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ORIGINAL ARTICLE

Comparability of haemoglobin mass measured with different carbon monoxide-based rebreathing procedures and calculations

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Abstract

Background. Measurements of haemoglobin mass (Hb_{mass}) with the carbon monoxide (CO) rebreathing method provide valuable information in the field of sports medicine, and have markedly increased during the last decade. However, several different approaches (as a combination of the rebreathing procedure and subsequent calculations) for measuring Hb_{mass} are used, and routine measurements have indicated that the Hb_{mass} differs substantially among various approaches. Therefore, the aim of this study was to compare the Hb_{mass} of the seven most commonly used approaches, and then to provide conversion factors for an improved comparability of Hb_{mass} measured with the different approaches. ***Methods.*** Seventeen subjects (healthy, recreationally active, male, age 27.1 ± 1.8 y) completed 3 CO-rebreathing measurements in randomized order. One was based on the 12-min original procedure ($CO_{original}$), and two were based on the 2-min optimized procedure (CO_{new}). From these measurements Hb_{mass} for seven approaches ($CO_{originalA\text{-}E}$; $CO_{newA\text{-}B}$) was calculated. ***Results.*** Hb_{mass} estimations differed among these approaches ($p < 0.01$). Hb_{mass} averaged 960 ± 133 g (CO_{newB}), 981 ± 136 g (CO_{newA}), 989 ± 130 g ($CO_{originalE}$), 993 ± 126 g ($CO_{originalA,D}$), 1030 ± 130 g ($CO_{originalB}$), and 1053 ± 133 g ($CO_{originalC}$). Procedural variations had a minor influence on measured Hb_{mass}. ***Conclusions.*** The relevant discrepancies between the CO-rebreathing approaches originate mainly from different underlying calculations for Hb_{mass}. Provided Hb_{mass} enabled the development of conversion factors to compare average Hb_{mass} values measured with different CO-rebreathing approaches. These factors can be used to develop reasonable Hb_{mass} reference ranges for both clinical and athletic purposes.

Key Words: *Carboxyhaemoglobin, CO-rebreathing, comparison of methods, red cell volume, blood volume*

Introduction

Measuring the total haemoglobin mass (Hb_{mass}), red cell volume (RCV) and blood volume (BV) is important in clinical, sports medicine, and athletic contexts [1,2]. The prevailing direct determination methods for these parameters were, until the early 1990s, based on radioactive markers and were therefore invasive and associated with potential side effects. With the possibility of an accurate and convenient method to measure blood carboxyhaemoglobin (HbCO in %) with a new generation of multiwavelength spectrophotometers [3,4], the carbon monoxide (CO) rebreathing method for estimating Hb_{mass} (first described by Grehant and Quinquard in 1882) has experienced a revival. Within the last 20 years, there has been a growing interest in this form of estimating Hb_{mass}. This can be explained by the fact that this method is non-invasive and has the lowest measurement error in comparison with other blood volume parameter estimation techniques [5].

However, at least seven different approaches to Hb_{mass} measurement have been published and used during the last few years [1,6–11]. An approach is first characterized by a specific CO-rebreathing procedure, and subsequently, by specific calculations to estimate Hb_{mass}. Each new approach has attempted to incorporate better suitable constants (e.g. Hüfner's number, the binding capacity of haemoglobin for CO) and correction factors (CO allowances due to CO resting in the system (VCO_{system}), CO being exhaled ($VCO_{exhalation}$), and CO flux to myoglobin ($VCO_{myoglobin}$)) to the anterior protocols and to thereby improve the preciseness and reliability of the CO-rebreathing method. In addition, the most recent rebreathing procedures have claimed to be more athlete-friendly by reducing the rebreathing period

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from at least 10 min with the 'original' ($CO_{original}$) procedure [3,6,7] to 2 min with the 'optimized' (CO_{new}) procedure [10] and by using exclusively capillary instead of venous blood samples.

To our knowledge, only two studies have compared Hb_{mass} estimated with different procedures to date: Gore et al. [1] and Schmidt and Prommer [10] compared Hb_{mass} measured with both the $CO_{original}$ and the CO_{new} procedures and observed no differences in Hb_{mass}.

However, we have observed substantial variations in Hb_{mass} measured and calculated with Heinicke et al.'s [9] original approach, as well as with Schmidt and Prommer's [10] optimized approach in our laboratory. We hypothesized that these disparities occur due to the use of dissimilar parameters for Hb_{mass} calculations.

It is important to know whether the diverse approaches for estimating Hb_{mass} truly result in different values for Hb_{mass}. If the approaches vary in terms of Hb_{mass}, then this information must be considered when comparing study results, as well as for establishing reasonable Hb_{mass} reference ranges for athletic purposes.

There were two specific aims of the present study. Our first goal was to compare absolute levels of Hb_{mass} estimated with the most frequently used CO-rebreathing approaches to date, and to highlight the influence of different protocols, parameters and constants for Hb_{mass} calculations. The second aim was to provide conversion factors based on experimental measurements for the comparison of Hb_{mass} measured with the diverse approaches.

Materials and methods

Study design

In all subjects, Hb_{mass} was measured three times in randomized order with a 24–48 h time lag between the tests. One measurement was based on a 12-min procedure ($CO_{original}$), and two measurements were based on a 2-min procedure (CO_{new}). In addition to the measurement of the HbCO blood concentration differences before and after the CO inhalation (ΔHbCO in %), various additional measurements were made, so that five different Hb_{mass} calculations for the 12-min procedure, and two different calculations for the 2-min procedure could be made. This resulted in five original approaches ($CO_{originalA}$ – $CO_{originalE}$) and two optimized approaches (CO_{newA}, CO_{newB}).

Due to the concentration on different calculation parameters' influence, two important procedure-based factors were held constant for all three tests: the volume of CO administered and the use of only capillary blood. The amount of CO (1.2 mL × kg^{-1}, maximal dose: 100 mL) was chosen to reach sufficient measurement sensitivity [7]. Only capillary blood samples were used, as taking capillary blood is more athlete-friendly and is as accurate and reliable as using venous blood to estimate Hb_{mass} [12].

Subjects

Seventeen healthy and recreationally active male volunteers (age 27.1 ± 1.8 years (mean ± SD), height 178.5 ± 5.3 cm, weight 75.6 ± 7.3 kg) participated in this study. All participants provided written informed consent, and the study was approved by the institutional review board of the Swiss Federal Institute of Sport. The study was carried out according to the recommendations of the Helsinki Declaration.

$CO_{original}$

$CO_{original}$ procedure. The 12-min CO-rebreathing procedure was carried out as described by Heinicke et al. [9] based on Burge and Skinner's [7] method with minor modifications [13] and additional measurements. Briefly, after the subjects spent 5 min in the sitting position, three capillary blood samples (~35 μL) were taken from an earlobe and were analysed immediately for HbCO (ABL 800flex, Radiometer A/S, Copenhagen, Denmark) and haemoglobin oxygen saturation (sO_2). Originally, the HbCO blood content was analysed with venous blood samples. However, Ashenden et al. [14] and Hütler et al. [12] measured Hb_{mass} with capillary blood samples and showed good conformity with the obtained venous blood sample values. The mean value of the three HbCO, as well as the mean of the sO_2 values, was taken as either the baseline HbCO or sO_2 value, respectively.

Then, the subjects were connected to a Krogh spirometer (Student Spirometer, Harvard Apparatus, Holliston, Massachusetts, USA). The spirometer (volume: 5 liters) was first flushed with oxygen and then filled with a mixture of oxygen and CO (1.2 mL × kg^{-1}, maximal dose: 100 mL). The subjects inhaled the gas mixture after an end-tidal exhalation, and then they started rebreathing in the closed circuit for 12 min. Blood samples from the earlobe were taken at min 8, 10, and 12, and were immediately analysed for HbCO and sO_2. The 'min 10' HbCO and sO_2 concentrations were calculated as the mean of the min 8, min 10, and min 12 blood samples. VCO_{system} was calculated after termination of the rebreathing procedure by determining the spirometer volume (spirometer volume + volume of the tubes (3.5 L) + residual volume of the lung) and multiplying the volume of the system with the CO concentration (CO Single Gas Detector, BW Technologies, Calgary, Canada) in the spirometer.

The reproducibility of Hb_{mass} with the 12-min procedure described has previously been measured and evaluated three times in our laboratory. The coefficient of variation (CV) for Hb_{mass} was between 1.4% and 1.7% [13,15], which corresponds

to the accuracy of the method observed by other authors [5,7].

$CO_{original}$ calculations

$CO_{originalA}$. The first equations for calculating Hb_{mass} for CO-rebreathing techniques that worked with a new generation of diode-array spectrophotometers were developed by Fogh-Andersen et al. [3] and Thomsen et al. [6]. These authors have calculated the monomeric amount of haemoglobin in the blood in mmol (nHb_m) (*Formula 1*).

$$nHb_m \text{ (mmol)} = 100 \times n\text{CO} \times 0.978/\Delta\text{HbCO} \quad (1)$$

where nCO is the amount of the administered CO in mmol and ΔHbCO is the difference between the baseline and HbCO levels after 10 min in %; 0.978 is the correction factor for the CO remaining in the system (2.2% of administered CO) [3].

The rebreathing procedure lasted 10 min, and blood was sampled from an antecubital vein. Unfortunately, the authors did not provide exact information regarding how to adjust the given amount of CO (ATPD) to STPD conditions. Burge and Skinner [7] substantiated Fogh-Andersen et al.'s [3] and Thomsen et al.'s [6] approaches, and calculated the tetrameric amount of Hb in the blood in mmol (nHb_t) from Formula 1, by dividing Hb in the blood (mmol) by four (*Formula 2*).

$$nHb_t \text{ (mmol)} = 25 \times n\text{CO}/\Delta\text{HbCO} \quad (2)$$

where $n\text{CO} = ((P_B/1013.25) \times V\text{CO}/R \times (273 + T)) \times 0.978$. nCO is the administered amount of CO in mmol, P_B is the barometric pressure in mbar, VCO is the volume of CO in mL, R is the gas constant (0.08206), T is the temperature in °C, 0.978 is a correction factor for the CO remaining in the system (if not measured) [6], and ΔHbCO is the difference between the baseline and HbCO levels after 10 min in %.

To obtain values with units that are comparable to newer approaches, the molar amount of Hb must be converted into grams with the help of the molecular weight (64450 g/mol) of Hb [19,20]. The tetrameric amount of Hb in mmol from Formula 2 is therefore multiplied by the factor 64.45. Results from this approach were included under the name of $CO_{originalA}$ in this study.

$CO_{originalB}$. Gore et al. [8] were the first to directly calculate Hb_{mass} in grams with an adopted formula for the same 10-min procedure, and therefore required a Hüfner's number of 1.34 (*Formula 3*).

$$CO_{originalB}: Hb_{mass} \text{ (g)} = k \times V\text{CO} \times 0.978 \times 100/(\Delta\text{HbCO} \times 1.34) \quad (3)$$

where $k = (P_B \times 273)/1013.25 \times (273 + T)$, P_B is the barometric pressure in mbar, T is the temperature in °C, VCO is the volume of the CO administered in ml, 0.978 is a correction factor for the CO remaining in the system, ΔHbCO is the difference between the baseline and HbCO levels after 10 min in %, and 1.34 is Hüfner's number (1 g Hb binds 1.34 ml O_2).

$CO_{originalC}$. Both Friedmann et al. [21] and Heinicke et al. [9] have adopted Formula 3 without the use of a correction factor for the CO remaining in the system (*Formula 4*).

$$CO_{originalC}: Hb_{mass} \text{ (g)} = k \times V\text{CO} \times 100/(\Delta\text{HbCO} \times 1.34) \quad (4)$$

where VCO is the volume of the CO administered in ml without a correction factor for CO remaining in the system. For more details, see Formula 3.

$CO_{originalD}$. Furthermore, the formula for Hb_{mass} estimation changed in several investigations. Instead of a Hüfner's number of 1.34, 1.39 was used [1,10,22]. Thus, Formula 3 changed to Formula 5:

$$CO_{originalD}: Hb_{mass} \text{ (g)} = k \times V\text{CO} \times 0.978 \times 100/(\Delta\text{HbCO} \times 1.39) \quad (5)$$

where VCO is the volume of CO administered in ml and 1.39 is the Hüfner's number (1 g Hb binds 1.39 ml CO). For more details, see Formula 3.

$CO_{originalE}$. Instead of a constant allowance of 2.2% of administered CO, the CO resting in the system has been measured and subtracted from the CO administered in previous investigations [10] ($CO_{originalE}$).

Twelve-minute approaches do not make allowances for CO flux to myoglobin, as recommended by Burge and Skinner [7].

CO_{new}

CO_{new} procedure. The two optimized 2-min CO-rebreathing measurements (CO_{new} 1; CO_{new} 2) were performed using a procedure based on Schmidt and Prommer's protocol [10,11], with some additional measurements. The procedure is briefly described here. After the subjects had spent 5 min in the sitting position, three capillary blood samples (35 µL) were taken from an earlobe and analysed immediately for both HbCO and sO_2 (ABL 800flex, Radiometer A/S, Copenhagen, Denmark). The mean of the three HbCO and sO_2 concentrations was taken as either the baseline HbCO or sO_2 value, respectively. Then the subject was connected via a mouthpiece and a tube to a CO-gas detector with parts-per-million sensitivity (Dräger PAC 7000, Dräger Safety, Lübeck, Germany). After complete exhalation to the residual volume, the end-tidal CO concentration was measured.

Subsequently the subjects inhaled a bolus of CO (1.2 mL × kg^{-1}, maximal dose: 100 mL). The gas was administered via a 100-mL plastic syringe

(Omnifix ®, B|Braun, Melsungen, Germany) connected to a specific glass spirometer (Blood Tec GbR, Bayreuth, Germany) with a 3.5 L anaesthetic bag filled with oxygen. The spirometer system had been flushed preliminarily with oxygen. After inhaling the CO and oxygen, subjects held their breath for 10 s before they began rebreathing in the closed circuit for 1 min 50 s. Two minutes after the rebreathing was completed, the end-tidal CO concentration was measured again as described above. To account for the CO exhaled from rebreathing termination to the midway point between the final two blood samples ($VCO_{exhalation}$), the difference between the end-tidal CO concentrations before and after the rebreathing procedure were multiplied by the estimated alveolar ventilation of 5.25 L/min [16]. Six and 8 min after inhalation of the CO [11], two final blood samples were taken from an earlobe and analysed for HbCO and sO_2. The mean value of the two HbCO and sO_2 values was taken as either the HbCO or sO_2 plateau.

To quantify the volume of CO that had not been absorbed by the body, the CO concentration in the anaesthetic bag was measured with the same CO-gas detector to measure the end-tidal CO concentration by connecting a tube to the glass spirometer. The measured CO concentration was then multiplied by the bag volume and the subject's residual volume.

The accuracy of the CO_{new} procedure was estimated with the two CO_{new} measurements ($CO_{new}1$ and $CO_{new}2$). The typical error obtained for Hb_{mass} in this study was 13.5 g (1.4%). This is in line with other publications that have measured the CV for the optimized CO-rebreathing method [1,10,17].

CO_{new} calculations. In contrast to the original 12-min procedures, the 2-min optimized procedure was designed to work only with capillary blood (i.e. earlobes or fingertips) from the beginning. The recommendations for the blood sampling time points after the rebreathing period differ among investigations [1,10,11], but the most obvious discrepancies are observed either in calculating or measuring CO not absorbed by the body. Further, the time point for the end-tidal exhalation and the estimation or calculation of the alveolar ventilation influenced the volume of CO exhaled after disconnection from the spirometer. To estimate the VCO_{system}, the residual volume (RV) of a subject's lung must be either measured or predicted and added to the spirometer volume. This total volume is then multiplied by the CO concentration that has been measured in the spirometer. The volume for CO flux from blood to myoglobin and other nonvascular tissue seems to have considerable inter-subject variability [11]. The allowances made in the published studies vary considerably from 0 mL to 0.3% × min^{-1} of administered CO in mL [11].

CO_{newA}. Hb_{mass} for the 2-min procedure was originally estimated by Schmidt and Prommer [10] as follows:

$$CO_{newA}: Hb_{mass}\ (g) = k \times MCO \times 100/(\Delta HbCO \times 1.39) \quad (6)$$

where MCO is the CO volume administered to the system (VCO) in mL minus CO volume not bound to haemoglobin in mL (CO_{system} + $CO_{exhalation}$). ΔHbCO is the difference between the baseline and HbCO levels after rebreathing in %; Hüfner's value for the CO-binding capacity of haemoglobin is 1.39. For k, see Formula 3.

Schmidt and Prommer [10] recommended correcting the CO bound to Hb by −1% to account for CO flux to myoglobin.

CO_{newB}. The formula with the latest recommendations for Hb_{mass} estimation with the 2-min procedure measures HbCO 6 and 8 min after the start of the rebreathing period, and allows for a $VCO_{myoglobin}$ of 0.3% × min^{-1} of administered CO [11] (*Formula 7*).

$$CO_{newB}: Hb_{mass}\ (g) = k \times MCO \times 100/\Delta HbCO \times 1.39) \quad (7)$$

For details, see Formula 6 and Formula 3. MCO is additionally reduced with a factor for CO flux to myoglobin (0.3% × min^{-1} of administered CO), which results in an allowance of 2.1%.

For further analysis and comparison with the 12-min approaches, the average of the two estimations of Hb_{mass} from the CO_{new} procedures was used.

Influence of sO_2 on HbCO

The influence of sO_2 on measured HbCO due to sO_2 variations between the procedures was additionally assessed. HbCO plateau levels for the $CO_{original}$ procedure were therefore corrected according to Hütler et al. [18], due to dissimilar plateau sO_2 levels in comparison to the CO_{new} procedure.

Lung residual volume

On the first test day, the lung residual volume (RV) of each subject was measured 30 min after the CO-rebreathing procedure with a helium-dilution method [23] (Masterscreen PFT, Viasys Healthcare GmbH, Hoechberg, Germany). Additionally, the RV was estimated with an age and gender specific formula [24] to assess the importance of actual vs. estimated RV for calculating Hb_{mass}.

Statistics

Differences in Hb_{mass}, ΔHbCO, allowances for CO not bound to Hb, and sO_2 parameters between the different

approaches were evaluated with a multivariate analysis of variance (MANOVA) approach for univariate repeated measures. Bonferroni post hoc tests with control for inflation of family-wise type I error for multiple comparisons were used to evaluate significant pairwise differences between the approaches. The Student's T-test for paired samples was used to analyse differences between measured and estimated residual volume. To compare Hb_{mass} from the two 2-min tests conducted as well as to compare the 12-min procedure calculations for Hb_{mass} with the calculations from the 2-min procedures, Bland and Altman plots [25] were applied.

The reliability of the optimized CO-rebreathing method was evaluated according to Hopkins' method [26]. Briefly, the typical error (TE) for blood volume parameters was calculated as the standard deviation of the different scores divided by $\sqrt{2}$. The CV was calculated as the percentage of TE compared with the mean of the two measurements. All statistical tests were done with the SPSS statistical package 14.0 (SPSS, Chicago, IL). Significance was set as $p < 0.05$. Values are reported as mean ± SD unless otherwise indicated.

Results

Hb_{mass}

The Hb_{mass} estimations differed among the approaches (Wilk's Lambda = 0.005, F(6,11) = 381.04, $p < 0.001$) (Figure 1). The mean Hb_{mass} ranged from 960 ± 133 g (CO_{newB}) to 1053 ± 133 g ($CO_{originalC}$), which corresponds to a 9.7% difference. The Hb_{mass} for $CO_{originalA}$ and $CO_{originalD}$ was identical (993 ± 126 g), and there was no difference compared to $CO_{originalE}$ (989 ± 130 g). The Hb_{mass} of $CO_{originalA}$ was lower than that of $CO_{originalB}$ (1030 ± 130 g; $p < 0.001$) and $CO_{originalC}$ ($p < 0.001$), which exhibited the highest values for Hb_{mass} values among all approaches.

The lowest amounts for Hb_{mass} were observed with CO_{newB}. The values for this approach were different from those for $CO_{orignalA\text{-}D}$ and CO_{newA} ($p < 0.05$) while there was no difference between CO_{newB} and $CO_{originalE}$ ($p = 0.07$).

Figure 2 illustrates the individual differences between the $CO_{original}$ approaches and CO_{newB} in the Bland-Altman plots. A mean bias ± 95% limits of agreement of 33 ± 76 g was apparent for $CO_{originalA,D}$ compared to CO_{newB}. The biases for $CO_{original\ B}$, $CO_{original\ C}$, and $CO_{original\ E}$ were 70 ± 76 g, 93 ± 77 g, and 30 ± 75 g, respectively.

Differences in ΔHbCO, sO_2 and CO volumes not bound to Hb between $CO_{original}$ and CO_{new} approaches

The volume of inspired CO yielded a ΔHbCO of 5.25 ± 0.40% for the 12-min procedure, 5.30 ± 0.41% for the first 2-min procedure and 5.27 ± 0.38% for the second 2-min procedure (Table I). The three ΔHbCO values did not differ (Wilk's Lambda = 0.919, F(2,15) = 0.664, $p = 0.53$). Oxygen saturation of Hb before the rebreathing procedure ($sO_{2\ pre}$) was not dependent on the procedure (Wilk's Lambda = 0.69, F(2,15) = 3.378, $p = 0.062$), while values after the rebreathing procedure ($sO_{2\ post}$) (Wilk's Lambda = 0.14, F(2,15) = 513.2, $p < 0.001$) and the discrepancy between $sO_{2\ pre}$ and $sO_{2\ post}$ both differed (Wilk's Lambda = 0.48, F(2,15) = 148.3, $p < 0.001$). Correcting HbCO of the $CO_{original}$ approaches, due to higher $sO_{2\ post}$ values in comparison to CO_{new}, led to

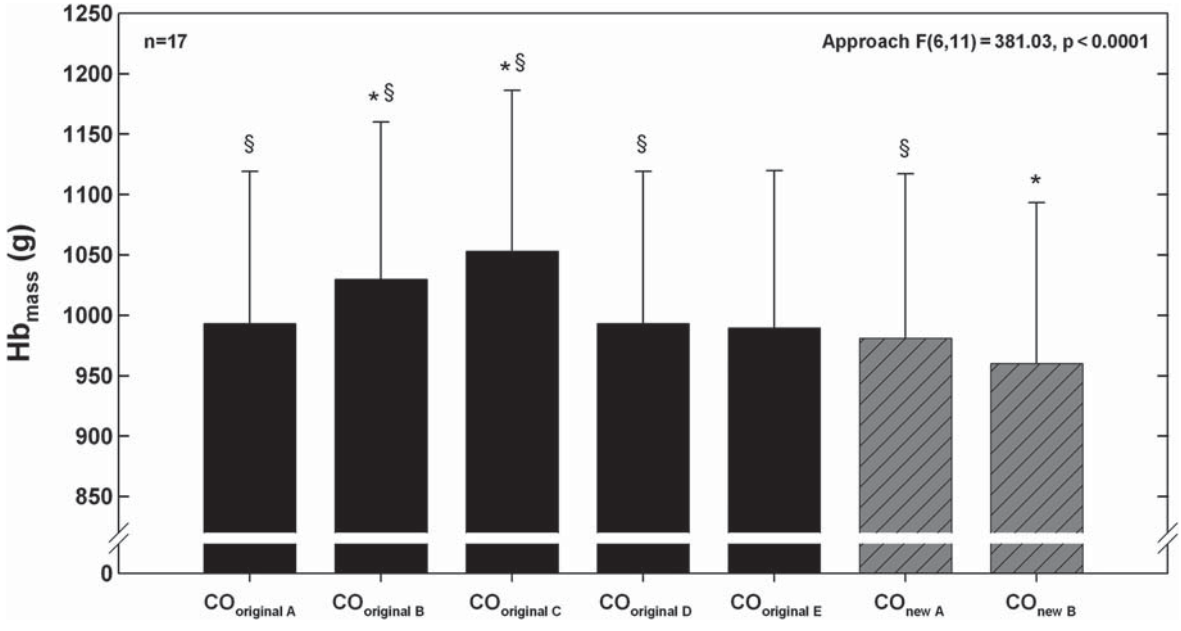

Figure 1. Haemoglobin mass (mean ± SD) for different CO-rebreathing approaches (12-min procedure: $CO_{originalA\text{-}E}$; 2-min procedure: $CO_{newA,B}$). For a detailed description of the approaches, see Methods section. *Significant difference between the approach CO_{newA} and other approaches ($p < 0.01$). §Significant difference between the approach CO_{newB} and other approaches ($p < 0.05$).

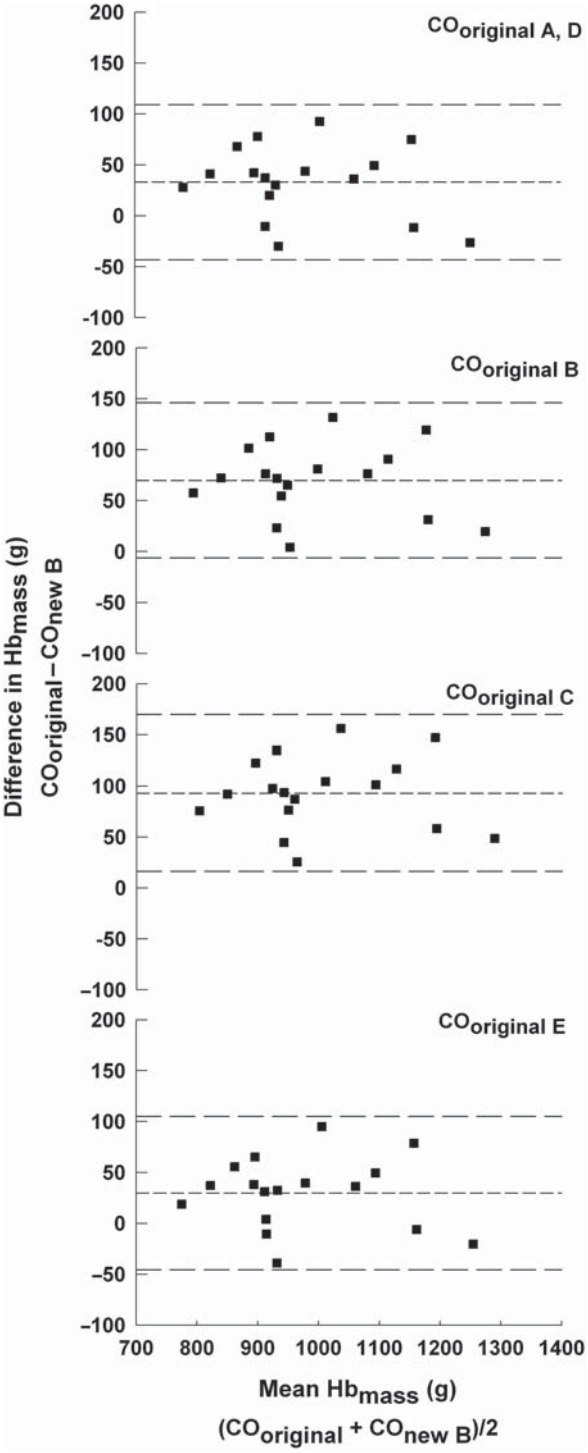

Figure 2. Haemoglobin mass (Hb_{mass}) estimated with the CO_{newB} approach in comparison to the $CO_{original}$ approaches demonstrated in Bland-Altman diagrams ($n = 17$). Short dashed horizontal lines (- - - -) indicate the mean difference between two measurements. Long dashed horizontal lines (– – – –) indicate 95% limits of agreement.

a ΔHbCO of 5.21 ± 0.40%. This value did not differ from the values of the two CO_{new} procedures (Wilk's Lambda = 0.843, F(2,15) = 0.1.40, $p = 0.277$). Corrected values of $CO_{original}$ approaches due to the sO_2 effect for Hb_{mass} were ~0.7% higher than those without correction, and amounted to 1000 ± 127 g for $CO_{originalA\ and\ D}$, 1038 ± 132 g for $CO_{original\ B}$, 1061 ± 135 g for $CO_{original\ C}$, and 997 ± 132 g for $CO_{original\ E}$.

The CO volumes not bound to Hb for the different approaches are described in Table II. The amounts of CO not bound to Hb differed among the approaches (Wilk's Lambda = 0.006, F(4,13) = 526.03, $p < 0.001$).

Measured residual volume (2.03 ± 0.37 L) was higher ($p < 0.001$) than RV predicted with Miller et al.'s formula (1.51 ± 0.13 L). Using the value for the predicted RV reduces the volume of VCO_{system} for the CO_{new} procedure from 1.40 to 1.28 mL ($p < 0.001$), and hence increases the mean Hb_{mass} by 1.35 ± 0.79 g.

Conversion factors for the comparability of Hb_{mass}

Conversion factors for Hb_{mass} between the different approaches are shown in Table III. These factors roughly aid the comparison of average Hb_{mass} between those investigations which did not use the same approach to measure Hb_{mass}.

Discussion

The present study demonstrates that the absolute level of Hb_{mass} depends on the specific approach used. Mean Hb_{mass} for all seven approaches ranged from 960 ± 133 g (CO_{newB}) to 1053 ± 133 g ($CO_{originalC}$), which corresponds to a relevant difference for the absolute Hb_{mass} ranging up to approximately 10%. While we observed only a minor influence of the procedure (ΔHbCO, sO_2) on measured Hb_{mass}, allowances for CO-volumes not bound to Hb varied significantly between the approaches. Thus, they influenced the calculation of the resulting Hb_{mass}.

Differences in Hb_{mass}

We observed differences for Hb_{mass} measured with various CO-rebreathing approaches (of up to 9.7%), which is practically relevant when results of studies utilizing different approaches are compared and reference ranges for athletic purposes should be established. Because estimating Hb_{mass} always depends on the procedure and the subsequent calculations, these two factors can both influence the resulting amount of Hb_{mass}. While the procedures' influence has already been discussed in detail [1,10,11], the influence of diverse calculations has not yet been precisely reported. The influence of the procedure is therefore only discussed briefly, while we focus on the different Hb_{mass} calculations to a greater extent.

Differences due to the procedure

To accurately estimate Hb_{mass}, a reproducible and valid measurement of the difference between the

Table I. Increase in carboxyhemoglobin and change of oxygen saturation caused by the three rebreathing procedures ($n = 17$ for every procedure).

	ΔHbCO	$sO_{2\ pre}$%	$sO_{2\ post}$	ΔsO_2
Procedure	%	%	%	%
$CO_{original}$	5.25 ± 0.40	94.8 ± 0.8	99.7 ± 0.2*§	5.00 ± 0.83*§
CO_{new} 1	5.30 ± 0.41	95.4 ± 0.9	96.7 ± 0.5*	1.28 ± 0.86*
CO_{new} 2	5.27 ± 0.38	95.0 ± 0.6	96.5 ± 0.5§	1.42 ± 0.66§

Values are means ± SD. ΔHbCO and ΔsO2 for $CO_{original}$ procedure: difference between the mean of three initial values and the mean of the values of min 8, 10 and 12 of the rebreathing procedure. ΔHbCO and ΔsO_2 for CO_{new} procedures: difference between the mean of three initial values and the mean of the values at min 6 and 8. $sO_{2\ pre:}$ average for haemoglobin oxygen saturation of blood measurements before the rebreathing procedure. $sO_{2\ post:}$ average for haemoglobin oxygen saturation of blood measurements after the rebreathing procedure. *§Significant difference between the measurements ($p < 0.05$).

baseline and HbCO levels after the rebreathing procedure (ΔHbCO) is a prerequisite. Only when this level of ΔHbCO among diverse procedures is comparable can subsequent calculations of Hb_{mass} lead to similar results. ΔHbCO is mainly influenced by the rebreathing time, the moment of blood sampling after the rebreathing procedure [1,10,11], the amount of CO administered [7,27], and by the oxygen saturation of Hb [18].

ΔHbCO measurements did not differ between procedures in our investigation, which is in line with Schmidt and Prommer's observations [10], although they measured HbCO 4 and 6 min after the rebreathing procedure. For the CO_{new} procedure, we observed slightly higher ΔHbCO values than for the $CO_{original}$ procedure. This small difference in ΔHbCO would decrease an Hb_{mass} of 1000 g calculated with $CO_{originalA}$ (20°C, 920 mbar) to 994 g, in which the procedure explains less than 1% of the differences in the estimated Hb_{mass}. The discrepancy is more pronounced when HbCO values of the $CO_{original}$ procedure are corrected due to the higher sO_2 at the end of the measurement. In our investigation, this adjustment elevated Hb_{mass} for every approach by about 8 g (< 0.8 %). An adjustment of the HbCO values by reason of the sO_2 effect is not the default in the published calculations for capillary blood measurements, and there are small sO_2 variations between the procedures. Therefore, we neglected the influence of sO_2 on HbCO for the development of the conversion factors.

The estimated amount of Hb_{mass} seems to be influenced by the underlying Hb_{mass} calculations to a greater degree than by the procedure.

Differences due to the calculations

$CO_{original}$. Because all $CO_{original}$ approaches used the same measurement, and hence the same individual ΔHbCO values, the differences in Hb_{mass} were derived only from the difference in the Hüfner's number (1.34 vs. 1.39) for the CO-binding capacity of haemoglobin and the assessment of the CO remaining in the spirometer.

Hüfner's number is not obvious for all $CO_{original}$ approaches, because the results are partially stated in either monomeric or tetrameric amounts of Hb in the blood. To assess the Hüfner's number that would have been used to directly estimate Hb_{mass} in grams, the indicated gas constant leads us to the volume of 1 mole of ideal gas at STP (22,414 mL). Because 1 mole of Hb weighs 64,450 grams and combines with 4 moles of CO, the Hüfner's number in the formula would have been 89,656 mL/64,450 g = 1.39 mL × g^{-1} [19,20]. Gore et al. [1] used this number for the $CO_{originalD}$ approach, which is why $CO_{originalA}$ and $CO_{originalD}$ yielded equal amounts of Hb_{mass}.

Nonetheless, Gore et al. [8] previously introduced a formula with a Hüfner's number of 1.34 ($CO_{original\ B}$), which corresponds more to the *in-vivo* oxygen binding capacity of Hb than to the CO-binding capacity [19]. Gore et al.'s approach was adopted by Friedmann et al. [28] and Heinicke et al. [9], with no allowances for CO remaining in the system ($CO_{original\ C}$).

The relevant influence of a lower Hüfner's number on estimated Hb_{mass} becomes obvious when $CO_{originalA,D}$ is compared to $CO_{originalB}$. Estimates for Hb_{mass} for $CO_{originalB}$ were about 4% higher than estimated with the $CO_{originalA,D}$ approaches.

Table II. Carbon monoxide volumes not bound to haemoglobin for different CO-rebreathing approaches.

	VCO_{system}	$VCO_{exhalation}$	$VCO_{myoglobin}$	$VCO_{not\ bound\ to\ Hb}$
Approach	mL	mL	mL	mL
$CO_{original\ A,\ B,\ D}$	2.0 ± 0.2			2.0 ± 0.2§
$CO_{original\ C}$				0.0 ± 0.0*
$CO_{original\ E}$	2.3 ± 0.5			2.3 ± 0.5
$CO_{new\ A}$	1.4 ± 0.3	1.1 ± 0.1		2.5 ± 0.3
$CO_{new\ B}$	1.4 ± 0.3	1.1 ± 0.1	1.9 ± 0.2	4.4 ± 0.4*

Values are means ± SD. VCO_{system}, CO volume remaining in the spirometer system and the residual volume. $VCO_{exhalation}$, CO volume exhaled between min 2 and 7 for the optimized rebreathing procedure. $VCO_{myoglobin}$, CO flux from blood to myoglobin (extravascular tissue) during the rebreathing period (0.3% × min^{-1} of administered CO) [11]. $VCO_{not\ bound\ to\ Hb}$, sum of the CO volumes not bound to Hb (VCO_{system}, $VCO_{exhalation}$, $VCO_{myoglobin}$). *Significant difference for $VCO_{not\ bound\ to\ Hb}$ compared to all of the other approaches ($p < 0.001$). §Significant difference for $VCO_{not\ bound\ to\ Hb}$ between $CO_{originalA,B,D}$ and CO_{newA} ($p < 0.001$).

Table III. Conversion factors for haemoglobin mass for different CO-rebreathing approaches (as a combination of the rebreathing procedure and the subsequent calculation of Hb_{mass}).

Approach	$CO_{original\ A}$	$CO_{original\ B}$	$CO_{original\ C}$	$CO_{original\ D}$	$CO_{original\ E}$	$CO_{new\ A}$	$CO_{new\ B}$
$CO_{original\ A}$	–	0.964	0.943	1.000	1.003	1.012	1.034
$CO_{original\ B}$	1.037	–	0.978	1.037	1.041	1.050	1.073
$CO_{original\ C}$	1.061	1.023	–	1.061	1.064	1.073	1.097
$CO_{original\ D}$	1.000	0.964	0.943	–	1.003	1.012	1.034
$CO_{original\ E}$	0.997	0.961	0.940	0.997	–	1.009	1.031
$CO_{new\ A}$	0.988	0.953	0.932	0.988	0.991	–	1.022
$CO_{new\ B}$	0.967	0.932	0.912	0.967	0.970	0.978	–

Conversion factors based on experimental measurements for haemoglobin mass (Hb_{mass}) for different approaches (see Methods for further details). Hb_{mass} that is measured with a specific approach (column header) can be multiplied by the number in this column to obtain comparable data to the approaches in the row header.

If the spirometer volume and CO concentration in the spirometer after the rebreathing period are not measured, then the proposed allowance of 2.2% of the administered CO used for the approaches $CO_{originalA,B,D}$ seems to be a good approximation of the true amount of CO remaining in the system ($CO_{originalE}$) (Table II). Therefore, measuring CO concentration in the spirometer led to only slightly lower Hb_{mass} values (-3.4 ± 6.7 g) for $CO_{originalE}$ compared to those for $CO_{originalA,D}$. Without an allowance for VCO_{system}, an additional 23.2 ± 2.9 g (+2.3%) of Hb_{mass} ($CO_{originalC}$ vs. $CO_{originalB}$) was observed. The combination of a Hüfner's number of 1.34 and not to account for CO remaining in the spirometer ($CO_{originalC}$) yielded 6.1% higher amounts of Hb_{mass} compared to $CO_{originalA,D}$.

CO_{new}. Due to the fact that the optimized CO-rebreathing procedure lasts only 2 min, exhalation of CO has to be considered. The allowance for CO exhalation is influenced by alveolar ventilation assumptions and the precision of measuring the subject's end-tidal CO concentration. With an alveolar ventilation of 5.25 L/min [16] and the measurement of the end-tidal CO concentration before and 4 min after the inhalation of the CO bolus, an allowance of 1.1 ± 0.1 mL was made. Prommer and Schmidt [11] observed a CO exhalation of 0.23 ± 0.9 mL/min when measuring the volume of CO exhaled in Douglas bags after the CO_{new} rebreathing procedure. This ratio would yield an allowance of 1.15 mL for the CO_{new} procedure (5 min $\times$ 0.23 mL/min), which is almost equal to the measured value. For practical reasons, Prommer and Schmidt [11] therefore recommended that $VCO_{exhalation}$ should be calculated via measurement of the end-expiratory CO concentration and alveolar ventilation estimation rather than measuring $VCO_{exhalation}$ directly.

Because $VCO_{exhalation}$ was subtracted from the CO administered for both CO_{new} approaches, the significant difference for Hb_{mass} (21.1 g) between CO_{newA} and CO_{newB} must be due to the additional allowance for CO flux to myoglobin. Based on a publication by Bruce and Bruce [27], who predicted the rate of uptake of carbon monoxide from blood by extravascular tissue with a multicompartment model, Schmidt and Prommer [10] recommended correcting the CO volumes bound to Hb by −1%. Gore et al. [1] increased the allowance to 2%, while the latest recommendations have used a correction factor of 0.3% $\times$ min^{-1} of administered CO [11]. These recommendations have more recently been supported with enhanced models that have confirmed the compatibility of the range of blood-tissue conductance for CO with Prommer and Schmidt's observed values [29,30].

The allowance for $CO_{myoglobin}$ calculated with the recommended correction factor averaged 1.9 ± 0.2 mL of administered CO. This loss of CO during the CO_{new} procedure was slightly higher than that calculated by Prommer and Schmidt (1.68 mL) [31], primarily due to the fact that we administered 1.2 mL CO/kg, in comparison to the 1.0 mL CO/kg chosen by Prommer and Schmidt [31]. The opposing development of a decreasing MCO while ΔHbCO remains constant was responsible for a lower Hb_{mass} calculated with CO_{newB}. Prommer and Schmidt [11] observed quite similar differences (22 g, mean Hb_{mass} 950 g) between two CO_{new} approaches (one with an allowance for $VCO_{myoglobin,}$ the other without).

$CO_{original}$ vs. *CO_{new}*. The two previous paragraphs illustrate that variations of Hb_{mass} are mainly due to different Hüfner's numbers as well as varying allowances for CO not bound to Hb. While there is no relevant difference (< 1%) on the procedure side (when ΔHbCO is not adjusted), the volumes for CO not bound to Hb differed significantly among the approaches (Table II). We revealed differences for $VCO_{not\ bound\ to\ Hb}$ ranging from 0 to 4.4 mL of administered CO, which has a relevant influence on the subsequent estimation of Hb_{mass}. The CO administered for CO_{new} approaches is at least always reduced by VCO_{system} and $VCO_{exhalation}$. Therefore, the lower values for Hb_{mass} estimated with CO_{new} are a combination of a slightly higher ΔHbCO and a lower volume of MCO. Measuring the blood 8 and 10 min after the inhalation of the CO bolus of CO_{new}, as recommended by Gore et al. [1], probably would

have provided more identical results, as no obvious HbCO plateau is reached after CO inhalation when the CO_{new} procedure is used, in contrast to the $CO_{original}$ procedure [11]. Our own unpublished observations revealed that measuring HbCO at min 8 and 10 instead of min 6 and 8 after CO bolus inhalation would reduce ΔHbCO by about 0.1%, and hence increase the estimated Hb_{mass} by about 1–2%, which corresponds to Gore et al.'s results [1]. Considering the higher volume of CO exhaled in the procedure's 2 additional min, the differences between the CO_{newA} and $CO_{originalA,D,E}$ approaches would become negligible. These considerations for Hb_{mass} indicate that the $CO_{original}$ and CO_{new} approaches produce similar results when we used the same Hüfner's number of 1.39, an allowance for CO remaining in the spirometer, and an allowance for CO exhalation (CO_{new}). This is in accordance with the results of the only two studies comparing CO_{new} and $CO_{original}$ approaches [1,10], and using formulas with these underlying characteristics. However, Schmidt and Prommer [10] described performing the $CO_{orignal}$ method according to Heinicke et al. [9], which would have yielded significant differences between the approaches.

The original approaches never used an allowance for $VCO_{myoglobin}$ because Thomsen et al. [6] and Burge and Skinner [7] argued that the extravascular loss of CO to myoglobin is negligible and no corrections are required.

For the CO_{new} method, the CO flux to myoglobin during the rebreathing procedure was quantified [11], and it was confirmed that there is a loss of CO to myoglobin, which was supported by models of whole-body uptake and the distribution of CO in humans [27,30]. Therefore, the most recent recommendations for estimating Hb_{mass} for CO_{new} include an allowance for $CO_{myoglobin}$. Using the same $VCO_{myoglobin}$ factor ($0.3\% \times min^{-1}$) for the $CO_{originalD}$ approach as the CO_{newB} approach would result in an Hb_{mass} of 959 ± 127 g, which is almost identical in comparison to the CO_{newB} approach (960 ± 133 g).

Measuring RV for VCO_{system} estimation seems to be of minor importance when allowances for VCO_{system} to estimate Hb_{mass} are required. Although the RV differed significantly between the measured and predicted values (average difference 0.52 L, maximal difference: 1.01 L), the RV's influence on estimated Hb_{mass} seems to be too small for measurement of the RV with a time-consuming method. Either an assumption [10] or an estimation of the RV with a formula [24] for the estimation of CO_{system} is absolutely sufficient because the differences between calculations of Hb_{mass} with the measured or estimated RV are less than 0.15% (maximal individual difference: 0.27%).

This underlines the conclusion that $CO_{original}$ and CO_{new} result in almost identical estimations of Hb_{mass} when the calculation parameters are the same. Furthermore, a change in either a parameter or factor (e.g., $CO_{myoglobin}$) for the Hb_{mass} calculation only yields a systematic shift of the Hb_{mass} and blood volume parameters.

Conversion factors for Hb_{mass}

Due to the observed differences among the various approaches for measuring Hb_{mass}, our intention was to provide conversion factors that facilitate the comparability of mean Hb_{mass} values from one specific approach to another. Although several approaches used for this investigation are no longer utilised and the conversion factors are only accurately valid when the experimental conditions are essentially the same, the factors in Table III should provide a simple tool to compare the average Hb_{mass} values measured and calculated with different approaches. It should be emphasized that such factors are only valid for mean value comparisons because they can never consider factors' inter-subject variability (e.g. CO flux to myoglobin [11]).

Because these conversion factors are proportional, they are also applicable to relative haemoglobin mass, as the reference values are primarily reported in relative Hb_{mass} (per kg body weight). As an example, mean Hb_{mass} values from Gore et al.'s investigation [8] ($CO_{originalB}$) must to be reduced by approximately 6–7% (factor = 0.932) to make them comparable to estimations with the optimized approach CO_{newB} (Table III).

Most valid CO-rebreathing approach for measuring Hb_{mass}

There is currently no gold standard for measuring Hb_{mass}. Thus, we do not have reliable reference data for the validity of the CO-rebreathing approaches under investigation in this study. Therefore, we cannot determine the most effective approach with certainty.

Fogh-Andersen et al. [3] and Thomsen et al. [6] were able to find excellent agreement (r = 0.97) between blood volume measured simultaneously with the original CO rebreathing approach ($CO_{originalA}$) and a method that labels radioactively erythrocytes (^{99m}Tc) [6]. Good agreement of $CO_{originalA}$ has been confirmed with additional validation studies that used different labelling techniques (^{51}Cr to measure the RCV [33–35], Evans' blue or labelling of albumin with ^{125}I [36–38] to measure PV). The accuracy of all other approaches used in our investigation has never been approved with a comparison to criterion methods for estimating red cell volume of the International Committee for Standardization in Haematology [32].

CO_{new} was therefore compared with $CO_{originalA}$ by Schmidt and Prommer [10] and by Gore et al.

[1], and both groups affirmed good accordance between CO_{newA} and $CO_{originalA}$ as observed in the present investigation. Adequate precision and sensitivity of the optimized method was further confirmed with several investigations that measured Hb_{mass} before and after a blood donation and then compared the measured loss of Hb_{mass} with the calculated loss of Hb_{mass} [10,39–41].

Presently, we do not know which approach leads to the most valid estimation of Hb_{mass}. Nonetheless, assuming that each new approach added more suitable constants and correction factors to the previous, and thereby improves the preciseness and reliability, we recommend either: the use of the already widely used 'optimized' procedure with calculations for CO_{newB} or the use of the $CO_{original}$ with an allowance for the $VCO_{myoglobin}$ in the range of 1% of the administered CO volume per 5 min of rebreathing [1].

Conclusion

Hb_{mass} that is measured and calculated with various CO-rebreathing approaches varies up to approximately 10%. These differences primarily stem from the different calculations for Hb_{mass}, and less from the variety of CO-rebreathing procedures used. This observation suggests that, to compare the mean Hb_{mass} values of different investigations, either the presently developed conversion factors can be used or the same basic principles for calculating Hb_{mass} should be applied. Furthermore, the exact descriptions of the procedure and the calculations for estimating Hb_{mass} in the methods section of a paper are prerequisites for enhancing the comparability of study results.

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