%) \) and \( r(0\%) \), obtained by dividing the value of \( E_{421}(100\%) \) or \( E_{421}(0\%) \), respectively, by the mean value of the absorbances at the two isosbetic points, are constant.

Therefore, the equation (4) is changed as follows and the determination of CO-Hb % becomes much easier.

\[
C_{CO\text{-Hb}(x\%)} = \frac{r(x\%) - R(0\%)}{R(100\%) - R(0\%)} \times 100
\]

where \( R(0\%) \) and \( R(100\%) \) are the constants obtained by repeated examinations, and the CO-Hb % is determined from the absorbances at the
Fig. 3. Schema diagram of spectrophotometric determination of carboxy-hemoglobin in blood.

wavelengths of 413.2 nm, 421 nm and 431.3 nm against water as the zero reference standard.

**Analytical Method (Fig. 3)**

1) Blood samples
   Approximately 1 ml of human blood is collected in the heparinized tube from the median vein.

2) Diluting solution I
0.04% ammonia solution (this solution, prepared with distilled water, can be useful for 3 to 4 days, if stored in a dark and cool place).

3) Diluting solution II (saturated with CO)\(^{10}\)

100% CO gas is bubbled through the diluting solution I for 20 minutes at the rate of 50 ml per minute. This CO-saturated solution contains 0.005 to 0.010 ml of CO per one ml of the solution.

4) Procedure

Prepare three glass tubes, A, B and C, and 10 ml of solution I is placed in each of the two tubes of A and C and 10 ml of solution II in tube B, and 0.1 ml of the blood is dissolved in each of the three tubes. This solution contains blood at the proportion of 1:101 and is a clear red color.

If the solution is not clear, solution II should be checked and renewed, or add 1 or 2 drops of 0.1% ammonia solution to make it clear.

Then 100% oxygen is bubbled into the solution of tube C, for about 15 minutes at the rate of 50 ml per minute. Three kinds of solutions, A, B and C, are put into a cuvette (path length of 1 mm), respectively, and their absorbances are measured at the three wavelengths (413.2, 421 and 431.3 nm) against water as the zero reference. Then each absorbance value is put into the equation (3, 4). The absorbance is measured on a double-beam spectrophotometer (Shimazu model UV-200).

5) Calculation

A small computer (Olivetti Programma 101) is used for the calculation substituting the nine measured absorbance values in the equation (3, 4). As described above, the values of \(r(100\%)\) and \(r(0\%)\) in the equation (3) for the oxygen-saturated solution and CO-saturated solution are stable, and \(R(100\%)\) and \(R(0\%)\) can be respectively a constant factor, under the stable condition of the diluting factors of blood, calibration by the spectrophotometer and path length of the cells.

Once this procedure of determining \(R(0\%)\) and \(R(100\%)\) is carried out, it is unnecessary to repeat the CO and \(O_2\) gas bubbling in each determination. For the determination of the CO-Hb % in the blood, the blood solution (A) is prepared first, and the absorbances of the solution (A) are read at the three wavelengths, then the value of \(r(x\%)\) is calculated by the equation (3), and finally the CO-Hb % is obtained by the equation (5). In this calculation, \(R(0\%)\) and \(R(100\%)\) are two known constant factors, and there are three measured values of \(E_{421}(x\%), E_{421.3}(x\%) \) and \(E_{431.3}(x\%)\), which are to be substituted in the equation (5) for calculation.
A comparison of the CO-Hb % in the series of 5 prepared samples containing 0 to 100% of CO-Hb, measured spectrophotometrically by the method described above, using the equation (5) with the measurements on the same samples by Commins and Lawther’s method, was made to test
Table 2. CO-Hb contents of smokers and nonsmokers obtained by the isosbestic method

<table>
<thead>
<tr>
<th>Nonsmoker CO-Hb %</th>
<th>No. of cigarettes per day</th>
<th>Cigarettes-smoker smoking experiences before test</th>
<th>CO-Hb %</th>
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N : 30
Mean: 0.72
S.D. : 0.67

N : 28
Mean: 3.45
S.D. : 1.44

the lowest determinable concentration of CO-Hb, and accuracy, reproducibility and stability for the measurement.

In the series of samples mentioned above, the average concentrations by the present method and by Commins and Lawther’s method are shown in Fig. 4 and Table 1. Relationship between both methods are linear (r=0.96) even at the CO-Hb concentration of 0 to 10%.

It was noticed that the determination by this method showed a smaller
standard deviation than by Commins and Lawther's method, though there was no significant difference ($P=0.05$) between the results by these two methods with the same samples.

Then the present procedure was used to determine the CO-Hb % in a series of fifty-eight healthy male and female subjects, including twenty-eight smokers. The mean value and standard deviation was $3.45\pm1.44\%$ in twenty-eight smokers and $0.72\pm0.67\%$ in the other nonsmokers (Table 2).

The reason for using the wavelengths of 418.2 nm and 431.3 nm was: at the wavelength of 418.2 nm and also at 431.3 nm, the absorbances of any concentration of CO-Hb presented the same measurement value (isobestic point)\(^{11}\).

The wavelength of 421 nm was adopted here as the maximum absorbance point because the differences of absorbances are greater than that of 420 nm, and showed a lineality with the concentrations of CO-Hb.

Also, the blood was diluted to 1:101 by this method, but using a common cuvette of a path length of 1 cm, the solution can be diluted ten times in volume, i.e. about 1:1,000 solution of the original sample blood is prepared. In other words, the absorbance at the wavelength of 421 nm should be within the measurable range of the available spectrophotometer.

The solution II (CO-saturated solution) contains 5 to 10 ml of CO per liter, after being bubbled. The hemoglobin content of the human blood is about 15 to 20 g per 100 ml, and the volume of the oxygen combined with the hemoglobin is reported to be 1.34 ml per g. Therefore, 0.027 ml of CO gas is needed to saturate 0.1 ml of blood (100% CO-Hb). And in 10 ml of the solution II, 0.05 to 0.10 ml of CO gas is contained, so this amount will be enough to saturate the blood with CO gas. The results of the experiment showed that in every sample of the solution prepared to be 100% CO-Hb, the absorbance was always the same, so the solution seemed to be the 100% CO-Hb saturated one.

Moreover, $R(0\%)$ and $R(100\%)$, as the constant factors for 0% CO-Hb and 100% CO-Hb, calculated by the data of Small\(^{15}\), are:

$$
R(\ 0\%)=1.238\pm0.015 \\
R(100\%)=2.087\pm0.015
$$

The value of $R(0\%)$ and $R(100\%)$, calculated by our method from 12 samples, are as follows (Table 3):

$$
R(\ 0\%)=1.222\pm0.006 \\
R(100\%)=2.035\pm0.016
$$

The values $R(0\%)$, $R(100\%)$ are similar to those of Small, and the coefficient of variation is 0.5% in $R(0\%)$ and 0.8% in $R(100\%)$, which show more stable values. So the determination of CO-Hb % in the blood
Table 3. Stability of constants $r(0\%)$ and $r(100\%)$ at 0% and 100% CO–Hb concentrations

<table>
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<th>No.</th>
<th>$r(0%)$ *1</th>
<th>$r(100%)$ *2</th>
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<tr>
<td>2</td>
<td>1.207</td>
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<td>9</td>
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<tr>
<td>11</td>
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</tr>
<tr>
<td>12</td>
<td>1.224</td>
<td>2.046</td>
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Mean 1.222 2.035
S.D. 0.006 0.016
Coefficient of variation ±0.5% ±0.8%

*1 $r(0\%) = E_{431}(0\%) / \frac{1}{2}(E_{413.2}(0\%) + E_{431.3}(0\%))$
*2 $r(100\%) = E_{431}(100\%) / \frac{1}{2}(E_{413.2}(100\%) + E_{431.3}(100\%))$

by the method using the equation (5) can be considered to be accurate. Therefore, the method described above is one of the spectrophotometries useful for determining the CO-Hb% in the blood.

**CONCLUSION**

We designed a new rapid and accurate method for the determination of the CO-Hb concentration, even of low concentration in the blood, especially to examine samples of a large mass in a short time.

Several prominent points of our method are as follows:

1) Water can be used as the zero reference standard.
2) Only three absorbances are measured.
3) CO and O₂ gas bubbling into each sample can be omitted.
4) Accurate determination of CO-Hb % is carried out by the equation (5) with three absorbances.
5) Pasing through the above procedures, time for treating one sample can be cut down about 30 minutes.
6) Compared to Commins and Lawther's method, our method is as accurate as their method.
7) The rapid and simple determination enable us to examine multiple samples in a short time.
Acknowledgements

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References


