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Total Blood Carbon Monoxide: Alternative to Carboxyhemoglobin as Biological Marker for Carbon Monoxide Poisoning Determination

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Abstract

As one of the most abundant toxic contaminants in the atmosphere, carbon monoxide (CO) plays a significant role in toxicology and public health. Every year, around half of the accidental non-fire-related poisoning deaths are attributed to CO in the USA, UK and many other countries. However, due to the non-specificity of the symptoms and often encountered inconsistency of these with the results obtained from measurements of the biomarker for CO poisonings, carboxyhemoglobin (COHb), there is a high rate of misdiagnoses. The mechanism of toxicity of CO includes not only the reduced transport of oxygen caused by COHb but also the impairment of cellular respiration and activation of oxidative metabolism by binding to other proteins. Therefore, in this study we propose the measurement of the total amount of CO in blood (TBCO) by airtight gas syringe–gas chromatography–mass spectrometry (AGS–GC–MS) as an alternative to COHb for the determination of CO exposures. The method is validated for a clinical range with TBCO concentrations of 1.63–104 nmol/mL of headspace (HS) (0.65–41.6 μmol/mL blood). The limit of quantification was found between 2 and 5 nmol/mL HS (0.8 and 2 μmol/mL blood). The method is applied to a cohort of 13 patients, who were exposed to CO under controlled conditions, and the results are compared to those obtained by CO-oximetry. Furthermore, samples were compared before and after a "flushing" step to remove excess CO. Results showed a significant decrease in TBCO when samples were flushed (10–60%), whereas no constant trend was observed for COHb. Therefore, measurement of TBCO by AGS–GC–MS suggests the presence of more dissolved CO than previously known. This constitutes a first step into the acknowledgment of a possibly significant amount of CO present not in the form of COHb, but as free CO, which might help explain the incongruences with symptoms and decrease misdiagnoses.

Introduction

Carbon monoxide (CO) is a highly toxic gas produced through incomplete combustion of hydrocarbon-based materials and fuels. It is one of the most abundant air pollutants, hence every individual is exposed to it on a daily basis through the innate and essential act of breathing. However, CO in small amounts is also produced endogenously, mainly through catalysis of heme and heme-containing proteins and through processes such as lipid peroxidation and photo-oxidation. Therefore, very low levels of CO are present in each individual, even though levels can vary according to physiological as well as pathological conditions (1). The burden of CO in air varies according to the amount and type of CO sources present in the living or working area of an individual, the season, which determines whether there are higher number of fires and barbecues (summer) or a high use of wood- or fuel-fired stoves and heaters (winter), and whether the individual is a smoker or exposed to passive smoke. Typically, CO concentrations in

indoor air are below 30 ppm, they are around double the amount in outdoor air (2). Even though the World Health Organization (WHO) and the national health and safety institutions of most countries have clear guidelines on the tolerance limits for CO exposure (3–5), CO poisoning is the main cause of accidental non-fire-related poisoning deaths in Western countries (6, 7) and leads to a high number of emergency department (ED) admissions worldwide (6–16). However, these numbers of fatalities and ED admissions likely underestimate the real burden of CO on the population. This is due to the characteristics of CO and the clinical, non-specific symptoms of CO intoxication. CO is an odorless, tasteless and colorless gas, making it difficult to detect if above normal concentrations and without the appropriate measuring devices (17). Clinical diagnosis of CO poisoning is difficult due to the non-specific symptoms it presents: dizziness, nausea, fatigue, headaches (18); often the causes of CO poisonings are misdiagnosed and attributed to other diseases or disturbances (19). Misdiagnoses can also arise from errors due to the analytical measurement techniques and parameters used (measurement error) and the related incongruence with symptoms reported by patients (19–23).

The current biomarker for CO exposure is carboxyhemoglobin (COHb). Once CO enters the bloodstream, it binds to hemoglobin (Hb) competitively to oxygen (O_2) (24, 25). This very high affinity of CO for Hb (200–250 times higher than O_2) causes a strong bond between CO and Hb and leads to the inhibition of oxygen transport through Hb and delivery to tissues, also a suggested mechanism for the resulting hypoxia in brain and heart, primary consequences of CO exposure (3). However, the presence of CO in blood only in the form of COHb does not explain the inconsistency repeatedly reported between COHb% results and symptoms (26). One hypothesis, suggested in previous studies (23, 27), is that a random and possibly significant amount of CO in blood is not bound to Hb but is present in free form and can thus distribute to other tissues, leading to pathophysiological effects through direct cellular toxicity. Around 10–15% of the absorbed CO has been previously documented to bind to proteins other than Hb (28, 29). These other proteins include myoglobin, cytochromes and guanylyl cyclase. Binding to myoglobin reduces the availability of oxygen in the heart and may lead to arrhythmias and cardiac dysfunctions as well as causing direct toxicity of skeletal muscle (30). CO binding to mitochondrial cytochrome oxidase, which was reported for *in vitro* studies (31, 32), impairs cellular respiration by inactivation of mitochondrial enzymes and also initiates an oxidative metabolism by generating oxygen-free radicals (29, 30, 33). Even though the affinity of CO for cytochrome oxidase is relatively low, the dissociation is very slow, leading to a prolonged impairment of oxidative metabolism, which may help explain the tardive and sustained effects of CO toxicity even after COHb has been cleared from the system through hyper- or normobaric oxygen therapy (28, 29). The loss of consciousness associated with CO poisoning may be caused by the stimulation of guanylyl cyclase, since increases in cyclic guanosine monophosphate result in cerebral vasodilation (34, 35).

In general, the role of direct cellular CO toxicity seems to be underestimated in the prevailing CO poisoning literature, given that most studies date back several decades. But given the numerous pathophysiological mechanisms, it is important to be able to determine the total amount of CO and not only COHb in CO poisoning cases.

However, the current measurement methods are based on COHb, either through spectrophotometric techniques, such as UV-spectrophotometry and CO-oximetry (36–41), or gas chromatographic techniques, such as gas chromatography–mass spectrometry (GC–MS) or GC–flame ionization detection (GC–FID) (1, 42–48), where the CO measured is backcalculated and correlated to COHb. The issue with optical methods is the dependency of the technique on the quality of the sample. Alterations of the sample, which can occur through degradation due to a time-delayed analysis or/and unsatisfactory and uncontrolled storage conditions (39, 45, 49–53), make the optical analysis difficult to interpret or in some cases infeasible. Gas chromatographic methods can overcome these problems in most cases. The CO present in blood is released through a releasing agent and then analyzed either directly (GC–MS) (42, 44–46) or after transformation into methane (GC–FID) (47, 54–57) in its gaseous form. One important step that has been applied in previous studies includes the "flushing" of the calibrator in order to target only CO bound to Hb. To achieve that, blood is subjected to a stream of nitrogen gas for an amount of time, to remove all excess CO (45, 47, 48, 52, 55, 56, 58) and, therefore, this approach does not consider the amount of excess CO as relevant, but only that bound to Hb. This could lead to mis- or underestimation of the total load of CO in blood. For this reason, in a previous study an approach that is based on the measurement of the total amount of CO in blood (TBCO) by airtight gas syringe(AGS)–GC–MS was developed and validated, however only for a concentration range with postmortem application (23).

With this research, we aim to test the hypothesis of the presence of CO in free form in blood, by comparing the analysis results of flushed and unflushed blood samples obtained at bedside from patients that were exposed to CO. This represents the first step in expanding the knowledge of the true CO burden. Furthermore, we want to improve the TBCO measurement technique by validating it for a concentration range applicable also in clinical settings.

Materials and Methods

Chemicals and reagents

Formic acid (reagent grade, purity ≥95%) was purchased from Sigma-Aldrich (St Louis, USA) and CO gas (99%) was from Multigas (Domdidier, Switzerland). To prevent degradation, all formic acid solutions were prepared on a daily basis. The internal standard formic acid (13 C, 99%) was ordered from Cambridge Isotope Laboratories (Cambridge, UK). Sulfuric acid (≥97.5%) was purchased from Fluka (Buchs, Switzerland). Human blood samples were obtained from volunteers participating in a study at the Department of Nephrology of the University Hospital of Geneva (HUG) in Switzerland. Blood of non-smokers before exposure to CO was used as a blank matrix. For the *in vitro* study of CO-fortified blood, bovine blood was obtained from a local butcher.

Materials

The AVOXimeter 4000 Whole Blood CO-Oximeter and cuvettes were obtained from International Technidyne Corporation—ITC (Edison, USA). S-Monovettes of the following types: 2.6 mL K3E (Ethylenediaminetetraaceticacid, EDTA), 3 mL 9NC (sodium citrate, NaCit), 2.7 mL FE (sodium fluoride, NaF), 2.6 mL KH (lithium heparin, LiH) were obtained from Sarstedt (Nürnbrecht, Germany). Precision sampling gas syringes equipped with a press button valve and with capacities of 500 µL (for dilution) and 2 mL (for injection) were purchased from VICI (Baton Rouge, LA, USA). Aluminum caps were from Milian (Vernier, Switzerland). All extractions were carried out in 20-mL headspace (HS) vials from Agilent Technologies (Santa Clara, CA, USA).

Instruments and GC–MS conditions

AVOXimeter 4000 Whole Blood CO-Oximeter from ITC was used for all COHb analyses. The instrument uses five wavelengths for quantitative analysis, namely, 520.1, 562.4, 585.2, 597.5 and 671.7 nm. Following parameters can be measured: total hemoglobin (tHb), oxyhemoglobin (O_2Hb), COHb and Methemoglobin (MetHb). In this study, manufacturer guidelines were followed to obtain COHb concentrations (59, 60).

Agilent 6890 N GC (Palo Alto, USA) equipped with a HP Molecular Sieve 5 Å PLOT capillary column (30 m × 0.32 mm × 30 µm) purchased from Restek (Bellefonte, USA) was used for gas chromatographic analysis. The temperature program used was as follows: 50°C, held for 4 min; injector temperature was set at 180°C, the injector used in splitless mode, and the MS interface at 230°C. Helium was employed as a carrier gas, at a flow rate of 40.0 mL/min. A solvent delay of 1.8 min was introduced.

An Agilent 5973 mass spectrometer (Palo Alto, USA) was used for detection, operating in electron ionization (EI) mode at 70 eV. Selected ion monitoring mode was used to acquire the signal for CO at *m/z* 28 and ^{13}CO at *m/z* 29, both at the same retention time of 3.5 min.

Sample preparation

Calibration standards

An aliquot of human blood from non-smokers, which was previously controlled by CO-oximetry and found at 0% COHb, is used as a matrix for calibration. Calibration standard working solutions of formic acid (43 µmol/mL) and working solutions of the internal standard isotopically labeled formic acid (84 µmol/mL) were prepared daily *de novo* in order to prevent degradation. Calibration points were set in a working range between 0 and 104 nmol/mL HS, with points at 1.63, 3.25, 6.5, 13, 26, 52 and 104 nmol/mL HS (equivalent to 0.65, 1.3, 2.6, 5.2, 10.4, 20.8 and 41.6 µmol/mL in blood). Matrix effects were evaluated by preparing a blank sample with the matrix without any reagent. A total of 10 µL of the working internal standard solution were added to each calibration sample before extraction, leading to a final concentration of 42 nmol of ^{13}CO/mL HS. All standards and samples were stored at +4°C when not in use.

Quality controls

Quality controls were performed with five internal control samples, at concentrations of 2, 5, 10, 30 and 60 nmol/mL HS (0.8, 2, 4, 12 and 24 µmol/mL blood), which were prepared daily with formic acid obtained from a different lot diluted with deionized water.

Additionally, validity of the method was tested with external controls, which were prepared by the dilution of pure CO gas at two concentration levels, low and high, respectively, 5 and 60 nmol/mL HS.

Extraction procedure

100-µL aliquots of blood were introduced in a 20-mL HS vial, followed by 10 µL of the internal standard solution. For calibration points, the respective aliquots of formic acid solution were added. Aluminum caps of 11 mm (i.d.) were first filled with 100 µL of sulfuric acid and then carefully introduced into the HS vial. The vial was immediately hermetically sealed with PTFE/silicone septum caps of 20 mm (i.d.). In order to ensure complete mixing of the liquids contained in the vial, the samples were vigorously shaken and vortexed. Extraction was finalized by heating the vials at 100°C for 60 min.

Analysis procedure

CO-oximeter

Approximately 50–100 µL of blood were sampled from the sampling tube and placed into an Avoximeter 4000 Whole Blood CO-oximeter cuvette, which was then introduced in the Avoximeter 4000 Whole Blood CO-oximeter for analysis.

CO in blood

One milliliter of HS was sampled from the 20-mL HS vial containing the extract and injected in the GC–MS for analysis.

To ensure that no contamination from CO contained in the air affected the measurements occurred, a 1-mL aliquot of air in the analysis room was additionally analyzed prior to sample analyses. Air samples were collected with the AGS and analyzed by GC–MS with the same conditions as the blood samples.

Validation procedure

The validation was performed according to the guidelines of the "French Society of Pharmaceutical Sciences and Techniques" (SFSTP) (61) and included the following validation parameters: response function (calibration curve), linearity, selectivity, trueness, precision (repeatability and intermediate precision), accuracy, limit of detection and limit of quantification (LOQ).

The response function, also defined as the calibration curve, is described as the relationship between the concentration of the analyte in the sample and the corresponding instrument response.

Linearity of the method is assessed by fitting backcalculated concentrations of control samples against the theoretical concentrations through the application of the linear regression model and evaluating the resulting regression coefficient.

Trueness, also defined as bias, describes the closeness between the average of the experimental value and the calculated target value. It is expressed as percent deviation from the calculated target value.

Precision is defined as closeness of agreement (degree of scatter) between a measurement series obtained from multiple sampling of the same homogenous sample under the prescribed conditions and is determined by calculating the repeatability (intra-day precision) and intermediate precision (inter-day precision) for each control sample. The repeatability is determined by calculating the intra-day variance (S^2r) and the intermediate precision through the sum of intra- and inter-day variances (S^2_{IP}).

Accuracy describes the closeness of agreement between the conventional true value or an accepted reference value and the value experimentally found. It is expressed as the sum of trueness (systematic error) and precision (random error).

Calibrators and QCs were used for the validation experiments performed on three non-consecutive days ($p = 3$) not within the same week. The validation approach is based on the use of a β-expectation-interval tolerance of 80%, indicating that the intervals for each experimental point include an average of 80% of the total values. The tolerance intervals (TIs) were defined as $TI = X \pm k \times \sqrt{(S^2_r + S^2_R)}$, where S^2_r is the standard deviation of repeatability and S^2_R is the standard deviation of reproducibility. In the β-expectation-interval tolerance approach, $k = t_v \times \sqrt{(1 + [1 / (I \times J \times$

B^2)]), where I is the number of series, J is the number of repetitions and B^2 is a coefficient. This coefficient is given as $B^2 = (R + 1)/[J \times (R + 1)]$ with $R = S^2_r/S^2_R$. t_ν is Student's coefficient with degrees of freedom ν defined as $\nu = (R + 1)^2 / \{[(R + 1/J)^2/(I - 1)] + [(1 - 1/J)/(I \times J)]\}$.

Samples from volunteers

CO-rebreathing method

The CO-rebreathing method consists of a closed-circuit breathing system containing oxygen mixed with a certain amount of CO. Patients breathe in and out through a mouthpiece linked to the circuit. In the study from which we acquired the blood samples, patients were lying in horizontal position. After insertion of the mouthpiece, an adjustment period of 2 min was used to deliver O_2 only, before starting the delivery of the O_2–CO mixture for a period of 10 min (62). The volumes of CO delivered in O_2 were between 57 and 105 mL, calculated according to the body mass index of each volunteer, in order to reach a target COHb value of ~10%.

Blood collection and preparation

Blood samples were obtained from a cohort of 13 patients (9 men, 4 women) under treatment at the Nephrology Department of the University Hospital in Geneva (HUG), Switzerland.

Three blood samples were taken from volunteers, one before and two directly after exposure to CO. Samples were immediately analyzed by CO-oximetry. Half of the samples taken after exposure of the individual to CO were flushed with a nitrogen stream for 2 min, with a flow rate below 5 mL/min. Two needles were inserted in the rubber septum of the blood tube. One needle was plunged in blood and provided the nitrogen stream, whereas the other was placed in the HS of the blood tube in order to relieve the pressure in the blood tube built with the release of CO and the nitrogen flush. After flushing, the samples were analyzed by CO-oximetry. Simultaneously, all samples (before exposure, flushed and not flushed after exposure) were prepared for analysis with AGS–GC–MS and analyzed in triplicates. All sampling and testing performed on volunteers were approved by ethical committee under the study number CCER-2017-00421.

In vitro study of CO-fortified samples

To test the relevance of the validated clinical range of CO concentrations for the blood samples obtained from the volunteers, *in vitro* CO-fortified blood samples were prepared. Bovine blood was obtained freshly from a local butcher and immediately transferred in containers with anticoagulants (EDTA, LiH, NaF, NaCit) to avoid coagulation during transport to the laboratory. After arrival at the laboratory, CO-oximetric analyses of the blood were performed to confirm the baseline CO content. Fortification with pure CO gas was then carried out by bubbling the gas directly into the blood for varying amounts of time. The COHb saturation levels were checked in 10-min intervals with the CO-oximeter until the desired initial COHb% level was reached. To ensure homogenization, the bottles were agitated for 20 min after fortification and the final COHb concentration was subsequently measured. After reaching the designated COHb% saturation (10–20, 30–40 and 60–70%), blood was transferred to the sampling tubes and subsequently analyzed without any flushing, in order to respect the physiological conditions of blood sampling and analysis when an individual is suspected of a CO poisoning.

Statistical analyses

Mean, standard deviation and interquartile ranges were determined for all measured variables. Paired Student's t-test with an α-error of 0.05 was performed for comparison of the group means. All data treatment and statistical analyses were performed with R (version 3.3.1, 2016-06-21).

Results

Validation of the method in a clinical range

Results of the validation for all criteria are summarized in Table I.

Calibration curves for CO determination were obtained by using CO negative human blood as a blank matrix. Calibration curves, which represent the response function, were acquired on three non-consecutive days ($p = 3$), in triplicates ($n = 3$) and at seven concentration levels ($k = 7$): 1.63, 3.25, 6.5, 13, 26, 52 and 104 nmol/mL HS (equivalent to 0.65, 1.3, 2.6, 5.2, 10.4, 20.8 and 41.6 µmol/mL blood). For each calibration point, calculated concentrations were compared to the target values and found to be within ±20%. The relationship between the CO concentration from samples spiked with formic acid and the measured response was found to be linear. Validation coefficients for the calibration curves are shown in Table I(**I**).

On each non-consecutive day ($p = 3$), control samples at five different concentrations ($k = 5$), namely, 2, 5, 10, 30 and 60 nmol/mL HS (0.8, 2, 4, 12 and 24 µmol/mL blood), were measured in triplicates ($n = 3$). The concentrations of the control samples were calculated by using the calibration curve determined for each analysis day. As shown in Table I(**II**), a satisfactory linearity was obtained, with a slope of 1.05 and a regression coefficient of 0.99 in the range of 2–60 nmol/mL HS (0.8–24 µmol/mL blood).

Selectivity of the AGS–GC–MS method was previously confirmed in the work by Oliverio *et al.*, hence was not repeated in this study (23).

Trueness was found to be lower than the acceptance criteria (within ±20% of the accepted reference value and within 20% at the LOQ), as is shown in Table I(**III**), which is satisfactory for validation according to SFSTP guidelines (61).

Table I(**IV**)shows that the relative standard deviation (RSD) for repeatability and intermediate precision are in a range between 0.50% and 3.55%.

Figure 1 represents the accuracy profile for CO. The mean bias (%) confidence interval limits for the control samples were within the acceptability limits of ±30%. Taking into consideration the acceptability limits of ±30%, the LOQ within validation criteria was found between 2 and 5 nmol/mL HS (0.8 and 2 µmol/mL blood). Thus, the method is confirmed to be accurate within the range of 5 and 60 nmol/mL HS (2–24 µmol/mL blood) according to the β-interval tolerance accuracy profile.

In addition, the external controls gave an excellent accuracy, with an RSD below 15% (Table I(**V**)).

Analyses of flushed samples

Blood samples of 13 patients were analyzed before and after exposure to CO as well as with and without a flushing step. Triplicates were acquired for analyses with CO-oximetry and AGS–GC–MS. Results of all analyses are found in Table II and represented in Figure 2.

For all samples analyzed, a relative increase of both TBCO and COHb can be observed when comparing the values before and after

Table I. Validation results for CO determination in blood by AGS–GC–MS—(**I**) represents the mean coefficients of the calibration functions obtained from analysis of the calibrators; (**II**) represents the coefficients of the linear regression function obtained from analysis of the QCs against their theoretical value; (**III**) represents the trueness obtained from the QC analyses expressed in %; (**IV**) represents the precision obtained from QC analyses expressed in relative standard deviation % (RSD%); (**V**) shows mean and RSD% of the external controls

(I) Response function (1.63–104 nmol/mL HS) ($k = 7$, $n = 3$, $p = 3$)			
	Day 1	Day 2	Day 3
Slope	0.03	0.02	0.03
Intercept	0.59	0.63	0.61
r^2	0.98	0.99	0.98

(II) Linearity (2–60 nmol/mL HS) ($k = 5$, $n = 3$, $p = 3$)	
Slope	1.05
Intercept	−0.48
r^2	0.99

(III) Trueness (relative bias) ($k = 5$, $n = 3$, $p = 3$)	
Levels (nmol/mL HS)	Trueness (%)
2.0	−11
5.0	−10
10	1
30	−18
60	−19

(IV) Precision (RSD%) ($k = 5$, $n = 3$, $p = 3$)	
Levels (nmol/mL HS)	Repeatability and intermediate precision[a]
2.0	0.5
5.0	0.6
10	1.7
30	1.6
60	3.6

(V) External controls ($k = 2$, $n = 3$, $p = 1$)		
Levels (nmol/mL HS)	Mean (confidence interval)	RSD (%)
5	4.49 (3.93, 5.06)	12.0
60	65.5 (60.0, 73.9)	6.57

k, number of concentration levels; n, number of repetitions for each level; p, number of non-consecutive days

[a]Inter-series variance (S2g) is negligible for all levels, resulting in same values for repeatability and intermediate precision

CO exposure (Figure 2 and Table II). Increases were confirmed and found to be significant by comparing the means with Student's *t*-test (Table III).

Important observations can be made when comparing the COHb and TBCO levels before and after a flushing step (Tables II and III). For the levels of COHb, there is no consistent trend that can be observed, with a COHb increase in some patients and a decrease in others. The 25th and 75th interquartile ranges (Table III) for COHb before and after flushing (range includes 0) as well as the results of the *t*-test (*P*-value > 0.05) confirm this result. This behavior could be explained by the known analytical variability of CO-oximeters at such low COHb saturations (63, 64).

Conversely, TBCO shows a consistent trend: for all samples analyzed, TBCO before flushing is higher than after flushing. Statistical

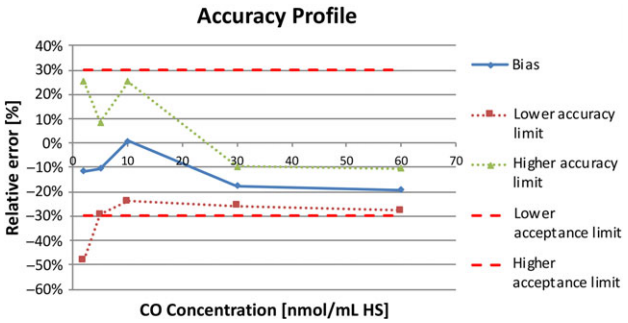

Figure 1. Accuracy profile for CO determination using a simple linear regression model within the range of 2–60 nmol/mL HS (0.8–24 µmol/mL blood). The continuous line represents the trueness (bias), the dashed lines represent the acceptance limits set at ±30% and the dotted lines are the relative lower and upper accuracy limits.

significance was confirmed by performing a paired Student's *t*-test of the means (*P*-value = $8.955e^{-06}$). Mean and interquartile range for TBCO differences before and after flushing additionally confirm the positive relationship (Table III).

Discussion

An improved AGS–GC–MS method for CO determination in blood was validated for a clinical concentration range (1.63–104 nmol/mL HS/0.65–41.6 µmol/mL blood) and was successfully applied to the analysis of blood samples coming from individuals with controlled CO exposures. The observed increase in both COHb and TBCO before and after CO exposure was to be expected, since all individuals were breathing in a mixture of O_2 and CO. Patients with a CO burden before exposure, namely, patients with ID number 3, 4, 9, 11, 12 and 13, admitted to be smokers. Smokers are known to have a higher baseline CO level, which varies depending on the frequency (65–67), hence it explains the presence of CO in several patients before exposure.

A significant variability of CO burden after exposure is found between individuals. Even though the volumes of CO administered were adapted to the weight and height of the patients, other factors involving the respiratory system and blood circulation, such as ventilation rate, tidal volume, inspiratory and expiratory reserve volume, alveolar ventilation, cardiac rhythm and cardiac output, influence the net amount of CO that enters the circulation (68). In addition, malfunctioning of the rebreathing system can lead to altered amounts of CO effectively being administered, resulting in the observed inter-patient variations.

The samples were all subjected to the same storage and sample treatment conditions (immediately after blood collection) and analyzed with the same parameters and measurement method (within 48 h after blood samplings). It is therefore not very likely that the differences in detected concentrations are due to any error in the measurement technique or used parameters, but mainly to the removal of CO through a constant nitrogen stream. This legitimizes the hypothesis that there is a significant amount of CO present in free form in blood from an individual, who was subjected to CO exposure. The amount of free CO on average ranges between 10% and 60% compared to the initial TBCO burden.

Additionally, when plotting the results of the 13 clinical samples with the results obtained from *in vitro* CO-fortified samples (Figure 3), it can be seen that the clinical samples all comply with

Table II. Differences in measurement results for COHb (%) and TBCO (μmol/mL) for before and after CO exposure and before and after flushing; for each individual, sex and volume of CO administered are provided

Patient ID	Sex	Volume CO (mL)	COHb (%)		TBCO (μmol/mL)	
			Difference after–before CO exposure	Difference not flushed–flushed	Difference after–before CO exposure	Difference not flushed–flushed
1	M	84.3	1.90	−2.20	0.11	0.11
2	M	89.0	7.30	−1.50	1.95	0.81
3	F	87.9	7.30	−1.30	2.20	0.30
4	M	59.3	6.80	−0.90	1.84	0.75
5	F	48.3	5.20	0.60	0.75	0.34
6	M	104	6.10	−0.90	0.93	0.67
7	M	86.7	7.70	−2.60	0.74	0.33
8	M	89.1	8.10	−0.90	1.06	0.31
9	F	71.2	10.90	1.20	1.99	0.30
10	M	57.4	6.80	1.50	0.78	0.66
11	F	99.5	6.90	2.50	1.51	0.49
12	M	60.8	6.20	2.10	1.63	0.23
13	M	65.3	8.70	0.10	3.05	0.57

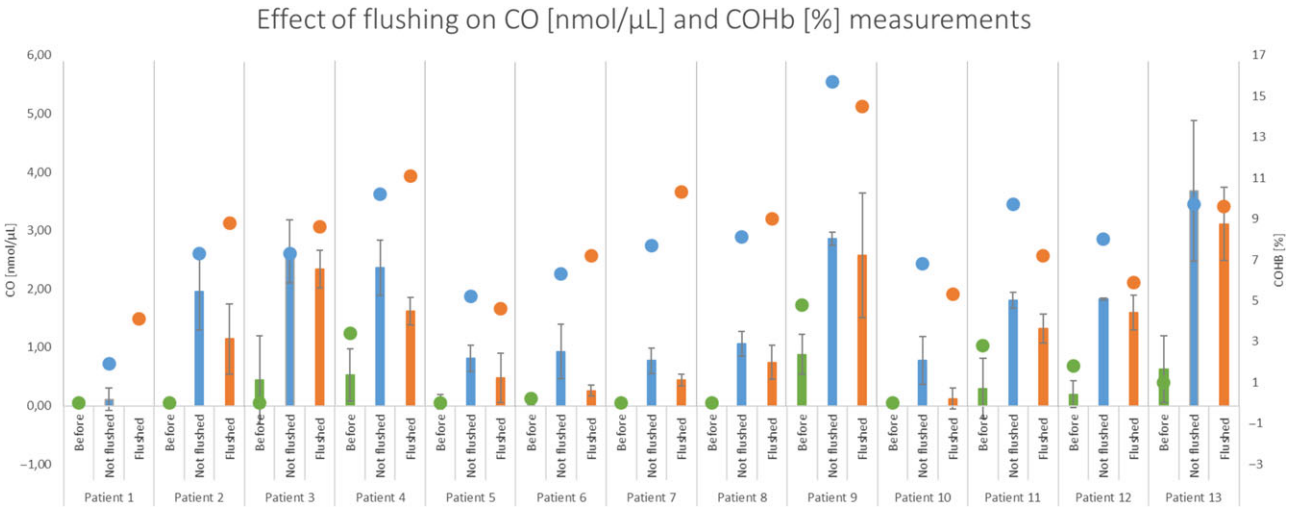

Figure 2. Results for the total CO concentration in blood (TBCO) in μmol/mL (bars, left axis) measured by AGS–GC–MS and the COHb saturation in % (dots, right axis) measured through CO-oximetry in blood for 13 patients before (green) and after (blue) CO exposure and after a flushing step (orange).

the *in vitro* measurements, further diminishing the probability of errors in the measurement and strengthening the assumption of CO being eliminated through flushing. This suggests that with the execution of a flushing step a significant amount of CO is removed from the analyzed sample. Thus, the result is biased not only from an analytical but also from a clinical point of view: the excess amount of CO may have a more significant pathophysiological activity than previously suspected. The direct cellular toxicity of molecular CO through impairment of cellular respiration and generation of free radicals, which are known to be tumor cell promoters, was reported in previous studies (28, 30–35). Yet, the importance given to its implications in the direct adverse effects in CO poisonings was held to a minimum. Most likely this was because the presence of CO dissolved in blood in free form was never clearly demonstrated before.

Furthermore, the acknowledgment of dissolved CO represents one possible argument for explaining the discrepancy between reported symptoms and measured COHb. Considering only the amount of CO bound to Hb when determining a CO poisoning may lead to underestimation of the true burden CO poses, explaining why in many cases the measurements are inconsistent with the symptoms a patient is showing and also why several patients show symptoms with a certain time delay. The amount of CO that is dissolved and not bound to Hb may be the missing quantity that gets closer to the true CO concentration in blood and burden on the body of an exposed individual. CO toxicity at cellular level may not only explain some of the symptoms of acute CO poisonings, but, due to the slow dissociation rate from cellular proteins, it may also elucidate the reasons behind the delayed neurological effects reported hours or days after COHb was removed from the system through oxygen therapy and after low level chronic exposures.

Limitations

This study constitutes a preliminary study that aims to demonstrate the existence of CO dissolved not bound to Hb. This hypothesis has been tested with a cohort of 13 individuals through bedside blood

Table III. Summary statistics and results of paired *t*-test with 95% confidence interval (CI) for the differences for COHb and TBCO before (B) and after (A) exposure and before (NF) and after flushing (F); *P*-values in **bold** are significant (below 0.05)

	COHb diff A/B (%)	COHb diff NF/F (%)	TBCO Diff A/B (µmol/mL)	TBCO diff NF/F (µmol/mL)
Mean	6.92	−0.18	1.43	0.45
SD	2.06	1.64	0.79	0.22
First quartile	6.20	−1.30	0.78	0.30
Third quartile	7.70	1.20	1.95	0.66
P-value	**6.02*e^{-07}**	0.71	**2.93*e^{-05}**	**8.96*e^{-06}**
95% CI	5.98; 9.53	−1.17; 0.81	0.95; 1.90	0.32; 0.58

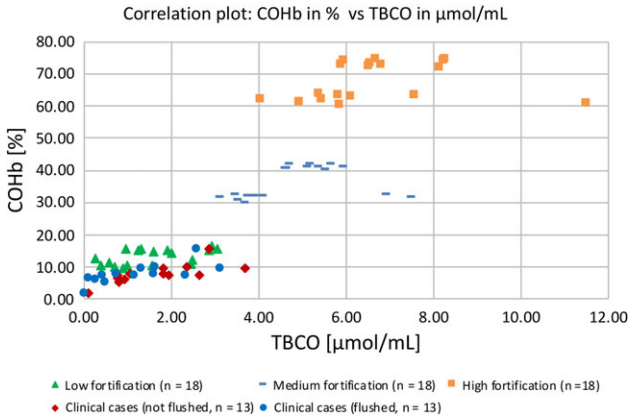

Figure 3. Correlation plot between TBCO in µmol/mL measured by AGS–GC–MS vs. the COHb saturation in % measured through CO-oximetry for two groups of samples: stored blood samples fortified with CO (squares for high fortification level 60–70%, lines for medium fortification level 30–40% and triangles for low fortification level 10–20%, $n = 54$) and real case samples (circles for flushed and diamonds for not flushed samples, $n = 26$).

collection. However, these findings have to be verified on a higher number of volunteers, even if the experimental design is complex to perform due to time-dependent analysis. Moreover, there is no clear evidence about a constant amount of CO dissolved, since in each patient the difference between TBCO before and after flushing varies. These variations are most likely due to interindividual variability: several factors such as pre-existing cardiovascular or respiratory conditions, metabolic rate, ventilation rate and volumes, sex and age can play a role in the behavior and amount of CO in blood. Additional measurements with more individuals will lead to higher statistical significance and will reduce the interindividual variability. In addition, analytical parameters affecting the storage as well as biological phenomena taking place after sampling of blood are known to potentially alter the measurement results, even if we reduced them to a minimum in this study. Further investigations into these TBCO pharmacodynamics and pharmacokinetics are needed to account for this behavior.

Conclusion

This study presents the validation of an improved CO analysis method in human blood, based on AGS–GC–MS, for a range of 1.63–104 nmol/mL HS (0.65–41.6 µmol/mL blood), which is applicable to clinical CO exposure cases. The method was applied to a cohort of 13 patients, who were exposed to controlled amounts of CO, and the results were compared to measurements by CO-oximetry. Furthermore, a flushing step was performed on samples after CO administration. Results seem to support the hypothesis that TBCO may be an alternative to COHb as a biomarker for determination of CO poisoning, since consideration of only CO bound to Hb may underestimate the total burden of CO in blood. By comparing flushed and unflushed samples, it was determined that there is a significant amount of CO present in blood in free form (10–60%) at the sampling time post-exposure.

This represents an important finding for the understanding of the true role played by CO in poisoning cases and for the explanation of the discrepancy often encountered by clinicians between symptoms and results and the onset of delayed neurological sequaelae, even after complete removal of COHb from the system after normo- or hyperbaric oxygen therapy, possibly leading to a decrease in the number of misdiagnoses. Nevertheless, before application of the method in clinical settings, this hypothesis needs to be verified by more numerous cohorts and in-depth statistical analyses, to increase statistical power. Additionally, further investigation into the biochemical mechanisms behind the distribution and behavior of dissolved CO in human blood is required.

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