Spectrophotometric measurement of carboxyhaemoglobin

An evaluation of the method of Commins and Lawther

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Lily, R. E. C., Cole, P. V., and Hawkins, L. H. (1972). *Brit. J. industr. Med.*, **29**, 454-457. **Spectrophotometric measurement of carboxyhaemoglobin. An evaluation of the method of Commins and Lawther.** The spectrophotometric method of Commins and Lawther for the measurement of carboxyhaemoglobin (COHb) concentration was evaluated for both accuracy and sensitivity. The method was calibrated by equilibrating diluted blood samples with known concentrations of carbon monoxide (CO) in air and measuring the resultant COHb saturations. Over the range 0-40% COHb saturation the mean error was 0.67% COHb (SD 0.65). It is considered that the accuracy of the method is more than sufficient for measurement of both the high COHb levels found in clinical situations and the lower COHb saturation resulting from exposure to urban air and cigarette smoking.

The spectrophotometric method of Commins and Lawther (1965) for the determination of carboxyhaemoglobin (COHb) has been severely criticized in a recent Air Pollution Control Administration (APCA) publication (National Air Pollution Control, 1970). This laboratory has been concerned with various aspects of the physiology, pathology, and epidemiology of carbon monoxide (CO) for the past five years. In that time about 4 000 estimations for COHb by the method of Commins and Lawther have been carried out and we have no reason to believe that such criticisms could be valid.

There have been several reviews of available methods for COHb estimation (Douglas, 1962; Coburn, Danielson, Blakemore, and Forster, 1964; Gramer, 1961; Blackmore, 1970). Accurate methods include spectrophotometric determination of dissolved COHb, analysis of alveolar air, and colorimetric estimations of substances liberated or used up during oxidation of CO or precipitated in the presence of CO. Accurate but less convenient are manometric, volumetric or infrared or gas chromatographic methods of analysis, the measurement in the

latter case being either the heat or the CO₂ liberated during the oxidation of CO.

Spectrophotometric methods have several advantages, principally that most of the procedures are simple and that the sample volume required for analysis is generally small. The basis of the method of Commins and Lawther (1965) is the comparison of the spectra of a sample solution containing COHb with that of a reference solution in which all the haemoglobin has been converted to oxyhaemoglobin (O₂Hb). The method is quick and easy to use with good reproducibility, and separate determinations of haemoglobin concentrations are not required. The method is ideal for the measurement of large numbers of blood samples as these have been shown to remain stable for at least a week if stored in the dark at 3°C.

A criticism of the method has been that there is a tendency for the COHb to dissociate, particularly when dissolved oxygen is present. Beeckmans in 1967 derived a theoretical relationship between the true and apparent COHb saturations which he confirmed experimentally using samples of blood saturated

with CO and aerated solvent. He then derived a series of factors varying with the oxygen tension of the solvent.

The purpose of the present study is to examine the APCA criticisms and to evaluate Commins and Lawther's method by illustrating its sensitivity and accuracy over a large range of blood COHb levels, with particular emphasis on values below 3% saturation, i.e., the levels expected from exposure of people to urban atmospheres.

Method

In Commins and Lawther's method of COHb measurement the blood sample (which may be taken from a finger prick) is dissolved in 10 ml of 0.04% ammonia solution. The solution is divided into two halves, and oxygen is bubbled through one half to convert any COHb into O₂Hb. The concentration of haemoglobin is indicated by the difference between the optical density of the oxygenated sample at 575 nm and at 559 nm (the wavelengths of maximum and minimum absorbances respectively of O₂Hb). This value is designated *d*. The concentration of COHb is indicated by the difference between the optical density of the COHb and that of the equivalent amount of O₂Hb, the sample containing COHb being placed in the sample beam and the oxygenated sample in the reference beam. This value, designated *h*, is measured near the peak absorbance for both COHb and O₂Hb at 420.2 nm, with the baseline at 414 nm and 426 nm respectively.

The limiting value of haemoglobin concentration, which is the maximum concentration allowing sufficient transmission of light, must first be found for the particular spectrophotometer used. In the present study, a Beckman DB-G grating spectrophotometer with a tungsten lamp as source was used for all measurements. Solutions containing various concentrations of O₂Hb were prepared and part of each was saturated with CO. For each of the varying values of *d* the corresponding value of *h* for 100% saturation of COHb was measured. The results in Fig. 1 show a linear relationship below a critical level of *d*, above which the linear relationship breaks down. For this particular instrument the critical value of *d* was taken as 0.05, which corresponds to approximately 6 µl of blood in 10 ml of solvent.

A series of mixtures of air and carbon monoxide was prepared in plastic Douglas bags by diluting known volumes of pure carbon monoxide with air from a high pressure cylinder (Hawkins, 1967). The final concentrations of carbon monoxide were estimated by means of a Hartmann and Braun infrared analyser designed to measure the range 0 to 600 ppm. The zero point of the analyser was set with pure oxygen (tolerance 0.5 ppm CO) and the span with 200 and 400 ppm CO (Rank Precision Industries). To reduce error due to the presence of water vapour in the gas mixture of 25 ppm and under, both calibrating gases and gas mixtures were passed through tubes packed with magnesium perchlorate. Twenty-nine different gas concentrations between 0 ppm and 690 ppm CO in air were used, 10 of which were below 30 ppm.

Samples of red cells from 1 ml of fresh blood were washed with saline, haemolysed, and diluted with 9 ml of

FIG. 1. Relationship between optical height *h* derived from measurements at 414 nm, 420 nm, and 426 nm, and difference *d* between absorbances at 575 nm and 559 nm for the Beckman DB-G grating spectrophotometer.

0.1 M phosphate buffer. This sample was then equilibrated in a 'bubble' tonometer (Adams and Morgan-Hughes, 1967) for three to four hours until serial analysis for COHb indicated that a plateau had been reached. The mean of duplicate analyses was calculated and corrected for any error due to COHb in dilute solution by Beekmans' (1967) method.

Theoretical COHb percent values were calculated from the Haldane equation:

$$(1) \quad (\text{COHb}) = \frac{M(\text{O}_2\text{Hb}) \text{ Pco}}{\text{Po}_2}$$

where (COHb) and (O₂Hb) are the concentrations of the haemoglobin compounds expressed in the present study as percentage saturation, and *M* is the relative affinity constant taken as 210 (Sendroy, Liu, and van Slyke, 1929). As the blood samples are fully saturated:

$$(2) \quad \text{O}_2\text{Hb} \% = (100 - \text{COHb}) \%$$

Pco and Po₂ are the partial pressures of CO and O₂ mmHg and can be calculated from the following equations:

$$(3) \quad \text{Pco} = \frac{\text{CO ppm}}{10^6} (\text{P}_B - \text{P}_{\text{H}_2\text{O}})$$

$$(4) \quad \text{Po}_2 = \frac{\text{O}_2}{100} \text{ percent } (\text{P}_B - \text{P}_{\text{H}_2\text{O}})$$

where P_B is the barometric pressure, P_{H₂O} is the partial pressure of water vapour at 37°C – numerically 47 mmHg and O₂ percent is the percentage concentration of oxygen in air – numerically 20.93%. Hence, substituting equations (2), (3), and (4) in equation (1):

$$\text{COHb percent} = \frac{100}{1 + \frac{997}{\text{CO ppm}}}$$

Measured COHb% values were then plotted against theoretical COHb% values.

Results

The errors resulting from dissociation of COHb when in dilute solution were very small, though greater with high COHb saturations. The maximum difference between the measured values and those corrected by Beekmans' method was 1.45%.

The final results are shown in Figure 2. The inset shows 10 values below 3.0% COHb saturation on an enlarged scale. The straight line of the graph denotes equality between theoretical and measured values, viz., the position of 100% correlation. Theoretical values of COHb percent ranged from 0 to 40.9%.

Over the total range of saturations measured the mean error was 0.67% COHb (SD 0.65). For values below 3% the mean error was 0.14% COHb (SD 0.13).

FIG. 2. Relationship between measured COHb% saturation after Beekmans' correction and theoretical COHb saturation derived from Haldane's equation. Inset shows 10 values below 3% COHb on an enlarged scale. The straight line represents equality between measured and theoretical COHb values.

Discussion

Spectrophotometric methods for estimation of carboxyhaemoglobin have often been criticized because they tend to produce errors in the presence of other blood pigments, particularly methaemoglobin. The method of Commins and Lawther is not susceptible to this type of error for the following reasons: the concentrations of methaemoglobin in both reference and sample solutions are identical and hence effects are cancelled out; the wavelengths used to estimate O₂Hb do not coincide with the absorbance peaks of methaemoglobin; finally, the COHb level is expressed in terms of total available haemoglobin

and not total haemoglobin (normal levels of methaemoglobin rarely exceed 2% of total haemoglobin). Similarly, interference from certain combining pigments such as myoglobin is important only if their concentration is very high.

The criticisms of Commins and Lawther's method listed in the APCA publication will be discussed in the order in which they are listed in that publication:

1. 'The use of finger prick blood is unsuitable because the varying amounts of tissue fluid contained in the different samples may lead to inaccuracies due to variations in the true volume of blood and interferences in the absorption peaks.'

If a measure of the total quantity of COHb in the sample was required, finger prick samples may lead to error. However, this error would result in a falsely low value for total haemoglobin and not from the measurement of the fraction of haemoglobin combined with CO. Commins and Lawther's method measures COHb as a percentage of the total available haemoglobin present in the sample. It follows that if the sample is diluted, COHb and total haemoglobin would be reduced to the same extent and the resultant COHb expressed as a percentage of the total haemoglobin would remain precisely the same.

2. 'The method of preparing standard solutions of 100% COHb and 100% O₂Hb involves bubbling CO or oxygen into the solutions. This produces an excess of dissolved gases; hence, when solutions containing less than 100% COHb were prepared they were inaccurate.'

It is agreed that a solution containing a relatively large amount of dissolved CO will be produced by bubbling pure CO through the dilute solution of blood. If this solution is then diluted 1:1 with a second solution containing pure O₂Hb, the resultant solution contains not 50% COHb but 100%, there being enough dissolved CO to saturate completely all of the available haemoglobin. However, Commins and Lawther do stress this very point in their paper describing the method (1965). They state:

'Solutions of O₂Hb and COHb of known composition cannot be prepared by mixing the corresponding proportions of oxygenated and carboxygenated solutions of blood, because the haemoglobin solution saturated with CO contains dissolved uncombined gas as well as COHb' (p. 141).

In order to produce a solution containing 100% COHb and no dissolved CO it is necessary to bubble CO through whole blood (not a solution of haemoglobin) and then to remove the dissolved CO by further shaking the blood in a tonometer with fresh room air for a short period (about 5 min). This results in the removal of the excess dissolved gas but does not result in any appreciable reduction in the COHb.

3. 'When mixed venous blood from heavy smokers and non-smokers was assayed for COHb, small amounts of unsaturated haemoglobin in the sample caused substantial interference between wavelengths 416 and 410 nm. When oxygen was bubbled through the non-smokers' blood sample, the interference disappeared, showing that the haemoglobin had become saturated. Interference caused by the unsaturated haemoglobin corresponded to about 10% COHb at 420 nm.'

Interference from reduced haemoglobin is not a problem in this method. The blood sample is prepared in 0.04% ammonium hydroxide solution. As this is alkaline, the O₂Hb dissociation curve is shifted so far to the left that all of the haemoglobin which is not COHb (or one of the other non-oxygen carrying forms) becomes O₂Hb at a relatively low P_{O₂}. In fact, the pH of the blood-ammonia solutions is about 9.7 and at this pH the haemoglobin would be fully saturated at a P_{O₂} of less than 10 mmHg. The P_{O₂} of the ammonia solution after being prepared with boiled distilled water is usually about 30 to 40 mmHg, which is well in excess of that required to ensure that no reduced haemoglobin remains in the solution.

The above conclusion can be confirmed experimentally by determination of the total absorption spectrum of a venous blood sample (originally containing about 50% reduced haemoglobin) made up in the 0.04% ammonia solution. The results show no evidence of the presence of reduced haemoglobin and no change in the spectrum after bubbling oxygen through the solution for 5 minutes.

The conclusion in the APCA publication was that spectrophotometric methods are unsuitable for the measurement of low levels of COHb. However, the results of the present evaluation show that the method of Commins and Lawther is quite suitable for the

measurement of both high and low concentrations of COHb with excellent accuracy within the range 0-40% COHb. There is little evidence after prolonged use of the method that the criticisms have any foundation. It is considered to be a useful technique for measurement of COHb levels resulting from exposure to the urban atmosphere and from cigarette smoking, and should not be limited to rough clinical diagnosis as the author of the critique suggests.

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