Research Paper

Computational analyses of CO-rebreathing methods for estimating haemoglobin mass in humans

Kinnera E. Chada and Eugene N. Bruce

Center for Biomedical Engineering, University of Kentucky, Lexington, KY, USA

Measurement of haemoglobin mass (M_{Hb}) is used to quantify alterations in oxygen delivery during exercise training or acclimatization to altitude. Uptake of carbon monoxide by haemoglobin is the basis of the common non-radioactive methods to determine M_{Hb} in humans. This study used a validated mathematical model to simulate CO uptake during rebreathing protocols and to determine sources of errors in estimation of M_{Hb}. Our previously published model was validated using experimentally measured carboxyhaemoglobin levels (%HbCO) from arterial, capillary and venous blood sites of human subjects during CO-rebreathing protocols. This model was then used to simulate various CO-rebreathing protocols in 24 human subjects with known M_{Hb}. Using variables generated by the model, M_{Hb} was estimated on the basis of assumptions typically made for calculating the volume of CO bound to myoglobin, the volume of CO exhaled and the volume of CO in the rebreathing system. It was found that inaccurate estimation of the volume of CO bound to myoglobin was the major source of error in determination of M_{Hb}. Additionally, the size of the error was found to depend on the site of blood sampling because of differences in %HbCO. Regression equations were developed to improve the estimation of volume of CO bound to myoglobin, and a new protocol that is less dependent on the site of blood sampling is proposed.

(Received 13 May 2011; accepted after revision 20 September 2011; first published online 23 September 2011)
Corresponding author E. N. Bruce: Center for Biomedical Engineering, University of Kentucky, Lexington, KY 40506-0070 USA. Email: ebruce@uky.edu

In clinical and sports medicine, measurements of haemoglobin mass (M_{Hb}) are used to determine the effects of adaptation to exercise training or environmental stresses, or the effects of illness or traumatic blood loss, on oxygen-carrying capacity of the blood. Determination of M_{Hb} from injection of a radioactive marker, such as ^{51}Cr, is the most reliable approach, but this approach cannot be used for sequential measurements. Recently, Gore *et al.* (2005) concluded that the determination of M_{Hb} from the uptake of carbon monoxide during rebreathing of a mixture of CO and oxygen has an error comparable to that of the radioactive methods. Methods that use CO uptake depend on the strong binding of CO to haemoglobin (Hb), and Hb mass is estimated as a ratio of the change in the amount of CO bound to haemoglobin to the change in carboxyhaemoglobin (HbCO) level in the blood. Two concerns limit the accuracy of these methods. First, one must determine the amount of CO bound to Hb (V_{COHb}) indirectly by accounting for all of the CO lost from the rebreathing circuit that is not bound to Hb. Second, this estimate of V_{COHb} is correct only if all of the blood has the same percentage carboxyhaemoglobin (%HbCO) as that at the site of blood sampling. That is, the HbCO concentration must be equilibrated throughout the vascular system.

In the various CO-rebreathing methods, a known volume of CO (V_{CO_total}) is first injected into a bag (or spirometer) containing 100% O_2. During the subsequent rebreathing, administered CO is taken up by pulmonary blood flow and can either remain in the vascular space, primarily as HbCO, or diffuse into extravascular compartments (Bruce & Bruce, 2003). In some published protocols, rebreathing is followed by a period of spontaneous breathing of room air, during which CO is removed from the body by exhalation (Schmidt & Prommer, 2005). When equalization of arterial and venous HbCO levels occurs, CO is assumed to be well mixed in the vascular space, and a blood sample is acquired 1–2 min

DOI: 10.1113/expphysiol.2011.059436

later for measurement of %HbCO (Burge & Skinner, 1995; Hutler *et al.* 2000; Schmidt & Prommer, 2005; Garvican *et al.* 2010).

In practice, after mixing of CO in the vascular space is deemed to be complete at time $t = t_{mix}$, M_{Hb} is calculated via eqn (1), assuming that a blood sample is acquired at a later time, $t = t_{sample}$, as follows:

$$M_{Hb} = K V_{COHb} \left(\frac{100}{1.58 \Delta \%HbCO} \right) \quad (1)$$

where K is a unit conversion factor [$K = 1$ when all values are at body temperature and pressure when saturated with water vapour (BTPS)], Δ%HbCO is the change in %HbCO between the start of rebreathing ($t = t_0$) and t_{sample} and 1.58 is Hüfner's constant (maximal millilitres of CO per gram of Hb at BTPS; Gorelov, 2004). The parameter V_{COHb} is the change in volume of CO bound to Hb and is calculated as follows:

$$V_{COHb} = V_{CO\_total} - V_{CO\_Lungs+System} - V_{CO\_exhaled} - V_{COMb} \quad (2)$$

where $V_{CO\_Lungs+System}$ is the volume of CO remaining in the lungs and rebreathing circuit at the time of blood sampling, $V_{CO\_exhaled}$ is the volume of CO exhaled up to the time of blood sampling and V_{COMb} is the change in volume of CO bound to myoglobin (Mb) between t_0 and t_{sample}. All values are expressed in BTPS units.

Experimentally, current analysis methods are not reliable to determine differences between %HbCO levels of less than 0.1%. Consequently, any practical method to assess the time at which vascular mixing is complete (t_{mix}) will be likely to underestimate the true mixing time. Thus, the sampling time (t_{sample}) is usually taken as 1–2 min after t_{mix}. The correct values for t_{mix} or t_{sample} are debated in the literature (Schmidt & Prommer, 2005; Gore *et al.* 2006); estimated t_{mix} values range from 3 to 12 min (Burge & Skinner, 1995; Schmidt & Prommer, 2005; Garvican *et al.* 2010). One anticipates that the duration of CO rebreathing, the volume of CO administered, the site of blood sampling and variability among subjects may be some of the factors contributing to the wide range of t_{mix} values reported in different studies. In addition, for a given subject the values of Δ%HbCO from typical sites for blood sampling (arterial, capillary and venous) are different, resulting in different estimates of M_{Hb} (Garvican *et al.* 2010). Such disparities must reflect the fact that CO content in the vascular system is not uniform, i.e. mixing is not complete, and it is unclear which site for blood sampling best reflects the %HbCO that will prevail when mixing is complete. Furthermore, the calculation of V_{COHb} is dependent on determining accurate values of $V_{CO\_Lungs+System}$, $V_{CO\_exhaled}$ and V_{COMb}. Various experimental approaches to resolving these issues have been attempted (Schmidt & Prommer, 2005; Gore *et al.* 2006; Prommer & Schmidt, 2007; Garvican *et al.* 2010), but all suffer from the limitation that the M_{Hb} of the subjects was not determined by an accurate standard method.

Prior to 2007, estimation of M_{Hb} from CO-rebreathing methods assumed no loss or minimal loss of CO (e.g. 1% of V_{CO_total}) from the blood to Mb (Burge & Skinner, 1995; Hutler *et al.* 2000; Schmidt & Prommer, 2005; Gore *et al.* 2006). However, Bruce & Bruce (2003) used their multicompartment model to simulate a CO-rebreathing method (Burge & Skinner, 1995) and concluded that Mb in muscle tissue binds significant amounts of CO. Inspired by these findings, Prommer & Schmidt (2007) derived a formula to estimate V_{COMb} and concluded that ~2% of V_{CO_total} is bound to Mb. This result was derived on the assumption that there is a continuous, non-varying flux of CO from blood to the tissues containing Mb. The errors introduced in determination of M_{Hb} due to using this formula are not known; however, underestimation of CO lost from Hb to Mb will lead to an overestimation of M_{Hb}. Additionally, the published CO-rebreathing methods make various untested assumptions in calculating $V_{CO\_Lungs+System}$ (Burge & Skinner, 1995) and $V_{CO\_exhaled}$ (Schmidt & Prommer, 2005). Conceptually, errors in determining these variables may lead to additive or compensatory effects on errors in estimated M_{Hb}.

Thus, the main aim of the present study was to determine sources of errors in estimation of M_{Hb} by using a validated mathematical model to simulate common CO-rebreathing protocols (Burge & Skinner, 1995; Schmidt & Prommer, 2005) for a variety of healthy subjects. Initially, for validation purposes, experimentally measured %HbCO levels acquired from three different blood sites (arterial, capillary and venous) in nine healthy human subjects during two commonly used CO-rebreathing protocols (Garvican *et al.* 2010) were compared with the %HbCO levels calculated by the model when each subject was simulated individually. Then, for evaluating magnitudes and sources of errors, we simulated the CO-rebreathing protocols in each of 15 healthy subjects (Benignus *et al.* 1994) for whom we had individual values for many of the major physiological parameters (Bruce & Bruce, 2006; Erupaka *et al.* 2010). Also, a new CO-rebreathing method that is predicted to estimate Hb mass with low errors, independent of the site of blood sampling, is proposed, and modifications to two common CO-rebreathing methods are suggested to improve estimation of M_{Hb}.

Methods

Model description

The mathematical model used in this study is described in detail, with its equations, in previous

publications (Bruce, *et al.* 2008; Erupaka *et al.* 2010) and in the online Supplemental material for this paper. The model predicts time-varying %HbCO levels, carboxymyoglobin (%MbCO) levels and O_2 tensions in various tissue and blood compartments for a variety of CO exposure conditions. The present model comprises several uniformly mixed compartments, including one identified as 'non-muscle', which represents all of the body tissues that do not contain myoglobin. The model is an extension of an earlier one which introduced the concept of representing a single, uniform muscle tissue compartment as two tissue subcompartments interacting with three vascular subcompartments, the latter assumed to represent the arterial, capillary and venous blood pools in muscle tissue (Bruce *et al.* 2008). Both models were shown previously to reproduce experimental data from a transient CO exposure (Benignus *et al.* 1994) and from one of the CO-rebreathing methods (Burge & Skinner, 1995). That is, the values for %HbCO predicted by the model for arterial blood and for venous blood of skeletal muscle tissue were in agreement with experimentally measured arterial and venous %HbCO values (Bruce *et al.* 2008; Erupaka *et al.* 2010). It should be noted that the model does not include mass balance analyses for CO_2.

Two common CO-rebreathing protocols were simulated: that of Burge & Skinner (1995), referred to as the Burge protocol, and that of Schmidt & Prommer (2005), which was later modified by Prommer & Schmidt (2007), referred to as the Prommer protocol. The Burge protocol comprises a baseline period of several minutes of breathing 100% O_2, followed by rebreathing for ∼40 min from a bag initially containing ∼50–100 ml of CO [1 ml CO (kg body mass (BM))$^{-1}$] in O_2. The Prommer protocol starts from a baseline of room air breathing, followed by 2 min of rebreathing from a bag initially containing ∼50–100 ml of CO [1 ml CO (kg BM)$^{-1}$] in O_2 (after an exhalation to residual volume), followed by breathing of room air for ∼18 min. In both protocols, soda lime is introduced into the rebreathing system to remove CO_2.

Equations (3–5) were added to the model to determine exact values of the variables V_{COMb}, $V_{\mathrm{CO\_Lungs+System}}$ and $V_{\mathrm{CO\_exhaled}}$ needed for calculating the Hb mass with no errors in these variables, referred to as the exactmodel_M_{Hb}_estimate. The volume of CO bound to myoglobin at time t is calculated in the model from the tissue volumes and MbCO concentrations of skeletal and cardiac muscle subcompartments, as follows:

$$V_{\mathrm{COMb}}(t) = \left\{ \begin{array}{l} V_{\mathrm{m1}}\mathrm{MbCO_{m1}}(t) + V_{\mathrm{m2}}\mathrm{MbCO_{m2}}(t) \\ +V_{\mathrm{cm1}}\mathrm{MbCO_{cm1}}(t) + V_{\mathrm{cm2}}\mathrm{MbCO_{cm2}}(t) \end{array} \right\} \quad (3)$$

where V_{m1} and V_{m2} are tissue volumes of skeletal muscle subcompartments, V_{cm1} and V_{cm2} are tissue volumes of cardiac muscle subcompartments, $\mathrm{MbCO_{m1}}$ and $\mathrm{MbCO_{m2}}$ are time-dependent MbCO concentrations in skeletal muscle subcompartments, and $\mathrm{MbCO_{cm1}}$ and $\mathrm{MbCO_{cm2}}$ are time-dependent MbCO concentrations in cardiac muscle subcompartments. Note that in experimental studies, $V_{\mathrm{COMb}}(t)$ is estimated indirectly in various ways because the MbCO values are not measurable (Gore *et al.* 2006; Prommer & Schmidt, 2007).

The volume of CO exhaled from the end of rebreathing ($t = t_1$) up to time t is given by:

$$V_{\mathrm{CO\_exhaled}}(t) = \int_{t_1}^{t} \dot{V}_{\mathrm{A}}(t) \times C_{\mathrm{A,CO}}(t)\mathrm{d}t \quad (4)$$

where $\dot{V}_{\mathrm{A}}(t)$ is the alveolar ventilation and $C_{\mathrm{A,CO}}(t)$ the alveolar CO concentration. The particular value required is the volume of CO exhaled from the end of rebreathing until the time at which a blood sample is taken, t_{sample}. In experimental studies, this calculation would be equivalent to collecting expired gas and determining how much CO is present; however, instead of collecting expired gas, different investigators approximate this value in various ways (Schmidt & Prommer, 2005; Garvican *et al.* 2010). Note that $V_{\mathrm{CO\_exhaled}}$ is zero in the Burge protocol.

The volume of CO in the lungs + rebreathing circuit at time t is given by:

$$V_{\mathrm{CO\_Lungs+System}} = (V_{\mathrm{RB}} + V_{\mathrm{LV}}) \times C_{\mathrm{A,CO}}(t) \quad (5)$$

where V_{RB} is the volume of the rebreathing circuit and V_{LV} the lung volume at the start of rebreathing (i.e. functional residual capacity for the Burge protocol and residual volume for the Prommer protocol). The value of $V_{\mathrm{CO\_Lungs+System}}$ is required at $t = t_{\mathrm{sample}}$ (Burge protocol) or at the end of rebreathing (Prommer protocol).

Data sets

This study comprised simulations of two separate data sets. The first, from the study by Garvican *et al.* (2010), was a comparative study of two CO-rebreathing methods. It included multiple measurements of %HbCO at several blood-sampling sites during the procedures; these data were used to validate the model for the CO-rebreathing protocols. The second data set is from the study by Benignus *et al.* (1994), in which subjects were exposed transiently to a high CO level. As we had obtained the values of numerous physiological parameters that the investigators had measured in each subject (V. A. Benignus, personal communication), simulations of these subjects were used to analyse the errors in estimated Hb mass in a healthy population.

The Garvican data set. Garvican *et al.* (2010) compared kinetics of CO uptake and distribution in CO-rebreathing

protocols based on those of Burge & Skinner (1995) and Schmidt & Prommer (2005). The Garvican study population consisted of seven healthy men and two healthy women. Two men were cyclists, one man and one woman were distance runners, and one man and one woman were strength trained; the remaining three were recreationally active (L. Garvican, personal communication). Garvican *et al.* (2010) reported the age, body mass, height, volume of CO dose administered and Hb concentrations for each subject. Also provided to us were the time-varying %HbCO levels measured in blood samples taken simultaneously from a forearm artery, a warmed fingertip (arterialized capillary) and a forearm vein at 12 time points from 0 to 40 min during the experiment.

The Benignus data set. Benignus *et al.* (1994) exposed 15 healthy male subjects to 6683 p.p.m. CO for ∼5 min, followed by washout with room air for ∼4 h. Values for age, body mass, height, blood volume, haemoglobin concentration, cardiac output, ventilation, initial %HbCO and lung diffusivity coefficient for CO were provided for each subject (V. A. Benignus, personal communication). In addition, values of %HbCO in arterial and antecubital venous blood samples were reported at 11 time points. We have previously demonstrated that our model can quantitatively reproduce these experimental measurements of %HbCO from a given subject when this transient CO exposure is simulated using the physiological parameters measured in that subject (Bruce & Bruce, 2006; Bruce *et al.* 2008; Erupaka *et al.* 2010).

Model validation studies

To validate the ability of the model to estimate %HbCO levels in specific blood compartments during CO-rebreathing protocols, we simulated each subject of Garvican *et al.* (2010) individually. In the model, total blood volume (V_B) of each subject was adjusted so that the product of V_B and Hb concentration (provided by L. A. Garvican) equalled the M_{Hb} reported for the subject. For both simulated protocols, using M_{Hb} calculated by Garvican *et al.* (2010) from the Burge and Skinner method resulted in better agreement between the model predictions of %HbCO levels and the experimental data than did M_{Hb} values determined from the Schmidt and Prommer method; therefore, we used the former values. The rebreathing bag volume, ambient temperature and ambient pressure were set to the experimentally measured values. Total body oxygen consumption was assumed to be 3.2 ml kg^{-1}, and cardiac output was estimated from a regression equation (Equation C.3 in Appendix C of Erupaka *et al.* 2010).

Our previous models were developed to simulate normal subjects (Bruce *et al.* 2008; Erupaka *et al.* 2010). However, reports in the literature indicate that capillary density, mitochondrial content, heart rate and stroke volume at rest differ significantly in athletically trained *vs.* non-athletic subjects (Andersen & Henriksson, 1977; Brodal *et al.* 1977; Tibes *et al.* 1977; Ingjer, 1979; Kalliokoski *et al.* 2001; Zoladz *et al.* 2005; Sagiv *et al.* 2007). Thus, to simulate Garvican's subjects, all of whom exercised regularly, the muscle diffusion coefficient of CO ($D_{m,CO}$) and capillary density of the skeletal muscle were increased by 34% (Brodal *et al.* 1977; Zoladz *et al.* 2005). The $D_{m,CO}$ was also scaled in proportion to muscle mass, based on an elevated (by 34%) normalized value of 0.302 ml min^{-1} mmHg^{-1} kg^{-1} (Bruce *et al.* 2008). Resting cardiac output was not assumed to be elevated, but a lowered heart rate of 51 beats min^{-1} was assumed (Tibes *et al.* 1977; Kalliokoski *et al.* 2001; Sagiv *et al.* 2007) to estimate myocardial oxygen consumption and myocardial blood flow (Erupaka *et al.* 2010). Other parameters at rest, such as muscle blood volume, muscle blood flow, cardiac output, metabolic rate or ventilation, did not differ statistically between the trained and untrained groups (Tibes *et al.* 1977; Kalliokoski *et al.* 2001; Sagiv *et al.* 2007). Values for these and other remaining parameters were either provided by the investigators, as noted above, or were those used and referenced in our previous publications (Bruce *et al.* 2008; Erupaka *et al.* 2010).

Our model does not specifically represent the typical sites for blood sampling in experimental studies (i.e. the radial artery, antecubital vein or fingertip capillaries); therefore, we assumed that certain compartments in the model could represent the following sites: the arterial compartment of the model was assumed to represent all arterial blood (subscript 'art'); blood entering the second vascular subcompartment of skeletal muscle tissue (Bruce *et al.* 2008) was assumed to be 'arterialized capillary blood' and thus the equivalent of prewarmed fingertip blood (subscript 'cap_mus'); venous blood exiting from the skeletal muscle compartment was assumed to represent antecubital vein blood (subscript 'ven_mus'); and venous blood from the non-muscle tissue compartment was assumed to represent earlobe blood (subscript 'ven_nonmus'). Model estimates were compared to Garvican's measurements of $\%HbCO_{art}$, $\%HbCO_{cap\_mus}$ and $\%HbCO_{ven\_mus}$ at 12 time points for each of the nine subjects for both the Burge and Skinner and the Schmidt and Prommer protocols. These comparisons provide the primary validation of the capability of the model to reproduce the temporal variations in CO uptake and distribution during CO-rebreathing protocols.

In addition, Hb mass was estimated from the simulations both by directly calculating V_{COHb} based on eqns (3–5) and by using the experimental approximations for $V_{CO\_Lungs+System}$, $V_{CO\_exhaled}$ and V_{COMb} as implemented by Garvican, *et al.* (2010). The 'exact model-estimated'

Hb mass estimates (exactmodel_M_{Hb}_estimate) were based on exact values calculated using eqns (1–5) at the t_{sample} and the blood sites specified by the investigators. That is, no assumptions or approximations were made concerning $V_{CO\_Lungs+System}$, $V_{CO\_exhaled}$ or V_{COMb}. These results established the smallest errors that can be expected in the estimates of Hb mass for the given t_{sample} and sampling site. Finally, the experiment-based Hb mass estimates (experiment_M_{Hb}_estimate) were calculated using the t_{mix}, t_{sample}, blood sites and approximations to eqns (3–5) specified by Garvican *et al.* (2010). Values obtained for exactmodel_M_{Hb}_estimate and experiment_M_{Hb}_estimate were compared with the Hb mass specified in the model for each subject.

Analysis of errors in estimates of M_{Hb}

Both protocols were simulated for each of the 15 subjects in the Benignus *et al.* (1994) data set. For each individual subject, corresponding parameters in the model were set equal to physiological parameters that had been measured in that subject. As blood volume and haemoglobin concentration had been measured, M_{Hb} in the model for each subject equalled that determined experimentally. Total body oxygen consumption was assumed to be 3.2 ml kg^{-1}. The $D_{m,CO}$ was scaled in proportion to muscle mass, using a normalized value of 0.225 ml min^{-1} mmHg^{-1} kg^{-1} (i.e. $D_{m,CO} = 7.2$ for a 'typical' muscle mass of 32 kg in a 70 kg male subject). Values for other parameters are given in our previous publications (Bruce *et al.* 2008; Erupaka *et al.* 2010). The volume of CO administered to a given subject was scaled by body mass and was the same in both protocols (i.e. 1 ml CO (kg BM)$^{-1}$).

The model was used to determine the uptake and distribution of CO and the time-varying values of %HbCO in the various vascular compartments, and the simulation results for each subject were analysed to calculate exactmodel_M_{Hb}_estimate and experiment_M_{Hb}_estimate, which were then compared with the Hb mass that was specified as a parameter of the model. The first comparison ensured that, for each subject, the approach implemented in the equations would yield small errors if exact values for the variables were used. The second comparison identified errors in the estimation of M_{Hb} as it is done in the laboratory. The major sources of error were determined by evaluating the effects of errors in $V_{CO\_Lungs+System}$, $V_{CO\_exhaled}$ and V_{COMb} on Hb mass estimates.

To determine t_{mix} for each subject, the time-dependent differences between the values of %HbCO of art, cap_nonmus, cap_mus and ven_mus blood compartments, considered pairwise, were plotted along with reference lines at ±0.1. The longest time at which any pairwise difference last crossed (and remained within) the ±0.1 reference lines was considered as t_{mix}. The t_{sample} was then set to 1.5 min after t_{mix}.

Next, to estimate M_{Hb} from eqn (1), the change in volume of CO bound to Hb (V_{COHb}) was calculated for each blood sampling site using the basic equation (eqn 2), in which the rightmost three terms were evaluated using one of two different approaches. On the one hand, V_{COHb} was calculated using exact values from model eqns (3–5), referred to as exactmodel_V_{COHb}. On the other hand, the formulae (eqns 6–8) published by the authors of the Burge and the Prommer protocols were used to determine the experiment-based estimate, experiment_V_{COHb}. Note that these latter formulas are not the same for the two protocols, as indicated below.

In the Burge protocol, the authors assumed that $V_{CO\_Lungs+System}$ is 2.2% of the volume of CO injected into the rebreathing circuit, as follows:

$$V_{CO\_Lungs+System} = \left(\frac{2.2}{100}\right) \times V_{CO\_total} \quad (6)$$

where V_{CO_total} is the total volume of CO administered.

In the Prommer protocol, $V_{CO\_Lungs+System}$ was estimated based on eqn (5) evaluated at $t = 2$ min, the time at which rebreathing ends.

In the Burge protocol, $V_{CO\_exhaled}$ is zero, whereas in the Prommer protocol the following equation was used (Schmidt & Prommer, 2005):

$$V_{CO\_exhaled} = \dot{V}_A \times \Delta t \times C_{A,CO,t=20} \quad (7)$$

where $\dot{V}_A$ is the alveolar ventilation (assumed to be 5 l min^{-1}), Δt is $t_{sample} - 2$ and $C_{A,CO,t=20}$ is the alveolar CO concentration at $t = 20$ min.

The value of V_{COMb} was estimated using eqn (8) in both of the protocols (Prommer & Schmidt, 2007; Garvican *et al.* 2010), as follows:

$$V_{COMb} = V_{COMb\left(t_{mix} - t_{sample}\right)} \times \left(\frac{t_{sample}}{t_{sample} - t_{mix}}\right)$$

where

$$V_{COMb\left(t_{mix} - t_{sample}\right)} = \left(V_{CO\_total} - V_{Lungs+System} - V_{CO\_exhaled}\right)_{t_{sample}} - \left(\frac{\Delta HbCO_{t_{sample}}}{\Delta HbCO_{t_{mix}}}\right) \times \left(V_{CO\_total} - V_{Lungs+System} - V_{CO\_exhaled}\right)_{t_{mix}} \quad (8)$$

ACSL^{TM} version 11.8 (AEgis Technologies Group, Huntsville, AL, USA) was used for implementing the model. For numerical integration, a Runge–Kutta–Fehlberg variable step-size algorithm with error flagging was used, and the maximal allowable step size was 0.001 min. A 12 min stabilization period was allowed at the start of every simulation to achieve a stable, steady-state baseline before rebreathing. A separate rebreathing

circuit was not implemented in the model; instead, to simulate rebreathing the lung volume was augmented by the volume of the rebreathing circuit and ventilation was set to zero. Also, for simulating both protocols an oxygen inflow at a rate equal to the metabolic uptake was added to the rebreathing system to avoid hypoxia. It was assumed that CO_2 was absorbed from the rebreathing circuit.

Results

Model validation

The mathematical model was used to simulate the CO-rebreathing experiments described by Garvican *et al.* (2010). The time course of %HbCO levels for a single subject from five blood sites for both protocols are shown in Fig. 1. In both cases, arterial %HbCO peaks within the first 2 min and then decreases. The capillary %HbCO rises initially and then reaches a plateau. Venous %HbCO increases slowly and then reaches values similar to that of capillary %HbCO in both protocols. For the same V_{CO_total}, peak $\%HbCO_{art}$ is higher in the Prommer protocol than in the Burge protocol.

The model reproduces the experimental measurements of the time-varying %HbCO levels in three different blood compartments during the two CO-rebreathing protocols. The model-predicted mean %HbCO ± SD ($n = 9$) values from the arterial, capillary and venous blood compartments at different time points fall within the 95% confidence limits of the experimental data (Fig. 2). For individual subjects, the predicted %HbCO levels showed good agreement with the experimentally measured values at corresponding time points in six of the nine subjects. In three subjects, however, the rise in %HbCO levels of the muscle venous compartment during the initial 5 min was faster than the experimental data for both protocols, but thereafter the predicted and experimental values were similar. In these three subjects, we were able to match the predicted $\%HbCO_{ven\_mus}$ levels to the experimental values by decreasing the muscle blood flow (by 20%) and increasing the blood volume of muscle vascular subcompartment 3 (the muscle venous compartment) by 20% of the total mixed venous blood volume (see Discussion). These parameter values had been based initially on formulae derived from average data from one or more study populations, and it is reasonable to assume that some subjects might deviate significantly from such averages Therefore, for these three subjects only, we changed these parameter values in the manner described above. Thus, the model is able

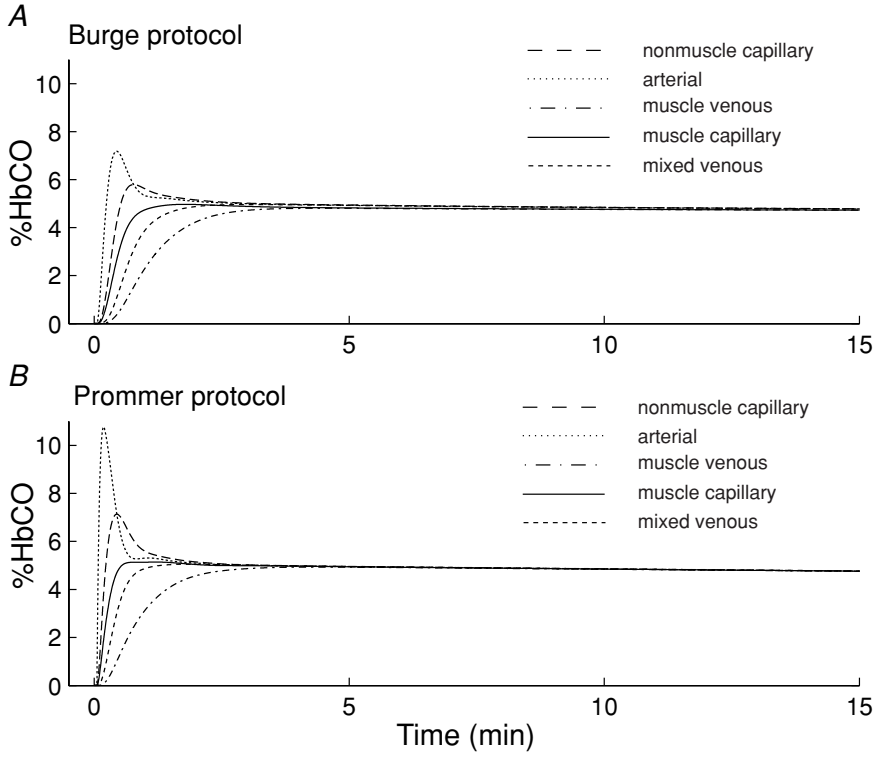

Figure 1. Uptake kinetics of CO in one subject in the Burge protocol (*A*) and the Prommer protocol (*B*)
The percentage carboxyhaemoglobin (%HbCO) level *versus* time in vascular compartments of the model: arterial, muscle capillary, non-muscle capillary, muscle venous and mixed venous. Carbon monoxide rebreathing starts at $t = 0$.

to reproduce the experimental measurements of time-varying %HbCO levels in individual subjects during CO-rebreathing protocols.

A second tier of model validation involved calculating Hb mass estimates for the nine subjects of Garvican *et al.* (2010) using data generated by the model and the t_{sample} and blood sampling sites specified by those investigators. Exactmodel_M_{Hb}_estimate underestimated the Hb mass by $0.32 \pm 0.8\%$ using simulations of the Burge and Skinner protocol and by $2.2 \pm 0.49\%$ using simulations of the Prommer and Schmidt protocol. We conclude that the model is sufficiently accurate to be used as a tool for evaluating methods of estimating Hb mass based on CO rebreathing. Furthermore, both protocols are capable of determining Hb mass with small errors when the indirect evaluation of V_{COHb} is replaced by exact values for $V_{CO\_Lungs+System}$, $V_{CO\_exhaled}$ and V_{COMb}.

In order to determine lower bounds on errors produced by these methods for estimating Hb mass, we sampled the predicted %HbCO values at every minute during the simulations and calculated estimated M_{Hb} using exact values for V_{COHb}, the latter being calculated directly as the sum of the CO content of all the vascular compartments in the model. As shown in the Supplemental material, the Prommer and Schmidt method is theoretically capable of producing Hb mass estimates with errors less than 1% using any blood sampling site after about 7 min into the procedure. The Burge and Skinner method is equally capable (after ~10 min) when blood is sampled from arterial or non-muscle capillary sites; however, the continuing flux of CO from blood into muscle compartments during rebreathing (Fig. 3) prevents equilibration within the time frame of the protocol, causing estimates based on muscle capillary sampling, in particular, to have a systematic error.

As a final validation step, exactmodel_M_{Hb}_estimate was calculated for both CO-rebreathing protocols for each subject of the data set of Benignus using an 'experiment-based' criterion for determining t_{sample}, as described in the Methods. Exactmodel_M_{Hb}_estimates are in close agreement with the measured M_{Hb} of these subjects (Table 1 and Fig. S2), with mean errors less than 2%. These results demonstrate both that the model is internally consistent and that the procedure for determining t_{mix} does not degrade the estimation of Hb mass if exact values for the required variables are available.

Errors in experiment_M_{Hb}_estimates

We first evaluated the accuracy of M_{Hb} estimates in simulations of the study by Garvican *et al.* (2010). When using their t_{sample} values and blood sampling sites, experiment_M_{Hb}_estimates underestimated measured M_{Hb} by $2.2 \pm 0.9\%$ for the Burge and Skinner method and by $5.8 \pm 0.5\%$ for the Prommer and Schmidt method. Note

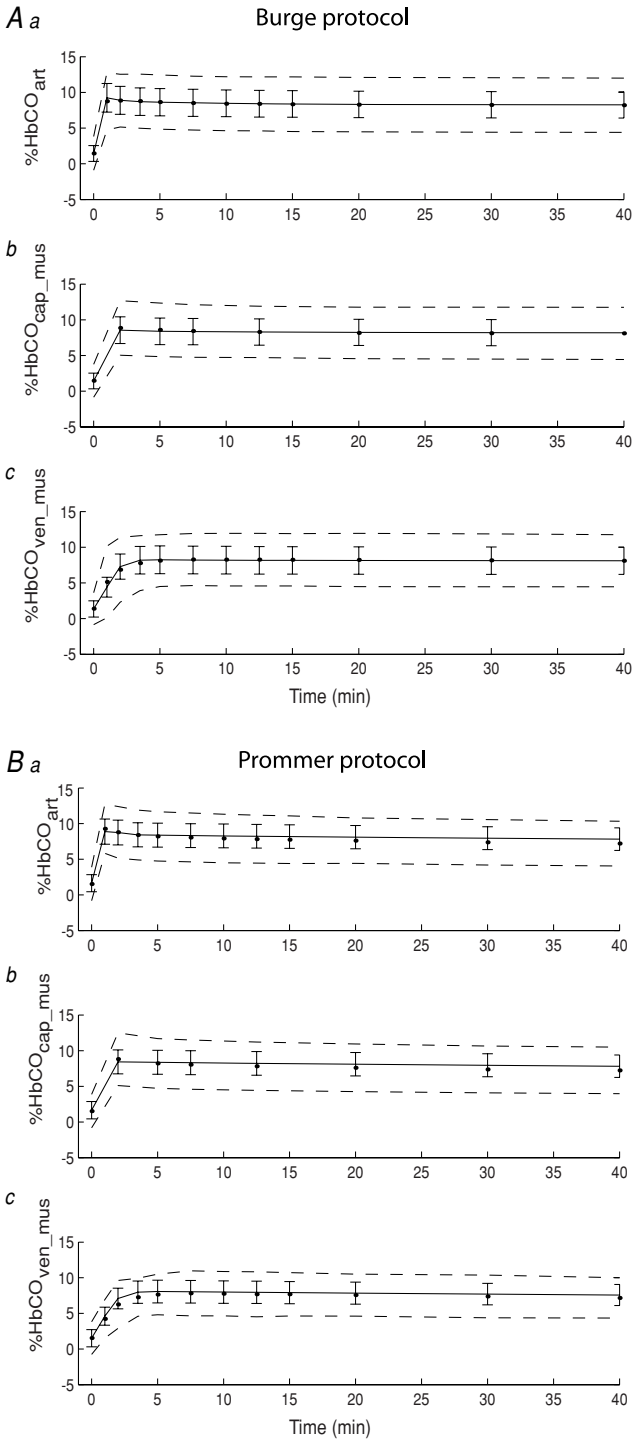

Figure 2. Comparison of model predictions of %HbCO with experimental data from arterial (*a*), muscle capillary (*b*) muscle venous blood (*c*) for the Burge protocol (*A*) and the Prommer protocol (*B*)
The continuous lines with error bars are the predicted mean %HbCO ± SD. Mean %HbCO from the experiments is represented by the symbol '•'. The dashed lines are the 95% confidence limits of the experimental data.

that these mean errors are significantly larger than those reported above for these same subjects when exact values for $V_{CO\_Lungs+System}$, $V_{CO\_exhaled}$ and V_{COMb} were used.

For the data set of Benignus *et al.* (1994), experiment_M_{Hb}_estimates for the two CO-rebreathing protocols were calculated from each of the four blood compartments using the approximation formulas of eqns (6–8). As there were no corresponding experimental data for these subjects, t_{mix} for each subject was determined based on the paired differences between values of %HbCO (see Methods). The mean errors from the different blood sites are shown in Table 2. Data for individual subjects using the Burge protocol are presented in the Supplemental material. For the Burge protocol, the largest errors are seen at the muscle capillary and muscle venous blood sites, at which the average errors exceed 4.5%. This estimate uses Prommer and Schmidt's formula (Prommer & Schmidt, 2007) to calculate V_{COMb}, and this formula assumes a constant flux of CO from blood to muscle tissue that is equal to the average flux between t_0 and t_{sample}. In fact, this flux varies with time (Fig. 3), causing an underestimation of the true volume of CO bound to myoglobin (Fig. 4) in the Burge protocol and an overestimation of both V_{COHb} and Hb mass. This underestimation of V_{COMb} is more prominent at blood site 'ven_mus'.

For the Prommer protocol, experiment_M_{Hb}_estimates from the arterial, non-muscle capillary and muscle capillary blood sites are close to actual M_{Hb} (Table 2). From blood site 'ven_mus', however, Hb mass is overestimated by 4.9%. These findings are correlated with errors in estimation of V_{COMb} (Fig. 4). This protocol also underestimates the volume of CO exhaled up to t_{sample} (Fig. 5). At the arterial, muscle capillary and non-muscle capillary blood sites, overestimation of V_{COMb} is partly compensated by the underestimation of $V_{CO\_exhaled}$, thereby resulting in estimates close to actual M_{Hb}. Thus, the overall errors are smaller due to compensating intermediate errors. At the blood site 'ven_mus', however, underestimation of both V_{COMb} and $V_{CO\_exhaled}$ results in consistent overestimation of actual M_{Hb}.

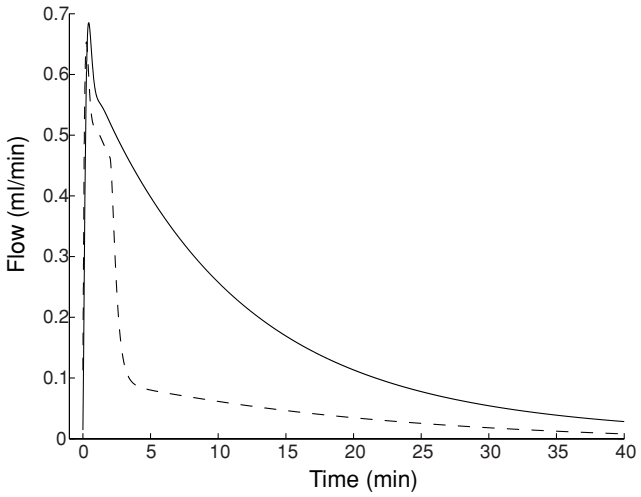

Figure 3. Carbon monoxide flux from blood to muscle tissues varies with time in the Burge protocol (continuous line) and the Prommer protocol (dashed line)

Table 1. Mean percentage error in exactmodel_M_{Hb}_estimate associated with blood sampling from four different sites, based on Benignus's 15 subjects

Site	Protocol	Mean percentage error
Arterial	Burge	0.118 ± 0.151
	Prommer	−0.865 ± 0.363
Non-muscle capillary	Burge	0.057 ± 0.140
	Prommer	−0.974 ± 0.365
Muscle capillary	Burge	1.619 ± 0.079
	Prommer	−0.769 ± 0.365
Muscle venous	Burge	1.883 ± 0.209
	Prommer	−0.392 ± 0.402

Table 2. Mean percentage error in experiment_M_{Hb}_estimate associated with blood sampling from four different sites, based on Benignus's 15 subjects

Site	Protocol	Mean percentage error
Arterial	Burge	2.051 ± 1.599
	Prommer	−0.056 ± 0.370
Non-muscle capillary	Burge	1.901 ± 1.582
	Prommer	−0.474 ± 0.410
Muscle capillary	Burge	4.870 ± 1.378
	Prommer	−0.159 ± 0.490
Muscle venous	Burge	5.479 ± 1.288
	Prommer	4.958 ± 0.702

Proposed modifications to improve CO-rebreathing protocols

The Burge protocol offers the advantage of determining Hb mass estimates close to the actual values if V_{COMb} is estimated accurately. However, the long duration of rebreathing and a longer t_{mix} are disadvantages. The Prommer protocol has a shorter t_{mix} and rebreathing duration, but the estimates of V_{COMb} and $V_{CO\_exhaled}$ are inaccurate and the apparent greater accuracy of Hb mass estimates depends on compensating errors in intermediate steps of the calculations. Thus, there is a need to determine a CO-rebreathing method with a shorter t_{mix} which estimates M_{Hb} without compensating errors and has low errors irrespective of the blood sampling site. To develop such a CO-rebreathing method, simulations were run for varying durations of CO rebreathing in 100% O_2

(2, 3.5, 5, 7.5 and 10 min) and varying durations of breathing room air or 100% O_2 after the CO rebreathing. In addition, the effect of administering 100% O_2 before CO rebreathing *versus* room air breathing was also tested. Every combination of factors was simulated for one typical subject (i.e. a subject for whom the errors in Hb mass estimates already discussed were close to average values), and the errors of Hb mass estimates were determined. Based on these simulation results, a new protocol was defined. In this new protocol, which is a modification of the Prommer protocol, prior to rebreathing the subject breathes 100% O_2. Then 1 ml of CO (kg BM)$^{-1}$ in 3 litres of oxygen is rebreathed for 3.5 min, followed by ∼8.5 min of room air breathing. The uptake kinetics of CO for this protocol are similar to those shown in Fig. 1 for the original Prommer protocol.

New protocol. The modified Prommer protocol was simulated for the 15 subjects of Benignus *et al.* (1994) and the nine subjects of Garvican *et al.* (2010). The values of t_{mix} and t_{sample} were determined from the methods described previously; t_{sample} ranged from 6.5 to 8.0 min in these 24 subjects. It is assumed that the volume of CO exhaled is determined by collecting exhaled gas over the duration of breathing room air, and the partial pressure of CO in the lungs and spirometer at the end of rebreathing is measured in order to calculate $V_{CO\_Lungs+System}$. Thus, these variables are known exactly. The volume of CO bound to myoglobin is estimated from a regression relation based on the model-calculated V_{COMb}. To develop this regression relation, the calculated values for V_{COMb} (in millilitres at BTPS) from the 24 subjects were estimated as functions of t_{sample} (in minutes) and V_{CO_total} (in millilitres at BTPS) by multilinear regression (SYSTAT, version 9). Predicted V_{COMb} values are close to the actual values calculated by the model (Fig. 6). See the online Supporting information for a discussion of possible sources of error when using this regression equation. The errors in experiment_M_{Hb}_estimates are less than 1% from this protocol (Table 3) from any blood sampling site. Thus, the modified Prommer protocol results in lower errors in

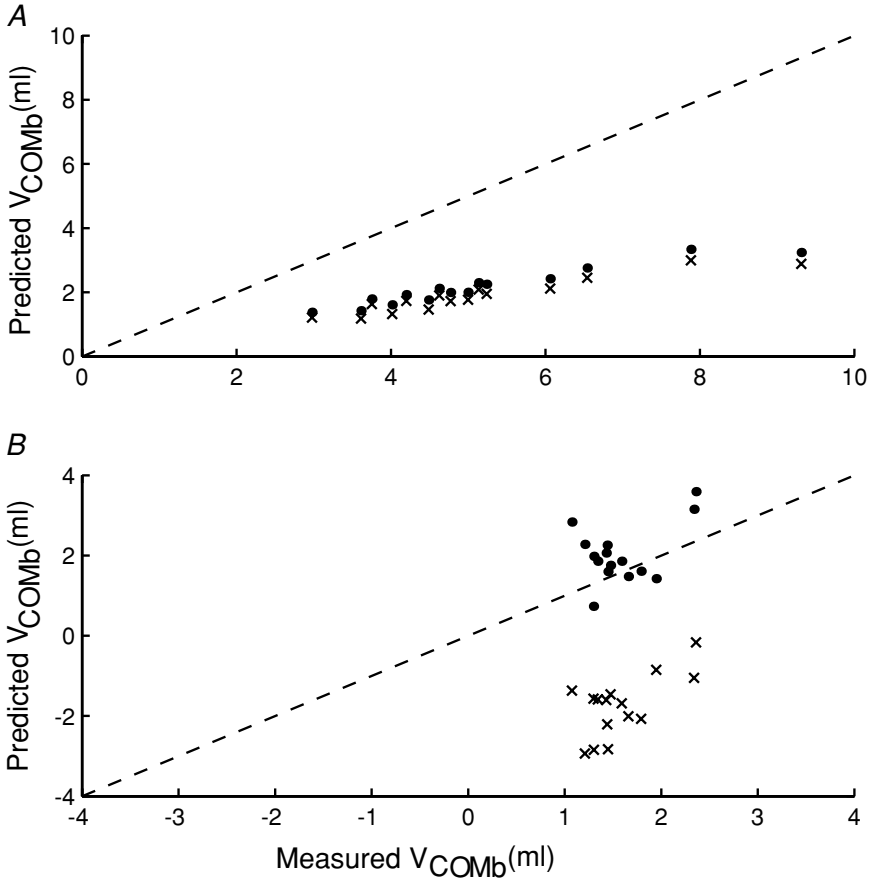

Figure 4. Comparison of V_{COMb} calculated from the model (abcissa) with V_{COMb} predicted by the Prommer and Schmidt formula (ordinate) for cap_mus (filled circles) and ven_mus blood sampling sites (crosses)
A, Burge protocol; *B*, Prommer protocol. Dashed lines are identity lines.

estimation of M_{Hb} compared with the original Burge and Prommer protocols without involving any compensatory errors in the calculations.

As it seemed likely that an improved estimate of V_{COMb} would decrease the errors associated with the original protocols, we developed predictors of this variable for these other protocols in the same fashion as described above. The regression relations for all three protocols are given in eqn (9), and the goodness of fit of each prediction is shown in Fig. 6.

$$V_{COMb} = \begin{cases} 0.223 t_{sample} + 0.024 V_{CO\_total} - 1.129; \\ \text{modifiedPrommerprotocol...9}a \\ 0.400 t_{sample} + 0.057 V_{CO\_total} - 4.685; \\ \text{Burgeprotocol...9}b \\ 0.091 t_{sample} + 0.013 V_{CO\_total}; \\ \text{Prommerprotocol...9}c \end{cases} \quad (9)$$

For the Burge protocol, the errors in estimation of M_{Hb} are less than 1.1% (independent of blood site sampled) when the regression equation is used (eqn 9b and Table 3). For this protocol, it is recommended that the volume of CO in the rebreathing circuit be measured at t_{sample}. For the original Prommer protocol, errors less than 1% (Table 3) without involving any compensatory effects are obtained when the regression equation (eqn 9c) is used. It is suggested that the actual volume of CO exhaled be measured at t_{sample}.

Additional analyses of the simulations of the three CO-rebreathing protocols were done to determine the effects of t_{sample} and the sampling blood site on estimation of M_{Hb}.

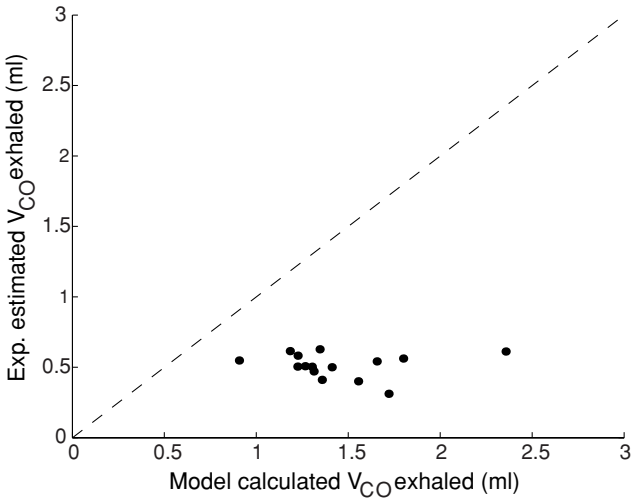

Figure 5. Comparison of $V_{CO\_exhaled}$ (ordinate) estimated by the Prommer and Schmidt method and exact $V_{CO\_exhaled}$ calculated from the model (abcissa) in the Prommer protocol
Dashed line is the identity line.

In general, simulations suggest that the combination of using a sample taken from blood sites 'art', 'cap_nonmus' or 'cap_mus' (but not 'cap_mus' for the Burge protocol) at 9 min and estimating V_{COMb} from the proposed regression equations (eqn 9) will provide estimates of M_{Hb} having small errors. We also examined the effects of changing various factors, such as myoglobin concentration, V_{CO_total}, $D_{m,CO}$, duration of rebreathing, muscle blood flow and muscle blood volume, on t_{mix}, and thus on t_{sample}. An increase in concentration of Mb results in a larger t_{mix}. Thus, non-athletic subjects and populations with lower muscle mass will have a smaller t_{mix} when compared with trained subjects and populations with larger muscle mass. Administering smaller doses of CO also results in a smaller t_{mix}.

Discussion

The main aim of this study was to use a validated mathematical model to determine potential sources of errors in estimation of M_{Hb} from CO-rebreathing methods. Estimated Hb mass was determined using the exact values from the model for $V_{CO\_Lungs+System}$, $V_{CO\_exhaled}$ and V_{COMb} (exactmodel_M_{Hb}_estimate) and using approximated values based on published formulae for calculating $V_{CO\_Lungs+System}$, $V_{CO\_exhaled}$ and V_{COMb} (experiment_M_{Hb}_estimate). It was found that the values of exactmodel_M_{Hb}_estimate were close to actual Hb mass (i.e. errors $< 2\%$) independent of the blood sampling site, while the values from experiment_M_{Hb}_estimate exhibited errors as large as 6% that were dependent on the blood sampling site. Experiment_M_{Hb}_estimate either inaccurately estimated actual M_{Hb} or closely approximated it based on compensating errors in intermediate calculations. Inaccurate estimation of volume of CO bound to myoglobin was found to be the major source of error in experiment_M_{Hb}_estimate. We also propose modifications to the Prommer method and suggest enhancements to the published methods for improving the calculation of V_{COMb}.

Model limitations

Model validation of %HbCO levels. The model was validated by comparing predicted and experimentally measured values of %HbCO from three blood sites (arterial, muscle capillary and muscle venous; Garvican *et al.* (2010)). The predicted mean %HbCO values from the three blood compartments at different time points were all within the 95% confidence limits of the experimental data (Fig. 2). In three of the nine subjects, in order to match the predicted $\%HbCO_{ven\_mus}$ with experimental values early during rebreathing it was

Table 3. Mean percentage error in experiment M_{Hb} esimate using corresponding regression equations to estimate V_{COMb}, based on Benignus's 15 subjects

	Modified Prommer	Burge	Prommer
Arterial	−0.249 ± 0.502	−0.694 ± 1.026	0.322 ± 0.504
Non-muscle capillary	−0.305 ± 0.488	−0.755 ± 1.014	0.212 ± 0.486
Muscle capillary	−0.730 ± 0.576	0.793 ± 0.897	0.419 ± 0.485
Muscle venous	−0.056 ± 0.453	1.053 ± 0.774	0.801 ± 0.424

necessary to alter the muscle blood flow and the resident blood volume in the muscle from the average parameter values reported in the literature (Bruce *et al.* 2008; Erupaka *et al.* 2010) that we had used in our model. Physiological values in any given subject probably differ from average values. Furthermore, in the model the separation of venous volume into an intratissue venous compartment and a mixed venous compartment was somewhat arbitrary, having been based initially on matching predicted muscle venous %HbCO to experimentally measured antecubital venous values for a 'typical' subject. Thus, altering these parameters by 20% (for these three subjects only) seems justifiable.

Model parameters. Values for many critical parameters in the model were either provided by investigators or estimated from regression equations developed from healthy populations (Bruce *et al.* 2008; Erupaka *et al.* 2010). Otherwise, average values from the literature for healthy populations were used. Intersubject variability was indirectly taken into account for some parameters whose values were not provided to us by investigators (Benignus *et al.* 1994; Garvican *et al.* 2010). For example, when the subject-specific alveolar ventilation was not measured (Garvican *et al.* 2010), the ventilation in each subject was adjusted so that an arterial P_{O_2} in the model of 98 mmHg was obtained during steady-state breathing of room air

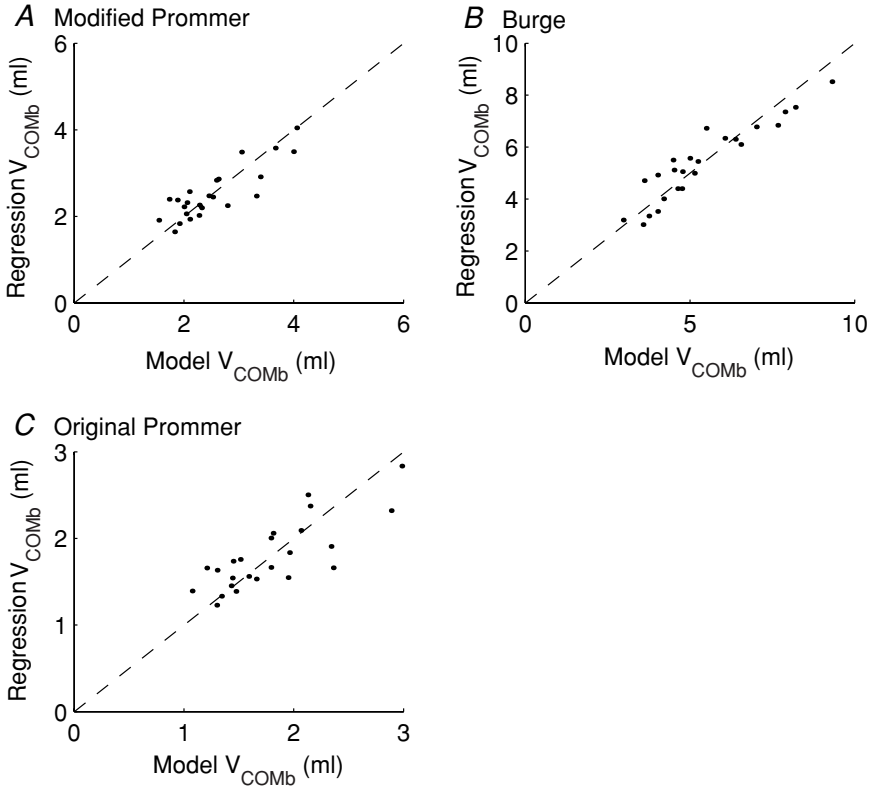

Figure 6. Proposed regression equations to estimate V_{COMb} for calculation of M_{Hb} from modified Prommer protocol (*A*), Burge protocol (*B*) and original Prommer protocol (*C*)
Abscissa is V_{COMb} calculated exactly from the model; ordinate is V_{COMb} estimated from t_{sample} and V_{CO_total} by regression. Dashed lines are identity lines. Regression equations are as follows: *A*, $V_{COMb} = 0.223t_{sample} + 0.024V_{CO\_total} - 1.129$, SEE = 0.39, $r = 0.85$; *B*, $V_{COMb} = 0.40t_{sample} + 0.057V_{COt\_total} - 4.685$, SEE = 0.72, $r = 0.89$; and *C*, $V_{COMb} = 0.091t_{sample} + 0.013V_{CO\_total}$, SEE = 0.33, $r = 0.96$.

at rest. In addition, $D_{m,CO}$ reflects the total diffusion coefficient for muscle and it should differ between subjects; thus, $D_{m,CO}$ was varied as a function of muscle mass to take into account intersubject variability (Benignus *et al.* 1994; Garvican *et al.* 2010). An average value of lung volume was used for simulating the three CO-rebreathing protocols. To determine the effects of changes in lung volume on estimation of M_{Hb}, the modified Prommer protocol was simulated in 15 subjects, and lung volume was varied as a function of age, body mass and height of the subject (Petersen *et al.* 1975). No significant differences were found compared with the exactmodel_M_{Hb}_estimate calculated using the same lung volume in every subject (data not shown). It is likely that this finding applies also to the other protocols. This negligible influence of lung volume on estimation of M_{Hb} has also been confirmed by the experiments and calculations of Steiner & Wehrlin (2011).

Effects of various factors on estimation of haemoglobin mass

Volume of CO bound to myoglobin. Simulation results predict that ∼6, 2 and 3% of V_{CO_total} is bound to myoglobin in the Burge, Prommer and modified Prommer protocols, respectively. On average, if V_{COMb} is ignored in the estimation of V_{COHb}, then the Burge, Prommer and modified Prommer protocols overestimate M_{Hb} by ∼7, 2.2 and ∼3.3%, respectively. In the Prommer and modified Prommer protocols, CO is exhaled after rebreathing ends, causing a decrease in the amount of CO entering the tissues and resulting in lower values for V_{COMb} at any t_{sample} when compared with the Burge protocol. Thus, the same underestimate of V_{COMb} will result in a higher estimate of M_{Hb} from the Burge protocol. In all protocols, if the volume of CO bound to myoglobin is estimated accurately, then the estimates of M_{Hb} can be close to the correct values of M_{Hb} (Tables 1 and 3).

In developing their formula to calculate V_{COMb}, Prommer & Schmidt (2007) assume that there is continuous flow of CO from Hb to Mb at a constant rate which can be quantified by estimating this CO flow between the times t_{mix} and t_{sample}. Certainly, there is continuous flow of CO from Hb to Mb (Bruce & Bruce, 2003), but the rate of flow of CO is not constant (Fig. 3). This assumption causes inaccuracies in calculation of V_{COMb} which may result in overestimation of M_{Hb} (Table 2), underestimation of M_{Hb} (see below) or correct estimation of M_{Hb} based on compensatory errors (Table 2). Also the formula (eqn 8) proposed by Prommer & Schmidt (2007) is greatly influenced by the choice of site of sampling, due to differences in %HbCO levels, and values for t_{mix}, and t_{sample}, due to the assumption of constant flow.

In the Burge protocol, the $\%HbCO_{cap\_mus}$ or $\%HbCO_{ven\_mus}$ levels rise slowly, resulting in a larger t_{mix} (Fig. 1). In this protocol, the assumption of the same constant flux of CO from Hb to Mb between t_0 and t_{mix} as between t_{mix} and t_{sample} results in underestimation of V_{COMb}. Thus, using Prommer and Schmidt's formula (eqn 8) to estimate V_{COMb} overestimates M_{Hb} in the Burge protocol. In the Prommer protocol, using this formula to estimate V_{COMb} from venous blood samples results in a similar overestimation of M_{Hb}, due to underestimation of V_{COMb}. For other blood sampling sites in the Prommer protocol, estimated V_{COMb} depends (eqn 8) on the ratio of $HbCO(t = t_{sample})$ to $HbCO(t = t_{mix})$. As this ratio is often less than one, V_{COMb} is often overestimated. In contrast, $V_{CO\_exhaled}$ is underestimated in the Prommer protocol, meaning that estimation of M_{Hb} from these sampling sites is based on compensating errors.

We proposed regression equations to estimate V_{COMb} from t_{sample} and V_{CO_total} for different protocols using V_{COMb} values calculated by the model. In the case of trained subjects, the volume of CO bound to Mb will be greater than for non-athletic subjects because t_{sample}, muscle mass and V_{CO_total} are all larger in trained subjects; however, the model incorporates these differences. The $D_{m,CO}$ might also be increased in athletic subjects if muscle capillary density is larger. Changing $D_{m,CO}$ (±50%) causes an error of approximately 25% in the opposite direction in the estimate of V_{COMb} from our regression equation. Thus, intersubject variability will still play a role in creating errors in estimated Hb mass when our predicted V_{COMb} is used.

Mixing time of CO in the vascular space (t_{mix}). We approximated the experimentally based method of determining mixing time by ascertaining when the magnitudes of paired differences between calculated %HbCO values from various blood sampling sites were all <0.1. The mixing times predicted by the model are in agreement with the literature (Burge & Skinner, 1995; Schmidt & Prommer, 2005; Gore *et al.* 2006). The observed effects of Mb concentration and dose of CO on t_{mix} can be explained by considering the rate of diffusion of CO from the vascular space to myoglobin-containing tissues, which is dependent on $D_{m,CO}$ and the partial pressure gradients of CO between blood and tissue compartments. The lower the rate of diffusion of CO, the faster will be the apparent equilibration of HbCO among the blood compartments (as determined using the 0.1% criterion). A large mass of Mb means that tissue P_{CO} is maintained at a low value and CO flux into tissues is increased, whereas when smaller doses of CO are administered, smaller CO pressure gradients between blood and tissue compartments are formed and CO flux is reduced. Likewise, for any CO-rebreathing protocol a lower value for $D_{m,CO}$ results in lower CO flux and a smaller t_{mix}. The Prommer protocol

has a small t_{mix} because the period of air breathing removes CO from the body and drives arterial HbCO down at the same time that venous HbCO is still rising, thereby reducing the partial pressure gradient. Variations in these factors may explain the range of values reported for t_{mix} in the literature.

Blood sampling site. Errors in experiment_Hb_estimate are dependent on blood sampling site. In both protocols, the errors were lower from arterial, non-muscle capillary (e.g. earlobe) and muscle capillary (e.g., fingertip) blood sampling sites than from muscle venous sampling (e.g. antecubital vein). These results are due to incomplete mixing of CO in blood and the effects of compensatory errors in the Prommer protocol and are in agreement with experimental studies (Gore *et al.* 2006; Garvican *et al.* 2010).

Predicted sampling sites to obtain low errors in estimation of M_{Hb} for the Burge protocol are arterial blood or ear lobe. For the original and modified Prommer protocols, arterial blood, ear lobe or fingertips are the predicted sites to obtain low errors. These predictions are in agreement with the preferred sampling blood sites of Garvican *et al.* (2010). For a given CO-rebreathing method, Garvican *et al.* (2010) chose their blood sampling site in each subject based on the smallest variation in estimated Hb mass with time. In contrast, suggestions for reliable blood sampling sites from our study are based on obtaining lowest errors in estimates of M_{Hb} when compared with actual Hb mass. Thus, our simulations validate the indirect approach taken by Garvican *et al.* (2010). We suggest that venous sites should be avoided because this compartment takes more time to reach equilibration with other compartments.

Proposed modifications to the common CO-rebreathing methods

Burge protocol. In this method, inaccuracies in estimation of V_{COMb} result in larger errors in experiment_M_{Hb}_estimate when compared with other CO-rebreathing methods. However, using the suggested regression equation (eqn 9*b*; Fig. 6) to estimate V_{COMb} will lower the errors (Table 3) when compared with using Prommer and Schmidt's formula (eqn 8) or ignoring V_{COMb}. Despite a longer t_{mix}, making a measurement from arterial or earlobe (other tissue capillary) blood sites at a sampling time of 9 min (see Supporting information) will allow estimation of Hb mass with low errors. This method has the advantage that no measurement of $V_{CO\_exhaled}$ is necessary. Also, we suggest that the volume of CO in the rebreathing circuit be measured at the suggested sampling time (volume of CO in the lungs can be estimated because errors here have minor effects).

Original Prommer protocol. The advantages of this method are that it has a shorter t_{mix}, shorter duration of CO rebreathing and lower volume of CO bound to myoglobin when compared with other rebreathing methods. However, the choice of 2 min duration of rebreathing in this method was not based on experimental or theoretical results. Furthermore, the values of experiment_M_{Hb}_estimate are based on compensatory errors in calculation of V_{COMb} and $V_{CO\_exhaled}$ (Table 3). To minimize errors, we suggest that $V_{CO\_exhaled}$ should be measured during the experiment and V_{COMb} should be calculated using the regression equation proposed for this protocol (eqn 9*c*; Fig. 6).

Conclusion

In this study, we applied a validated mathematical model to determine potential sources of errors in estimation of Hb mass using CO-rebreathing methods. In theory, all protocols could produce estimates that are within 2% of actual Hb mass if ideal data were available. In practice, common assumptions lead to errors that may be >5%. Inaccurate estimation of volume of CO bound to myoglobin was found to be the major source of error. In addition, the original Prommer method involves compensating errors in intermediate calculations. In order to estimate M_{Hb} with low errors and facilitate comparison of haemoglobin mass from different studies, we propose either using a modified Prommer rebreathing method or applying the suggested modifications for estimating volume of CO bound to myoglobin to the published CO-rebreathing methods. In all cases, recommended sampling times and blood sampling sites should be carefully considered.

References

Andersen P & Henriksson J (1977). Capillary supply of the quadriceps femoris muscle of man: adaptive response to exercise. *J Physiol* **270**, 677–690.

Benignus VA, Hazucha MJ, Smith MV & Bromberg PA (1994). Prediction of carboxyhemoglobin formation due to transient exposure to carbon-monoxide. *J Appl Physiol* **76**, 1739–1745.

Brodal P, Ingjer F & Hermansen L (1977). Capillary supply of skeletal muscle fibers in untrained and endurance-trained men. *Am J Physiol Heart Circ Physiol* **232**, H705–H712.

Bruce EN & Bruce MC (2003). A multicompartment model of carboxyhemoglobin and carboxymyoglobin responses to inhalation of carbon monoxide. *J Appl Physiol* **95**, 1235–1247.

Bruce EN, Bruce MC & Erupaka K (2008). Prediction of the rate of uptake of carbon monoxide from blood by extravascular tissues. *Respir Physiol Neurobiol* **161**, 142–159.

Bruce MC & Bruce EN (2006). Analysis of factors that influence rates of carbon monoxide uptake, distribution, and washout from blood and extravascular tissues using a multicompartment model. *J Appl Physiol* **100**, 1171–1180.

Burge CM & Skinner SL (1995). Determination of hemoglobin mass and blood-volume with CO: evaluation and application of a method. *J Appl Physiol* **79**, 623–631.

Erupaka K, Bruce EN & Bruce MC (2010). Prediction of extravascular burden of carbon monoxide (CO) in the human heart. *Ann Biomed Eng* **38**, 403–438.

Garvican LA, Burge CM, Cox AJ, Clark SA, Martin DT & Gore CJ (2010). Carbon monoxide uptake kinetics of arterial, venous and capillary blood during CO rebreathing. *Exp Physiol* **95**, 1156–1166.

Gore CJ, Bourdon PC, Woolford SM, Ostler LM, Eastwood A & Scroop GC (2006). Time and sample site dependency of the optimized CO-rebreathing method. *Med Sci Sports Exerc* **38**, 1187–1193.

Gore CJ, Hopkins WG & Burge CM (2005). Errors of measurement for blood volume parameters: a meta-analysis. *J Appl Physiol* **99**, 1745–1758.

Gorelov V (2004). Theoretical value of Hüfner's constant. *Anaesthesia* **59**, 97.

Hutler M, Beneke R & Boning D (2000). Determination of circulating hemoglobin mass and related quantities by using capillary blood. *Med Sci Sports Exerc* **32**, 1024–1027.

Ingjer F (1979). Effects of endurance training on muscle fibre ATP-ase activity, capillary supply and mitochondrial content in man. *J Physiol* **294**, 419–432.

Kalliokoski KK, Oikonen V, Takala TO, Sipila H, Knuuti J & Nuutila P (2001). Enhanced oxygen extraction and reduced flow heterogeneity in exercising muscle in endurance-trained men. *Am J Physiol Endocrinol Metab* **280**, E1015–E1021.

Petersen MR, Lapp NL & Amandus HE (1975). The relationship of several ventilatory capacities and lung volumes to age, height, and weight. *J Occup Med* **17**, 355–356.

Prommer N & Schmidt W (2007). Loss of CO from the intravascular bed and its impact on the optimised CO-rebreathing method. *Eur J Appl Physiol* **100**, 383–391.

Sagiv M, Goldhammer E, Ben-Sira D & Amir R (2007). What maintains energy supply at peak aerobic exercise in trained and untrained older men? *Gerontology* **53**, 357–361.

Schmidt W & Prommer N (2005). The optimised CO-rebreathing method: a new tool to determine total haemoglobin mass routinely. *Eur J Appl Physiol* **95**, 486–495.

Steiner T & Wehrlin JP (2011). Comparability of haemoglobin mass measured with different carbon monoxide-based rebreathing procedures and calculations. *Scand J Clin Lab Invest* **71**, 19–29.

Tibes U, Hemmer B & Boning D (1977). Heart rate and ventilation in relation to venous [K^+], osmolality, pH, PCO_2, PO_2, [orthophosphate], and [lactate] at transition from rest to exercise in athletes and non-athletes. *Eur J Appl Physiol Occup Physiol* **36**, 127–140.

Zoladz JA, Semik D, Zawadowska B, Majerczak J, Karasinski J, Kolodziejski L, Duda K & Kilarski WM (2005). Capillary density and capillary-to-fibre ratio in vastus lateralis muscle of untrained and trained men. *Folia Histochem Cytobiol* **43**, 11–17.

Acknowledgements

This work was supported by grant OH008651 from the National Institute of Occupational Safety and Health. The authors thank Dr Vernon Benignus (US Environmental Protection Agency, Research Triangle Park, NC, USA) and Drs Laura Garvican and Chris Gore (Australian Institute of Sport, Canberra, ACT, Australia) for providing both data published in their papers and unpublished measurements of parameter values from their subjects.

Supporting Information

Additional Supporting Information may be found in the online version of this article

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.