DOT/FAA/AM-02/15

Office of Aerospace Medicine
Washington, DC 20591

An Accurate Method for the Determination of Carbon Monoxide in Postmortem Blood Using GC/TCD

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August 2002

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-02/15		

4. Title and Subtitle	5. Report Date
An Accurate Method for the Determination of Carbon Monoxide in Postmortem Blood Using GC/TCD	August 2002
7. Author(s)	6. Performing Organization Code
Lewis, R.L., Johnson, R.D. and Canfield, D.V.	
9. Performing Organization Name and Address	8. Performing Organization Report No.
FAA Civil Aerospace Medical Institute P.O. Box 25082 Oklahoma City, Oklahoma 73125	10. Work Unit No. (TRAIS)
12. Sponsoring Agency name and Address	11. Contract or Grant No.
Office of Aerospace Medicine Federal Aviation Administration 800 Independence Ave., S.W. Washington, DC 20591	13. Type of Report and Period Covered
15. Supplemental Notes	
This work was accomplished under the approved task AM-B-02-TOX-204.	14. Sponsoring Agency Code

16. Abstract

During the investigation of aviation accidents, postmortem samples from accident victims are submitted to the FAA's Civil Aerospace Medical Institute for toxicological analysis. To determine if the accident victim was exposed to an in-flight/post crash fire or faulty heating/exhaust system, the analysis of carbon monoxide (CO) is conducted. While our laboratory predominantly uses a spectrophotometric method for the determination of carboxyhemoglobin (COHb), we consider it essential to confirm with a second technique based on a different analytical principle. Our laboratory encountered difficulties with many of our postmortem samples while employing a commonly used GC method. We believed these problems were due to elevated methemoglobin (MetHb) concentration in our specimens. MetHb does not bind CO, thus elevated MetHb levels will result in a loss of CO binding capacity. Since most commonly employed GC methods determine %COHb from a ratio of unsaturated blood to CO-saturated blood, a loss of CO binding capacity will result in an erroneously high %COHb value. Our laboratory has developed a new GC method for the determination of %COHb that incorporates sodium dithionite, which will reduce any MetHb present to Hb. Using numerous fresh human blood controls ranging from 1% to 67% COHb, we found no statistically significant differences between %COHb results from our new GC method and our spectrophotometric method. We then applied our new GC method to putrefied and non-putrefied postmortem samples. To validate the new GC method, postmortem samples were analyzed with our existing spectrophotometric method, a GC method commonly used without reducing agent, and our new GC method with the addition of sodium dithionite. As expected, we saw errors up to and exceeding 50% when comparing the unreduced GC results with our spectrophotometric method. With our new GC procedure, which incorporates a reducing agent, the error was virtually eliminated.

17. Key Words	18. Distribution Statement
Combustion Toxicology, Carbon Monoxide, Carboxyhemoglobin, Methemoglobin, Aircraft Accident Investigation	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	9	

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

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INTRODUCTION

During the investigation of aviation accidents, postmortem samples from accident victims are submitted to the FAA's Civil Aerospace Medical Institute for toxicological analysis (1). The analysis of carbon monoxide (CO) is conducted to determine if the accident victim was exposed to an in-flight/post crash fire or faulty heating/exhaust system (2, 3, 4).

Carbon monoxide (CO) is a deadly, odorless, tasteless gas that is a product of the incomplete combustion of carbon-containing materials (5). While our laboratory predominantly uses a spectrophotometric method for the determination of carboxyhemoglobin (COHb) (6), we consider it essential to confirm with a second technique based on a different analytical principle. The second technique chosen was gas chromatography (GC) with a thermal conductivity detector (TCD).

There are many different GC methods employed for the determination of CO in blood (7-12). The most common and rapid GC method determines %COHb from a ratio of the CO peak area of CO-unsaturated blood to CO-saturated blood (13). When working with fresh or well preserved blood samples, this GC-ratio method is extremely accurate. It does encounter limitations, however, when analyzing postmortem blood that may have experienced some degree of deterioration or may have been improperly stored.

It is well known that methemoglobin (MetHb) can be elevated in a postmortem blood sample. The oxidation of hemoglobin to MetHb (14) can result from postmortem deterioration due to a lack of timely preservation (11), exposure to heat (15), and as a result of the freezing of a blood sample at and above temperatures of –30°C (16, 17). MetHb does not bind CO (18). Therefore, elevated levels of MetHb in a sample will result in a loss of CO binding capacity and will produce an erroneously high %COHb value. Dominguez et al. (19) in 1964, when determining %COHb in various dog tissues, incorporated a reducing agent to reduce any potential MetHb back to hemoglobin. Dubowski et al. (20) also made reference to the need for a reducing agent when determining %COHb in decomposed postmortem blood when using the GC-ratio method. Clearly, the importance of using a reducing agent has been demonstrated in an animal model. This paper describes an improved GC method for the determination of COHb in human postmortem blood using a reducing agent to eliminate any error due to substantial MetHb levels.

Materials and Methods

Reagents and Supplies

The chemicals used for these experiments were sodium dithionite, saponin, sulfuric acid, and ammonium hydroxide, which were obtained from Fisher Scientific (Fischer Scientific Co., Fair Lawn, NJ). Distilled-deionized water was prepared using a Millipore model Milli-QT bench-top purification device (Millipore, Continental Water Systems, El Paso, TX) and was used for all reagent preparations.

The reducing agent used for the GC procedure was 0.287 M sodium dithionite. The CO liberating agent used was 1 M sulfuric acid with 1.5% (w/v) saponin. The 10 mL headspace vials (MicroLiter Analytical, Suwanee, GA) were sealed with aluminum crimp caps fitted with a silicon septa (Supelco, Bellefonte, PA).

GC Method for CO Analysis

The analysis of blood for CO by GC was achieved using a Varian Micro-GC model CP-2003P fitted with a thermal conductivity detector. The inlet temperature was 110°C. The GC was equipped with a Varian 20 m molsieve 5 Å, 0.32 mm ID column module, which was held isothermally at 120°C. The carrier gas used was He and the column pressure was set at 35 psi. A sampling time of 40 sec was used, followed by a 100 msec injection time. The TCD sensitivity was set at high for all analyses. The total chromatographic run time was 195 sec. The GC system control and data analysis were accomplished on a Dell™ Latitude™ C600 laptop computer using Varian Star© software.

Blood Controls

Blood CO controls were prepared by saturating human CO-negative blood with 99.95% CO gas in the following manner. Fifty mL of CO-negative human blood was placed in a 100 mL glass bottle. The headspace was purged with CO for 5 min. The bottle was then sealed and rocked front to back on a tube rocker for 45 min. The bottle was removed, re-purged, and rocked for an additional 45 min. The bottle was

then flushed with He for 5 min. Diluting various amounts of the CO-saturated blood with CO-negative blood made multiple levels of CO controls, including 16, 25, 35, 50, and 67%. The controls were stored in vials with zero-headspace to avoid any changes in blood CO levels (11).

GC Analysis of CO

To determine the amount of CO in a sample, 0.5 mL of unsaturated blood was placed in a 10 mL headspace vial followed by 0.5 mL of reducing agent, and the vial was sealed. A different aliquot from the same sample was used to prepare a CO-saturated blood by placing 1 mL blood and 1 mL reducing agent in a 10x75 mm test tube. The headspace was purged with CO gas for 30 sec; the tube was capped and placed on a rocker for 30 min. The tube was again purged with CO for 30 sec and rocked for an additional 30 min. The headspace was then purged with He for 10 sec. One mL of this saturated blood mixture was transferred to a 10 mL headspace vial and sealed. CO was liberated from both saturated and unsaturated blood samples by adding 1 mL of liberating agent. Following the addition of liberating agent, the samples were agitated at room temperature on a shaker (Glas-Col, Terre Haute, In) for 40 min. The samples were then analyzed using the GC.

Following the analysis, the COHb concentration was calculated by the ratio of the chromatographic peak areas from the unsaturated sample and the CO-saturated sample. The CO peak area of the unsaturated specimen (CO) was divided by the peak area of the CO-saturated specimen (SatCO) and then multiplied by 100 to get the percent concentration of COHb (%COHb=[CO/SatCO]*100).

Spectrophotometric Analysis of Carboxyhemoglobin

A detailed description of the spectrophotometric method used has been previously published (6, 21). Briefly, the absorption of the blood sample was determined following its dilution with sodium dithionite and ammonium hydroxide. A UV spectrum of the sample was collected from 450 to 700 nm. Carboxyhemoglobin has an absorbance maximum at 540 nm. There is an isosbestic point at 579 nm through which multiple species of hemoglobin pass (9). A ratio of the absorbance at 540 and 579 nm was used to determine the % COHb. A Hewlett Packard 8453 UV spectrometer was used for the above analysis.

RESULTS AND DISCUSSION

The GC/TCD method described here is a modified version of that described by Van Dam et al. (13). The method provides an excellent separation of CO from all other common gases, as can be seen in Figure 1. The thermal conductivity detector, described extensively by Goldbaum et al. (7) and Van Dam et al. (13), provided superb sensitivity. CO eluted at approximately 2.5 min, thus a total run time of 3.25 min was used. The CO liberating agent used was 1 M sulfuric acid with 1.5% (w/v) saponin. Saponin was included to ensure complete breakdown of all red blood cells in the sample. Using a wide range of COHb blood controls, it was determined that a 40 min liberation time was sufficient for complete CO release. This data supports what Van Dam et al. (13) have previously reported.

A reducing agent is commonly employed in the spectrophotometric analysis of COHb to reduce any oxyhemoglobin and MetHb present in the blood, thereby eliminating their potential interference with the absorbance of COHb (6, 11, 21, 22). Reduction of blood has also been reported for use with a CO-oximeter (23). However, to the best of our knowledge, no one has yet reported the determination of %COHb in human postmortem blood using the GC-ratio procedure in conjunction with the use of a reducing agent. This reduction is extremely important in postmortem specimens, which have the potential to contain high levels of MetHb. This is important because the commonly employed GC-ratio method determines %COHb from a ratio of CO liberated from unsaturated blood to CO-saturated blood. Since MetHb does not bind CO, significant levels of MetHb in a blood sample will result in a reduction of total CO binding capacity. This less than complete hemoglobin CO saturation produces an erroneously high %COHb value. By treating a sample with a reducing agent, MetHb is converted back to hemoglobin, thus restoring the total CO binding capacity of the sample.

The appropriate amount of sodium dithionite needed to reduce any potential MetHb present in postmortem blood was investigated. Various concentrations of sodium dithionite, ranging from 0.0287 M to 1.0 M, were used to reduce human blood with a MetHb level of approximately 40%. These samples were analyzed using a CO-oximeter to determine the

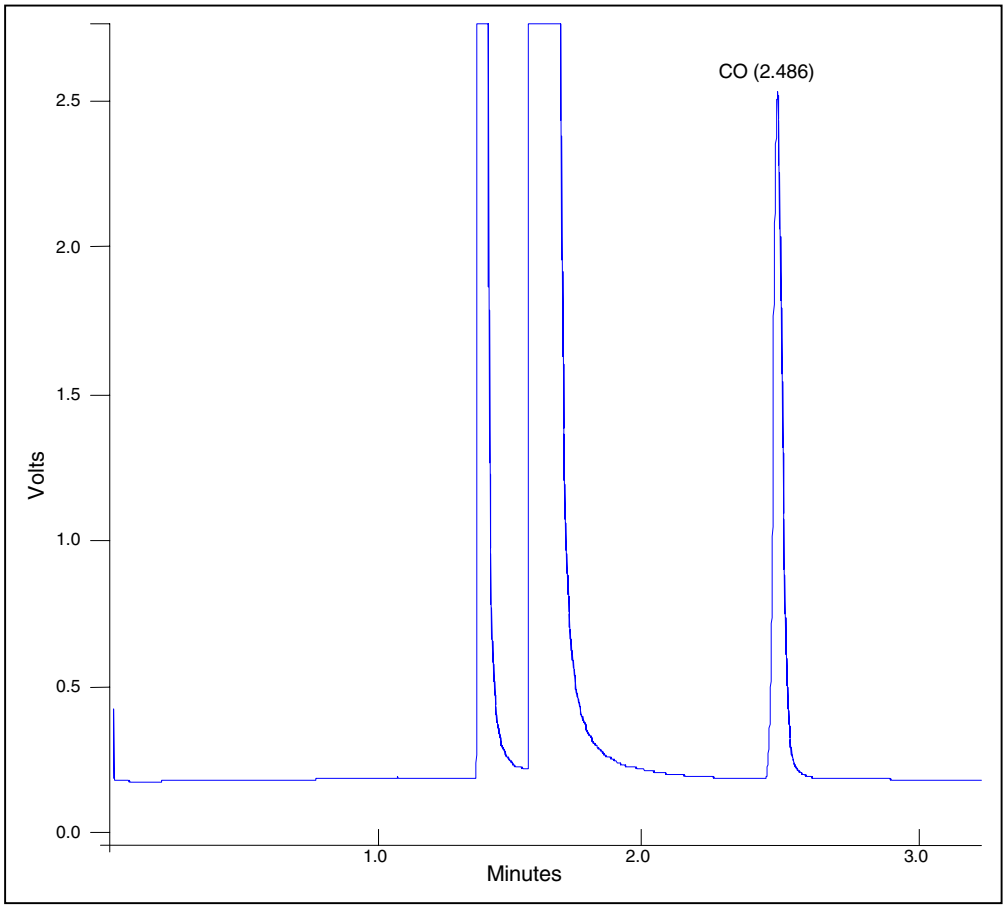

Figure 1. A representative GC chromatogram showing the separation of CO from oxygen and nitrogen.

MetHb concentrations (24). The optimum sodium dithionite concentration was determined to be 0.287 M. Concentrations less than this value did not supply enough reduction capacity, while concentrations substantially greater than this were found to interfere with the binding of CO during saturation. Sodium dithionite at 0.287 M had no effect on existing levels of COHb. To determine if sodium dithionite had any effect on the release of CO from a sample, a wide range of COHb blood controls, both with and without reducing agent added, were analyzed using the GC. Comparing the CO area counts between the reduced and unreduced samples yielded relative errors of less than 1%. This demonstrates that 0.287 M sodium dithionite had no effect on the release of CO from a sample. Additionally, the analysis of a combination of sodium dithionite and liberating agent alone revealed no unique peaks.

The new GC method was tested for its accuracy and precision. The results obtained from the GC were compared with results obtained from the UV spectrophotometric method we commonly employ in our laboratory. As seen in Table 1, the GC and UV results were found to be virtually identical, demonstrating the accuracy of the GC method. Additionally, the coefficient of variation for each control level was less than 10%, indicating good precision.

After showing that 0.287 M sodium dithionite had no effect on an existing concentration of COHb or the release of CO from a sample and proving that the method was both accurate and precise, the GC method was applied to real postmortem blood samples. Blood samples from various fatal aviation accident victims that had previously tested positive for CO were analyzed. The amount of MetHb present in the samples was determined using a CO-oximeter. The samples were found to have MetHb values ranging from 5% to 64%. The %COHb in the specimens was then determined using our existing spectrophotometric method and the GC method with and without reducing agent. We anticipated that, as the amount of MetHb increased, so would the %COHb error associated with

Table 1. Accuracy and precision of the GC method compared with the spectrophotometric method.

Method used for %COHb ± s.d.	Target %COHb					
	Neg	16%	25%	35%	50%	67%
GC*	0.9 ± 0.1	16 ± 1	25 ± 2	38 ± 3	54 ± 2	69 ± 5
UV**	1.1 ± 0.4	16.0 ± 0.4	23.7 ± 0.3	32.6 ± 0.4	49.6 ± 0.5	66.7 ± 0.4

* n=5 for both saturated and unsaturated samples.
** n=4

Table 2. Postmortem blood results from aviation accident victims.

Sample number	UV %COHb	GC with reduction %COHb	GC without reduction %COHb	Predicted unreduced %COHb	%MetHb
1	27	27	30	29	5
2	37	36	47	44	15
3	7	7	9	9	26
4	17	16	27	26	35
5	29	30	46	47	38
6	41	38	74	73	44
7	22	20	45	47	52
8	7	7	16	18	64

the GC method not utilizing reducing agent. The results listed in Table 2 indicate that our hypothesis was correct. As shown in the table, the GC method without sodium dithionite experienced errors up to and exceeding 50% when compared with the spectrophotometric method. However, by incorporating sodium dithionite into the GC method, virtually all errors between the GC method and the spectrophotometric method were eliminated. This illustrates that the discrepancy between the %COHb values determined using the GC method without reduction and the %COHb values determined using the GC method with reducing agent is directly attributable to the MetHb present in a sample.

To further illustrate that MetHb accounts for the %COHb error found between unreduced and reduced samples, we mathematically predicted the unreduced %COHb values using the spectrophotometric %COHb results and MetHb values. The equation for predicting %COHb from unreduced samples is: Predicted %COHb = [((UV %COHb)/(100 − %MetHb))∗100]. As MetHb levels increase, the CO binding capacity, i.e., the denominator, decreases. Since %COHb is calculated as a ratio of peak areas of the unsaturated and saturated samples, if the denominator decreases, the %COHb value increases. For example, sample number 7 from Table 2 had 22% COHb, as determined by our spectrophotometric method. Additionally, the sample contained 52% MetHb, thus leaving only 48% of the total hemoglobin available for CO saturation. With these values, we would predict that the unreduced GC method would yield 47% COHb. The unreduced method actually gave us 45% COHb. Not only does this show that MetHb accounts for the error between reduced and unreduced samples, it exemplifies the potentially significant error caused by not incorporating a reducing agent when determining %COHb.

CONCLUSION

This study is concerned with the interpretation and the significance of CO findings in cases where human postmortem specimens are analyzed. Through the analysis of blood samples with varying amounts of MetHb, this GC method has proven to be accurate, precise, and reliable for the determination of %COHb in postmortem and antimortem specimens. Admittedly, the GC procedure without reduction can be used for CO detection; however, it is clear that as MetHb levels increase in postmortem samples so does the error introduced in the determination of %COHb. Therefore, when analyzing postmortem blood using the GC-ratio method, the inclusion of a reducing agent is essential in obtaining meaningful and reliable COHb values.

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