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Originals

RAPID DETERMINATION OF CARBOXYHEMOGLOBIN BY ABSORBANCE DIFFERENCE BETWEEN DOUBLE WAVE LENGTH.

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Abstract……A spectrophotometric determination of CO-Hb content was investigated on the basis of double wavelength spectrophotometry. Absorbance differences at the two wavelengths at which O_2-Hb has the same absorbance reflect only the CO-Hb component because the O_2-Hb component is nulled out from the mixture of O_2-Hb and CO-Hb. After measurement of absorbance difference, the measuring solution was saturated with pure CO and remeasured. CO-Hb % was calculated from the ratio of the absorbance differences.

Since temperature significantly influences the spectrum of hemoglobin pigment, the wavelength pair changed with temperature. For accurate measurement, it was necessary to control temperature of diluting solution before dilution. Liberation of CO from CO-Hb was observed at more than 80% CO-Hb, and 5% and 2% liberation from 100% CO-Hb occured at 100-fold dilution with 10 mm cuvette and 20-fold dilution with 2 mm cuvette respectively.

Advantages of this method are that the standard curve or equation for calculation and accurate dilution are not necessary because the interfering component is nulled out and 100% CO-Hb of the standard sample is made with the same sample; and measurement is very rapid, taking less than 2 min.

Key words: Carboxyhemoglobin, Double wavelength spectrophotometry, Temperature effects on spectrum, CO liberation from CO-Hb, Absorbance differences.

INTRODUCTION

The determination of the carboxyhemoglobin (CO-Hb) in blood is usually documented by measuring it with the spectrophotometer or indirectly by measuring the amount of CO.

Methods for the measurement of CO-Hb with spectrophotometry can be divided into

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several groups according to the wavelength ranges, i. e. 390-440 nm (Soret region) and 500 -600 nm ; and according to the number of wavelengths used, i. e. the single and multiple wavelength methods.

Spectrophotometric determination of CO-Hb using the single wavelength of 419 nm according to Akiya method (Akiya and Tanimura, 1952) and its modification (Kozuka et al., 1969) have been adopted in Japan as official standard methods of chemical analysis in poisonings. However, it has been shown that the data obtained by these methods disagree with those by other methods (Hayashi et al., 1977 ; Miyauchi and Sakaki, 1977 ; Sakai and Kojima, 1978). Maehly (1962) reviewed the methods by which CO-Hb is measured by the absorbance ratio from two or three wavelengths between 430 nm and 579 nm. This principle was applied in the CO-Oximeter which automatically calculated the absorbance ratio using a computer (Maas et al., 1970 ; Freireich and Landau, 1971).

Double wavelength spectrophotometry was first proposed by Chance (1951) for the measurement of turbid samples in biological analysis. This is a convenient method for determining one component in a mixture of two components. The instument has two

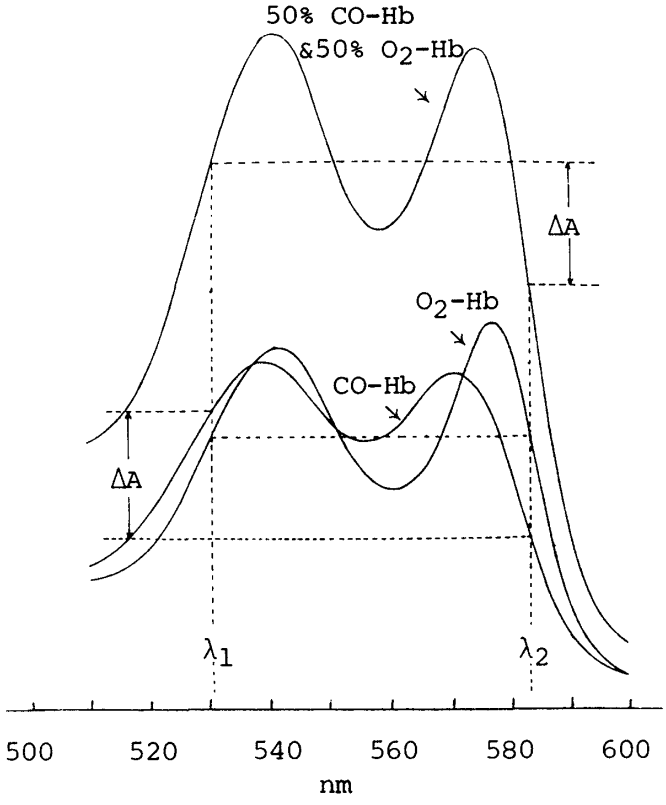

Fig. 1. Absorption curve of blood containing 50% CO-Hb composes of the spectra of both CO-Hb and O_2-Hb components. Since absorbances of O_2-Hb at λ_1 and λ_2 are the same value, absorbance difference ΔA shows only that of CO-Hb component.

monochromators in the same instrument and passes light beams of different wavelengths through a single cuvette: the photometer measures the absorbance difference (ΔA). Application in the determination of CO-Hb was first proposed by Ramieri et al. (1974). However, since double wavelength spectrophotometer is not so widely available, we tried measuring with a conventional spectrophotometer using this principle and measuring conditions were examined.

Principle of the method. There are three main hemoglobin derivatives (O_2-Hb, CO-Hb and Hb) in CO-intoxicated blood. Since Hb is converted to O_2-Hb with dissolved O_2 in the diluting solution, hemoglobin components in the measuring solution are O_2-Hb and CO-Hb. Absorption curve of blood which contained 50% CO-Hb is shown at the top of Fig. 1. This spectrum is constituted of the total of the absorbances of 50% CO-Hb and 50% O_2-Hb. The wavelength pair of λ_1 and λ_2 is determined as that O_2-Hb shows the same absorbance, but CO-Hb does not. At these wavelengths the absorbance difference (ΔA) between λ_1 and λ_2 results from only CO-Hb component, because that of O_2-Hb component is nulled out. After determining ΔA, the sample is saturated with pure CO to determine the absorbance difference (ΔA_2) at same wavelengths. The ratio $\Delta A/\Delta A_2$ is the percentage of CO-Hb in the blood. The principle of the method is shown in Fig. 1 and 2.

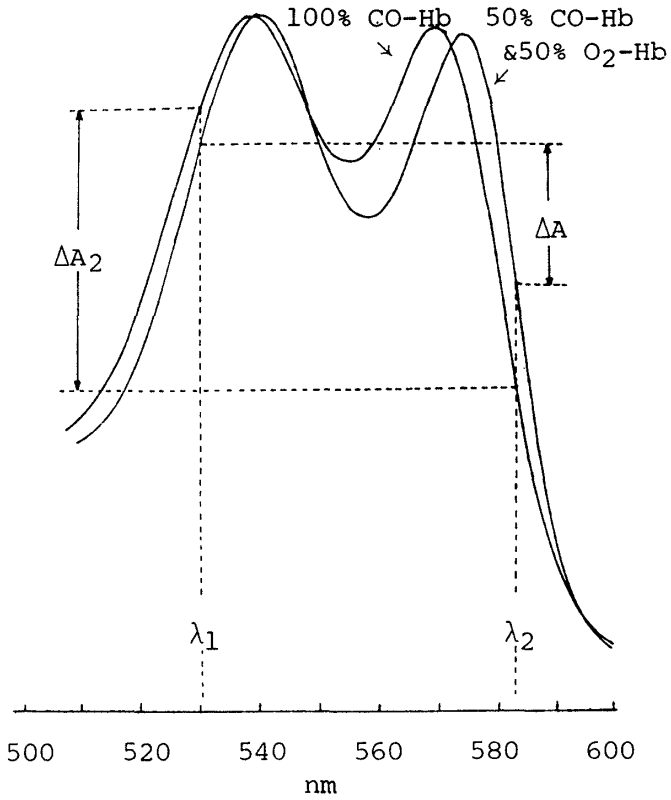

Fig. 2. After measurement of ΔA, CO is bubbled in a cuvette to make 100% CO-Hb and absorbance difference is remeasured. The ratio $\Delta A/\Delta A_2$ is the percentage of CO-Hb in the blood.

MATERIALS AND METHODS

Reagents. Preserved blood was used in this experiment and pure CO was made from formic acid and sulfuric acid. Diluting solution was made by bubbling N_2 gas in distilled water for 10 min to remove dissolved O_2 to make 7 mmol/liter NH_4OH solution (28% NH_4OH 0.48 ml/1000 ml distilled water) and stored anaerobically in a disposable syringe of 50 ml to prevent contact with air.

Apparatus. Spectrophotometric measurements were made with a Shimadzu UV-200 double beam spectrophotometer with cuvettes having 10 mm and 2 mm light paths. Gas chromatographic determination was performed by head space method (Sakata and Haga, 1980) with Simadzu GC-6APTF gas chromatograph with thermal conductivity detector, equipped with 3 mm×1 m glass column containing molecular sieve 5A, 80/100 mesh.

Determination of the wavelength pair. In this method, for fine wavelength trimming it is always necessary to adjust in moving the dial from the long wavelength side. The wavelength scale of the spectrophotometer was accurately adjusted by means of the emission maximum of the hydrogen discharge lamp at 486.0 nm and 656.1 nm.

Ramieri et al. (1974) proposed the wavelength pair of 530.6 nm (λ_1) and 583.0 nm (λ_2) at which O_2-Hb absorbance are the same strength. To confirm whether the reference wavelength pair was suited to a conventional spectrophotometer, a wavelength which had the same absorbance as that which was found at 583.0 nm was decided upon. In order to study the effects of temperature on absorbance change, the temperature in a cuvette was measured with thermistor thermometer (Toyo Electric Co.).

Analytical procedure. 40-50 μl of blood is added to 5 ml of diluting solution in a disposable syringe (5 ml) with microsyringe. After attaching the needle, the syringe is shaken gently several times to mix with small bubbles of air throughly. Then the solution is transfered gently to the cuvette for spectrophotometric measurement through the needle. After measurement of λ_1 and λ_2 within a period of 1 min, 10 ml of pure CO is bubbled into the cuvette for several seconds through a 22 G needle to convert the hemoglobin into CO-Hb. Then absorbance difference (ΔA_2) is remeasured. The percentage of CO-Hb in blood is calculated as follows:

$$\text{CO-Hb}\ (\%) = \Delta A/\Delta A_2 \times 100$$

Because wavelength scale is sometimes discrepant or small differences may exist between different spectrophotometers, characteristic λ_1 and λ_2 should be determined with blood of a nonsmoker. In the measurenment of the blood of experimental animals, the wavelength pair should be also determined independently because the absorption curve is slightly different.

Linearity of method and liberation of CO from CO-Hb during dilution. The linearity of method was checked by making six measurements of each samples of 100%, 80%, 60%, 40%, 20% and 0% CO-Hb blood which had been prepared by dilution of CO saturated blood (100%) with calculated amounts of unsaturated preserved blood. Saturated blood was

prepared by shaking with pure CO for 10 min followed by removal of dissolved CO with N_2 according to the procedure described in our previous paper (Sakata and Haga, 1980).

Liberation of CO during dilution was examined with two kinds of diluting solutions; 7 mmol NH_4OH solutions prepared with distilled water and deoxygenated water. Liberation effects of CO caused by dilution was checked by two methods; 1) 100-fold dilution with 10 mm cuvette and 2) 20-fold dilution with 2 mm cuvette.

RESULTS

Determination of wavelength pair. Preserved blood containing 0.7% CO-Hb was diluted with deoxygenated distilled water to measure the absorbance at 583.0 nm first, then from 530.0 nm to 530.6 nm at 0.1 nm intervals. After measurement the solution was saturated with CO and remeasured. Temperature of diluting water was controlled at 20°C before dilution. Room temperature was 20°C at measurement. Calculated % saturations at various wavelengths are shown in Table 1.

Table 1. Calculated CO saturations at various wavelengths.

CO saturation of the blood (GC method): 0.7%
Temperature of diluting water: 20°C
Room Temperature: 20°C

Wavelength (nm)	λ_2	583.0 nm						
	λ_1	530.0	530.1	530.2	530.3	530.4	530.5	530.6
Calculated CO saturation (%)		0.25	0.75	1.5	2.0	2.7	3.2	4.2

Table 2. Variation of absorbance during measurement.

CO saturation of the blood (GC method): 0.7%
Temperature of diluting water: 20°C
Room temperature: 20°C Temperature in the photometer: 27°C

Time after Dilution (min)	λ_1 (530.1 nm)	λ_2 (583.0 nm)	ΔA
0	0.666	0.663	0.003
1	0.665	0.665	0
2	0.664	0.666	−0.002
3	0.662	0.667	−0.005
4	0.662	0.670	−0.008
5	0.662	0.670	−0.008
cooled to first stage	0.667	0.664	0.003

Variation of absorbance during measurement. Previous experiments indicate that wavelength pair of 530.1 nm (λ_1) and 583.0 nm (λ_2) at 20°C should be used. At this wavelength pair, absorbance was measured continuously keeping the cuvette in a spectrophotometer for 5 min. Since absorbance of λ_1 decreased and λ_2 increased gradually, ΔA indicated negative value after 2 min. Cooling of the solution in a cuvette was tried after 5 min, and absorbance almost returned to the first stage level. Since the temperature in the photometer was 27°C, this phenomenon means that the temperature of a cuvette varied during measurement so as to move the spectrum. (Table 2)

The variation of absorbance difference according to temperature. The initial temperature of the diluting solutions was controlled at various levels (from 20°C to 30°C at 2°C intervals), untreated preserved blood (0.7% CO-Hb) was measured at 583.0 nm for λ_2 and 530.1 nm and 530.6 nm for λ_1. Absorbance differences and calculated CO saturations are shown in Table 3. Optimum wavelengths for λ_1 which have the same absorbance as 583.0 nm for λ_2 were shown as 530.1 nm at 22-24°C or 530.6 nm at 27-28°C.

Table 3. The variation of absorbance differences with temperature and calculated CO saturation.

CO saturation of the blood (GC method): 0.7%
Temperature Room: 20°C In the photometer: 27°C

Temperature in a cuvette	λ_1 530.1 nm λ_2 583.0 nm		Calculated CO saturation	λ_1 530.6 nm λ_2 583.0 nm		Calculated CO saturation
Initial/Final	ΔA	ΔA_2	%	ΔA	ΔA_2	%
20−23°C	12	409	2.9	27	420	6.4
22−24°C	3	418	0.7	20	429	4.6
24−25°C	1	450	0.2	17	463	3.7
26−27°C	−7	376	−	8	386	2.1
28−27°C	−10	395	−	4	407	0.9
30−29°C	−23	479	−	−3	493	−

Linearity of method and liberation of CO from CO-Hb during dilution. Measured values of serially diluted samples are shown in Table 4. Theoretical CO saturations were calculated from 0.7% CO-Hb of untreated preserved blood. Measuring conditions were 20°C for initial diluting temperature and 530.1 nm (λ_1) and 583.0 nm (λ_2) for wavelength pair. Standard deviations were under 1% at every point. Good linearity was observed up to 80% CO-Hb with deoxygenated diluting solution in both dilution ratios, but at more than 80% a slight curvature was observed. Liberations of 2% and 5% were observed in saturated blood in a deoxygenated diluting solution with a 2 mm and 10 mm cuvette respectively. On the other hand, a liberation of 10% at every point of saturation was observed with a diluting solution containing dissolved O_2.

Table 4. Linearity of method and liberation of CO from CO-Hb by diluting ratio.

Temperature of diluting water: 20°C
Room temperature: 20°C
Wavelength pair: λ_1 530.1 nm; λ_2 583.0 nm

Theoretical CO saturation	Calculated CO saturation (%)	
	20-fold dilution with 2 mm cuvette	100-fold dilution with 10 mm cuvette
100 %	98.6±0.8	95.1±0.3
80.1%	79.3±0.4	78.8±0.4
60.3%	60.8±0.7	59.7±0.4
40.4%	41.1±0.5	40.3±0.4
20.6%	20.7±0.6	19.4±0.4
0.7%	0.7±0.3	0.8±0.3

DISCUSSION

The difference of this method is that the interfering O_2-Hb component is able to be nulled out from a mixed absorbance from other multiple wavelength methods in which CO-Hb is calculated from the absorbance ratio at multiple wavelengths.

First, we tried measuring the untreated preserved blood (0.7% CO-Hb measured by gas chromatographic method) with the wavelength pair of 530.6 nm and 583.0 nm proposed by Ramieri (1974), and found an estimated 3.7% CO-Hb and an absorbance variation occured during measurement. Therefore, the wavelength pair was reevaluated. In order to keep constant measuring conditions, the temperature of the diluting solution was maintained at 20°C and all the measurements were performed within a 2 min period. λ_1 of 530.1 nm had the same absorbance as that of 583.0 nm under those conditions, but absorbance varied gradually during measurement. It was clear that variation of absorbance was caused by change of temperature. The temperature significantly influences the spectrum of the hemoglobin pigment (Refsum, 1957) and absorption spectra for O_2-Hb and Hb at the temperatures of 17°C and 37°C were reported to shift the spectrum toward a higher wavelength at higher temperatures (Andersen et al., 1972).

Temperature in the spectrophotometer and working solutions in the cuvette were measured with a thermistor thermometer which is able to measure the temperature instantaneously. Since temperature of the spectrophotometer was 27°C at an environmental temperature of 20°C, the temperature of working solution was first controlled at 20°C and increased to 23°C at the end of measurement. These temperature changes during measurement depend upon the differences of the temperature between the inside of spectrophotometer and the working solution. As shown in Table 3, wavelength pair of 530.6 nm and 583.0 nm proposed by Ramieri (1974) may be adopted at 27-28°C in this experiment. Most

methods are based on measurements at room temperature without temperature constancy, even though room temperature sometimes differs as much as from 15°C in winter to more than 30°C in summer. Therefore, differences of temperature may be responsible for error when measurement is performed. The use of thermostatted cuvette holder is advantageous but relatively satisfactory data was obtained if the initial temperature of the diluting solution was carefully controlled.

As shown in Table 4, measured values were in close agreement with the theoretical value (less than 1% error) and that of gas chromatographic method (Sakata and Haga, 1970) under 80% CO-Hb, while some liberation of CO from CO-Hb was observed at more than 80%.

It has been indicated that CO is liberated during dilution (Paul and Theorell, 1942) and the more dilution occurs the more liberation. To avoid liberation, a special cuvette of 0.1 mm or 1 mm light path was used (Small et al., 1971 ; Siggaard-Andersen et al., 1972). At 2,000-fold dilution it was reported that 20-30% of CO was liberated during dilution (Miyauchi and Sakaki, 1977). In the present experiment, dissolved O_2 was removed by bubbling N_2 to make the diluting solution, slight liberation at 80% CO-Hb and 5% liberation at 100% CO-Hb were observed with 10 mm cuvette (100-fold dilution) and only 2% liberation occured with 2 mm cuvette (20-fold dilution). In all cases, liberation did not occur at every concentration, but rather at higher concentrations of CO-Hb. It is sufficient to use a 10 mm cuvette with O_2 free diluting solution, because such a high concentration of more than 80% CO-Hb is rare even in routine measurement of forensic autopsy cases.

In this method, fine wavelength trimming to exactly null out interfering component is necessary for a high degree of accuracy because absorption curve has a steep slope at those wavelengths. It is recomended that the wavelength which has the same absorbance as 583.0 nm should be decided first using the blood of a nonsmoker under the same measuring conditions, because wavelength matching with the scale of a spectrophotometer is sometimes discrepant or small differences may exist between different spectrophotometers.

Advantages of this method are 1) the standard curve or equation for calculation and accurate dilution are not necessary because the interfering component is nulled out and 100% CO-Hb standard is made with the same sample and 2) measurement is very rapid, taking less than 2 min.

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