A new method of carboxyhaemoglobin determination

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ABSTRACT A quick and accurate method of determining the concentration of carboxyhaemoglobin (COHb) in blood has been developed. The method uses a dual wavelength double beam spectrophotometer in the 1st derivative mode, linked to a digital voltmeter (DVM), with the two beams set 3 nm apart around an isobestic point of reduced haemoglobin (Hb_{red}) and carboxyhaemoglobin at 579 nm. The 1st derivative mode measures the slope, and this slope is proportional to the concentration of COHb.

Techniques currently used to measure COHb levels in blood include gasometric methods, colorimetry, gas chromatography, precipitation techniques, infrared analysis and spectrophotometric methods. Not one of these methods (described in the papers listed in the Reference section) is both rapid and accurate. In an effort to combine accuracy and rapidity, attention has been concentrated on spectrophotometric methods.

Theory

The absorbance spectra of reduced haemoglobin (Hb_{red}) and COHb (Figure 1) between 600 and 500 nm have similar curves with three isobestic points. At the isobestic point around 579 nm the absorbance slope alters with concentration (Figure 2). Closer examination of absorbance curves (Figure 3) between 585 and 575 nm shows that the curves of Hb_{red} and COHb are straight lines but have different slopes. If the slope can be measured, then the concentration of COHb can be calculated: this is the basis of the method described here.

Figure 4 shows schematically the absorbance curve of COHb with the *x* axis representing wavelength and the *y* axis absorbance.

Absorbance = *C* - *pW* where *C* = constant intercept of *x* on *y*.

The slope is given by the 1st derivative

$$= \frac{dA}{dW} = -p$$

$$\text{Now, } \frac{dA}{dW} \approx \frac{\Delta A}{\Delta W}$$

$$\text{But if } \frac{d^2A}{dW^2} = 0 \text{ (2nd derivative)}$$

that is, a curve is a straight line over ΔW ,

$$\text{then, } \frac{dA}{dW} = \frac{\Delta A}{\Delta W}$$

This condition holds between 576 and 580 nm

If the % of COHb $\propto p$

$$\therefore \% \text{COHb} = Kp = - \frac{KdA}{dW} = -K \frac{\Delta A}{\Delta W}$$

But $\Delta W = \text{constant}$

Thus, $\% \text{COHb} = K * \Delta A$

Method

Using a Perkin Elmer 356, double beam, dual wavelength spectrophotometer, 1st derivative measurement is possible. Figure 5 shows the conventional absorbance spectrum of Hb_{red} between 600 and 500 nm with its 1st derivative curve superimposed upon it. First derivative absorbance curves of various concentrations of COHb with the two beams set 1 nm apart (Figure 6), demonstrated that the greatest difference between concentrations is registered around the isobestic point at 579 nm. When the wavelengths at which the beams are set are fixed and the spectrophotometer is attached to a digital voltmeter (DVM), a digital value for a blood

Fig. 1 Absorbance spectra of Hb_{red} and $COHb$ between 600 and 500 nm.

sample is obtained, proportional to the concentration of $COHb$.

CARBOXYHAEMOGLOBIN ESTIMATION

To measure the $COHb$ concentration in human or animal (dog, rat) blood the sample was collected into EDTA, and 100 μ l was diluted with 0.4% ammonium hydroxide to a final dilution of 1/100. After thorough mixing, 5 ml was transferred to a 6 ml plastic tube containing 50 mg sodium dithionite to reduce the haemoglobin, and another 5 ml was converted to 100% $COHb$ by passing carbon monoxide gas through the solution for two minutes. Each tube was then capped and the blood well mixed. Readings were taken from these two samples on the spectrophotometer using the DVM. The plastic cuvettes containing the samples were read empty and then filled, to eliminate variations attributable to differences between cuvettes. Blood from control animals not exposed to carbon monoxide was examined alongside the test samples in an identical manner. From the results

Fig. 2 Absorbance spectra of various concentrations of $COHb$ between 600 and 500 nm.

obtained with control and test samples the concentration of $COHb$ was calculated, using the 0% and 100% samples.

CALCULATION

(In all cases the empty cuvette reading for the sample was subtracted from the filled cuvette reading on the DVM).

Test sample

$$100\% \text{ COHb} = A$$

$$\text{Test result} = B$$

Control sample

$$100\% \text{ COHb} = C$$

$$0\% \text{ COHb} = D$$

% $COHb$ in test sample =

$$\frac{B - \frac{A \times D}{C}}{A - \frac{A \times D}{C}} \times 100$$

Fig. 3 Absorbance spectra of Hb_{red} and COHb between 585 and 575 nm.

Results

LINEARITY

To verify that the concentration of COHb against the 1st derivative readings was linear, various concentrations were prepared. Using a sample of blood from an animal not known to have had contact with any source of carbon monoxide, a master dilution of blood was made. One half of this diluted sample was converted to COHb by bubbling carbon monoxide through the solution for 20 minutes followed by nitrogen gas for 30 minutes, to remove excess dissolved carbon monoxide, although this does cause some dissociation of the COHb (about 10%). The remainder of the diluted sample had oxygen gas bubbled through it for 20 minutes to remove any COHb, followed by nitrogen gas to remove excess dissolved oxygen. The oxygenated sample was then dispensed accurately in known quantities into sodium dithionite, the reducing agent. The COHb solution was then added accu-

rately in small known amounts. These were well mixed before being read as above. Typical results can be seen in Figure 7 which shows concentrations of COHb between 0 and 93.6%. The correlation between theoretical and observed values is excellent ($r = 0.999$). A similar experiment was carried out using samples of blood with a COHb concentration between 0% and 20% in 1% steps. The linearity of this was again excellent ($r = 0.999$).

REPRODUCIBILITY

Tests on reproducibility of the method between readings of the same subsamples, subsamples of the same dilution and dilutions of the same blood were carried out. The samples of blood used contained various concentrations of COHb. The study was carried out with the wavelengths set both 3 nm apart and 1 nm apart.

Reproducibility between readings

Twenty samples of blood were each diluted 1/100

Fig. 4 Schematic absorbance curve of COHb.

with 0.4% w/v ammonium hydroxide to give a final volume of 10 ml. Each dilution was mixed thoroughly and half of each sample was placed in a 10 mg/ml solution of the reducing agent sodium dithionite. Carbon monoxide was bubbled through the remainder for two minutes. Each of these samples was then read four times consecutively in the 1st derivative mode, initially with wavelengths set 3 nm apart (λ_{1579} ; λ_{2576}), then with wavelengths set 1 nm apart (λ_{1579} ; λ_{2578}). The results were analysed statistically and are shown in the Table.

Reproducibility between subsamples

Four samples of blood were each diluted 1/100 with 0.4% w/v ammonium hydroxide to give a final volume of 60 ml. Each dilution was mixed thoroughly and five portions of 10 ml removed from each. After mixing, half of each 10 ml sample was mixed with the reducing agent sodium dithionite and the remainder was gassed with carbon monoxide. Each of the 20 pairs of subsamples was read in random order in 1st derivative mode with the wavelengths set 3 nm apart. Statistical analysis of results is shown in the Table.

Reproducibility between dilutions

Two samples of blood were each diluted twenty times in the routine manner: 100 μ l blood was mixed with 0.4% w/v ammonium hydroxide to give a final dilution of 1/100. Each sample was well mixed, then 5 μ l was transferred into 50 mg sodium dithionite; the remainder was gassed with carbon

Table Analysis of variance of results showing reproducibility between readings, subsamples and dilutions, at different wavelengths

Reproducibility	Wavelength	
	λ_{1579}	λ_{2576}
Between readings	0.2190	0.0563
Between subsamples	—	0.2319
Between dilutions	—	—
4.161 COHb (low)	—	0.3351
13.168 COHb (high)	—	0.6006

Fig. 5 Absorbance spectrum of Hb_{red} between 600 and 500 nm with its inverse 1st derivative curve superimposed upon it.

monoxide for two minutes. Each of the 20 paired samples was read in random order in 1st derivative mode with wavelengths set 3 nm apart. Results were statistically analysed and are shown in the Table.

If only one dilution, one subsample and one reading are done on a blood sample using wavelengths λ_{1579} and λ_{2576} nm then in the worst possible case, the variance would be 0.33507 (SD = 0.05788) for low COHb concentrations and 0.60064 (SD = 0.077501) for high COHb concentrations. If two dilutions per sample are done with one subsample and one reading then the variance is halved.

The variance attributable to machine reading is

Fig. 6 Inverse 1st derivative spectra of various concentrations of COHb between 600 and 500 nm.

Fig. 7 Concentrations of COHb showing linearity between theoretical and observed values (correlation $r = 0.999$).

that predicted by the necessity of making a decision as to the last digit on the DVM reading, when it tends to flicker between two figures, so this variance could be attributed solely to the electronic limitations of the output device.

Variance in the subsamples is due to sampling error which may, in part, be due to very slight differences in the amount of sodium dithionite used for reducing haemoglobin. Dilution error was not statistically significant.

There was no statistical evidence that the variances differed between the higher concentrations of COHb and the lower concentrations. This result was anticipated from the high correlation coefficient.

Time

Another aspect of the method was assessed, using the results from the above experiments. The deviation from the mean plotted against the order of reading the subsamples and dilutions gave a measure of the effect of time on the correlations. There was a time effect in that the readings of both reduced and 100% COHb showed a fall, whereas the calculated COHb concentration showed no such fall. This demonstrates that, during the two-hour period over which the experiment was carried out, the samples showed significant changes in COHb.

Discussion

We believe the method described above is novel in that it employs digital read-out of a 1st derivative of an absorption curve. This combines the improved accuracy of a digital output with the sensitivity of 1st derivative recording. Although we have used this method for the measurement of carboxyhaemoglobin it is equally applicable to any mixture of two compounds with an isobestic point around which the slopes of both compounds are essentially even (in other words, 2nd derivative is zero).

The advantages of this method over those currently available are accuracy and speed. Our results show an accuracy of $\pm 0.5\%$ COHb over the whole range. The rapidity of the method is limited largely by the necessity to gas an aliquot of each sample with carbon monoxide, but a reasonable estimate for the output of two operators working together would be 150 samples during an ordinary working day.

The success of this method leads us to suggest that digital 1st derivative spectrophotometry may be an extremely useful tool in the estimation of carboxyhaemoglobin and possibly of other constituents of blood. Although the apparatus is rather expensive, it may already be found being used for other purposes in a number of laboratories.

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