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Interference of fetal hemoglobin in the determination of carboxyhemoglobin by spectrophotometry

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Abstract

Determination of carboxyhemoglobin (HbCO) is routinely performed in suspected cases of carbon monoxide intoxication and unexplained deaths. However, some authors have suggested that measured HbCO may be falsely elevated in infants (0–12 months) due to the presence of fetal hemoglobin (HbF). The purpose of this study was to evaluate the impact of fetal hemoglobin on the spectrophotometric determination of carboxyhemoglobin. The interference of HbF in the determination of HbCO in infants aged from 0 to 12 months was evaluated using 16 ante-mortem and 19 post-mortem blood samples. The %HbCO was quantified spectrophotometrically by calculating the 560 nm/530 nm absorbance ratio, using a dual beam spectrophotometer. The average measured HbCO in infants of 3 months of age or under was 17%, which is abnormally elevated. No significant difference in HbCO measurement was found between ante-mortem and post-mortem samples. These results highlight the fact that care must be taken in interpretation of carboxyhemoglobin measurements in infants when using a spectrophotometric method.

KEYWORDS:
Forensic toxicology, carboxyhemoglobin, fetal hemoglobin, spectrophotometry, neonates, false positive

1 | INTRODUCTION

Carbon monoxide (CO) is a gaseous product of the incomplete combustion of organic matter such as wood or fuel. When CO is inhaled, it binds with hemoglobin (Hb) to form carboxyhemoglobin (HbCO). Hemoglobin has an affinity for CO that is 250 times larger than oxygen (O_2); therefore, at high CO levels, hemoglobin cannot accomplish its oxygen delivery duties[1]. Although the mechanisms are not completely understood, myoglobin, cytochromes and chemical mediators are also suspected to be involved in CO toxicity[2]. Levels of HbCO up to 10% in smokers are considered normal, whereas levels between 20% and 50% are toxic and levels above 50% are lethal[3]. Carbon monoxide intoxication is typically observed in cases of residential or commercial fires, industrial accidents, heating systems (oil, propane, fireplaces) and suicide through car exhaust inhalation. HbCO can be quantified in blood by gas chromatography, with an oxygen electrode, or by obtaining the absorption spectrum using a spectrophotometer[4] or a CO-oximeter[5]. The spectrophotometric measurement is very popular because it is simple, accurate, rapid and accessible[6]. However, it is reported that spectrophotometric measurement of HbCO can be subject to interferences from fetal hemoglobin (HbF)[7,8]. Fetal hemoglobin is structurally similar to adult hemoglobin (HbA) but can bind oxygen with greater affinity[2]. At birth, HbF accounts for around 75% of total hemoglobin, decreasing drastically at 4 months of age and reaching a level close to zero in adult blood[9]. The absorption spectrum for fetal hemoglobin is very similar to adult hemoglobin, but the absorption coefficients differ[10], because of a hypsochromic shift in the oxygen dissociation curve of HbF compared to adult

hemoglobin[11]. These small differences are the likely cause of reported inaccuracy in the spectrophotometric quantification of HbCO in neonates[12]. Studies on this topic yield contradictory results. Vreman et al.[7] and Mehrotra et al.[8] report that spectrophotometric measurement of HbCO is falsely elevated by the presence of HbF, giving false positive results up to 8% when using a CO-oximeter. Zwart et al.[13] and Variend et al.[14] report that in the method proposed by Katsumata et al.[4], reduction of blood with sodium dithionite eliminates the interference of HbF, and thus should not give positive bias when using a basic spectrophotometer. In this paper, using the Katsumata et al.[4] method, the impact of the infant's age on determination of HbCO is studied in 35 cases under one year of age. Comparison between ante-mortem and postmortem blood samples is performed. The effect of increasing the concentration of sodium dithionite, as well as increasing the incubation time, is also examined.

2 | METHODS

2.1 | Materials

Sodium carbonate (certified ACS, 100.0%) and sodium hydroxide (certified ACS, 99.0%) were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Sodium dithionite (technical grade, 85%) was obtained from Sigma-Aldrich (St. Louis, MO, USA). HbCO standards were obtained from Instrumentation Laboratory (Bedford, MA, USA). A Perkin Elmer UV/Vis Spectrophotometer Lambda 35 was used to perform the spectrophotometric analysis. Minitab 17 (Minitab Inc., State College, PA, USA) and Excel (Microsoft, Redmond, WA, USA) were used to perform statistical calculations and generate plots.

2.2 | Ante-mortem and post-mortem blood sampling

All infants studied were under 1 year of age. Ante-mortem venous blood samples ($n = 16$) were obtained from the pediatric hospital as excess from routine blood work. Blood was collected in BD Vacutainer tubes (lavender cap) containing EDTA and stored at 4 °C. In post-mortem cases ($n = 19$), femoral and cardiac blood, when available, were sampled during the autopsy from infants who died in circumstances unrelated to the presence of carbon monoxide (e.g. shaken baby syndrome, dog attack, co-sleeping). Femoral blood was collected through a disposable needle inserted in the femoral vein. The femoral vein was unclamped during this operation and 1 to 10 mL of the available femoral blood was collected. Average femoral blood volume collected was 2 mL. Samples were stored at 4 °C in BD Vacutainer tubes (grey cap) containing 100 mg sodium fluoride and 20 mg potassium oxalate. Cardiac blood samples consisted of pooled cardiac blood (left and right ventricles) that flowed out of the heart after the aorta was sectioned. Average cardiac blood volume was 5 mL but ranged from 2 to 50 mL. Samples were stored at 4 °C in Starplex Leakbuster polypropylene containers without any preservative. Given the short time interval between the collection of the samples and the analysis (0–72 hours), no stability issue was expected.

2.3 | Spectrophotometric quantification

The HbCO quantification method was based on the work of Katsumata et al.[4,15]. Briefly, 50 μL of blood was diluted in 5 mL of 1 mg/mL sodium carbonate and vortexed. Then 20 mg of sodium dithionite and 500 μL of 1 N sodium hydroxide were added to the solution, with vortexing after each addition. The sample was allowed to rest for approximately 1 minute between the addition of sodium dithionite and that of NaOH. An aliquot of this solution was then transferred to a 1 cm path length disposable plastic absorbance cell. The absorbance spectrum from 500 nm to 650 nm was obtained using a double-beam spectrophotometer, with sodium carbonate solution as the reference sample. Absorbance maxima measured around 530 nm and 560 nm were used to quantify HbCO using Equation 1.

$$\text{HbCO}\ (\%) = (2.21 - A_{560}/A_{530}) \times 79 \tag{1}$$

The constants in Equation 1 were obtained empirically using prepared standards of 5%, 20% and 50% HbCO. This method was validated according to ISO 17025 and CAN-P-1578 guidelines (Canadian guidelines for the accreditation of forensic testing laboratories). Sodium dithionite should convert all forms of hemoglobin (e.g. hemoglobin, methemoglobin, oxyhemoglobin) into reduced hemoglobin (HHb) except for carboxyhemoglobin, providing a uniform system of HbCO and HHb. The addition of sodium hydroxide clears the turbidity of the sample and allows a better spectrophotometric separation of the peaks of reduced hemoglobin and carboxyhemoglobin. The spectra of the two components combine to form two distinct peaks at 530 nm and 560 nm. The absorbance ratio of these two peaks changes linearly with HbCO concentration in blood.

2.4 | Evaluation of sodium dithionite impact

The impact of sodium dithionite on the reduction of blood was evaluated by varying the mass added and the time of incubation after addition. Five samples from infants with a high %HbCO were analyzed according to the method described above except that the mass of sodium dithionite added to the sample was tripled (60 mg) and the incubation time was extended to 5 or 30 minutes after the addition and vortexing.

3 | RESULTS

3.1 | Quantification of HbCO in neonates

A typical spectrum of neonate blood is shown in Figure 1 . The calculated results for neonates studied are presented in Table 1 . The apparent levels of HbCO in infants under 3 months of age ($n = 30$) varies between 4.3% and 26.9%, while it is under 5% in infants between 4 and 11 months of age ($n = 5$) for both ante-mortem and post-mortem infants.

FIGURE 1 Absorption spectrum of a blood sample. (1) Peak absorbance of HbCO at 530 nm. (2) Peak absorbance of HHb at 560 nm.

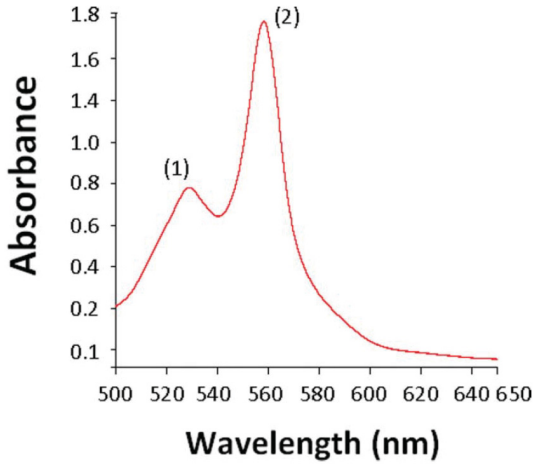

Figure 2 shows that apparent HbCO decreases with age in both groups, and correlates with the expected decrease of fetal hemoglobin concentration in blood[9]. Prediction lines at the 95% confidence level, which represents the range in which a single new observation is likely to fall given the predictors, is also shown in Figure 2 . It shows that HbCO values of 14% to 34% can be obtained at an age of 2 weeks, whereas this interval goes down to 0% to 21% at 3 months.

3.2 | Impact of sodium dithionite

Results of the tests with sodium dithionite are shown in Table 2 for the 5 cases studied. No significant change from baseline value was observed when increasing the quantity of sodium dithionite threefold and increasing the time of incubation to 5 minutes ($p = 0.447$) or 30 minutes ($p = 0.101$). The expected effect was that sodium dithionite would reduce HbF to HHb, therefore eliminating the HbF interference. This would then translate to a lower level of measured HbCO. However, in the majority of cases, an increase of the %HbCO was observed. This shows that sodium dithionite does not eliminate the HbF interference.

3.3 | Comparison of ante-mortem and post-mortem cases

The first order slope was obtained by least squares linear regression for %HbCO value against age in post-mortem cases and in ante-mortem cases. There was no significant difference between slopes of ante-mortem ($m = -3.54$) and post-mortem ($m =$

TABLE 1 Results for HbCO (%) in infants.

Case number	Ante/Post-mortem	Type of blood	Age (months)	% HbCO
1	Ante	Whole	1	24.7
2	Ante	Whole	2	10.6
3	Ante	Whole	0.75	20.0
4	Ante	Whole	7	1.9
5	Ante	Whole	2	22.9
6	Ante	Whole	0.5	24.0
7	Ante	Whole	6	1.6
8	Ante	Whole	0.5	13.0
9	Ante	Whole	0.5	19.0
10	Ante	Whole	0.5	18.0
11	Ante	Whole	0.75	20.0
12	Ante	Whole	3	8.1
13	Ante	Whole	0.75	17.0
14	Ante	Whole	0.5	22.0
15	Ante	Whole	4	1.3
16	Ante	Whole	1.75	16.0
17	Post	Cardiac	2	9.0
18	Post	Cardiac	1.75	10.0
		Femoral	1.75	12.0
19	Post	Cardiac	1.25	17.7
		Femoral	1.25	24.1
20	Post	Cardiac	3	13.4
		Femoral	3	26.9
21	Post	Cardiac	0.5	24.0
		Femoral	0.5	23.0
22	Post	Cardiac	2	18.0
23	Post	Cardiac	1.75	10.0
24	Post	Cardiac	0	24.0
		Femoral	0	24.0
25	Post	Cardiac	0.5	19.0
		Femoral	0.5	20.0
26	Post	Cardiac	2	8.3
27	Post	Cardiac	3	6.4
		Femoral	3	9.3
28	Post	Cardiac	1.5	19.0
29	Post	Cardiac	0.25	20.0
30	Post	Cardiac	0.25	25.0
31	Post	Cardiac	2.5	7.4
32	Post	Femoral	11	4.0
33	Post	Cardiac	1	19.0
		Femoral	1	24.0
34	Post	Cardiac	3	4.3
		Femoral	3	6.3
35	Post	Cardiac	6	3.0

−4.84) cases at a 95% confidence level ($p = 0.56$), showing that there is no significant difference in the rate of decrease of the interference with age. Additionally, there is no significant difference between the y-intercept of ante-mortem ($b = 22.0$) and post-mortem ($b = 23.7$) cases at a 95% confidence level ($p = 0.46$). In conjunction with the lack of significant difference between the slopes, this shows that ante-mortem and post-mortem cases suffer from similar interference due to the presence of HbF.

FIGURE 2 Quantified HbCO (%) in neonates of 0 to 11 months. Prediction interval lines are shown for the 0 to 3 months regression.

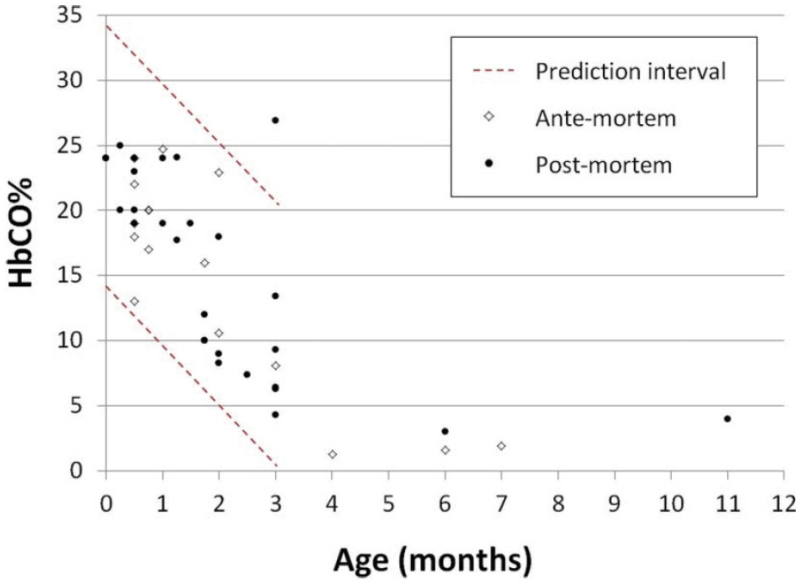

TABLE 2 Comparison of %HbCO between expected values and new conditions of blood reduction by sodium dithionite in 5 cases.

Case number	**Reference** 1x dithionite (20mg) Incubation : 1 min (%HbCO)	**Test #1** 3x dithionite (60mg) Incubation : 5 min (%HbCO)	**Test #2** 3x dithionite (60mg) Incubation : 30 min (%HbCO)
1	20	22	23
2	19	26	24
3	20	22	27
4	16	14	15
5	12	10	13

3.4 | Comparison of cardiac and femoral blood

A paired t-test was performed between cardiac and femoral HbCO (%). Results show that there is a significant difference ($p = 0.042$) in the concentration of HbCO at the 95% confidence level. However, the small number of cases ($n = 9$) where cardiac and femoral blood were both available and the proximity of the p-value to the significance threshold (0.05) should be kept in mind and temper the weight placed on these statistical conclusions. Further tests with larger sample sizes would be necessary to assess any significant difference between the two types of blood.

4 | DISCUSSION

4.1 | Quantification of HbCO in neonates

Normal HbCO levels have been found to be below 1.5% for non-smokers and up to 15% for cigarette smokers[16]. The fact that the apparent HbCO in infants under 3 months of age went up to 25% in this study strongly suggests that the results were biased towards positive values due to the presence and interference of HbF. This study focused on neonates under 4 months of age,

when the fetal hemoglobin proportion is higher[9]. Only 4 samples of infants aged from 4 months to 1 year were analyzed, therefore there is no sufficient experimental evidence to draw definitive conclusions regarding the measurement of HbCO in that age range. However, after 4 months, there is a rapid transition from HbF to HbA. In the results presented here, this transition translated into a rapid decrease of the apparent HbCO concentration as the age of the neonate increased. Therefore, HbF interference in HbCO measurements in infants of 4 months and older is unlikely. Prediction interval boundaries in Figure 2 showed the range of HbCO concentrations likely to be measured in neonate blood from 0 to 4 months. Measurement of additional samples would help refine these boundaries and help in the interpretation of HbCO measurements in neonate cases.

These results do not exclude other exogenous factors that could give rise to high levels of HbCO. HbF has a greater affinity with CO than adult hemoglobin (2.5–3 times)[17]. If the babies were exposed to a smoking environment prior to death or hospitalisation, it is likely that their HbCO level would be higher than normal. However, given that the half-life of carboxyhemoglobin is about 7 hours in fetal blood[17,18], this should not be an issue for ante-mortem samples, since they came from children hospitalised in a nonsmoking area. Given that the post-mortem results are not significantly different from ante-mortem results, we infer that, in aggregate, the post-mortem cases did not present elevated HbCO levels due to environmental exposure.

4.2 | Impact of sodium dithionite

Sodium dithionite is used to convert all forms of hemoglobin into reduced hemoglobin except for carboxyhemoglobin. Variend et al.[14] reported that reduction of blood with sodium dithionite eliminates the interference of HbF and should eliminate the positive bias. However, our results showed that addition of sodium dithionite did not completely eliminate the interference from HbF. In order to verify that this was not due to slow reaction kinetics, the quantity of dithionite was tripled (60 mg for 50 μL of blood) and incubation time was increased up to 30 minutes. The results did not change after these modifications. No difference between Variend et al. protocol and the one used here was found to explain the discordant results. The one notable difference is the alternative wavelengths used by some authors (534 nm and 579 nm instead of 530 nm and 560 nm). However, this is known to be a difference attributable to the absence of strong base (1 N NaOH) in the preparation, which shifts the spectrum and thus the peaks of absorption[15]. The only conclusion is that sodium dithionite does not allow reduction of all fetal hemoglobin and proper elimination of its interference.

4.3 | Comparison of ante-mortem and post-mortem cases

No significant difference between the measurements in ante-mortem and post-mortem cases was found. Therefore, future studies on the subject of HbCO in neonates could be conducted on ante-mortem blood, which is more easily available than post-mortem specimens. This would allow a larger sampling of the population and a more complete statistical analysis.

4.4 | Comparison of cardiac and femoral blood

No strong conclusion can be given as to whether there is a significant difference between the measurements in cardiac and femoral blood in infants. Additional tests should be done on a larger sample size in order to indicate if pooled cardiac blood could confidently be used to quantify HbCO in whole blood. This piece of information would be particularly important in neonate cases, where the volume of available femoral blood is much reduced (average of 2 mL collected). Femoral blood could therefore be kept for quantification of analytes more subject to post-mortem redistribution phenomena.

5 | CONCLUSION

The false elevation of neonates' carboxyhemoglobin, as determined by spectrophotometric measurements, is an important piece of information for forensic toxicologists, coroners, pathologists and health professionals. It can help in the determination of the cause of death, or in the use of an adequate treatment for carbon monoxide poisoning. Because of false positives, HbCO results for newborns under 4 months of age must be interpreted with care. We showed that HbCO values of 14% to 34% are likely to be obtained at 2 weeks of age, whereas this interval goes down to 0% to 21% at 3 months. Confirmation by a second method (e.g. gas chromatography) is strongly recommended for more accurate measurements. Another option would be the use of a

neonate specific calibration curve with the current spectrophotometric method. However, due to the rapid change in HbF levels in neonates, this is a difficult technique to set up. Ideally, the spectrophotometric method should be modified to be resistant to HbF interference.

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