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Carboxyhemoglobin Determination by Second-Derivative Spectroscopy

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In this procedure hemoglobin is converted to its reduced form and the magnitude of the zero-order spectral shift of the reduced hemoglobin peak at 430 nm to the carboxyhemoglobin peak at 418 nm is determined by second-derivative spectrum analysis. The method is simple, straightforward to set up, and rapid. A result may be obtained within 15 min of receiving the sample. It is sufficiently sensitive to differentiate carboxyhemoglobin concentration in the blood of smokers and nonsmokers.

Additional Keyphrases: *effects of tobacco smoking · derivative spectra · spectrophotometry*

The spectrum of a compound or mixture of compounds may be mathematically differentiated to produce derivative spectra. Although this concept has been known for many years (*1*) and its theory and potential usage have been well reviewed (*2–4*), and despite the availability of this technique on many modern recording spectrophotometers, it has found little application in clinical chemistry. Odd-numbered derivatives are of most use in determining the exact points of absorbance maxima of the original or zero-order spectrum and hence the qualitative properties of the substance under investigation; even-numbered derivatives are helpful in quantitative determinations (*2*). The latter prove particularly useful when the properties of the zero-order spectrum are changed owing to interferents such as baseline shift, change in baseline slope, the presence of other compounds with similar optical properties, or the presence of inert material such as air bubbles, insoluble material, or colloidal particles. Variations between samples because of baseline differences, refractive index, or reagent purity may also be compensated for. Despite these potential advantages, the reported applications in clinical chemistry are few, but include the measurement of porphyrins (*5*), bilirubin (*6*), Paraquat (*7*), and benzodiazepines (*8*).

The application of derivative spectroscopy to the measurement of carboxyhemoglobin is particularly useful, because the methods currently in use have a number of problems. Zero-order spectroscopy is insensitive and confused by the presence of reduced hemoglobin; the method of Tietz and Fiereck (*9*) is insensitive; and diffusion methods (*10*) are time consuming and unsuitable for routine use.

Materials and Methods

Preparation of calibrants. Two 10-mL samples of heparinized blood from a healthy nonsmoker were treated in separate 100-mL flasks (Jencons Scientific Ltd., Leighton Buzzard, LU7 8UA, U.K.), one with 100% oxygen and the other with 100% carbon monoxide (Phase Separations Ltd., Queensferry, Clwyd CH5 2LR, U.K.). Each flask was gassed for 15 min with gentle agitation, after which it was stopped and gently inverted for a further 15 min. Excess carbon monoxide was removed from the carbon-monoxide-treated sample by gassing with 100% nitrogen (BOC Ltd., London, SW19 3UF, U.K.) for 5 min with gentle agitation. These two preparations were used as 0 and 100% carboxyhemoglobin standards, from which intermediary standards were also prepared, in accordance with the procedure of Tietz and Fiereck (*9*).

Determination of carboxyhemoglobin. Reducing reagent is prepared immediately before use (because it is only stable for a few hours), as follows. Mix 1.6 mL of ammonia solution (relative density 0.88) and 0.25 g of sodium dithionite (both from B.D.H. Chemicals Ltd., Poole, BH12 4NN, U.K.) and dilute to 100 mL with water. Add 10 mL of the reducing reagent to 10 μL of fresh whole blood collected into an EDTA-containing blood tube, mix thoroughly by inversion, and allow to stand for 5 min. Measure the second-derivative spectrum between 390 and 450 nm (we used a Unicam P.U. 8800 scanning spectrophotometer; Pye Unicam Ltd., Cambridge, CB1 2PX, U.K.). Calibrants are similarly treated.

Results

Oxyhemoglobin is reduced by treatment with dithionite, which simplifies the situation because only the spectra of reduced hemoglobin and carboxyhemoglobin then need be considered. Figure 1 shows the zero-order spectra of (*a*) reduced hemoglobin with absorbance peaks at 430 and 555 nm; (*b*) carboxyhemoglobin with peaks at 418, 540, and 570

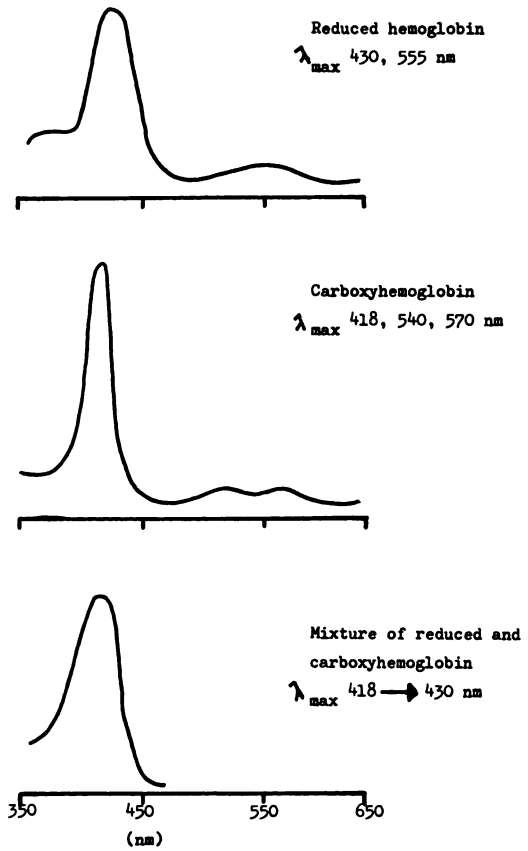

Fig. 1. Zero-order spectra of reduced hemoglobin and carboxyhemoglobin

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nm; and (*c*) a mixture of reduced and carboxyhemoglobin with one broad peak, having a maximum between 418 and 430 nm, its exact location depending upon the relative proportion of reduced and carboxyhemoglobin. Figure 2 shows the second derivative of this mixed spectrum and denotes an amplitude change A between 409 and 419 nm due to the zero-order carboxyhemoglobin peak at 418 nm, and an amplitude change B between 433 and 442 nm due to the zero-order reduced hemoglobin peak at 430 nm. Consequently, there is a direct relationship between carboxyhemoglobin and amplitude A and an inverse relationship with amplitude B. By using calibrants with different concentrations of carboxyhemoglobin, one can show that ln A/B is proportional to carboxyhemoglobin concentration (Figure 3).

Most recording spectrophotometers plot derivative spectra by calculating the mean rate of change of absorbance over a small defined change in wavelength. The resolution of the spectrum obtained is therefore affected by the size of the band width and the scanning speed. If these are too great, the resolution is poor; if they are too small, background noise is excessive. Optimum conditions are achieved only by trial and error and may vary from one instrument to another. For the estimation of carboxyhemoglobin with the P.U. 8800 we found a band width of 0.2 nm and a scan speed of 1 nm/s to be satisfactory.

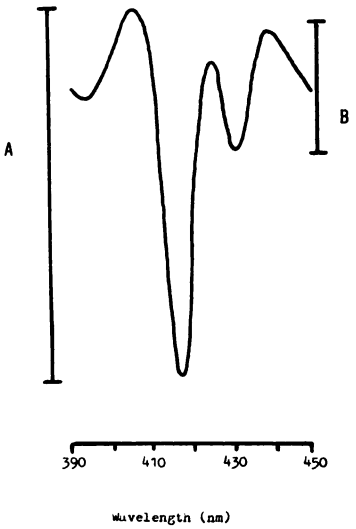

Fig. 2. Second-derivative spectrum of reduced hemoglobin plus carboxyhemoglobin

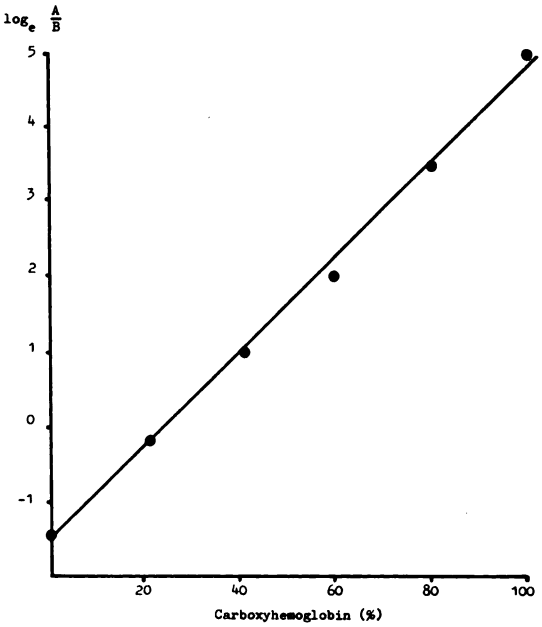

Fig. 3. Calibration curve for carboxyhemoglobin estimation

To determine the precision of the method, we made 15 replicate analyses of 20 and 50% carboxyhemoglobin standards within one day. The respective CVs were ≤2.9 and 3.3%. For 50 selected patients' samples, 25 smokers and 25 nonsmokers, results by this method were compared with those by the method of Tietz and Fiereck (*9*), and correlated well when the carboxyhemoglobin content exceeded 5%. Below 5% the Tietz and Fiereck method is too insensitive to produce a good correlation; 15 samples gave results of less than zero. On the basis of the data from nonsmokers, we established an upper limit of 2.0% for the reference interval (Figure 4).

Discussion

The application of derivative spectral analysis to the measurement of carboxyhemoglobin seems particularly relevant. Despite the discontinuance of coal gas as a domestic fuel source in many countries, automobile exhaust remains a source of carbon monoxide poisoning, both intentional and accidental, and the need to measure carboxyhemoglobin in blood remains. By the nature of the type of investigation the method needs to be fast and easy to carry out. Given an increasing awareness of environmental pollution factors, there is also a need for more sensitive measurements of carboxyhemoglobin concentrations below 10%. Derivative spectral analysis fulfills these criteria. The initial reduction is fast, and the spectral analysis is straightforward and takes only a few minutes to perform. The only problem is the instability of the reducing agent, which must be freshly prepared. We suggest that for emergency use pre-weighed portions of sodium dithionite be kept at hand, which then would require only the addition of ammonia solution and water for solubilization. The sensitivity of the method is demonstrated by the discrimination of smokers and nonsmokers in Figure 4.

Calibration and the preparation of standards is laborious, but these need not be done with every investigation. Repeated checks on the calibration curve show it is consistent for a given instrument. Because a ratio is being measured, the procedure is not operator dependent beyond the actual amplitude measurements of the spectrum. This is fortunate,

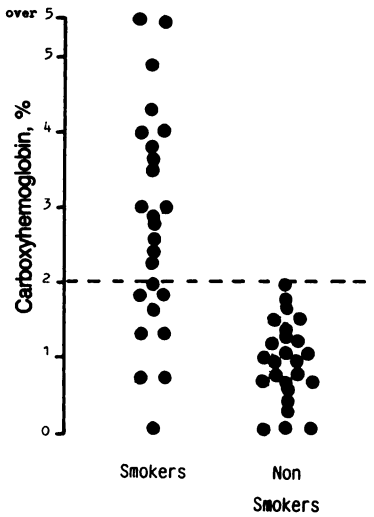

Fig. 4. Carboxyhemoglobin (%) in blood of smokers and nonsmokers
Dotted line: Upper reference limit for nonsmokers

because calibration checking is a relatively time-consuming procedure. The standards are not stable and therefore must be prepared immediately before use. The standard gassed with carbon monoxide is assumed to be converted to 100% carboxyhemoglobin, and although this has not been proven, the reproducible nature of the calibration curve has demonstrated consistency. However, this preparation must subsequently be treated sufficiently thoroughly with nitrogen to remove all dissolved carbon monoxide. We have not rigorously checked the effect of other hemoglobins but we do not believe that they will present a problem. Methemoglobin would be reduced to reduced hemoglobin and would therefore rightly contribute to the noncarboxylated fraction. Sulfhemoglobin would be unaffected but does not have an absorbance peak within the 390–450 nm region.

Although this method could be applied to samples collected post-mortem, it has not been used in this context; it would therefore be important to establish the absence of any interfering substances that are not present in living subjects.

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An Evaluation of the Du Pont *aca* IV: Does It Meet Medical Needs?

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A new bench-top analyzer from Du Pont, the "*aca* IV," was evaluated. To do so, we performed 10 different tests during an eight-week test period. The instrument is very precise (CVs <2%), results agree well with those obtained with the older *aca*s (II and III), linearity is good in the clinically important ranges, and there is very little drift or carryover. From a medical-needs viewpoint, we found the analyzer satisfactory for all tests except possibly for calcium, for which the medical-needs criteria are so strict that few laboratories can meet them. We reached the latter conclusions by using a statistical model, described here, and from the predictive value of an abnormal test for populations with various prevalences of disease.

A judgement as to the acceptability of a new instrument that is to be used to perform clinical chemistry tests has many facets, including reliability, precision, accuracy, stability, and serviceability. However, the primary basis for acceptability is the instrument's ability to produce "medically acceptable" results. What is medically acceptable? Several authors have addressed this question, e.g., Barnett (*1*), Harris (*2*), and Westgard (*3*). The answer is subjective and is dependent on the purposes of the test, the population being tested, and the cost to society of false-positive and false-negative results (*4*). Once the medical priorities have been set, then a more objective answer is possible as to medical needs, and the suitability of an instrument can be determined largely from laboratory experiments.

Here we follow the goal setting described by Harris (*2*) for testing a population to identify individuals with a biochemical abnormality. This appears to be the most prevalent use of the Du Pont *aca* IV, hence our choice of this model. Our choice is arbitrary; as described by Harris, goal setting could equally well be based on the detection of life-threatening biochemical abnormalities or on the determination of within-person trends with time of an analyte.

Materials and Methods

Statistical Model

The model for goal setting assumes a gaussian distribution of results for both the well and the sick, and an overlap of the results such that both clinical sensitivity and specificity are always less than one (*5*). Overlapping populations are the usual case; however, the distributions are usually not gaussian (*6*). Nevertheless, in this context it is a useful device to use in making an estimate of medical needs. We assumed that the optimum decision point between well and sick is at the midpoint of the overlapping values, that an increased value is abnormal, and that 2.3% of the well have values exceeding the decision point, and 2.3% of the sick have values below the decision point. In this model, the

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