DOT/FAA/AM-98/21

Office of Aviation Medicine
Washington, D.C. 20591

Preparation of Carboxyhemoglobin Standards and Calculation of Spectrophotometric Quantitation Constants

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August 1998

Final Report

U.S. Department
of Transportation

Federal Aviation Administration

NOTICE

Technical Report Documentation Page

1. Report No.	2. Government Accession No.	3. Recipient's Catalog No.
DOT/FAA/AM-98/21		

4. Title and Subtitle	5. Report Date
Preparation of Carboxyhemoglobin Standards and Calculation of Spectrophotometric Quantitation Constants	August 1998
	6. Performing Organization Code
7. Author(s)	8. Performing Organization Report No.
Canfield, D.V., Smith, M.D., Ritter, R.M., and Chaturvedi, A.K.	
9. Performing Organization Name and Address	10. Work Unit No. (TRAIS)
FAA Civil Aeromedical Institute P.O. Box 25082, Oklahoma City, OK 73125	11. Contract or Grant No.
12. Sponsoring Agency name and Address	13. Type of Report and Period Covered
Office of Aviation Medicine Federal Aviation Administration 800 Independence Ave., S.W. Washington, DC 20591	14. Sponsoring Agency Code

15. Supplemental Notes

Work was accomplished under approved task AM-B-96-TOX-202

16. Abstract

The Toxicology and Accident Research Laboratory of the Federal Aviation Administration is required to analyze specimens from all pilots involved in fatal aircraft accidents for the presence of carbon monoxide poisoning to determine if carbon monoxide caused pilot incapacitation leading to the accident. Therefore, reliable and stable carbon monoxide controls are needed to assure the accuracy of the analysis of carbon monoxide. A method was developed for the preparation of carboxyhemoglobin standards, which were stable for more then 4 months with the prepared control remaining within acceptable limits during this time. A mathematical equation was developed to more accurately determine the constants A and B used in the equation COHB% = 100[(C-B)/(A-B)], where B = 0% COHB peak ratio at 540nm and 579nm; A = 100% COHB peak ratio at 540nm and 579nm; and C = the peak ratio at 540nm and 579nm for the blood being analyzed. The following equations were developed to calculate A and B: $B = P_{avg} - (P)[(P_{avg} - N_{avg})/(P - N)]$; $A = B + (P_{avg} - N_{avg})/(P - N)$, P_{avg} = average peak ratio 540/579 for the positive standard run on the spectrophotometer; P = average decimal concentration measured on the CO-OXIMETER for the positive standard; N_{avg} = average peak ratio 540/579 for the negative standard; N = average decimal concentration measured on the CO-OXIMETER for the negative standard. The new equations provided results consistent with those obtained from a CO-OXIMETER.

17. Key Words	18. Distribution Statement
Carboxyhemoglobin, Analysis, Standards	Document is available to the public through the National Technical Information Service, Springfield, Virginia 22161

19. Security Classif. (of this report)	20. Security Classif. (of this page)	21. No. of Pages	22. Price
Unclassified	Unclassified	11	

Form DOT F 1700.7 (8-72) Reproduction of completed page authorized

Preparation of Carboxyhemoglobin Standards and Calculation of Spectrophotometric Quantitation Constants

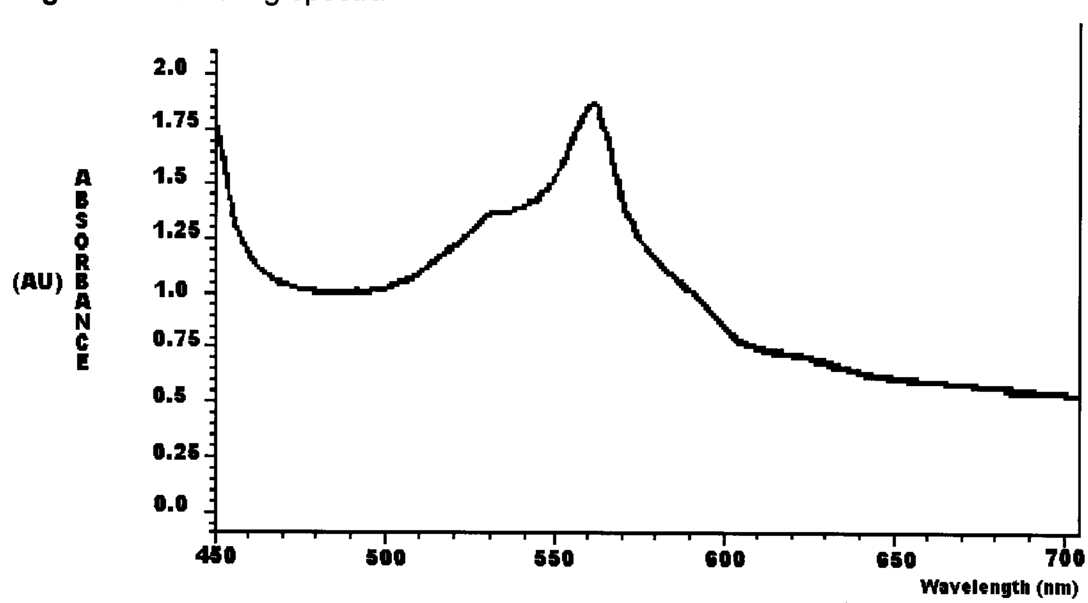
INTRODUCTION

The Toxicology and Accident Research Laboratory of the Federal Aviation Administration is required to analyze specimens from all pilots involved in fatal aircraft accidents for the presence of carbon monoxide poisoning to determine if carbon monoxide caused pilot incapacitation leading to the accident. Therefore, reliable and stable carbon monoxide controls are needed to assure the accuracy of the analysis of carbon monoxide. The determination of the A and B UV/VIS constants in the calculation of carboxyhemoglobin concentration is complicated by the fact that the A and B constants are based on an assumed 100% carbon monoxide saturated solution of hemoglobin and a 0% carboxyhemoglobin solution. Research has shown that this assumption is not always true and could be problematic when attempting to interpret results (1). In late 1995 commercially available whole blood carboxyhemoglobin standards, used to calibrate a UV spectrophotometer and to serve as controls, were discontinued. This required that a stable and reliable standard and calibration control be developed for carboxyhemoglobin analysis in postmortem whole blood by UV/Vis spectrophotometry. The samples received for analysis by the laboratory do not lend themselves to analysis by CO-OXIMETER due to the presence of clots and occasionally to interfering absorbance (Figure 1). Procedures found in the literature (1-7) for the preparation of controls were difficult to use and the assumed concentrations could not easily be confirmed, leading to questionable instrument calibration when compared with commercially available controls.

METHODS

In this experiment, fresh whole blood (non-smoker) was drawn into four 10 mL gray top vacutainer tubes (VENOJECT, Terumo Medical, Elkton, MD, part #LK3S) containing 100 mg sodium fluoride and 20 mg potassium oxalate on the day the carboxyhemoglobin standards were to be prepared. The gray top tubes are used by the Forensic Toxicology Laboratory in actual cases to collect postmortem blood. The collection of whole blood in gray top tubes adequately simulates the biological matrix received by the laboratory for analysis. The gray top tubes are used by most forensic toxicology laboratories to prevent clotting and preserve the specimen. Attempts were made to use blood obtained

Figure 1. Interfering spectra



from a local blood bank; however, the blood contained fibrils which resulted in problems with reproducibility of carboxyhemoglobin concentration on the CO-OXIMETER 282 manufactured by Instrumentation Laboratory, Lexington, MA. The fresh whole blood collected should be treated immediately after collection. The blood collected was placed on a tube rocker and rocked for 30 minutes after collection to make sure the samples were well mixed with the anti-coagulant and the anti-bacterial agents. All of the samples were then placed into a 25 x 150 mm tube and mixed for an additional 30 minutes.

A "NEGATIVE CO" standard was prepared by placing the fresh blood sample into a 200 mL volumetric flask. The flask was placed in a horizontal position and rotated, while < 1.0 mL/minute nitrogen flow was used to purge the flask for 2-3 minutes. A portion of this negative control was pipetted into small plastic 1.5 mL standard micro centrifuge tubes made by Elkay Products, Inc, cat. #000-micr-150. Each tube was filled to capacity and sealed with an attached plastic cap. These "NEGATIVE CO" standards were stored overnight at 4° C and tested the next day on a CO-OXIMETER to obtain the average concentration for the "NEGATIVE CO" control.

The "POSITIVE CO" standard was prepared under a biological ventilated hood by placing the remaining "NEGATIVE CO" blood into a 200 mL volumetric flask. The flask was placed in a horizontal position and rotated, while < 1.0 mL/minute carbon monoxide flow was used to purge the flask for 20 minutes. This approximately 100% carboxyhemoglobin (COHB) standard was diluted to approximately 45% COHB with a portion of the negative control prepared earlier. A portion of this approximately 45% positive control was pipetted into small plastic 1.5 mL standard micro centrifuge tubes. Each cup was filled to capacity and sealed with an attached plastic cap. These "POSITIVE CO" standards were stored overnight at 4° C and tested the next day on a CO-OXIMETER to obtain the average concentration for the "POSITIVE CO" control. It is important to allow the specimen to equilibrate overnight in order to reach complete equilibrium between bond and unbound CO. Kinetic experiments made during this research indicate that it takes several hours after the preparation of the standard for the CO saturated blood to reach equilibrium.

In the preparation of the test reagent, bottles of Ammonium Hydroxide and Sodium Dithionite were allowed to equilibrate at room temperature for more than 15 minutes. In a 1000 mL volumetric flask, 500 mL of deionized water was added, along with 5 grams of sodium dithionite. The solution was mixed and allowed to completely dissolve before filling the flask to the 1000 mL mark with deionized water. The solution was then placed under a ventilated hood and 4 mL of concentrated Ammonium Hydroxide was pipetted into the solution. The solution was then poured into a brown bottle pipetor and permitted to equilibrate at room temperature for more than 15 minutes. The solution has a shelf life of no more then 4 hours.

Each NEGATIVE and POSITIVE standard was run on the CO-OXIMETER 10 times to obtain an average carboxyhemoglobin level and a standard deviation. These levels were then used to calibrate the HP 8453 UV Visible Spectrophotometer, equipped with an HP 89052B Sipper System, an HP 89072A Autosampler, and controlled by the HP UV-Visible Chemstation Revision A.02.05, which were supplied by Hewlett-Packard, Palo Alto, California. This method uses the equation COHb% = 100*[C-B)/(A-B)] and the isosbestic points at 540 nm and 579 nm to calculate the concentration of Carboxyhemoglobin in the blood. The following equations were developed by the laboratory to calculate the constant values of A and B: $B = P_{avg} - (P)[(P_{avg} - N_{avg})/(P - N)]$; $A = B + (P_{avg} - N_{avg})/(P - N)$, P_{avg} = the average peak ratio 540/579 for the positive standard run on the spectrophotometer; P = average decimal concentration measured on the CO-OXIMETER for the positive standard; N_{avg} = average peak ratio 540/579 for the negative standard; N = average decimal concentration measured on the CO-OXIMETER for the negative standard.

Once the instrument was calibrated, the whole blood samples received for analysis were prepared in duplicate. For each specimen, there were two samples prepared and placed into two 16 x 100 mm tubes. Each tube contained 10 mL of the prepared reagent (Sodium Dithionite/Ammonium Hydroxide) along with 100ml of the unknown blood sample (clot free). Parafilm was placed over the top of each tube and properly mixed. NEGATIVE and POSITIVE standards were prepared in the same way as samples received for analysis.

The samples were then placed into the Hewlett Packard 89072A Autosampler on the Hewlett Packard 8453 Diode Array Spectrophotometer for analysis in the following order: Reference Blank, Positive, Reference Blank, Negative, Reference Blank, Unknown (1a), Reference Blank, Unknown (1b), Reference Blank.

The instrument was started and data were collected on samples. Absorbance was obtained at 540 nm and 579 nm for all specimens (Figure 2) and used to calculate the % COHB.

A comparison of the prior method for calculating the A and B constants used in the quantitation of CO were made by taking a portion of the "NEGATIVE CO" standard and placing it into a 200 mL volumetric flask. The flask was placed in a horizontal position and rotated for 20 minutes, while < 1.0 mL/minute flow of carbon monoxide was pumped into the flask to prepare a theoretical 100% carboxyhemoglobin. Nitrogen was used to purge the flask for 2-3 minutes to remove unbound CO. The 0% carboxyhemoglobin was prepared by using oxygen instead of CO in the method provided for preparing the 100% carboxyhemoglobin standard. This blood was used to determine the A and B constants, and the results from these values were compared with values from the new way of calculating A and B.

RESULTS

The visible spectra for the positive control can be seen in Figure 2 and the negative control in Figure 3. In comparing the two instruments used in the experiment, the CO-OXIMETER (Table 1), and UV Spectrophotometer (Table 2, UV/VIS), clearly show that the calibrations were close in value for both the CO-OXIMETER and the UV Spectrophotometer with standard deviations of 0.9 and 0.3, respectively. Tables 3 and 4 show the results of a "negative control" run on the CO-OXIMETER and the UV/VIS. The results of tests run on the same control for over 5 months can be seen in Tables 5 and 6.

The new equation gave A = 1.543 and B = 1.128 for the UV constants and the old procedure gave an A = 1.516 and B = 1.142.

The A and B values calculated using the new equation gave results that were within 3% of the carboxyhemoglobin values measured with the CO-OXYMETER for a theoretical 100%, 50%, 25%, 12.5%, 6.25%, 3.13%, and 0%. The A and B values obtained using the old method gave results that were off by as much as 100% from the CO-OXYMETER values at low concentrations (Table 7).

Figure 2. 46.6% Carboxyhemoglobin

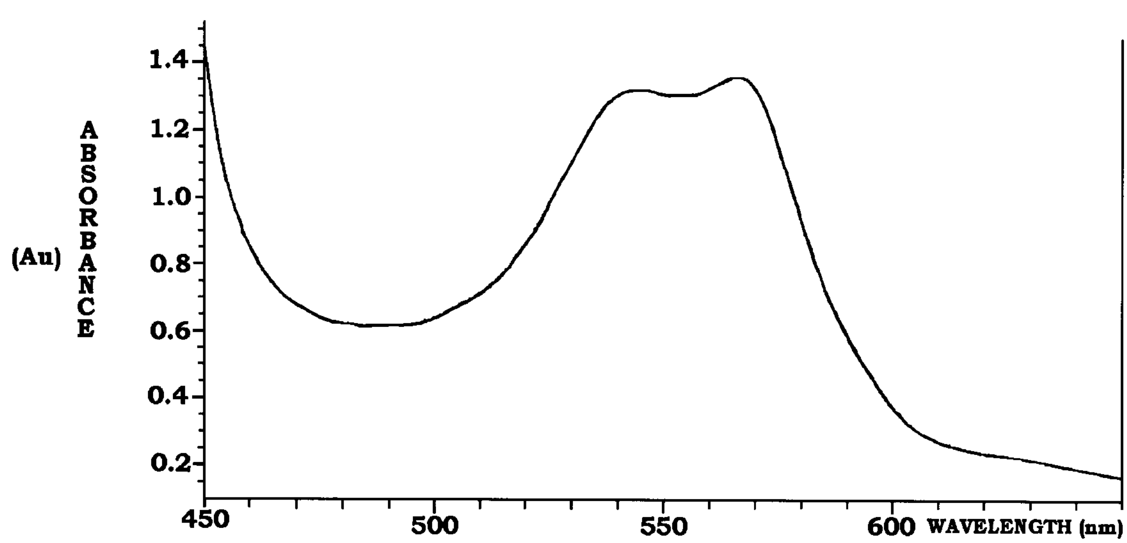


Figure 3. 2.9% Carboxyhemoglobin

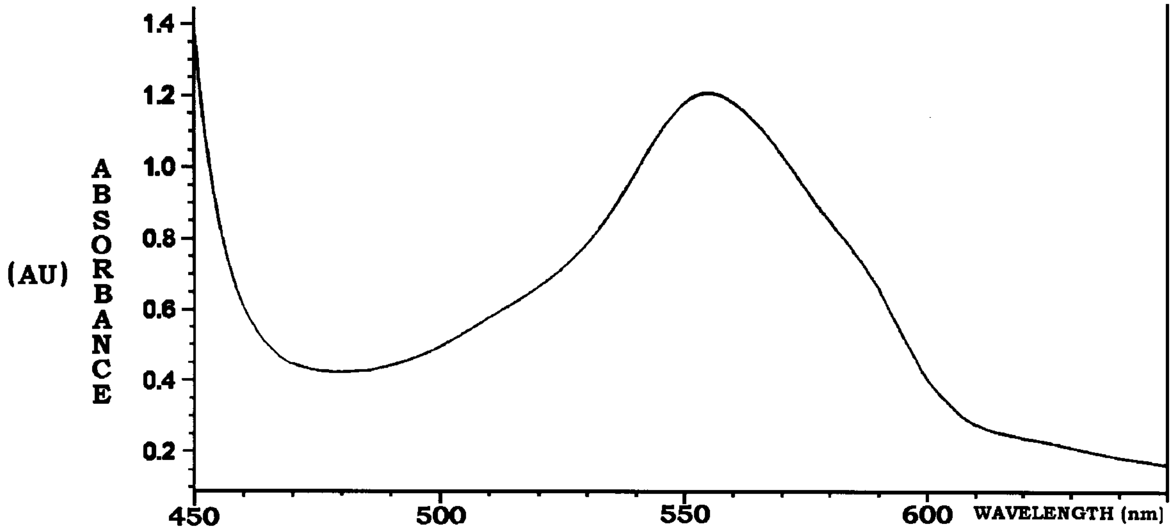


Table 1. Ten consecutive runs on the CO-OXIMETER for a positive control.

Observ. #	X	Levey-Jennings Chart		
1	44.4	Conf +99%	48.4	+3SD
2	45.6	Conf +95%	47.5	+2SD
3	43.8	Conf +68%	46.5	+1SD
4	45.5	Mean	45.6	
5	46.3	Conf -68%	44.7	-1SD
6	47.0	Conf -95%	43.8	-2SD
7	45.9	Conf -99%	42.8	-3SD
8	45.6			
9	45.8	Statistics		
10	46.2	N=	10.0	
		Mean	45.6	
		Variance	0.8	
		SD	0.9	
		CV	2.0	

Table 2. Ten consecutive runs on a spectrophotometer of the same sample run in Table 1.

Observ. #	X	Levey-Jennings Chart	
1	45.1	Conf +99%	46.8 +3SD
2	45.9	Conf +95%	46.5 +2SD
3	46.0	Conf +68%	46.2 +1SD
4	45.6	Mean	45.8
5	45.8	Conf -68%	45.5 -1SD
6	46.3	Conf -95%	45.2 -2SD
7	46.2	Conf -99%	44.8 -3SD
8	45.8		
9	45.9	Statistics	
10	45.8	N=	10.0
		Mean	45.8
		Variance	0.1
		SD	0.3
		CV	0.7

Table 3. Ten consecutive runs on the CO-OXIMETER for a negative control.

Observ. #	X	Levey-Jennings Chart	
1	1.1	Conf +99%	2.0 +3SD
2	1.1	Conf +95%	1.7 +2SD
3	1.1	Conf +68%	1.4 +1SD
4	1.5	Mean	1.2
5	1.8	Conf -68%	0.9 -1SD
6	1.0	Conf -95%	0.6 -2SD
7	1.2	Conf -99%	0.3 -3SD
8	0.8		
9	1.0	Statistics	
10	1.0	N=	10.0
SUM X	11.6	Mean	1.2
		Variance	0.1
		SD	0.3
		CV	24.8

Table 4. Ten consecutive runs on a spectrophotometer of the same sample described in Table 3.

Observ. #	X	Levey-Jennings Chart	
1	1.3	Conf +99%	1.9 +3SD
2	0.9	Conf +95%	1.7 +2SD
3	1.0	Conf +68%	1.5 +1SD
4	1.3	Mean	1.3
5	1.4	Conf -68%	1.0 -1SD
6	1.2	Conf -95%	0.8 -2SD
7	1.3	Conf -99%	0.6 -3SD
8	1.1		
9	1.4	Statistics	
10	1.6	N=	10.0
		Mean	1.3
		Variance	0.0
		SD	0.2
		CV	16.5

Table 5. Results from a 49.2% mean positive control run for over 5 months.

Observ. #	X	Levey-Jennings Chart	
1	46.3	Conf +99%	61.5 +3SD
2	46.3	Conf +95%	57.0 +2SD
3	53.2	Conf +68%	52.6 +1SD
4	54.6	Mean	48.1
5	54.3	Conf -68%	43.7 -1SD
6	50.9	Conf -95%	39.3 -2SD
7	49.8	Conf -99%	34.8 -3SD
8	50.9		
9	51.9	Statistics	
10	50.2	N=	44.0
11	51.5	Mean	48.1
12	51.7	Variance	19.7
13	51.4	SD	4.4
14	51.0	CV	9.2
15	51.5		
16	51.5		
17	53.1		
18	53.4		
19	50.7		
20	50.3		
21	41.8		
22	43.6		
23	43.3		
24	39.6		
25	42.1		
26	39.1		
27	42.0		
28	42.3		
29	44.9		
30	42.8		
31	40.8		
32	41.6		
33	49.9		
34	52.4		
35	51.4		
36	48.7		
37	51.6		
38	51.9		
39	48.7		
40	49.5		
41	49.1		
42	43.9		
43	45.7		
44	47.0		

Table 6. Results from a 1.6% mean "negative control" run for over 5 months.

Observ. #	X	Levey-Jennings Chart	
1	1.2	Conf +99%	6.9 +3SD
2	1.0	Conf +95%	5.1 +2SD
3	0.0	Conf +68%	3.3 +1SD
4	0.0	Mean	1.5
5	3.9	Conf -68%	-0.4 -1SD
6	6.0	Conf -95%	-2.2 -2SD
7	4.9	Conf -99%	-4.0 -3SD
8	3.3		
9	4.9	Statistics	
10	3.1	N=	29.0
11	4.6	Mean	1.5
12	0.1	Variance	3.3
13	0.9	SD	1.8
14	0.0	CV	125.8
15	0.0		
16	1.3		
17	1.4		
18	0.6		
19	0.0		
20	0.0		
21	0.0		
22	0.0		
23	0.0		
24	1.2		
25	1.7		
26	1.0		
27	1.0		
28	0.0		
29	0.0		

Table 7. Comparison of assumed COHB levels and actual measured levels using the CO-Oximeter, old method, and new method.

Assumed	Co-Ox	Old Way	New Way
100	92.4	99.2	92.8
100	92.8	99.7	93.2
100	92.4	99.3	92.9
100	92.5	100.3	93.8
100	91.8	100.9	94.3
50	50.5	53.2	51.4
50	51.0	52.3	50.5
50	51.0	52.6	50.7
50	51.1	52.9	51.0
50	51.2	53.0	51.2
25	25.0	23.4	24.4
25	24.9	23.1	24.2
25	25.1	23.3	24.4
25	24.3	22.8	23.9
25	24.8	23.3	24.4
12.5	12.1	9.1	11.6
12.5	12.5	9.2	11.6
12.5	12.0	9.5	12.0
12.5	12.3	9.3	11.8
12.5	12.0	9.7	12.1
6.25	5.8	2.6	5.7
6.25	5.5	2.6	5.7
6.25	5.7	2.9	6.0
6.25	5.6	2.5	5.6
6.25	5.5	2.3	5.4
3.13	4.0	0.5	3.8
3.13	3.8	0.5	3.8
3.13	4.0	0.3	3.7
3.13	3.7	0.3	3.6
3.13	3.6	0.4	3.8
0	3.2	0.0	3.3
0	3.4	0.0	3.2
0	3.3	0.0	3.1
0	3.6	0.0	3.0
0	3.5	0.0	3.4

CONCLUSION

The new method provided reliable whole blood carboxyhemoglobin standards stable for more then 4 months when stored at 4°C. This is a substantial improvement over the prior commercially available controls, which were stable for only 1 month. The necessary constants for use in calculating COHB by spectrophotometry are reliably and efficiently provided by the present derived equations for A and B. The calculated A and B constants gave values which were a substantial improvement over values measured using the A and B constants from the old method (Table 7).

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 ☆U.S. GOVERMENT PRINTING OFFICE: 1998 -660-088/80005