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Determination of Carboxyhemoglobin in the Presence of Other Blood Hemoglobin Pigments by Visible Spectrophotometry

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ABSTRACT: The convenience of the spectrophotometric method for the determination of carboxyhemoglobin has been tempered by the observation that the analysis of postmortem bloods is often biased by the presence of pigments other than oxyhemoglobin, carboxyhemoglobin, and reduced hemoglobin. These other pigments include most prominently methemoglobin and sulfhemoglobin. Using a microprocessor-controlled spectrophotometer, a method was developed depending on absorbance difference measurements at isosbestic points for oxyhemoglobin, carboxyhemoglobin, and reduced hemoglobin that is accurate down to 2% carboxyhemoglobin in fresh blood. A correction for the error caused by methemoglobin is part of the method. Qualitative confirmation of carboxyhemoglobin by examination of spectra details, sodium dithionite reduction, and first derivative spectra is described. The analysis of denatured and autolyzed bloods is examined in the context of postmortem case reports. A number of spectra are shown in detail, including methemoglobin, sulfhemoglobin, alkaline hematin, acid hematin, and mixtures of blood pigments containing varying concentrations of carboxyhemoglobin. The method has been shown to be precise, accurate, and reliable for fresh bloods. While accuracy for denatured bloods is diminished, reliability of carboxyhemoglobin identification is maintained. The analysis time is about 5 min for routine blood samples and the method is easily implemented with a precise microprocessor-controlled spectrophotometer.

KEYWORDS: forensic science, spectroscopic analysis, blood, carboxyhemoglobin analysis, spectrophotometry, methemoglobin, sulfhemoglobin, isosbestic points, denatured hemoglobin

Spectrophotometric methods for determination of carboxyhemoglobin have a history of approximately 80 years and presently are the most frequently used methods for routine blood carboxyhemoglobin analyses. Published spectrophotometric methods include those of Freireich and Landau [*1*], Amenta [*2*], Siggard-Anderson et al [*3*], Pannell et al [*4*], Blackmore [*5*], Hufner [*6*], Commins and Lawther [*7*], Heilmeyer [*8*], and Dubowski and Luke [*9*]. Some carboxyhemoglobin calibration techniques do not require saturation of the specimen of blood analyzed with carbon monoxide, while other methods specify measurements before and after treatment [*4*] or a difference spectrum [*7,10*]. A particularly appropriate method for postmortem bloods is the Wolff procedure as modified and described by Maehly [*11*, pp. 566-569] in which acetate buffer and heat isolate carboxyhemoglobin in solution while other pigments precipitate. Katsumata and coworkers [*12*] very recently reported a spectrophotometric absorbance ratioing method applicable to denatured bloods.

Blackmore [*5*] has suggested that gas chromatography of carbon monoxide released from

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hemoglobin offers more specificity and accuracy. However, gas chromatographic methods are time-consuming and do require tedious calibration. Errors are introduced from postmortem formation of methemoglobin and other pigments that cannot bind carbon monoxide, and the quantity of hemoglobin capable of binding carbon monoxide originally present in vivo is underestimated. Two questions were asked in initiating this study: (1) can carboxyhemoglobin be determined simply, accurately, and reliably to the 1 or 2% saturation level in fresh blood and (2) can other hemoglobin pigments that are especially prevalent in denatured blood be recognized spectrophotometrically and accounted for in the determination of carboxyhemoglobin? We present a rapid, accurate, and easily implemented method for determining carboxyhemoglobin in fresh clinical bloods and in moderately denatured bloods. A protocol for grossly denatured bloods is given.

The delta-absorbance (ΔA) technique, first published by Ramieri et al [*13*] and since by other authors [*14, 15*], is a key aspect of our approach. While Rameri and coworkers used a research grade double-monochrometer spectrophotometer, we conducted our studies on a routine double-beam, single monochrometer instrument. Validation data were sketchy in the three ΔA methods cited. Here, substantial data relating to precision, accuracy, and reliability are presented.

Materials

Spectrophotometer

A Hitachi Model 110 spectrophotometer provided all spectra and analytical data given in this paper. Specifications for this instrument include slit width (spectral band pass) control from 0.1 to 4 nm fixed, microprocessor control of operation parameters and scanning programs, ± 0.2 nm repeatability, ± 0.001 absorbance photometric accuracy, a digital meter and tape printer for recording data, and first and second derivative spectra recording capability. The absorbance repeatability (precision) was checked with holmium oxide at four wavelengths and wavelength accuracy was determined (see Table 1).

Chemicals and Reagents

The chemicals and reagents used included:

1. Ammonium hydroxide (NH_4OH), aqueous 0.4%; 4 mL of concentrated reagent grade NH_4OH diluted to 1 L with distilled water.

TABLE 1—*Instrumental settings for carboxyhemoglobin analysis and calibration data for the Hitachi Model 110 spectrophotometer.*[a]

Wavelength, nm	Absorbance Observed[b]
641.0	0.120 ± 0.001
536.2	0.266 ± 0.001
445.2	1.630 ± 0.003
360.4	0.529 ± 0.002

[a] Scan range 650–500 nm; scan speed 100 nm/min; chart span 0 to 2*A*; nanometre per division 50 (25 nm/cm); fixed slit position 1.0 nm; pen response 1 s; tungsten lamp; zero at 560 nm water versus water. Calibration with holmium oxide filter, 2-nm slit. Wavelength accuracy calibration: the 656.1-nm deuterium lamp emission line peaked at 656.0 on this instrument.

[b] Absorbance readings on 30 different days for the holmium oxide filter with one standard deviation indicated.

2. Carbon monoxide gas lecture bottle (Matheson).
3. Wolff's buffer, as described in Ref *11*, pp. 566-569.
4. Palladium chloride solution, as described in Ref *16*, p. 837.

Carboxyhemoglobin Standards

A screw-cap test tube with 3 to 10 mL of blood is flushed with carbon monoxide (CO) cylinder gas for a few seconds and then sealed and rotated for a few minutes. This process is repeated four or five times; a cherry-red color is produced as CO saturation approaches 100%. A drop of this blood in 0.4% NH_4OH is checked for an absorption wavelength maximum shift from 576.2 nm downward to approximately 568.3 nm, at which point the blood is maximally saturated. The blood is then purged for approximately 30 s with water-saturated nitrogen to clear plasma-dissolved CO, which is minimal by this saturation technique. Another portion of this blood, which is initially as low as possible in carboxyhemoglobin, is used as a counter mixture to prepare blood standards of convenient CO saturation contents. For this study, standards of 5, 10, 20, 25, 30, 40, 50, 60, 75, 80, 90, and 100% saturation were prepared to establish a calibration line for quantitative determinations. For the purpose of calculations, the maximally saturated blood was considered 99% and the untreated blood 1% saturated.

Experimental Method

Oxyhemoglobin, Reduced Hemoglobin, and Carboxyhemoglobin

Reduced hemoglobin (Hb_r) is formed by treating oxyhemoglobin (HbO_2) with a spatula tip full of sodium dithionite ($Na_2S_2O_4$). The spectra of the three major hemoglobin pigments, each near 100% purity, are shown in Fig. 1. The absorptivity values for five chemical forms of hemoglobin are shown in Table 2; composite absortivity is shown in Table 3. Instrument settings used when the absorptivities in Table 2 ($E^{1\%}_{1cm}$, or absorbance of 1 g specified hemoglobin per 100 mL solvent) were measured are indicated in Table 1. The absorbance at each wavelength is proportional to $[HbO_2] + [HbCO] + [Hb_r]$, where HbCO is carboxyhemoglobin. Measurements on known concentrations of HbO_2, Hb_r, and HbCO were made to obtain the data in Table 4 and isosbestic crossover points (illustrated in Fig. 1) were noted. Using the absorptivities at the crossover points (isosbestic points), equations for the three-component system (HbCO, HbO_2, and Hb_r) were developed.

Let λ = wavelength, x = grams HbCO per 100 mL, y = grams HbO_2 per 100 mL, z = grams Hb_r per 100 mL, and d = absorbance at nm indicated; a, b, and c are constants.

$$\lambda_1 = 548.2\,\text{nm} \qquad d_1 = a_1x + b_1y + c_1z$$

$$\lambda_2 = 568.1\,\text{nm} \qquad d_2 = a_2x + b_2y + c_2z$$

$$\lambda_3 = 578.0\,\text{nm} \qquad d_3 = a_3x + b_3y + c_3z$$

Using the absorptivities from Table 4 we have:

$$d_1 = 7.45x + 7.45y + 7.45z$$

$$d_2 = 8.7x + 6.7y + 6.7z$$

$$d_3 = 5.8x + 9.0y + 5.8z$$

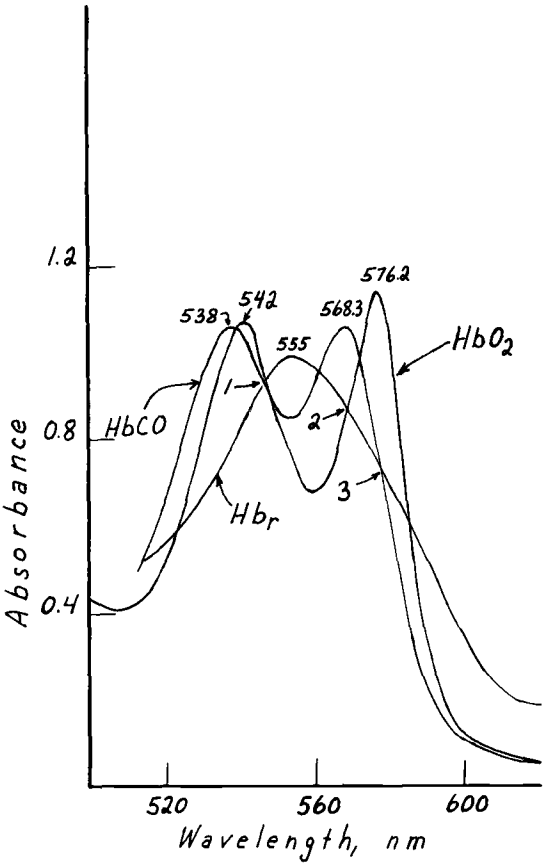

FIG. 1—*Triad of hemoglobin pigments scanned in 0.4% aqueous NH_4OH; HbO_2, HbCO, and Hb_r. Three isosbestic points and absorbance maxima are indicated.*

TABLE 2—*Absorptivity constants $E^{1\%}_{1cm}$ for various hemoglobin species in 0.4% aqueous ammonium hydroxide.*

Species	Symbol	Max Wavelength, nm	$E^{1\%}_{1cm}$
Oxyhemoglobin	HbO_2	576.2	9.25
Carboxyhemoglobin	HbCO	568.3	8.6
Cyanomethemoglobin	metHb-CN	541	6.84
Reduced hemoglobin	Hb_r	555	7.9
Methemoglobin	metHb	575	5.6

Solving for x, y, and z, Eqs 1, 2, 3, and 4 are obtained, permitting a composition calculation of the three component system.

$$x = (d_2 - 0.899d_1)/2.0 \tag{1}$$

$$y = (d_3 - 0.78d_1)/3.2 \tag{2}$$

$$z = (d_1 - 0.60d_2 - 0.38d_3)/1.2 \tag{3}$$

$$\%\text{HbCO} = [x/(x + y + z)]\,100 \tag{4}$$

TABLE 3—*Composite absorptivity* E* *for total hemoglobin at various HbCO contents in clinical bloods and in fresh postmortem bloods.*[a]

%HbCO	E^*	%HbCO	E^*
0	9.2	40	8.4
5	9.1	50	8.3
10	9.0	60	8.4
20	8.8	75	8.5
30	8.6	100	8.6

[a] A value for E^* taken from this table is used in Eqs 5, 6, and 8 for calculation of %HbCO. The parameter E^* is not a pure constant, but a composite of HbO_2, Hb_r, and HbCO that changes as HbCO changes; E^* is not applicable after reduction with $Na_2S_2O_4$.

TABLE 4—*Absorptivity constants for the major species of hemoglobin at the isosbestic crossover points in 0.4% aqueous* NH_4OH.

Wavelength of Isosbestic Point	E_{1cm} for Hemoglobin Species and Symbol[a]			
	HbCO (x)	HbO_2 (y)	Hb_r (z)	metHb[b]
548.2 (λ_1)	7.45 (a_1)	7.45 (b_1)	7.45 (c_1)	5.7
568.1 (λ_2)	8.7 (a_2)	6.7 (b_2)	6.7 (c_2)	5.3
578.0 (λ_3)	5.8 (a_3)	9.0 (b_3)	5.8 (c_3)	5.4

[a] The letter symbols in parentheses following the hemoglobin forms and absorptivity constants are used in developing Eqs 1 through 4.

[b] Methemoglobin (last column) is not isosbestic with the other hemoglobins at the wavelengths given, but is listed for comparison.

Equation 2 does not give the physiological HbO_2 content, since dilution with 0.4% aqueous NH_4OH (or water) increases the degree of oxygenation; the in vivo HbO_2 content is determined without dilution in narrow path cells [*3*].

Delta-Absorbance Method for Carboxyhemoglobin

Ramieri et al [*13*], using a Perkin-Elmer 156 double-wavelength spectrophotometer, noted that the HbCO concentration is proportional to the absorbance difference of 530.6 minus 583 nm, the two wavelengths being measured simultaneously by the dual monochrometer instrument. Our ΔA interval, obtained by measuring 528.7 and 583 nm in rapid succession, was found to be linear from 0 to 100% saturation. A typical plot of our data is shown in Fig. 2. The ΔA interval was not found to be precisely the same before and after treatment with $Na_2S_2O_4$. Equations 5 and 6 relate %HbCO to ΔA.

$$\%\text{HbCO} = \left(\frac{A_{528.7} - A_{583}}{3.7} \frac{E^*}{A_{max}} \right)_{Oxd.} 100 \qquad \text{(before } Na_2S_2O_4\text{)} \tag{5}$$

$$\%\text{HbCO} = \left(\frac{A_{530} - A_{583}}{4.2} \frac{E^*}{A_{max}} \right)_{Red.} 100 \qquad \text{(after } Na_2S_2O_4\text{)} \tag{6}$$

The value for composite absortivity E^* is taken from Table 3; E^*/A_{max} is the reciprocal of total hemoglobin concentration. To determine which value of E^* to use from Table 3, a rough estimate of %HbCO is obtained by Eq 7:

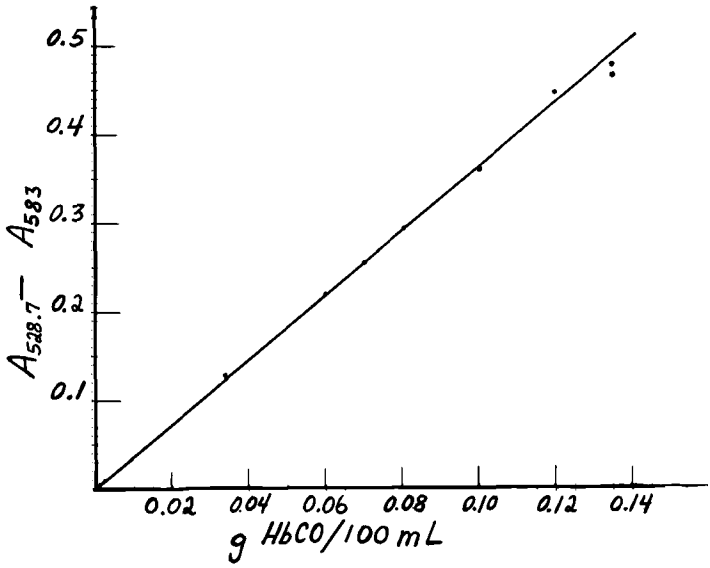

FIG. 2—*Linear regression plot of grams carboxyhemoglobin per 100 mL solution versus* ΔA *(absorbance at 528.7 minus absorbance at 583 nm). From plots such as this, Eqs 5, 6, 7, and 8 were developed.*

$$\%\text{HbCO} = [(A_{528.7} - A_{583})/A_{\max}]\,240 \tag{7}$$

where $A_{\max}$ is the absorbance maximum nearest 576 nm. This maximum in itself provides an estimate of %HbCO, as Fig. 3 illustrates. At less than 20% HbCO saturation, a y-intercept correction is made, as indicated in Eq 8.

$$\%\text{HbCO} = \{[(A_{528.7} - A_{583} - 0.007)/3.7](E^*/A_{\max})\}\,100 \tag{8}$$

Once a suitable calibration line has been established, routine HbCO determinations are accomplished as follows:

1. To 10 mL of 0.4% aqueous NH_4OH, add four drops of the test blood. If the composition of the original blood is desired, make an accurate hundredfold dilution here. Hemolysis is rapid; the absorbance maximum for typical whole blood will be 1 to 2A.
2. Set the spectrophotometer as indicated in Table 1.
3. Scan from 650 to 500 nm for total spectrum appearance. When methemoglobin is present, the blood is a darker red and the scan slope from 650 to 600 nm is steeper. An inflection point at 600 nm becomes evident above 20% methemoglobin (see Fig. 4), and the color becomes brown.
4. Obtain in sequence absorbances at 528.7, 583, and 576.2 nm; find precisely the maximum nearest 576.2 nm, which will nearly always be equal to or less than 576.2 nm.

Steps 1 through 4 provide a routine determination of HbCO. Qualitative and quantitative corroboration of these findings is achieved in Steps 5 through 8:

5. Obtain absorbances at 548.2, 568.1, 578, and 584 nm.
6. Add a spatula tip full of $Na_2S_2O_4$ to the sample and reference cells, mix, and rescan from 650 to 500 nm.
7. Measure absorbances at 530, 583, and 555 nm.
8. Set the instrument in the first derivative mode of operation (scan speed 100 nm/min, sensitivity factor of 2 or 3, and slit width of 1 nm) and record the first derivative of the absorbance versus wavelength scan ($\Delta A/\Delta\lambda$) from 650 to 500 nm.

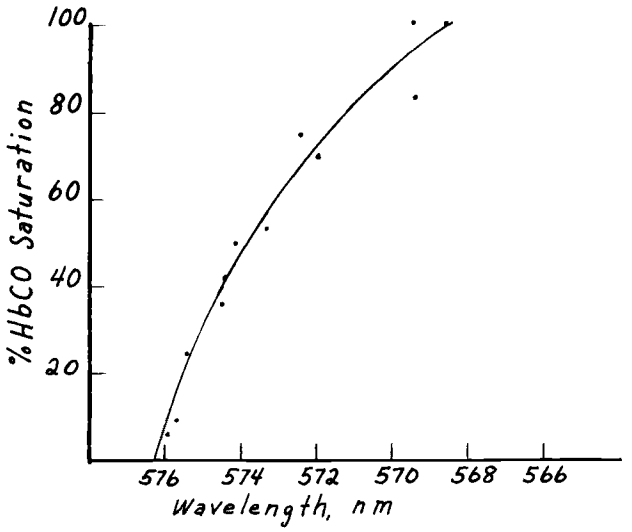

FIG. 3—*The position of the high wavelength maximum as a function of percent of total hemoglobin which is carboxyhemoglobin (%HbCO saturation).*

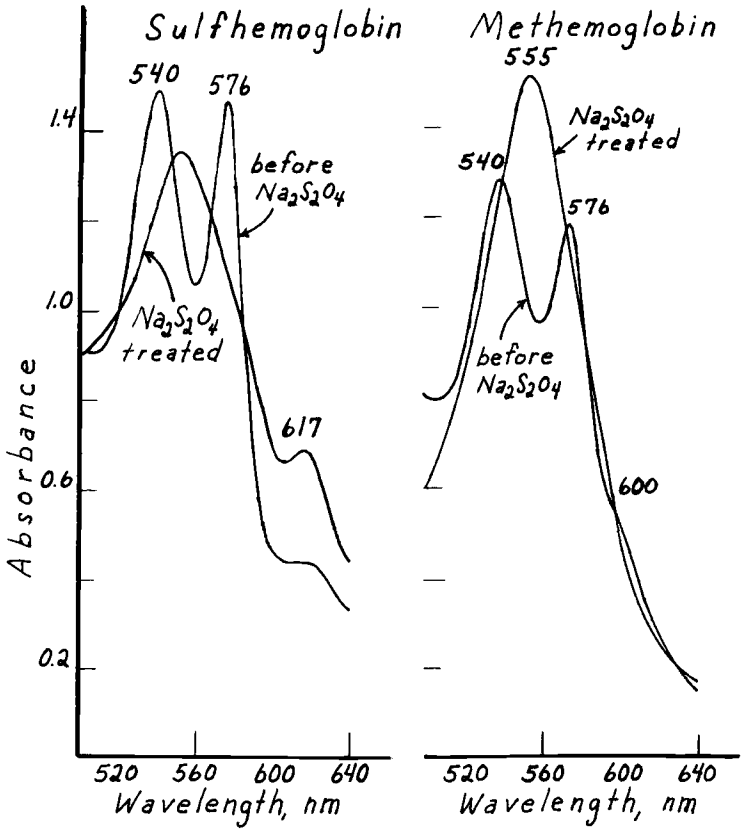

FIG. 4—*The visible absorption spectra of methemoglobin (right) and sulfhemoglobin (left), both in 0.4% aqueous NH_4O_4. The 617 maximum of sulfhemoglobin is not eliminated by treatment with $Na_2S_2O_4$.*

Use of the data obtained in these eight steps and application of the equations given will be demonstrated in the sections that follow.

Steps 5 through 8 are particularly applicable when methemoglobin is apparent; methemoglobin is reduced to Hb_r, somewhat negating this source of error. Figure 5 illustrates a series of wavelength-absorbance scans before and after reduction, and the first derivative scan of the reduced hemoglobin solution. Slight changes in spectrum shapes are apparent as low as 10% HbCO after reduction; by 40% HbCO the reduced solution has a saddle-shaped appearance. The first derivative scan shows peak development at 560 nm as %HbCO increases. An estimate of %HbCO is attained by comparing spectra of bloods analyzed to Fig. 5.

Bloods that are substantially denatured or autolyzed, as evidenced by spectral distortions and nonagreement of the different equations, are analyzed of Wolff's method [11, pp. 566-569]. In addition, a qualitative or semiquantitative analysis is carried out by diffusing carbon monoxide into palladium chloride, as described by Blanke [*16*].

Method Evaluation

Precision

The ΔA interval reproducibility of the Hitachi model 110 used is $\pm 0.0015A$, which is equivalent to approximately 4×10^{-4} g HbCO per 100 mL 0.4% NH_4OH solution, or about 0.3% HbCO in a typical analysis; therefore by our method the limit for reliable detection is approximately 1.5% HbCO. A blood with a measured carboxyhemoglobin content near 29% was analyzed in ten replicates (Steps 1 through 4 above) and its HbCO calculated by Eq 5. A mean of 26.68 ± 0.56% saturation (±2% coefficient of variation) was obtained with a range of 27.78 to 29.48%. Repeatability on a single solution of blood was ±0.2% saturation. The

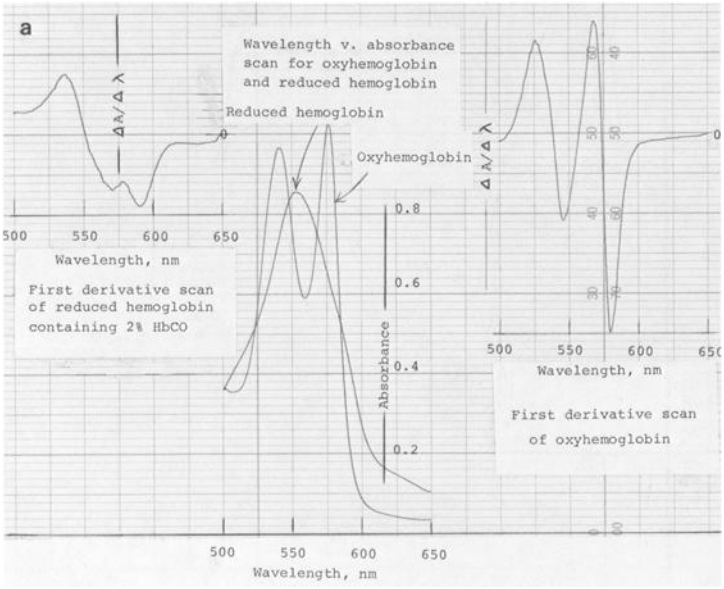

FIG 5—*A complete presentation of the absorption scans and first derivative ($\Delta A/\Delta\lambda$) scans in 0.4% aqueous NH_4OH of hemoglobin at varying compositions of HbO_2, HbCO, and HB_r. Carboxyhemoglobin at 2, 20, 45, 75, and near 100% saturation is shown. Treatment with $Na_2S_2O_4$ causes HbO_2 to become reduced but does not change the HbCO content. Carboxyhemoglobin is recognized by the formation of two absorption peaks after $Na_2S_2O_4$ treatment when HbCO is greater than 30% of the total hemoglobin and a peak at 560 nm in the first derivative presentation.*

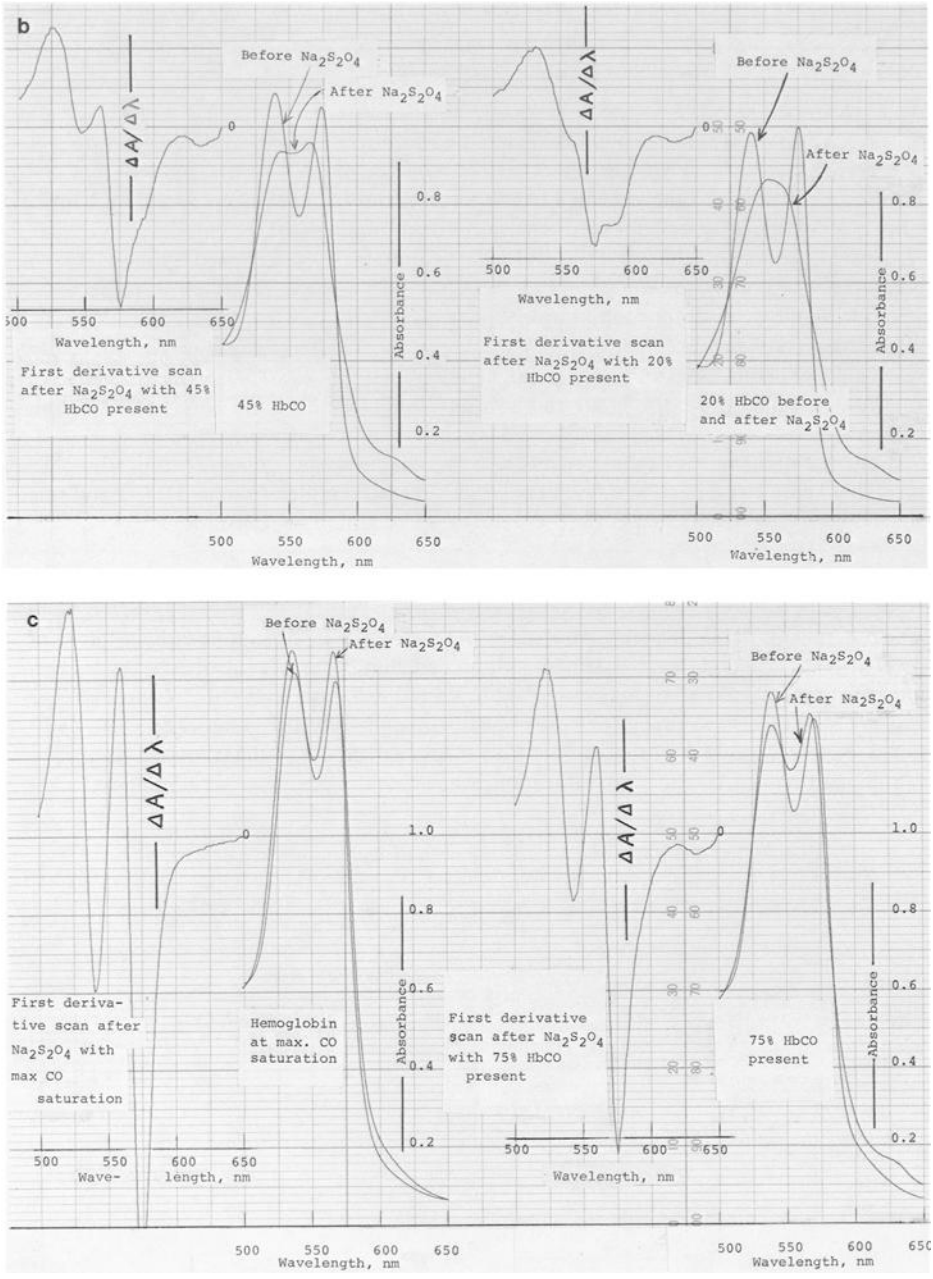

FIG. 5—*Continued.*

standard deviation of ±0.6% saturation remains constant throughout the concentration range of 1 to 85% saturation.

Accuracy

Several observations relating to accuracy were made:

1. Finger prick blood of nonsmokers was 0.5 to 3% saturated by the ΔA method.
2. Only 4 bloods of more than 1000 analyzed gave zero or negative ΔA values, indicating that a correct ΔA interval was chosen.
3. Finger prick blood ranged from 4 to 6% saturation for light smokers and from 5 to 9% for heavy smokers, in agreement with other published findings [*9, 13*].
4. Two quality control bloods of 30 and 60% saturation, as determined by more than 50 laboratories in a forensic toxicology proficiency study [*17*], yielded 32 and 60%, respectively, by Eq 5 with one analysis for each sample.
5. Internal consistency of the different equations presented here was observed with respect to both carboxyhemoglobin and total hemoglobin.
6. Close agreement with the Wolff and isosbestic points methods was observed, as Table 5 indicates.
7. Concentrations in car exhaust deaths and fire deaths ranged generally from 55 to 90% saturation. Table 6 compares the results of various methods of determining HbCO and total hemoglobin in four bloods that cover the usual range of HbCO concentrations.

Sources of Error

The ΔA value is pH dependent. Analysis in pH 6.6 buffer yields significantly different results. The $E^{1\%}_{1cm}$ constants were obtained with an exit slit of 1 nm; a slit of 2 nm yields constants of 0.1 to 0.2% lower but in the same ratio. Equation 6 was used for the purpose of specificity, not quantitative accuracy. The use of Eqs 1 through 4 for HbCO saturation below 10% resulted in greater errors than results from Eq 5. Close agreement of all estimating techniques was attained between 10 and 95% saturation. Accuracy and precision are diminished when

TABLE 5—*Comparison of the* ΔA *and three-wavelength isosbestic points methods with the Wolff's buffer method.*[a]

	Carboxyhemoglobin Found, %				
			Isosbestic Points		
Specimen	ΔA_{oxd}	A_{max}	Oxidized	Reduced	Wolff
51890	78	80	73	76	76
53215	76	75	68	74	65
46898	86	90	83	85	90
43095	79	82	79	76	75
43095	13	15	17	20	14
54504[b]	52	57	53	54	57
37914	32	30	27	34	28
45459	15	15	17	...	17
4131	51	55	47	51	46
4129	18	20	14	20	18

[a] The ΔA method uses Eq 5 (see text) to obtain the %HbCO found under ΔA_{oxd}; A_{max} results were estimated from Fig. 3; Eqs 1-4 before and after $Na_2S_2O_4$ treatment were used to calculate percent HbCO in the third and fourth columns of results; the Wolff's buffer isolation technique is described by Maehly [*11*, pp. 566-569].

[b] This blood specimen contained 10% methemoglobin.

TABLE 6—*Comparison of %HbCO and total hemoglobin Hb_t by the various quantitative techniques used in this study.*

Blood Specimen	Method of Quantitation	Result
Fresh blood nearly saturated with CO gas	A_{max} = 568.9 nm	95% HbCO
	ΔA_{oxd} by Eq 5	96% HbCO
	ΔA_{red} by Eq 6	99% HbCO
	Eq 4 before $Na_2S_2O_4$	94% HbCO
	Eq 4 after $Na_2S_2O_4$	94% HbCO
	$A_{max}/8.55 = Hb_t$	0.099 g per 100 mL
	Sum of Eqs 1-3 = Hb_t	0.095 g per 100 mL
Fresh Blood with minimal HbCO	A_{max} = 576.2 nm	<8% HbCO
	ΔA_{oxd} by Eq 5	0% HbCO
	ΔA_{red} by Eq 6	3% HbCO
	Eq 4 before $Na_2S_2O_4$	0% HbCO
	Eq 4 after $Na_2S_2O_4$	7% HbCO
	$A_{max}/9.2 = Hb_t$	0.104 g per 100 mL
	Sum of Eqs 1-3 = Hb_t	0.107 g per 100 mL
Clinical blood in greentop Vacutainer tube	A_{max} = 575.8 nm	12% HbCO
	ΔA_{oxd} by Eq 8	12% HbCO
	ΔA_{red} by Eq 6	11.7% HbCO
	Eq 4 before $Na_2S_2O_4$	10.9% HbCO
	$A_{max}/9.2 = Hb_t$	0.12 g per 100 mL
	Sum of Eqs 1-3 = Hb_t	0.12 g per 100 mL
Postmortem blood; CO exposure history	A_{max} = 573.8	50% HbCO
	ΔA_{oxd} by Eq 5	50% HbCO
	Diffusion into palladium chloride	50% HbCO
	Wolff method	47% HbCO

absorbance readings are outside the 1 to 2 absorbance range because of too much or too little dilution of the test blood.

Analysis of Denatured and Autolyzed Postmortem Bloods

The presence of forms of hemoglobin other than Hb_r, HbO_2, and HbCO cause analytical error if Eqs 1 through 5 and Eq 8 are applied as written. Equation 6 will correct for methemoglobin reasonably well up to about 15% methemoglobin. Methemoglobin interference is detected by an inflection in the absorbance scan at 600 nm (Fig. 4), or by a maximum at 628 nm in pH 6.6 buffer (Fig. 6). Methemoglobin (MetHb) is estimated in our analysis (in the 0.4% NH_4OH) by Eq 9, which applies before $Na_2S_2O_4$ treatments. Equations 10 and 11 are used to check results.

$$C_{metHb} = (A_1 - 0.52A_2)/2.77 \quad (9)$$

$$C_{HbO2} = (A_2 - 1.10A_1)/3.9 \quad (10)$$

$$C_{total} = A_1/4.8 \quad (11)$$

where A_1 = absorbance at 584 nm, A_2 = absorbance at 576 nm, and concentrations C are in g per 100 mL of 0.4% aqueous NH_4OH. After the quantity of methemoglobin per 100 mL NH_4OH solution is determined by Eq 9, ΔA in Eqs 5, 7, and 8 is corrected by subtracting $0.05A$ for every 0.1 g metHb per 100 mL. If no correction is made for metHb, the %HbCO from Eqs 5 and 8 is falsely elevated to the degree that methemoglobin is present.

Sulfhemoglobin, shown in Fig. 4, causes errors in the ΔA calculation, falsely elevating %HbCO. Sulfhemoglobin is rarely encountered in clinical bloods, but may occur when there

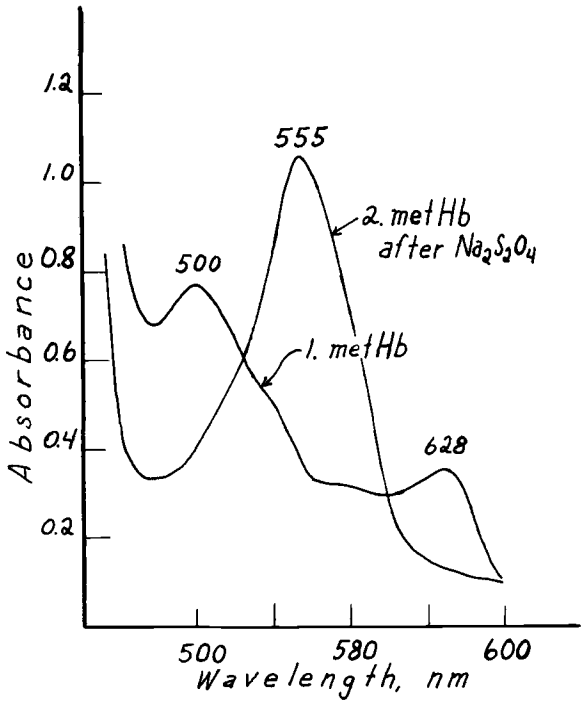

FIG. 6—*Methemoglobin in pH 6.6 buffer, characterized by 628- and 500-nm maxima* (Trace 1); *treatment with* $Na_2S_2O_4$ *creates the reduced hemoglobin scan* (Trace 2).

has been exposure to hydrogen sulfide [*11*, pp. 539–592]. We have observed that bloods from decomposed bodies in which the blood is putrid and cloudy in solution often contain sulfhemoglobin.

Acid and alkaline hematin, shown in Figs. 7 and 8, are hemoglobin forms produced by acid and base treatment of blood, respectively. Alkaline hematin responds to $Na_2S_2O_4$ (see Fig. 8), while acid hematin shows little change after $Na_2S_2O_4$. Once these pigments are formed, it becomes difficult to determine the %HbCO saturation that existed at the time of death, as these hematins do not bind carboxyhemoglobin.

Case Reports and Discussion

The precision and accuracy required in forensic toxicology depend on the questions being asked. Reliability of detection of toxicologically significant concentrations of carboxyhemoglobin is an overriding factor. Our methods allow the analyst to choose between a rapid screen within a few minutes or specific documentation of the percentage of the major hemoglobin pigments present. Approximately 20 bloods can be analyzed in 1 h by ΔA and Eq 5 or 8. Several case reports are presented below to illustrate the approaches and equations used for various situations.

Case 33024

The subject was killed in a motor vehicle accident; no alcohol or drugs were found present. The value for ΔA was $(A_{528.7} - A_{583}) = 0.038$; $A_{max} = 0.524$. Apparent HbCO by Eq. 5 was 14%; by Eq 6 after treatment with $Na_2S_2O_4$ the value was determined to be 13%. However,

$$C_{\text{metHb}} = (A_1 - 0.52A_2)/2.77 = [0.432 - 0.52\,(0.524)]/2.77$$

$$= 0.058 \text{ g per } 100 \text{ mL}$$

(see Eq. 9). Therefore

$$0.05A\,(0.058/0.1) = 0.023$$

and $0.038 - 0.023 = 0.015$ (corrected ΔA). Using 0.015 for ΔA in Eq 5, HbCO = 6%.

A 13 or 14% HbCO value would suggest that death was not instantaneous, providing grounds for a legal suit related to the degree of suffering before death. The 6% value is consistent with smoking or heavy traffic or both.

Case 65400

The subject died in a closed garage with the car running and windows open; ethanol and sedative drugs were negative. Blood was taken in a redtop tube on the day of death and analyzed the next day. Analyzing the data from Table 7,

1. HbCO = [(1.312 − 0.727)/3.7](8.5/1.582)100 = 84.5% (by Eq 5), and Hb_{total} = 1.582/8.5 = 0.186 g per 100 mL.
2. A_{max} = 569.0 for approximately 85 to 90% saturation (from Fig. 3).
3. HbCO by Eqs 1 to 4: x = 0.156 g per 100 mL, y = 0.024 g per 100 mL, z = 0.01 g per 100 mL, and $x + y + z$ = 0.190 g per 100 mL. Therefore HbCO = 82%.
4. HbCO by Eq 6 = 87.5% (after reduction by $Na_2S_2O_4$).
5. HbCO by Eqs 1 to 4 after reduction: x = 0.165 g per 100 mL, y = 0.000 g per 100 mL, z = 0.032 g per 100 mL, and $x + y + z$ = 0.197 g per 100 mL. Therefore HbCO = 83%.

The interpretation of death by carbon monoxide inhalation in this instance is difficult to challenge; the numbers obtained for the composition with respect to Hb_r, HbO_2, and HbCO are internally consistent, even though exact agreement is lacking. Figures 1 and 6 demonstrate the difference in the spectrum produced by reduction of HbO_2 and that produced by reduc-

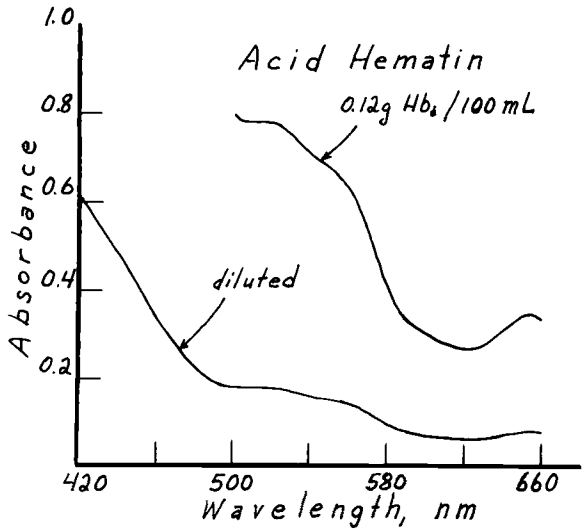

FIG. 7—*Acid hematin in aqueous solution, characterized by the absence of sharp absorption bands. Acidic conditions produce this hemoglobin pigment.*

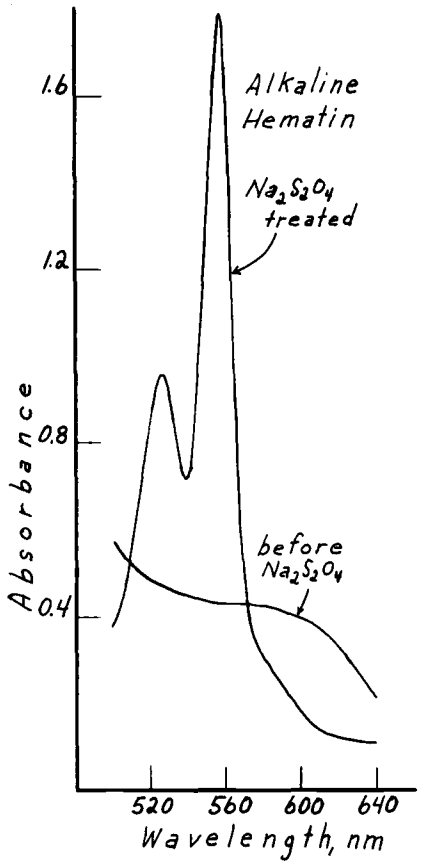

FIG. 8—*Alkaline hematin in aqueous solution, formed by making an aqueous solution of normal hemoglobin strongly basic with* NH_4O_4. *Reduction of alkaline hematin with* $Na_2S_2O_4$ *produces a more defined structure.*

tion of preformed methemoglobin. Dubowski and Luke [9] noted "other products" of this reaction. This may account for the slight differences in %HbCO calculated before and after $Na_2S_2O_4$ treatment, as in the example above. In addition, our studies show that the 548.2 nm position is not an exact isosbestic triple point for the three hemoglobin components Hb_r, HbO_2, and HbCO, which would cause some inaccuracies.

Case 21277

The blood in this particular case contained approximately 20% sulfhemoglobin or sulfmethemoglobin, but no particular history of CO exposure. Absorbance data are given in Table 8. By Eq 5, %HbCO = {[(0.728 − 0.629)/3.7](8.7/0.909)}100 = 25.6%. But A_{max} = 576.1 nm or HbCO = 10% (approximately) by Fig. 3. By Eq 6, after $Na_2S_2O_4$ treatment, (%HbCO = {[(0.620 − 0.537)/4.2](8.7/0.909)}100 = 19%. The presence of an abnormal hemoglobin pigment (sulfhemoglobin) in this blood caused an overestimate of %HbCO. From the calculations shown, one can only estimate the HbCO to be between 5 and 20% of total hemoglobin. It has been our experience that the presence of acid or alkaline hematin likewise causes %HbCO to be considerably overestimated.

The major advantages of our method lie in its broad applicability and ability to demonstrate specificity of the analysis, using a routine double-beam spectrophotometer. In a review article,

TABLE 7—*Absorbance readings in Case 65400.*

Initial Absorbance Readings		Absorbance Readings After $Na_2S_2O_4$	
Wavelength, nm	Absorbance	Wavelength	Absorbance
528.7	1.312	530.0	1.396
583.0	0.727	583.0	0.713
569.0 (A_{max})	1.582	...	...
548.2	1.400 (d_1)	548.2	1.415
568.1	1.573 (d_2)	568.1	1.604
578.0	1.168 (d_3)	578.0	1.090

TABLE 8—*Absorbance readings for Case 21277.*

Initial Absorbance Readings		Absorbance After $Na_2S_2O_4$	
Wavelength, nm	Absorbance	Wavelength, nm	Absorbance
528.7	0.728	530.0	0.620
583.0	0.629	583.0	0.537
576.1 (A_{max})	0.909	555.0	0.816
650.0	0.115	548.2 (d_1)	0.825
...	...	568.1 (d_2)	0.737
...	...	578.0 (d_3)	0.875

Maehly [*16*, p. 585] recommends a series of methods that employ both spectrophotometry and gasometric techniques, depending on the range of measurements and condition of the blood specimen. Maehly's own excellent but elaborate spectrophotometric technique was developed before the recent improvements in accuracy and precision of routine laboratory spectrophotometers. However, the ΔA null points (ΔA is zero at HbCO = 0%) and isosbestic points will vary from instrument to instrument, depending on wavelength calibration accuracy. Spectrophotometers that cannot give repeatable wavelengths to ± 0.1 nm will have larger inherent errors than the data presented here indicate. On the other hand, dual monochrometer instruments and those able to set wavelengths reproducibly to ± 0.05 nm may be accurate down to 0.2% carboxyhemoglobin in freshly drawn blood or in finger prick blood that is analyzed immediately.

While the method presented here is suitable for partially denatured bloods, those bloods containing substantial quantities (more than 25% of total) of acid or alkaline hematin or sulfhemoglobin are beyond the quantitative scope of the method. In such seriously denatured and autolyzed blood specimens, no one technique or methodology can stand alone in the forensic toxicology setting.

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