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RESEARCH ARTICLE

Stability of carboxy-hemoglobin during storage at different temperatures

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Abstract

Accurate determination of carboxy-hemoglobin (COHb%) is essential for the assessment of hemoglobin mass (Hbmass) by CO-rebreathing. To analyze blood samples for a certain period of time after blood collection, it is necessary to know the stability of the COHb% during storage. The aim of the study was to determine the stability of COHb% at different storage temperatures over a period of up to 3 months. Twenty-five milliliters of cubital venous blood was taken from five volunteers (three females and two males) before and after inhalation of 0.8/1.0 mL/kg carbon monoxide and stored at +20°C and +4°C for 6 days and at −70°C for 12 weeks. Within the first 6 days, the blood was analyzed daily, then weekly for 12 weeks. Additionally, Hbmass was determined in 13 endurance athletes immediately after blood collection and after storage for 3 days (eight cyclists) and 7 days (five swimmers) at +20°C or +4°C. COHb% before and after CO inhalation was 1.56 ± 0.48 and 5.86 ± 1.12%, respectively, and remained unchanged over 6 days, with no difference between storage at different temperatures. The standard deviation (STD) over time was between 0.07% and 0.12%. Similarly, storage at −70°C for 12 weeks did not change COHb%, whereas STD was 0.07%. Hbmass determined immediately and, after 3 or 7 days of storage, differed by 10 ± 7 g and 15 ± 11 g corresponding to a typical error of 0.8% and 1.1%. Blood storage at +20°C and +4°C for 6 days and at −70°C for 12 weeks does not affect COHb% and has, therefore, no influence on Hbmass assessment.

KEYWORDS
accuracy, carbon monoxide, CO-rebreathing, hemoglobin mass, reliability

1 | INTRODUCTION

The CO rebreathing method is a minimally invasive, simple, and accurate method for determining absolute hemoglobin mass and blood volume that has been continuously developed since its rediscovery by Thomson et al. in 1991.[1–4] Today, it has completely replaced the former gold standard method of blood volume determination, that is, use of radioactive tracers, in the field of sports. There is also a discussion of using Hbmass determination via CO rebreathing to detect blood manipulation.[5,6]

Meanwhile, the method is used for research purposes in the clinical area,[7] especially in patients with cardiac and renal insufficiency,[8] but also in other indications,[9] to clarify volume status and to differentiate between true and dilution anemia. It is expected that the method can be used routinely in the future.

An indispensable prerequisite for this is the accurate determination of the COHb% in the blood, which is carried out before and after a defined period of time following inhalation of the tracer gas CO. If this determination is carried out immediately after blood sampling, as has been the case up to now, the methodological measurement error (typical error, TE) of the Hbmass determination is small and amounts to between 1% and 2%.[10–12] Most hospitals possess an appropriate CO-oximeter, so that an immediate analysis can occur. However, CO rebreathing is often performed outside well-equipped laboratories, for example, during training camps or in clinical settings in decentralized laboratories, so that immediate analysis is not feasible. Furthermore, if CO rebreathing is to be used in the future for the detection of blood manipulation, it is necessary to have a second sample on hand for later analysis.[13]

In all these cases, it is desirable or even necessary to be able to store the blood samples and analyze them at a later time, possibly at another location. Since hemoglobin has a 200–300-fold higher affinity for CO compared with oxygen,[14] appropriate storage may well be possible.

To clarify this issue, studies have therefore already been carried out mostly after CO poisoning, which showed that the COHb% was unchanged after 7 days even at ambient temperature after postal dispatch of the samples,[15] and even in deceased persons, the COHb% in the blood remained stable after 4 months[16] and 2 years.[17] However, these data cannot be directly applied to the determination of Hbmass and BV, because a much more accurate determination of COHb% is necessary. For example, a change in COHb% of 1%, which is hardly important for the diagnosis of CO poisoning, would already lead to an inaccuracy of about 20% in the determination of Hbmass.

Therefore, the aim of the present study was to investigate the possible change in COHb% in blood samples taken before and after CO inhalation for the determination of Hbmass after different types of storage, that is, at room temperature (20°C), when cooling at +4°C, and when freezing at −70°C for up to 12 weeks. To check the practical applicability, the reliability of Hbmass determination was evaluated when COHb% was measured immediately after blood collection and after a storage period of 3 and 7 days.

2 | METHODS

2.1 | Subjects and study design

In the first part of the study, blood was collected from five young healthy subjects (two males and three females; see Table 1), stored at three different temperatures for different periods of time, and periodically analyzed for possible changes in COHb%. In the second part, test–retest measurements were performed with blood from 13 endurance athletes (five elite swimmers and eight cyclists; Table 1) before and after storage. The participants provided written consent after they were informed about the content of the study, the associated risks, and the possibility to withdraw without indication of any reason. The study was approved by the ethics committees of the Friedrich-Alexander-University Erlangen (part I) and the University of Bayreuth, both in Germany (part II).

2.2 | Blood sampling and blood storage

In the first part of the study, 25 mL of cubital venous blood was collected twice after at least 15 min of sitting. The first collection was performed without CO inhalation (low COHb%, LCO), the second 7 min after inhalation of 0.8 mL CO/kg body weight (females), or 1.0 mL/kg (males) (high COHb%, HCO). Inhalation of CO (99.97% carbon monoxide, Linde, Munich, Germany) was performed using a glass spirometer (Blood tec, Bayreuth, Germany), whereby a CO bolus was inhaled together with pure O_2 and ventilated for 2 min. Blood was collected in syringes prepared with 20-µL heparin. Immediately after blood collection, capillaries of 100-µL capacity were filled from each syringe and sealed at both ends with plastic plugs (Radiometer, Copenhagen, Denmark). In addition, the blood from each syringe was aliquoted into Eppendorf caps (1 mL of blood in caps with a volume of 1.5 mL).

For baseline values, COHb% was determined six times in the blood from both syringes immediately after collection and averaged. The capillaries from each of the two sampling times were stored at ambient conditions at a constant 20°C and in a refrigerator at +4°C, respectively. The Eppendorf caps were stored at −70°C in a freezer. On the following 5 days, the COHb% in the blood was determined from the capillaries stored at 20°C and at 4°C and from the Eppendorf caps frozen at −70°C. Subsequently, the measurement was

TABLE 1 Characteristics of participants.

	Sex/sports	Age (years)	Height (cm)	Body mass (kg)	BMI
Part I	Males ($n = 2$)	25.5 ± 3.5	177 ± 2	70.0 ± 1.4	22.5 ± 1.0
	Females ($n = 3$)	24.0 ± 1.0	167 ± 6	61.8 ± 4.0	22.4 ± 1.6
Part II	Cyclists				
	Males ($n = 8$)	28.5 ± 5.0	180 ± 4	75.1 ± 4.0	23.2 ± 1.1
	Swimmers				
	Males ($n = 4$)	23.0 ± 3.6	194 ± 8	86.8 ± 14.6	23.0 ± 2.0
	Females ($n = 1$)	19	182	75.2	22.7

performed weekly with the samples frozen at −70°C. The COHb% was determined twice from each blood sample, and the mean value was calculated.

2.3 | Hbmass determination

In the second part of the study, Hbmass was determined according to the protocol described by Schmidt and Prommer.[3,18] Briefly, subjects inhaled a bolus of CO along with pure O_2 via a glass spirometer for a period of 2 min. COHb% was determined immediately before and exactly 7 min after initial inhalation in capillary blood collected from the earlobe (analyzer cyclists: OSM3, swimmers: ABL 90, both Radiometer, Copenhagen, Denmark). At the identical time points before and after CO inhalation, two additional capillaries of blood were taken and stored for 3 days at ambient temperature (cyclists), or in a refrigerator for 7 days at +4°C (swimmers). During the rebreathing procedure, the CO volumes remaining in the spirometer as well as diffusing to myoglobin and being exhaled until the second blood draw were determined as described elsewhere.[18] These data were used to calculate Hbmass with the COHb% values determined immediately after blood drawing as well as after storage for 3 or 7 days, respectively.

2.4 | Statistics

In part 1, data for all time points are presented as means and standard deviations. Furthermore, the variance of COHb% was determined for each subject over the period of 6 days (all storage temperatures) and 12 weeks (−70°C), respectively. The mean of the individual variance of all subjects was then used to calculate the mean standard deviation and, as a measure of reliability, the typical error over time (TE).[19]

Analyses of variance were performed comparing the courses of COHb% under the different storage conditions over 6 days and over 12 weeks. Paired *t*-tests should be used in case of significant ANOVA results to specify the significance of differences between storage conditions and of differences from baseline. As no significance was achieved in the ANOVA, no post hoc tests were performed in part I of the study.

In part II, a paired *t*-test was applied to check the significance of differences between the Hbmass measurements performed immediately after blood drawing and after storage. The tests were two-sided, and statistical significance was set at $p < 0.05$. The storage-related error (TE) was determined according to Hopkins.[19]

3 | RESULTS

3.1 | COHb% during storage

Basal COHb% was 1.56 ± 0.48% without CO inhalation (LCO), and 5.86 ± 1.12% after CO inhalation (HCO). Over the 6-day storage period, there was no significant change in COHb% and no difference between storage modes (Figure 1a). The mean deviation from baseline (Table 2) ranged from −0.03 ± 0.11 (20°C, HCO) to 0.12 ± 0.08% (−70°C, HCO). The mean difference in COHb% between LCO and HCO was 4.31 ± 0.96% basally and varied by a maximum of −0.07 ± 0.12% (4°C) on average.

There was also no significant difference in COHb% during storage for 12 weeks at −70°C (Figure 1b). The mean difference was 0.03 ± 0.08% for LCO and 0.05 ± 0.04% for HCO (Table 2). The mean variation in the difference was 0.01 ± 0.08%.

The mean individual variation for storage over 6 days was approximately 0.09% and did not differ between different temperatures. When blood samples were stored at −70°C for 12 weeks, the mean STD was 0.07% (Table 2).

3.2 | Comparison Hbmass

The mean Hbmass of the cyclists was 1076 ± 79 g when COHb% was measured immediately and 1072 ± 79 g when measured after a 3-day storage of samples at 20°C. The STD of the differences was 11 g, and the TE was 0.8% (Table 3).

The mean Hbmass of the swimmers was 1173 ± 278 g 1 day after the test and 1175 ± 286 g when storing the samples for 7 days at 4°C. The STD of differences was 19 g, and the TE was 1.1%.

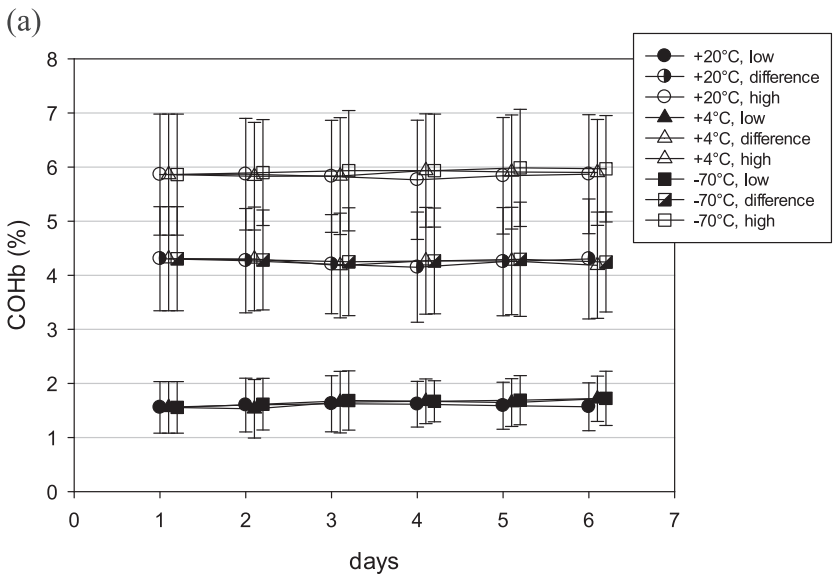
4 | DISCUSSION

The most important result is that storage of blood samples at ambient temperatures and at +4°C for 6 days and storage at −70°C until 12 weeks after collection does not affect COHb%.

4.1 | Stability of COHb%

In the literature, the stability of COHb% is described for two purposes, once, for short storage for rapid determination of blood gas status and acid–base balance[20] and, second, for a longer period of several weeks and months for diagnosis of CO poisoning.[15–17,21] For rapid determination, the maximum storage time of a blood sample is limited by the stability of PO_2, which is no longer guaranteed after 60 min even with ice cooling. In contrast, the COHb% remains unchanged after 3 h of storage at ambient conditions or on ice.[20]

Long-term storage studies have been conducted to exclude possible changes in COHb% when samples are sent by mail or stored post-mortem for forensic purposes in carbon monoxide poisoning cases. These studies showed massive decreases in COHb% over the course of several weeks when samples were stored unsealed.[16,21] In sealed containers and not in contact with ambient air, COHb% remained nearly constant with only a slight variation in the percentage range for 4 weeks when blood was stored under ambient (20°C) and cool (4°C) conditions[15,16] and at least 2 years when stored constantly at 3°C.



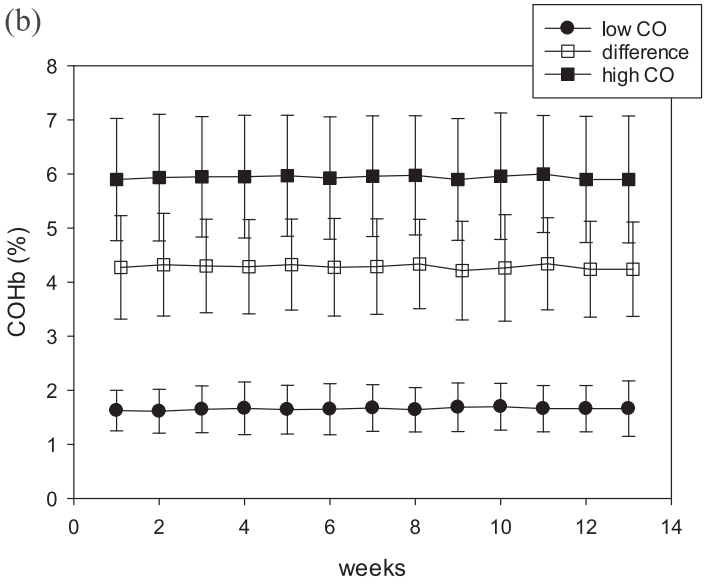


FIGURE 1 COHb% during storage at different temperatures. (a) Storage for 6 days at 20, 4, and −70°C; (b) storage for 12 weeks at −70°C. Low CO = blood samples without CO inhalation, high CO = blood samples after inhalation of a CO bolus (0.8 mL/kg in females, 1.0 mL/kg in males); difference = difference in COHb% between low and high CO.

TABLE 2 Basal COHb% and variation by storage at 20, 4, and −70°C for 6 days and at −70°C for 12 weeks.

			Initial value (%)	Mean difference from initial value (%)	Mean individual STD (%)
6 days	20°C	Low CO	1.56 ± 0.48	0.04 ± 0.11	0.09
		High CO	5.86 ± 1.12	−0.03 ± 0.11	0.07
		ΔCO	4.31 ± 0.96	−0.07 ± 0.05	0.12
	4°C	Low CO	1.56 ± 0.48	0.09 ± 0.08	0.11
		High CO	5.86 ± 1.12	0.02 ± 0.15	0.09
		ΔCO	4.31 ± 0.96	−0.07 ± 0.12	0.11
	−70°C	Low CO	1.56 ± 0.48	0.12 ± 0.08	0.09
		High CO	5.86 ± 1.12	−0.08 ± 0.13	0.10
		ΔCO	4.31 ± 0.96	−0.04 ± 0.11	0.09
12 weeks	−70°C	Low CO	1.56 ± 0.48	0.03 ± 0.08	0.07
		High CO	5.86 ± 1.12	0.05 ± 0.04	0.07
		ΔCO	4.31 ± 0.96	0.01 ± 0.08	0.08

TABLE 3 Hbmass data calculated with ΔCOHb% determined immediately after blood collection and after storage for 3 days or 7 days at +20°C (cyclists) or +4°C (swimmers).

		Hbmass (g)		
		Immediately	**After 3 days at +20°C**	**Difference**
Cyclists	#1	1114	1109	6
	#2	997	1005	(−) 8
	#3	1170	1178	(−) 8
	#4	1,125	1132	(−) 7
	#5	1053	1035	17
	#6	948	946	2
	#7	1040	1034	6
	#8	1158	1136	22
	Mean	**1076**	**1072**	**|10|** [a]
	STD	**79**	**79**	**|7|** [a]
	TE abs (g)			**8**
	TE %			**0.8**
		Immediately	**After 7 days at +4°C**	**Difference**
Swimmers	#9	1301	1301	0
	#10	1534	1553	(−) 19
	#11	1171	1146	25
	#12	1077	1099	(−) 22
	#13	781	774	7
	Mean	**1173**	**1175**	**|15|** [a]
	STD	**278**	**285**	**|11|** [a]
	TE abs (g)			**13**
	TE %			**1.1**

Note: TE abs = absolute typical error, TE % = percentage typical error, difference = difference from the first measurements, negative values are marked in brackets.
[a]Absolute value of the mean and corresponding standard deviation.

Although in these long-term studies, the COHb% was predominantly very high and minor changes of a few percent have no clinical impact, it is absolutely necessary to measure the COHb% very accurately for the determination of Hbmass. For example, with a Hbmass of 980 g (moderately trained young men[22]) and a COHb% difference of 4.5% following a CO rebreathing maneuver, a measurement inaccuracy of ±0.1% already leads to a change in calculated Hbmass of ±22 g, corresponding to 2.2%.

The reliability of COHb% measurement with the analyzer used here, which has a display accuracy of 0.1%, was determined by Alexander et al 2011[23] on arterial and venous blood samples. In duplicate determinations, the mean STD of repeated measurements ranged from 0.05% to 0.08% for both low (<2%) and high COHb% (2.5%–9%). With multiple determinations, the STD decreased to as low as 0.04% (fivefold determination). In the present study, which used duplicate determinations, the mean STD was only slightly above these values of Alexander et al.[23] in the 6-day experimental series (0.07%–0.11%) and very similar in the 12-week series (0.07%). When differences between a high and low COHb% value are calculated, as is common with the CO rebreathing method, there was no difference in reliability between the values measured directly after blood drawing (STD 0.07%–0.10%[23]) and the values determined here for the samples stored up to 12 weeks (STD 0.07%–0.09%). These data clearly show that no change in COHb% occurs as a result of the storage modes studied here. Physiologically, this stability can be explained by the consistently high affinity of the hemoglobin molecule for CO,[14] whereas other hemoglobin derivatives, for example, MetHb, are subject to change.[24]

4.2 | Practical significance

COHb% values unchanged for at least 1 week under normal ambient conditions and for at least 3 months when refrigerated allow blood samples to be shipped or stored for diagnostic and forensic purposes without any restrictions.

Similarly, a delayed determination of COHb% can be performed in the context of CO rebreathing tests to determine Hbmass. The mean STD of 0.08% for the difference between high and low COHb (Δ4.31%) corresponds to an analyzer error of 1.3%, which is 13 g for an assumed Hbmass of 980 g (example from above). These theoretical considerations are consistent with our Hbmass determinations before and after storage at 20 and 4°C. The TE in the cycling group was 8 g, corresponding to 0.8%. Similarly, in the swimmers, a TE of 1.1% was

found with a different CO oximeter (ABL 90). These methodological deviations correspond to the reliability of directly analyzed blood samples reported in the literature, which ranges from 1.1% to 1.6% for experienced users.[11,12,25]

The methodological error mentioned here in part I can even be reduced in the context of a CO rebreathing procedure by a higher number of repeated measurements and by a slightly higher CO application. For example, in Alexander's study,[23] at a ΔCOHb% of 5.5%, STD decreases by approximately 35% with fivefold measurements versus duplicate determinations reducing the analyzer error to 0.0095% corresponding to 9 g in the above example.

The use of stored blood samples, therefore, allows the CO rebreathing method to be performed without the presence of a CO analyzer on site and the COHb% to be performed elsewhere, for example, in central laboratories of larger hospitals. Furthermore, stored blood samples can be used as evidence, for example, as B-sample, in an anti-doping context. In the clinical context, stored samples can be used without restrictions for diagnostic and forensic purposes.

5 | CONCLUSION

In the course of a 6-day storage of blood samples at 20 and 4°C as well as a 12-week storage at −70°C, the COHb% is not changed and can therefore be used for the determination of Hbmass.

6 | LIMITATIONS

We investigated the storage of blood samples at ambient temperatures and at +4°C only for 6 days due to our practical experience. If, for example, no blood gas analyzer is immediately available at an altitude training camp, the samples can be usually analyzed within a few days (<1 week). If blood samples are stored for a longer period, they are usually frozen, which is what we have done for the longer storage period. Nevertheless, prolonged storage of blood samples at room temperature and at +4°C should also be investigated in order to clarify how long the COHb % value is stable, before the samples have to be stored frozen.

A simple freeze–thaw protocol results in massive hemolysis. We did not add glycerol or other substances such as dimethyl sulfoxide (DMSO) to any blood sample to prevent hemolysis and did not observe any obvious effects of erythrocytic stroma on COHb% in our measurements. The stability of COHb% observed by us is in good agreement with the data of Tang et al.,[26] who found no effects on hemoglobin concentration after 30 days of whole blood storage at −70°C without cryoprotectant. Nevertheless, the addition of cryoprotectants should be investigated in future studies to rule out any possible effects.

CONFLICT OF INTEREST STATEMENT

Walter F. J. Schmidt is a managing partner of the company Blood tec GmbH, but he is unaware of any direct or indirect conflict of interest with the contents of this paper. The other authors have no conflicts of interest to declare.

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