New Method for Rapid Determination of Carboxyhemoglobin by Use of Double-Wavelength Spectrophotometry

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We have developed a simple method for rapid determination of carboxyhemoglobin concentration in blood, by use of double-wavelength spectrophotometry. The absorbance of both oxyhemoglobin and reduced hemoglobin are instrumentally nullled out in this procedure, so that the absorption signal reflects HbCO and only a single spectrophotometric reading is required. This procedure is accurate in the range encountered in ambulatory persons as well as in the "toxic" range. Results correlated well with the dithionite reduction procedure. The stability of carboxyhemoglobin in solution was investigated and concentrations of carbon monoxide in groups of smokers and nonsmokers were determined.

Additional Keyphrases: comparison with dithionite method • CO in blood of smokers • CO poisoning • toxicology

Exposure to and poisoning from carbon monoxide is usually documented by directly measuring carboxyhemoglobin or indirectly by measuring carbon monoxide. In each case, it is the percentage of carboxyhemoglobin that correlates with clinical symptomatology.

Existing methods for the measurement of CO and HbCO\(^1\) in blood are variations of several basic quantitative methods (1). The classic manometric methods of Van Slyke and Salvesen (2) and the volumetric methods of Scholander and Roughton (3) have borne the test of time, and are accurate when done fastidiously; but are too demanding for routine or emergency measurements in the clinical laboratory. Hüfner (4) and Heilmeyer (5) proposed a spectrophotometric method based on the two-component system HbO\(_2\)-HbCO. In their determination the sample absorbance (A) is measured at 576, 560, and 541 nm. Ratios of \(A_{541}/A_{560}\) and \(A_{576}/A_{560}\) are calculated, and the percentage of HbCO is determined from a prior calibration. Hartmann (6), Drabkin (7), and Amenta (8) have advocated the use of similar or slightly modified methods. The "CO-Oximeter" (Instrumentation Laboratory Inc., Lexington, Mass. 02173) is based on the same principle (9). Klendshoj et al. (10) exploited the resistance of HbCO to reduction by sodium dithionite (sodium hydrosulfite, Na\(_2\)S\(_2\)O\(_4\)) to determine the HbCO present in blood. Siggaard-Anderson et al. (11) have recently published simple nomograms that expand on this technique to include the measurements of met- and sulfhemoglobin. Whitehead and Worthington (12) have described a spectrophotometric method that is based on the heat stability of HbCO. A microdiffusion method described by Feldstein and Klendshoj (13) involves the reduction of palladium chloride by carbon monoxide in acid solution. Other methods include infrared spectrophotometric (14), and gas-chromatographic techniques (15–17).

We have developed a method in which double-wavelength spectrophotometry is used to measure HbCO at high or low concentrations. This method effectively "nulls out" the interfering spectral absorption of HbO\(_2\) and HHb and requires only a single measurement.

Principle of the Method

Double-wavelength spectrophotometry was first described by Chance (18). Two monochromators in the same instrument pass light beams of different wavelengths through a single cuvette; the photometer measures the absorbance difference (\(\Delta A\)). If one component of a two-component system shows the same extinction at each of the two selected wavelengths, \(\Delta A\) will be proportional to the concentration of the second component. Important to this procedure is the ability to precisely null the interfering component by fine tuning of the two monochromators.

The absorption curves of HbCO and HbO\(_2\) are shown in Figure 1. Their spectra closely overlap so that these two hemoglobin components cannot be separated at any given wavelength. However, one can find wavelength pairs at which HbO\(_2\) shows the same extinction, but HbCO does not. At these wavelengths the absorbance of HbO\(_2\) is effectively cancelled by a double-wavelength measurement, and \(\Delta A\)

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\(^1\) Nonstandard abbreviations used: carboxyhemoglobin, HbCO; hemoglobin, Hb; oxyhemoglobin, HbO\(_2\); reduced hemoglobin, HHb; absorbance difference, \(\Delta A\); and EDTA, ethylenediaminetetraacetate.
reflects only HbCO concentration. Wavelengths \( \lambda_a \) and \( \lambda_b \) in Figure 1 are one such pair.

Although this approach is theoretically correct, all venous blood samples contain significant amounts of HHb, in addition to \( \text{HbO}_2 \) and HbCO, which interferes with the spectrophotometric methods. Figure 2 shows the spectral absorbance curves of \( \text{HbO}_2 \), HHb, and HbCO. In the present method we use the carefully chosen wavelengths \( \lambda_1 \) (530.6 nm) and \( \lambda_2 \) (583.0 nm), such that both HHb and \( \text{HbO}_2 \) absorbances are negated by a single double-wavelength measurement. Under these conditions, the resulting absorbance reflects only HbCO.

Reagents and Equipment

Spectrophotometric measurements were made with a Model 156 double-wavelength spectrophotometer (Perkin-Elmer Corp., Norwalk, Conn. 06856). Rectangular glass cuvettes (1-cm lightpath) were used.

*Venous blood samples* were collected in "Vacutainer" tubes containing ethylenediamine tetracetic acid (EDTA) as an anticoagulant (Becton-Dickinson Co., Rutherford, N. J. 07070).

*Ammonium hydroxide*, 7 mmol/liter. Dilute 0.48 ml of 28% \( \text{NH}_3 \) ("ammonia water, stronger") to 1000 ml with distilled water.

*\( \text{HbO}_2 \) and HbCO standards*. Packed cells obtained from blood collected in EDTA-containing tubes were used. To prepare \( \text{HbO}_2 \) and HbCO, respectively, technical grade 100% oxygen or 100% carbon monoxide was gently bubbled through samples for about 15 min. After the CO treatment of blood, it was washed with nitrogen to remove any carbon monoxide in solution not bound to hemoglobin. Plasma was then re-added to the system and the appropriate dilution made.

HHb was obtained by dithionite reduction of \( \text{HbO}_2 \).

**(a)** Standard curve: Dilute aliquots of nitrogen-washed HbCO with \( \text{HbO}_2 \). Determine total Hb as cyanomethemoglobin. Prepare a standard curve by plotting known HbCO, in g/dl, vs. \( \Delta A \). Measure the \( \Delta A \) in unknown specimens and convert to HbCO by using the standard curve. The HbCO divided by the total Hb gives the percentage of HbCO in the erythrocytes.

**(b)** A simpler procedure, which avoids the Hb measurement and preparation of a standard curve, is as follows: After determining \( \Delta A \) for the sample, bubble 100% CO into the cuvette for a few seconds, to convert all hemoglobin to HbCO. Then remeasure the absorbance difference (\( \Delta A_2 \)). The ratio, \( \Delta A/\Delta A_2 \) is the percentage of HbCO in the blood.

**Results**

Standard curves obtained by method (a) (Figure 3) were linear, both at high and low (<10%) concentrations of HbCO. These curves were reproducible with blood from different donors. Once the instrument had been zeroed with oxyhemoglobin, 20 different
oxygenated samples showed an average absorbance (ΔA) of 0.001. This corresponds to an HbCO concentration of less than 0.2%. An HbCO concentration of 0.1 g/dl will give a ΔA of 0.005 by this method, which corresponds to about 0.7% HbCO when the total hemoglobin concentration is normal.

As an internal check on the procedure, HbCO was determined both by methods a and b in 97 HbCO samples. A correlation coefficient of 0.997 (P < 0.0001) was obtained with essentially identical mean values (4.59 and 4.61% HbCO). The largest single-point difference was 0.4% HbCO.

The sodium dithionite method was chosen for comparison because it is based on a different chemical principle. Eighteen bloods of various HbCO concentration were analyzed by both the double-wavelength and the dithionite methods. The results are shown in Figure 4. The correlation coefficient was .991 (P < 0.0001) with a maximum difference of 5% (at 50% HbCO percentage).

The effect of experimental conditions on precision was studied. Sample stability proved to be an important factor, because the diluted sample was stable only for a short time: When identical diluted solutions were left undisturbed in the spectrophotometer cuvette holder at room temperature, the average decrease in absorbance was about 1.5% after 0.5 h, 3% after 1 h, 12% after 2 h, and 26% after 4 h. Whole blood in EDTA, on the other hand, was stable at 4 °C for two months. Intra-day precision studies on 22 samples of the same 80% HbCO blood, with use of the same Autopipette and instrument settings, showed a standard deviation of 0.46% HbCO. Duplicate analyses on similar bloods gave a standard deviation of 0.19% HbCO. Inter-day precision, as measured during 22 consecutive days, was 0.68% HbCO (standard deviation).

During many of the studies with carboxyhemoglobin, it became quite apparent that sample agitation and pouring were important variables. Ordinary pouring of a 3 ml diluted aliquot from a test tube to a cuvette diminished the % HbCO by as much as 2%. After 15 serial “pourings” over a period of 10–15 min as described, the sample absorbance decreased by an amount corresponding to 32% HbCO. Therefore, in all work, we were careful to minimize both the number of solution transfers and sample agitation during the transfer.

To validate the analytic method, we determined HbCO concentrations in the blood of 97 smokers and nonsmokers from an urban area (New Haven, Connecticut). All smokers had used at least one pack per day over the past several months. The results were consistent with the known effects of cigarette smoking. The 47 smokers had venous blood carboxyhemoglobin percentages ranging from 3.7 to 13.7% (mean, 7.22%; SD, 2.25%). The corresponding values for 50 nonsmokers were 0.20 to 3.35%, 2.01 g/dl, and 0.78 g/dl.

We also studied the effect of methemoglobin. Based on the spectral absorbance curve of this component under the conditions of the assay, the absorbance change between 530.6 and 583.0 nm was negative. Any methemoglobin present therefore effectively lowered the measured HbCO level. For each 1% methemoglobin in a sample of normal hemoglobin concentration the decrease is about 0.1% HbCO. In one patient studied with (9%) methemoglobinemia, the measured carboxyhemoglobin was 1.6% of the total Hb.

Discussion

The absorbance contribution of the two normal hemoglobin components, oxyhemoglobin and reduced hemoglobin, was eliminated by this procedure. In theory, double-wavelength spectrophotometry will resolve two-component systems, and many pairs of wavelengths other than those selected herein could be selected to resolve HbCO from HbO₂. Fortunate-
ly, we were able empirically to determine a wave-
length pair at which both HbO₂ and HHb showed a
Δλ of zero. Although the same results might theo-
retically be obtained by taking multiple readings with a
conventional spectrophotometer, fine wavelength
trimming to exactly null out the interfering com-
ponent is necessary for a high degree of accuracy. This
is particularly true if the interfering component (in
this case HbO₂) is present at high concentration,
and the measurement is made at a point where the
absorption curve has a steep slope. This procedure
permits a rapid, sensitive, single-measurement de-
termination of carboxyhemoglobin in blood. The sen-
sitivity (0.05 A/g per 100 ml of HbCO) is sufficient
for accurate work at proportions of less than 10%,
which may be important in studies of chronic toxici-
ty.

Other accurate, sensitive procedures are available
for measuring CO or HbCO, particularly the gas-
chromatographic procedures described by Rodkey
(15). However, the proposed procedure, which re-
quires only a single dilution of blood and a single
spectrophotometric measurement, is well suited for
clinical toxicology as well as for batch analysis of
large numbers of samples, such as might be required
for research or pollution control.

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