GST Final Report

PhD Research Project

„Carbon monoxide poisonings: exploring new approaches for quantification and evaluating measurement errors from an analytical and epidemiological point of view in retrospective and prospective exposure studies"

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Background

Every year, approximately 50,000 emergency department (ED) visits and 2,741 deaths in the US are due to carbon monoxide (CO) poisoning. In Europe, CO-related deaths average at 350 annually, while in non-European states such as Australia and Japan, this number reaches 250 and over 3,200, respectively. It is thus not surprising, even for laymen, that the most common analogy used to describe CO is the silent killer. Mortality data shows that the majority of deaths are due to accidental, non-fire related (ANFR) CO poisoning. In the UK alone, around 200 hospital admissions and 40 deaths are attributed to ANFR CO poisoning annually. The global burden of CO on morbidity and mortality seems to be very high, but this should not be surprising considering that CO is one of the major air pollutants with high human toxicity. Of the total global CO emissions, 60% are anthropogenic, while 40% originate from natural processes.

CO is a gaseous, diatomic molecule with no taste, colour or odour – at first 'sight', not a menacing substance. However, despite its toxicokinetic and toxicodynamic mechanisms still not being sufficiently understood, CO is known to have the potential of causing severe adverse health effects involving the respiratory, cardio-circulatory and neurological systems, which can, in the worst cases, lead to death.

One of the major issues with CO poisonings is the non-specificity of the reported symptoms, which often lead to misdiagnosis by both the patient and the clinician, who attribute the signs to other more common illnesses, such as the flu, general stress or fatigue and gastrointestinal issues. In correctly diagnosed cases, the most common therapeutic measure is to administer normo- or hyperbaric O_2, which helps flush out CO and restore physiological O_2-levels in the body of the individual. However, this treatment is not possible in instances where low-level, chronic exposures occur. On one hand, this is due to the mentioned lack of suspicion and specific symptoms, leading to misdiagnosis. On the other hand, when the clinician or patient might be aware of potential exposure to CO and, thus, correctly diagnose it, the measurement method used for detection may have insufficient accuracy, the storage conditions could impact the CO burden measurement and the reported levels might be underestimated.

These misdiagnoses and underestimations do not only affect the immediate health status of an exposed individual, but they also have implications into the health status of the population. Air quality guidelines and required occupational air pollutant levels are set up based on risk and exposure assessment studies. Exposure assessment studies typically sample a representative amount of people in a population subgroup, measure their exposure and with the aid of air pollution monitoring and environmental models, extrapolate the results to the population. Therefore, mistakes in exposure measurements have a widespread relevance,

ultimately having an impact on the population morbidity, mortality and risk rates derived from CO.

Aims and objectives of the project

This project aims at tackling the raised issues from a scientific standpoint by following a path that links the toxicological to the epidemiological investigation.

i. One of the objectives is to develop and validate an alternative analytical method for more accurate CO poisoning determination for clinical and forensic applications, to help decrease misdiagnosis due to the inconsistencies between symptoms and COHb levels.
ii. Moreover, this research intends to determine and quantify the influence of storage parameters on changes in COHb/CO concentrations over time and aid in creating a model that allows incorporation of these alterations during the interpretation of results from CO poisoning determinations in individual measurements. This should also decrease the number of cases erroneously not attributed to CO as a cause of morbidity or mortality.
iii. An additional aim is to identify gaps in CO exposure assessments, with a focus on determining and quantifying measurement error arising from recent methodological and toxicological advances, including part of the work conducted in this study. This will enable the development of an overview of current practices and frequent errors in CO exposure assessment as well as generating an approach to correct for a part of these errors.
iv. Finally, this project wants to determine the magnitude of the impact that improvements in methodological and analytical measurement and exposure assessment methods can have on the population estimates and, thus, on the relative risk related to CO. This is an essential step that can directly link the analytical improvements with population health.

Major outcomes

1) A novel method to detect CO in blood has been developed, which is based on a headspace method combined with Gas Chromatography-Mass Spectrometry (GC-MS). This method measures the total amount of CO in blood (TBCO), whereas when using a spectrophotometric method such as a blood gas analyser, only the CO bound to Hb is measured. The method was validated and successfully tested on real cases in both clinical and forensic settings. Results show that this method provides more accurate and reliable results and, when compared to the traditional method of detecting carboxyhaemoglobin (COHb), that previous results were significantly underestimated.

2) The novel method was further investigated and compared to the traditional spectrophotometric method by testing the influence that storage conditions can have on the obtained results. This part of the study showed that storage conditions, especially the choice of the preservative and the storage temperature, have a significant impact on the results, leading to either falsely elevated or reduced values. Furthermore, it was pointed out that these effects were more prominent for the measurement of COHb than of TBCO. Therefore, it is recommended to store samples for CO analysis in the appropriate conditions and, when possible, use GC-MS analysis as a confirmatory method. It is not yet cost-efficient and practical to employ GC-MS for all CO analysis, especially in a clinical setting.
3) Through a thorough search and investigation of the literature, it was determined that there are significant gaps in CO exposure assessments, especially for low indoor levels, which are the most challenging levels for clinicians. Not only is there an insufficient number of studies performing exposure assessments for CO, but the few studies that have completed exposure assessments did not comply with the required methodology to obtain valid and accurate results from an exposure assessment.
4) The literature search was also used to generate a systematic overview of current trends and practices in CO exposure assessments and their sources of error, which points out the complexity of exposure assessments and highlights the importance of taking into consideration all potential sources of error. It also shows that the work carried out in this study, which is mainly focused on measurement error, is only one part that completes the bigger picture that is CO exposure.
5) Based on the overview of sources of error and a study of Ben Armstrong, which helps calculating measurement error, we have developed a protocol to simulate measurement error calculations and have applied this protocol to a selected study. Results from the study were compared to simulated results if a more accurate detection method, such as the one developed in this project, would have been used and then the effect that such differences have on the relative risk (RR) of CO exposure. Results show that reducing measurement error that contributes 5% of the total error increases the observed relative risk by 1%, whereas improving measurement error of 30% increases the relative risk by 8%, from 1.22 to 1.33. If measurement error contributes 50% of the total error, a better exposure measurement method can increase the RR by 17% to 1.43. For a pollutant like CO that is always present in small quantities in the atmosphere, this is a significant improvement, which shows that even with small changes, important improvements can be made to population health.

Publications

The research carried out in this project lead to the publication of following articles in scientific journals:

1) Oliverio, S. and Varlet, V., 2018. Carbon monoxide analysis method in human blood by airtight gas syringe–gas chromatography–mass spectrometry (AGS-GC-MS): relevance for postmortem poisoning diagnosis. Journal of Chromatography B, 1090, pp.81-89. https://doi.org/10.1016/j.jchromb.2018.05.019.

2) Oliverio, S. and Varlet, V., 2019. Total blood carbon monoxide: alternative to carboxyhemoglobin as biological marker for carbon monoxide poisoning determination. Journal of Analytical Toxicology, 43(2), pp.79-87. https://doi.org/10.1093/jat/bky084.

3) Oliverio, S. and Varlet, V. 2019. What are the limitations of methods to measure carbon monoxide in biological samples?. Forensic Toxicology, 38, pp. 1–14 (2020). https://doi.org/10.1007/s11419-019-00490-1.

4) Oliverio, S. and Varlet, V., 2020. New strategy for carbon monoxide poisoning diagnosis: Carboxyhemoglobin (COHb) vs Total Blood Carbon Monoxide (TBCO). Forensic Science International, 306, p.110063. https://doi.org/10.1016/j.forsciint.2019.110063.

5) Another manuscript is currently being finalized and will soon be submitted with the results from the exposure assessment critical review and simulation of measurement error improvements.

Valorisation

Results of this project have been presented at a variety of conferences and meetings, including:

1) Poster presentation at annual meeting of the International Society of Environmental Epidemiology (ISEE) Young – Europe Chapter – 19th-20th March 2018 in Freising, Germany
2) Oral presentation at a meeting of the All Parliamentary Party COMed group – 2nd May 2018 in London, United Kingdom

3) Oral presentation at annual meeting of the Swiss Society of Legal Medicine (SSLM) – 25th-26th May 2018 in Bulle, Switzerland
4) Oral and poster presentation at annual meeting of the International Association of Forensic Toxicology (TIAFT) – 16th-30th August 2018 in Ghent, Belgium
5) Oral presentation at the annual Postgraduate Research Conference of the Brunel College of Health and Life Sciences – 5th December 2018 in London, United Kingdom
6) Oral presentation at the biannual meeting of the Society of toxicological and forensic chemistry (GtfCh) – 10th-11th April 2019 in Mosbach, Germany
7) Poster presentation at the annual meeting of the International Society of Environmental Epidemiology (ISEE) – 25th-28th August 2019 in Utrecht, Netherlands
8) Poster presentation at the annual meeting of the International Association of Forensic Toxicology (TIAFT) – 2nd-6th September 2019 in Birmingham, United Kingdom

An abstract was also submitted and accepted for the 20th UK & Ireland Occupational and Environmental Epidemiology Conference to be held on 16th March 2020 in Bristol, United Kingdom.

Conclusion

The work carried out in this doctoral research provides a strategical pathway to approach the CO issue from both toxicological and epidemiological point of view and link the two disciplines, highlighting the importance of always requiring the complete picture to be able to understand the issues and tackle them with appropriate measures. For CO exposure, this includes implementing more accurate and reliable measurement methods but also carrying out more thorough exposure assessments, which need to be considered already in the study design phase. As one of the first in its kind, this research not only provided the necessary data and information on both the analytical/toxicological and epidemiological issues related to CO exposure, it also employed these findings to generate a linkage between the two fields, allowing us to directly observe the effects that changes in the laboratory can have on the population health. A lot of work still needs to be carried out to improve CO exposure assessments and measurements even further and a lot more funding needs to go into research in this field to achieve results with even higher impact. Nevertheless, with this work we have established a foundation and provided some valuable tools to help future researchers to continue towards closing the knowledge gap on CO exposure and CO poisonings.

Acknowledgments

Finally, we would like to thank the Gas Safety Trust immensely for giving us the opportunity to carry out this very important research and achieve such exciting results, which can hopefully help ameliorate public health and give a foundation for future research into this field.
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Carbon monoxide analysis method in human blood by Airtight Gas Syringe – Gas Chromatography – Mass Spectrometry (AGS-GC-MS): Relevance for postmortem poisoning diagnosis

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ABSTRACT

Carbon monoxide is one of the most abundant toxic air pollutants. Symptoms of a CO intoxication are non-specific, leading to a high number of misdiagnosed CO poisoning cases that are missing in the disease statistics. The chemical nature of the molecule makes it difficult to detect for long periods and at low levels, thus requiring a very accurate and sensitive method. Current methods capable of accurate and sensitive analyses are available, however an inconsistency between results and symptoms are frequently reported.

Therefore, an improved method for the analysis of carbon monoxide in blood and in the headspace (HS) of the sampling tube with the use of Airtight Gas Syringe – Gas Chromatography – Mass Spectrometry (AGS-GC-MS) is hereby presented and validated, for CO concentrations in a range of 10–200 nmol/mL HS (2–40 µmol/mL blood). Analytical LOQ is found at 0.9 nmol/mL HS (0.18 µmol/mL blood) and LOD at 0.1 nmol/mL gas. Application to intoxicated samples from autopsies and comparison to previously published methods show that this method is more appropriate, since performed under fully controlled conditions. Results show higher CO concentrations compared to previous approaches, indicating that results might have been underestimating the true blood CO burden. Therefore, this approach has the potential to help reduce the misdiagnosed cases and the gap between measurement and diagnosis of CO poisonings.

1. Introduction

A very simple chemical structure (molecule built by two atoms), formation during incomplete combustion of hydrocarbons and high occurrence in fires, exhaust fumes of motor vehicles, industrial exhaust gases, cigarette smoke and wood-fired stoves – those are all characteristics of carbon monoxide (CO) [1]. This odourless, tasteless and colourless gas has been related to numerous hospitalizations and deaths, not only due to its high toxicity, but mostly because of its chemical characteristics: exposure to CO occurs without the awareness of an individual. It is inhaled through the lungs and from there directly transferred to the blood stream [2]. Current knowledge affirms that once diffused to blood, CO combines with the haemoglobin (Hb) present to form carboxyhaemoglobin (COHb) [3] and is also transported to the tissues [4]. Hb is the oxygen-carrying protein molecule present in red blood cells. One of the main characteristics of CO is its high affinity for Hb, being 200–250 times higher compared to the affinity of oxygen (O_2) [5]. This results in CO competing with and displacing O_2 from the binding sites on the haeme, leading to a reduced oxygen-carrying and -storage capacity of Hb [6]. The main organs suffering from the deriving hypoxia are the brain and heart, since they are the organs with the highest oxygen requirement [5].

Other known damages caused by CO include the inhibition of mitochondrial respiration, the excess-activation of platelets (resulting in inflammation-like effects), ischemic and anoxic brain injuries and the generation of free radicals, which are known to be mutagenic and tumour cells-promoters [7,8].

The severity of the damages caused to an individual exposed to CO is related to the quantity and time of exposure to CO. However, the symptoms of a CO poisoning, which include dizziness, nausea, headache and respiratory troubles, do not always present themselves immediately, but appear only after a certain time delay, and when they do, they are often attributed to other types of diseases or infections [4]. Therefore, it is of high importance to have accurate and reliable, but also rapid and simple methods to measure the levels of CO poisoning, especially in cases where the symptoms do not give a clear indication of

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the causes.

Due to the high affinity of CO to Hb, it is assumed that the majority of CO binds with Hb when introduced in the blood circulation, which resulted in COHb being the primary biomarker for CO poisonings [1]. CO is mainly eliminated unchanged through the lungs. Between 10 and 50% of CO in the organism is bound to tissue proteins, mainly myoglobine (Mb) and cytochrome *c* oxidase, and the rest is thought to be under bound form as COHb [9].

Until now, the most common technique used in clinical as well as post-mortem routine analyses is the measurement of the COHb-levels by CO-oximetry (blood analysis). CO-oximetry is a technique based on automated differential spectrophotometry, which measures the concentration of an analyte by relating it to the measured absorbance when exposed to light of different wavelengths, according to the Lambert-Beer-Law. With a CO-oximeter, the saturation levels of COHb (%), methaemoglobin (MetHb), oxyhaemoglobin (O_2Hb) and normal, non-carrying haemoglobin (HHb) are measured [10]. Pulse CO-oximeters (clinical finger monitoring) can additionally determine standard pulse oximeter parameters such as oxygen saturation, pulse rate and perfusion index [11]. The major advantage of pulse CO-oximetry is that the measurement is done continuously and is non-invasive, thus allowing the monitoring of the parameters in a clinical setting, without causing pain or damage to the patient. However, this technique cannot be used in postmortem samples, since an active blood circulation is needed to obtain results and clinical samples taken perimortem have an excessively significant sampling time delay.

A major drawback of spectrophotometric methods is the dependence on the optical state of the sample. Degradation of the sample due to storage as well as postmortem interferences, such as thermo-coagulation [12], contamination due to incomplete haemolysis, high lipid concentrations or thrombocytosis and putrefaction [13], can change the blood state and result in either an alteration of the measurement or the impossibility of the device to determine a value. Consequently, another biomarker of CO exposure should be investigated and its detection method should not be optical-based.

Therefore, techniques focused on direct CO rather than optical ones that focus on COHb, which are independent of the quality of the blood sample, have been investigated and developed. The most successful was found to be Gas Chromatography (GC) in combination with a variety of detection methods, such as thermo conductivity detector (TCD), flame-ionization detector (FID), Reduced Gas Analyzer (RGA) and Mass Spectrometry (MS).

In gas chromatographic CO detection, CO is released at gaseous state through a liberating agent, after lysis of the blood, and then analysed. Haemolysis is performed through the use of a haemolytic agent, the most common ones being saponine, Triton X-100 or other detergents. Liberation of the CO occurs through the reaction with a strong acid, which yields CO and water as the only products [12,14–17]. As releasing agents, sulphuric acid (H_2SO_4), hydrochloric acid (HCl) and potassium ferricyanide ($K_3Fe(CN)_6$) are generally used. Other acids such as lactic acid [18], citric acid [18,19] or phosphoric acid [19] have also been tested.

For the gas chromatographic separation, a capillary column with a 5 Å molecular sieve has been found to be specific for the separation of CO from other interfering gases such as carbon dioxide (CO_2), nitrogen (N_2), oxygen (O_2) and methane (CH_4) [20].

Detection of the analyte is achieved with numerous detectors linked to the gas chromatograph. The first detector applied to CO-determination was TCD [15], later replaced by other detectors, such as FID [20]. For detection with FID, CO is chemically reduced to methane with a methanizer and then detected. This method is very sensitive and specific and was the most popular detection system used in conjunction with CO [17–19,21–24]. Nevertheless, one of its major drawbacks is the fact that the addition of a methanizer to the apparatus is needed, which limits the use of the instrument only for CO-analysis. Therefore, another type of detector employed was MS. The developed MS methods are more simple, rapid, accurate, reproducible, in addition to the versatility of the instrument, since it can be used for all types of analysis and hence is useful in laboratories for routine analyses [13,25,26]. Furthermore, MS allows for a higher power of identification: additionally to the retention time, the compounds are identified with the mass spectrum, which allows quantification with a stable labelled isotope as internal standard.

One issue regarding all measurement methods is the calibration. Calibration of the techniques was performed either with pure CO gas, which was diluted appropriately, or with the fortification of blood with CO to reach different COHb% saturation levels. In the latter, 100%-saturation was confirmed with either UV-spectrophotometry or CO-oximetry. Reliability can be debated though, considering that, first of all, the spectrophotometric methods used at that time were only detecting at several wavelengths, while modern CO-oximeters analyse the full spectrum, leading to a possible error in the obtained values. Secondly, these optical methods only measure the CO bound to Hb, not taking into account possible dissolved CO present in the sample that was not taken into account when building the calibration curve, which could shift the 'real' curve into higher levels of CO poisoning.

An alternative calibration method was developed firstly in 1993, where Cardeal et al. used the reaction of formic acid with sulphuric acid to form CO [19]. Varlet et al. went another step further by developing an approach which uses isotopically labelled formic acid (^{13}HCOOH) to produce ^{13}CO as internal standard for a Headspace (HS)-GC-MS method [26].

The HS-GC-MS approach with isotopically labelled formic acid used for building of the calibration curve shows the most accuracy, sensitivity, specificity and reproducibility. However, after development and validation [26,27], no further research was carried out in the field.

An additional issue involves the currently existing correlation between the COHb%-levels and the symptoms developed by patients, which do not always agree: patients were found to have an elevated COHb% saturation level, but showed no signs of CO-intoxication, while other patients with a low COHb%-level lost consciousness or suffered severe delayed consequences [28]. Thus, there seems to be a great fallacy in the understanding of the true role played by CO in poisoning cases. This might be due to an underestimation of the total CO measured with the current techniques and the neglect of the possible presence of CO in dissolved state and not bound to Hb, which can have major implications in the role of CO in the pathophysiology of a CO-poisoning.

Therefore, an improved approach by Airtight Gas Syringe (AGS) followed by GC-MS for CO determination is hereby presented, which not only shows improved sensitivity and lower costs, but also takes into account the total amount of CO present in blood by analysing the CO in blood and in the headspace of the blood tube used to store the sample, with high importance from both an analytical and clinical point of view. This constitutes the first step to acknowledge the significance of total CO in blood as alternative biomarker for CO exposure.

2. Materials and methods

2.1. Chemicals and reagents

Calibration standard: formic acid (reagent grade, purity ≥ 95%) was purchased from Sigma-Aldrich (St Louis, USA) and CO gas (99%) from Multigas (Domdidier, Switzerland). All formic acid solutions were prepared daily to prevent degradation. Internal standard: formic acid (13C, 99%) was obtained from Cambridge Isotope Laboratories (Cambridge, UK). Sulphuric acid (≥97.5%) was from Fluka (Buchs, Switzerland). Bovine blood obtained at a local butchery is used as blank matrix for calibration.

2.2. Materials

The Avoximeter 4000 Whole Blood CO-Oximeter Cuvettes were purchased from International Technidyne Corporation - ITC (Edison, USA). S-Monovettes of following types: 2.6 mL K3E, 3 mL 9NC, 2.7 mL FE, 2.6 mL KH, were obtained from Sarstedt (Nürnbrecht, Germany). Precision sampling gas syringes equipped with a press button valve with capacity of 500 µL for dilutions and 2 mL for injections were from VICI (Baton Rouge, LA, USA). Aluminium caps from Milian (Vernier, Switzerland). All headspace extractions were carried out in 20 mL headspace vials from Agilent Technologies (Santa Clara, CA, USA).

2.3. Instruments and GC-MS conditions

For spectrophotometric analysis, AVOXimeter 4000 Whole Blood CO-Oximeter from ITC was used. Manufacturer guidelines were followed to obtain COHb analyses.

For gas chromatographic analysis, 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) obtained from Restek (Bellefonte, USA) was used. Following temperature programme was used: 50 °C, held for 4 min; the injector was set at 180 °C, used in splitless mode, and the MS interface at 230 °C. The employed carrier gas was helium at a flow rate of 40.0 mL/min. A solvent delay of 1.8 min was used.

For detection, Agilent 5973 mass spectrometer (Palo Alto, USA) was used, operating in electron ionization (EI) mode at 70 eV. Selected Ion Monitoring (SIM) mode was used to acquire the signal for CO at *m/z* 28 and ^{13}CO at *m/z* 29.

2.4. Sample preparation

2.4.1. Fortification of blood

Fortification of blank bovine blood was carried out by bubbling the tubes containing blank bovine blood with pure CO gas for a certain amount of time. The COHb% saturation levels were checked in 10-minute-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 by CO-oximetry. Because no flushing occurs after blood collections from intoxicated patients, this step was not planned in the experimental design in order to be the most representative of CO pathophysiology.

2.4.2. Calibration standards

An aliquot of freshly sampled bovine blood, which was previously analysed with CO-oximeter to guarantee absence of CO before use, is used as matrix for calibration. Fresh solutions of the working calibration standard formic acid (87 nmol/µL) and working internal standard isotopically labelled formic acid (84 nmol/µL) were prepared daily with deionised water to prevent degradation. Calibration points were set in a working range between 0 and 208 nmol/mL HS, congruent with CO-saturation in a range relevant for postmortem samples (based on the results obtained from available real postmortem samples), with points at 6.5, 13, 26, 52, 104, 156 and 208 nmol/mL HS (equivalent to 2.6, 5.2, 10.4, 20.8, 31.2 and 41.6 µmol/mL in blood). Matrix effects were evaluated by preparing a blank sample with the matrix without any reagent. 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/µL. All standards were stored at +4 °C when not in use.

2.4.3. Quality controls (QC)

QC samples were prepared daily with formic acid obtained from a different lot. Five QC solutions at concentrations of 10, 25, 80, 150 and 200 nmol/mL HS (2, 5, 16, 30 and 40 µmol/mL blood) were obtained from formic acid diluted with deionised water.

Additionally, the validity of the method was tested with an external control, which was prepared by dilution of pure CO gas at two concentration levels, low and high, respectively 20 and 150 nmol/mL HS.

2.4.4. Extraction procedure

100 µL of blood was introduced in a 20 mL HS-vial, followed by aliquots of the various formic acid solutions for the calibrators and 10 µL internal standard solution. Subsequently, an aluminium cap of 11 mm (i.d.) was first filled with 100 µL of sulphuric acid and then carefully introduced into the HS-vial. The vial was immediately hermetically sealed with magnetic PTFE/silicone septum caps of 20 mm (i.d.) and afterwards vigorously shaken and vortexed, in order to ensure complete mixing of the liquids contained in the vial. After preparation of all vials, extraction was completed by heating at 100 °C for 60 min.

Concerning real samples, two analyses were performed per tube: CO analysis in the HS before tube opening in order to measure an eventual CO release in tube HS during storage and CO analysis in blood after tube opening and blood sampling. For CO in HS-analysis, internal standard and sulphuric acid only were inserted into the HS-vial.

2.5. Analysis procedure

2.5.1. CO-oximeter

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

2.5.2. CO in HS

250 µL HS were sampled from the closed blood tube and inserted into a previously prepared 20 mL HS-vial with internal standard. Subsequently, 1 mL was sampled and injected in the GC-MS for analysis.

2.5.3. CO in blood

1 mL 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, a sample of air in the analysis-room was also analysed.

2.6. Validation procedure

The validation was carried out according to the guidelines of the "French Society of Pharmaceutical Sciences and Techniques" (SFSTP). Their validation criteria include the following: selectivity, response function (calibration curve), linearity, trueness, precision (repeatability and intermediate precision), accuracy, limit of detection (LOD) and limit of quantification (LOQ). The validation experiments were conducted with calibrators and QC samples on three non-consecutive days (p = 3) and in two separate weeks. The approach was based on the use of a β-expectation interval tolerance of 80%, meaning that the intervals for each experimental point contain an average of 80% of the total values. The tolerance intervals (TI) were defined as $TI = X \pm k \times \sqrt{(S_r^2 + S_R^2)}$, where S_r^2 is the standard deviation of repeatability and S_R^2 is the standard deviation of reproducibility. In the β-expectation interval tolerance approach, $k = t_\nu \times \sqrt{(1 + [1 / (I \times J \times B^2)])}$, where I is 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 $R = S_r^2 / S_R^2$. t_ν is Student's coefficient with degrees of freedom ν defined as $\nu = (R + 1)^2 / \{[(R + 1 / J)^2 / (I - 1)] + [(1 - 1 / J) / (I \times J)]\}$.

2.7. Postmortem samples

A set of three samples of both cardiac and peripheral blood from CO positive cases were analysed (Table A.1). The samples obtained during

Table A.1
Summary of relevant information on a set of real postmortem cases.

Sample	Sample type	Age	Sex	Manner of death
1	Cardiac and peripheral blood	22	F	Fire victim
2	Cardiac and peripheral blood	67	M	Fire victim
3	Cardiac and peripheral blood	44	M	Suicide by CO intoxication

autopsy were all with a postmortem-interval (PMI) of < 40 h, and analysed immediately after collection at the toxicology lab. Measurement with CO-oximeter and GC-MS were performed with the above mentioned conditions. The samples were then subdivided into sampling tubes with four different preservatives, namely ethylenediaminetetraacetic acid (EDTA), sodium fluoride (NaF), trisodium citrate (Cit) and lithium heparin (LiH), and stored at −20 °C for a period between 4 and 7 weeks. Subsequently, for all samples the COHb and CO concentrations were determined with CO-oximetry and GC-MS respectively, where GC-MS was used to determine the CO in both the headspace and the blood of the sample. Additionally, the validity of the proposed approach was tested by comparison with approaches previously published by Cardeal et al. [19] and Sundin et al. [22] by backcalculating the COHb%-levels from the obtained CO concentrations. These methods were found to give similar results to other methods found in the literature [29,30].

3. Results

3.1. Selectivity

The selectivity of the method was assessed with the measurement of samples obtained by the mixture of various intracadaveric gases with CO. These analyses were evaluated for co-eluting chromatographic peaks with possible interferences with either the CO or ^{13}CO detection. No interference peaks for any of the other gases were observed at CO *m/z* ratio of 28 or ^{13}CO *m/z* ratio of 29 (see Fig. B.1), which indicates that the method is sufficiently selective for determination of CO.

3.2. Response function (calibration curve)

The response function, also known as the calibration curve, is defined as the relationship between the concentration of the analyte in the sample and the corresponding response. An assay of calibration curves was performed for CO determination by using bovine blood as blank matrix and each point of the curve was defined as the area ratio of CO to ^{13}CO. The calibration curves were prepared on three non-consecutive days (p = 3), in triplicates (n = 3) and at seven concentration levels (k = 7): 6.5, 13, 26, 52, 104, 156 and 208 nmol/mL HS (equivalent to 1.3, 2.6, 5.2, 10.4, 20.8, 31.2 and 41.6 µmol/mL in blood). The calculated concentrations for each calibration point were compared to the target values and found to be within ± 20%. A linear relationship between the CO concentration from samples spiked with formic acid and the measured response was determined. Table A.2(I) shows the validation results for the calibration curves.

3.3. Linearity

The linearity of the model was evaluated by fitting back-calculated concentrations of control samples against the theoretical concentrations through the application of the linear regression model. On each non-consecutive day (p = 3), control samples at five different concentrations (k = 5), namely 10, 25, 80, 150 and 200 nmol/mL HS (2, 5, 16, 30 and 40 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 represented in Table A.2(II), a satisfactory linearity was obtained, with a slope of 0.9887 and a regression coefficient of 0.989 in the range of 10 to 200 nmol/mL HS (2–40 µmol/mL blood).

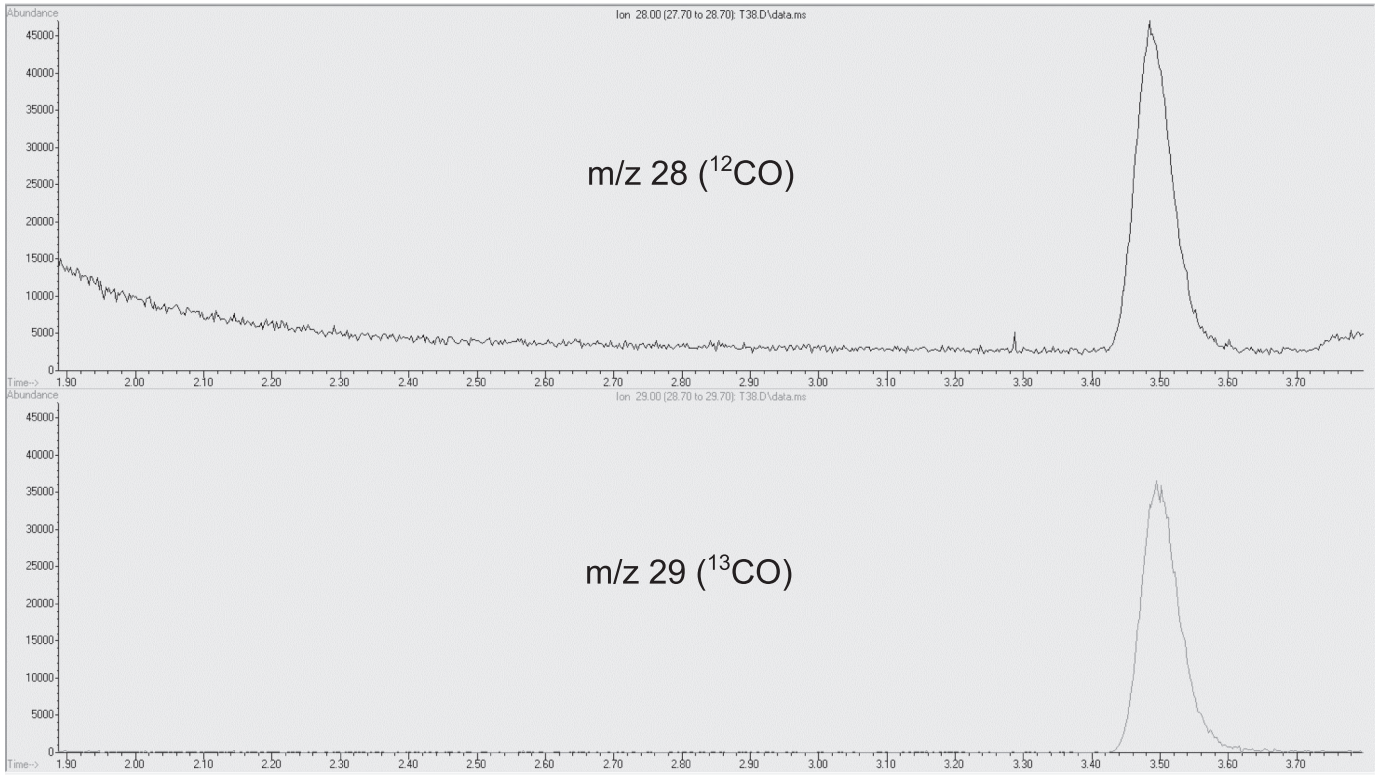

Fig. B.1. Extracted ion chromatograms for *m/z* ratios 28 (upper) and 29 (lower), corresponding to CO and ^{13}CO respectively, for selectivity tests.

Table A.2
Validation parameters.

(I) Response function [6.5–208 nmol/mL HS] (k = 7, n = 3, p = 3)			
	Day 1	Day 2	Day 3
Slope	0.0252	0.0219	0.0214
Intercept	0.4698	0.5803	0.4528
r^2	0.9892	0.9864	0.9920

(II) Linearity [10–200 nmol/mL HS] (k = 5, n = 3, p = 3)	
Slope	0.9887
Intercept	−0.5322
r^2	0.9962

(III) Trueness (relative bias %) (k = 5, n = 3, p = 3)	
Levels [nmol/mL HS]	Trueness (%)
10	−12
25	0
80	−2
150	−3
200	−5

(IV) Precision (RSD%) (k = 5, n = 3, p = 3)		
Levels [nmol/mL HS]	Repeatability	Intermediate precision
10	0.951	0.952
25	4.001	4.326
80	5.980	5.980
150	8.364	11.347
200	4.046	8.630

3.4. Trueness

The trueness, also known as bias, expresses the closeness between the experimental average value and the calculated target value and is expressed as the percent deviation from the calculated target value. Trueness was found to be lower than the acceptance criteria (within ± 15 of the accepted reference value and within 20% of the LOQ), as can be seen in Table A.2(III), and hence defined as satisfactory for CO determination.

3.5. Precision: repeatability and intermediate precision

Precision is designed to detect random errors and is defined as closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. It is assessed by calculating the repeatability (intra-day precision) and intermediate precision (inter-day precision) for each control sample. The repeatability variance was established by calculating the intra-day variance (S^2r) and the intermediate precision was determined through the sum of intra- and inter-day variances (S^2_{IP}). As can be seen in Table A.2(IV), the RSD for repeatability and intermediate precision are in a range between 0.95% and 11.95%.

3.6. Accuracy and limit of quantification

Accuracy expresses the total error defined by the sum of trueness (systematic error) and precision (random error). It is defined as the closeness of agreement between the conventional true value or an accepted reference value and the value found. The accuracy profile for CO, depicted in Fig. B.2, expresses the method's ability of providing analytical results by using both systematic and random errors, with a risk set at α = 5% for each concentration level. 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 limit of quantification within validation criteria was found at below 10 nmol/mL HS (2 µmol/mL blood). Thus, the method is confirmed to be accurate within the range of 10 and 200 nmol/mL HS (2–40 µmol/mL blood), considered as the relevant range for postmortem analyses. The analytical LOQ was later determined at 0.9 nmol/mL HS (0.18 µmol/mL blood).

3.7. Limit of detection

The LOD was determined by analysis of samples containing sulphuric acid and decreasing amounts of formic acid and assessed by using a signal-to-noise ratio of S/N > 3. The noise was estimated by measuring 15 blank samples. The resulting LOD for CO quantification was found at 0.1 nmol/mL gas.

3.8. Matrix effects

The possible presence of matrix effects was evaluated by comparing the results obtained from the analyses of blank samples containing only bovine blood (Bl-IS) and sulphuric acid and samples containing bovine blood, sulphuric acid and internal standard (Bl + IS). The Bl-IS samples show the generation of low amounts of CO, which is most likely due to the acidic conditions the reaction takes place as well as heat, which lead to decomposition and degradation of proteins contained in blood. This confirms what was previously reported by Varlet et al. in 2012 [26].

The Bl + IS samples allow for quantification of the matrix effects through interpolation of the calibration curves. The matrix effects were quantified as a mean concentration of 21,8 nmol/mL HS (4.5 µmol/mL blood), with a standard deviation (SD) of ± 4.3 nmol/mL HS (0.9 µmol/mL blood). With the use of an internal standard, this matrix effect is taken into account equally for all samples analysed.

3.9. Analyses of postmortem samples

Three postmortem blood samples were analysed in order to assess the performance of the method and its applicability on real human blood samples of people subjected to fatal CO intoxication. Changes due to storage were investigated by re-analysing each sample after a period of one month, in which the sample was stored at −20 °C with different preservatives (EDTA, NaF, Heparinate, Citrate). The results are presented in Table A.3. The results obtained for the three postmortem cases were also compared to the results obtained from the fortification of blank blood with CO in the range of 60%–80% COHb, as obtained by CO-oximetry analysis (Fig. B.3).

4. Discussion

4.1. Determination of CO through AGS-GC-MS

CO content of three cardiac blood and three peripheral blood samples was determined through AGS-GC-MS, showing significant amounts of CO. Measurements by CO-oximetry, when possible, also result in high amounts of COHb determined (all > 50%), indicating that CO intoxication was most likely the cause of death.

For cardiac blood of case #3, no measurement was possible with the CO-oximeter due to the poor quality of the blood sample, leaving peripheral blood as the only available sample for COHb%-determination. It was however possible to determine the CO content of cardiac blood through AGS-GC-MS, which resulted in a concentration of 50.5 nmol/mL HS (10.1 µmol/mL blood). Cardiac blood of samples 1 and 2, which when analysed by CO-oximetry revealed a COHb concentration of > 75%, resulted in CO concentrations of 142.5 nmol/mL HS (28.5 µmol/mL blood) and 68.8 nmol/mL HS (13.2 µmol/mL blood) respectively. Therefore, if compared with the results from cardiac blood

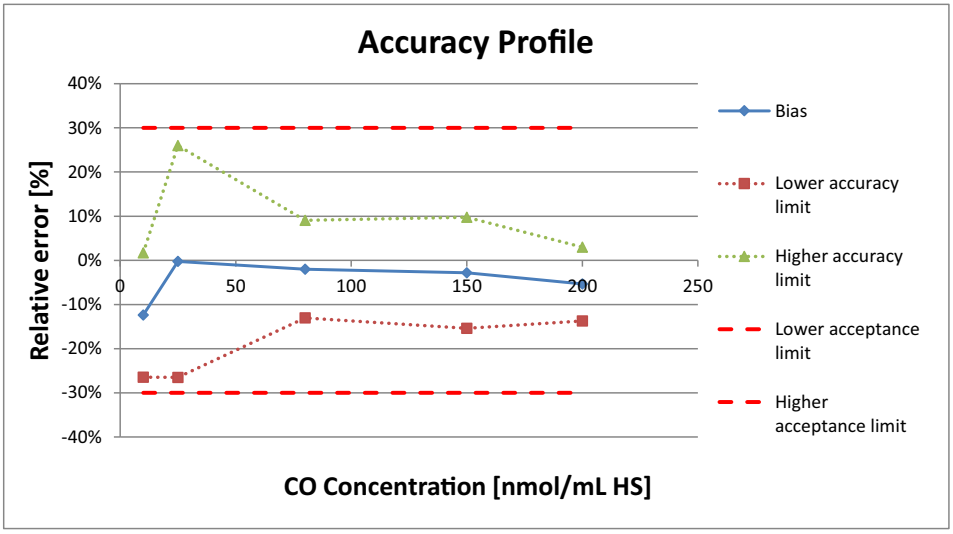

Fig. B.2. Accuracy profile for CO determination using a simple linear regression model within the range of 10–200 nmol/mL HS (2–40 µmol/mL blood). The continuous line represents the trueness (bias), the dashed lines represent the acceptance limits set at ± 30% and the dotted lines are the relative lower and upper accuracy limits.

Table A.3
Summary of data from the analyses of three real postmortem cases with suspicion of CO intoxication, analysed by CO-oximetry and AGS-GC-MS.

Sample		Preservative	COHb [%] CO-oximetry		CO in blood [nmol/mL HS] (µmol/mL blood) AGS-GC-MS	
			Day 0	Day 30	Day 0	Day 30
1		EDTA	> 75	> 75	142.45	80.00 (16.00)
	CB	NaF		> 75	(28.49)	78.04 (15.61)
		LiH		> 75		93.15 (18.63)
		Cit		> 75		77.73 (15.55)
1		EDTA	57,1	49.7	89.92	68.80 (13.76)
	PB	NaF		44.9	(17.98)	87.33 (17.47)
		LiH		61.8		62.38 (12.48)
		Cit		55.3		47.57 (9.51)
2		EDTA	> 75	70.7	68.77	95.55 (19.11)
	CB	NaF		64.8	(13.75)	108.10 (21.62)
		LiH		70.5		79.75 (15.95)
		Cit		71		57.10 (11.42)
2		EDTA	69.5	65.1	100.62	119.50 (23.90)
	PB	NaF		47.5	(20.12)	136.21 (27.24)
		LiH		64.7		81.94 (16.39)
		Cit		64.8		52.15 (10.43)
3[a]		NaF	NA	NA	50.48	47.60 (9.52)
	CB				(10.10)	
3[a]		NaF	> 75	70.8	113.09 (22.62)	64.15 (12.83)
	PB					

Samples: cardiac blood (CB), peripheral blood (PB); preservatives: ethylenediaminetetraacetic acid (EDTA), sodium fluoride (NaF), lithium heparin (LiH), trisodium citrate (Cit).

[a] For Sample 3, second analyses were carried out after 52 days.

of samples 1 and 2, the cardiac blood concentration in sample 3, with a concentration of 50.5 nmol/mL HS (10.1 µmol/mL blood), is significant and could be indicative of CO poisoning. This was further confirmed by the peripheral blood sample, which showed a COHb% level of > 75% and a CO concentration of 113.1 nmol/mL HS (22.6 µmol/mL blood).

Nevertheless, this case confirms the significant drawbacks of optical measurement methods. Without the peripheral blood sample, accurate determination of COHb levels would have not been possible, leaving the case unsolved, whereas total CO concentration by GC-MS was still possible, allowing interpretation. Furthermore, the importance of the development of an alternative method for CO determination, such as AGS-GC-MS, is highlighted, which might have the potential to also be of use in routine CO-poisoning determinations.

However, it is not yet possible to fully interpret the results obtained through AGS-GC-MS measurements from a diagnostic point of view, since correlations between the symptoms and COHb% levels are the only associations available in the literature. Until now, no correlation between total blood CO and the symptoms has been developed. To obtain that, a complete assay to study the link between total CO in blood, independent of Hb, and the symptoms is required.

Fig. B.3 illustrates that the CO concentration range associable to lethal doses (above 55% COHb) is hereby found to be above 45 nmol/mL HS (9 µmol/mL blood). This result is consistent with the CO concentration of 3 µmol/mL blood previously proposed to define CO as an actor in the cause of death [27].

4.2. Backcalculation of COHb% from CO

To assess the validity of the proposed approach, the results obtained for CO concentrations were backcalculated to COHb% saturation levels with formulae already published and compared to the values obtained through CO-oximetry. In fact, the correlation between the CO measured with GC and COHb% levels and the use of formulae to backcalculate the CO to COHb% is still under discussion. Several formulae have been published to calculate the equivalent COHb% from the values obtained through the analyses with gas chromatographic methods [13,16,19,22,29]. The application of these formulae is mainly due to apparent satisfactory correlation between the spectrophotometrically measured COHb% levels and CO levels obtained through the backcalculation from the GC-analyses. However, these correlations were obtained with different experimental designs. While CO levels were measured with GC-MS or GC-FID, COHb ranges did not cover the full range of expected COHb saturations: for example, Cardeal et al. [19] obtained their correlation formula with a dataset that included a CO concentration range of 0.005–16.85 nmol/mL HS (0.001–3.35 µmol/mL blood), which is equal to backcalculated COHb% levels of 0.01%–16.1%, therefore being exclusively in a low level, clinical range; Sundin et al. [22] have a range that covers even lower levels, between 0.5% and 5% COHb, which are within normal clinical levels for not intoxicated people.

Another difference concerns the calibrators, which were artificially generated *in situ*, either diluted from pure gaseous CO or prepared after fortification of blood with CO followed by flushing with an inert gas, intended for the removal of residual dissolved CO. However, through this flushing step, the presence at the time of analysis of CO dissolved in blood and not bound to Hb is neglected, which is not in accordance with the pathophysiology of CO intoxications [4]. Nevertheless, despite these alterations, a majority of coherent results were confirmed with the use of several formulae by Varlet et al. [26].

In the present study, the backcalculated COHb% values obtained by

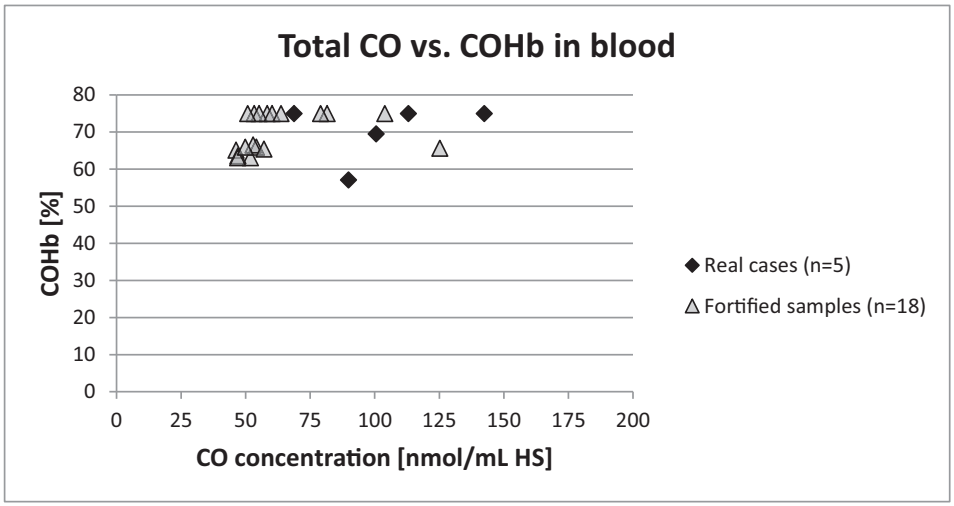

Fig. B.3. Correlation plot between the total CO concentration in nmol/mL HS (measured by AGS-GC-MS) *vs.* the COHb saturation in % (measured through CO-oximetry) measured in blood for two groups of samples: real case samples (n = 5) and samples fortified with CO stored in blood (n = 18).

applying the formulae by Cardeal et al. [19] and Sundin et al. [22] were found to be in a range between 83% and 274% and 82% and 285% respectively, which is prevalently outside the physiologically relevant range and not consistent with the results obtained with the analyses by CO-oximetry. While the lower limits are in conformity with the expected high COHb amount, the highest backcalculated COHb saturations are not relevant. Despite the fact that the number of samples analysed in this study is not statistically significant, the results cannot be disregarded and legitimates the investigation to identify the source of variation for higher backcalculated COHb results:

(i) Postmortem changes can lead to CO production through microbial metabolism and/or erythrocyte catabolism. However, all samples were obtained with minimal PMI, no postmortem changes could have occurred with our samples. Storage of the body and sampling was performed according to the regulations of Swiss forensic laboratories, therefore no degradation of the sample occurred due to poor storage conditions. However, no detailed information about the origin of the samples, PMI and storage conditions were given by Cardeal et al. [19], Sundin et al. [22] or Vreman et al. [29], even though these are important factors that need to be controlled to guarantee reproducibility and specificity of the results. Thus, this is a possible cause for the incongruence between approaches.

(ii) A part of blood CO burden is not bound to Hb in the tubes at the time of analysis. This might be explained by COHb dissociation during storage in tubes and/or during PMI in the body, or an existing, variable amount of CO dissolved at the time of sampling. The hypothesis of the presence in blood of CO dissolved during intoxications can be formulated because even though CO dissolved in blood binds preferably to Hb, an unknown part can remain dissolved until binding with tissue proteins (Mb) and intracellular distribution (mitochondrial distribution) occurs. So far, it was assumed that once in the blood system, CO binds almost completely to Hb, resulting in COHb as the used biomarker for CO intoxications. But the results obtained here provide some doubts in whether there is more CO present in blood than just COHb and that the CO dissolved might play a more important role than expected [31]. This can result in a highly relevant role from a physiological perspective, with possible pathophysiological effects caused by the amount of CO dissolved in blood that have not yet been investigated, but might be relevant for both clinical and forensic cases. This could also help finding an explanation for the disagreement between the symptoms reported and COHb% levels measured in individuals. For this purpose, the correlation between total CO in blood and reported symptoms could be investigated.

Furthermore, in PM blood samples, CO might also originate by dissociation from Hb, Mb or cytochromes, since binding of CO to these proteins is reversible with time. COHb measured optically in this type of sample would show only a part of CO present in blood and would not take into account this variable and unknown part of CO dissolved.

(iii) Finally, a part of the CO in blood not bound to Hb in the tubes at the time of analysis can originate from COHb dissociation during storage. When blood showing an important COHb saturation is exposed to air for significant time, the COHb measured at the end of exposure by optical methods was found to be lowered [32]. As time goes by during storage, CO dissociation from Hb can be hypothesized because the CO-Hb bond is reversible. Many studies have been led to investigate the influence of storage conditions on COHb measured by optical methods and an important diversity of results was obtained. However, to our knowledge, few data is available concerning the influence of storage conditions on CO measured by GC-MS. Moreover, the part of CO dissociated from COHb during storage could only partly explain the discrepancy between the results from the optical measurement methods and the backcalculated results – for values as high as 75% COHb, even if completely dissociated, theoretically the CO dissolved could not reach values that backcalculated are higher than 100%. Furthermore, COHb decreases did not exceed 20–25%.

Preliminary tests for the evaluation of the role played by preservatives in the stability of the sample were performed with two sets of samples from two cases. The COHb and CO concentrations obtained for the measurements with different preservatives (Table A.3) show the same trend for case 1, while for case 2 increased CO concentrations were observed for two of the preservatives (EDTA and NaF) and decreases for the other two preservatives (LiH and NaCit). Previous studies suggested that no significant effects or differences were observed when using EDTA or NaF tubes [33], which contradicts our results for case 2, where increases are observed in both cardiac and peripheral blood. No antecedent research was found on the other two preservatives LiH and NaCit. Therefore, our results do not give clear indications on whether preservatives play a relevant role in the stability of samples and given the limited number of samples, no significant conclusions can currently be drawn from our data. The role of preservatives might be of relevance, but further investigation is required. In order to be able to determine the significance of the influence with a

strong statistical relevance, a higher number of samples need to be tested and evaluated.

Additionally to the preservatives, there are several other factors that could influence the measurement of total CO in blood and have not yet been investigated. Storage conditions, such as temperature and volume of air in sampling container, are known to be of relevance in the measurement of samples in forensic and clinical cases and have specifically been studied for the measurement of COHb through spectrophotometric methods and gas chromatographic methods, which used calibrators with flushing of CO in excess prior to the analysis [14,32–34]. However, no data is available on storage conditions for the measurement of total CO in blood.

In the present study, a measurement of the amount of CO in the HS of the samples was also performed. However, the amounts detected were as low as 0.001% of total CO, indicating that no significant amounts of CO are released into the HS during frozen storage over one month. This implies that CO would remain under dissolved form in blood during storage. But given the limited number of samples, this assumption is not definitive and needs to be analysed further for confirmation.

Nevertheless, in the present study the discrepancy between optical measurements of COHb% by CO-oximetry and backcalculated COHb% from total CO measured by GC-MS is noticeable even in samples immediately analysed without any storage. As a result, the important backcalculated COHb saturations might in fact derive from variable amount of CO dissolved in blood, not bound to Hb at time of sampling. The CO-oximeter is only capable of quantifying the amount of CO bound to Hb. With this AGS-GC-MS approach the total amount of CO present in the blood sample is analysed, which is a sum of COHb and dissolved CO.

5. Conclusion

An AGS-GC-MS method for the quantification of the total amount of CO in blood from CO-poisoning cases, validated according to the 'β-expectation tolerance interval' accuracy profile as recommended by the SFSTP, was hereby exposed. The method presents improved sensitivity (lower LOD and LOQ) and lower costs due to reduced quantities of reagents compared to the previously published study by Varlet et al. from 2012 [26]. Moreover, the method is accurate and reliable (± 30%) for measurements of CO concentrations in a range from 10 to 200 nmol/mL HS (2–40 µmol/mL blood).

The main novelty of this study is the consideration of the totality of CO present in the blood, which includes CO bound to Hb as well as CO dissolved in blood and the CO released into the HS of the sampling tube. No flushing of the calibrators is hereby performed, which is in accordance with physiological principles. The results reported show a significant difference of the CO concentrations if compared to results from previously published works, suggesting that the AGS-GC-MS method might be a valid alternative to the use of COHb as a biomarker for CO exposures, with the latter possibly underestimating the true role played by CO in such an intoxication.

Additionally, this method could be of high importance in explaining numerous cases in which the reported COHb% levels did not correlate to the symptoms shown by intoxicated patients, with applications in both the clinical and forensic field, even though further research into this approach needs to be performed for confirmation. Furthermore, for applicability and validity in clinical cases, the validation of this method with a lower calibration range and with real clinical samples also needs to be completed.

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Conflict of interest

The authors declare no conflict of interest.

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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

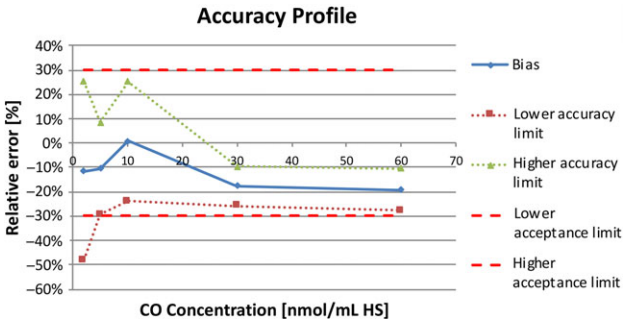

Figure 1. Accuracy profile for CO determination using a simple linear regression model within the range of 2–60 nmol/mL HS (0.8–24 µmol/mL blood). The continuous line represents the trueness (bias), the dashed lines represent the acceptance limits set at ±30% and the dotted lines are the relative lower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

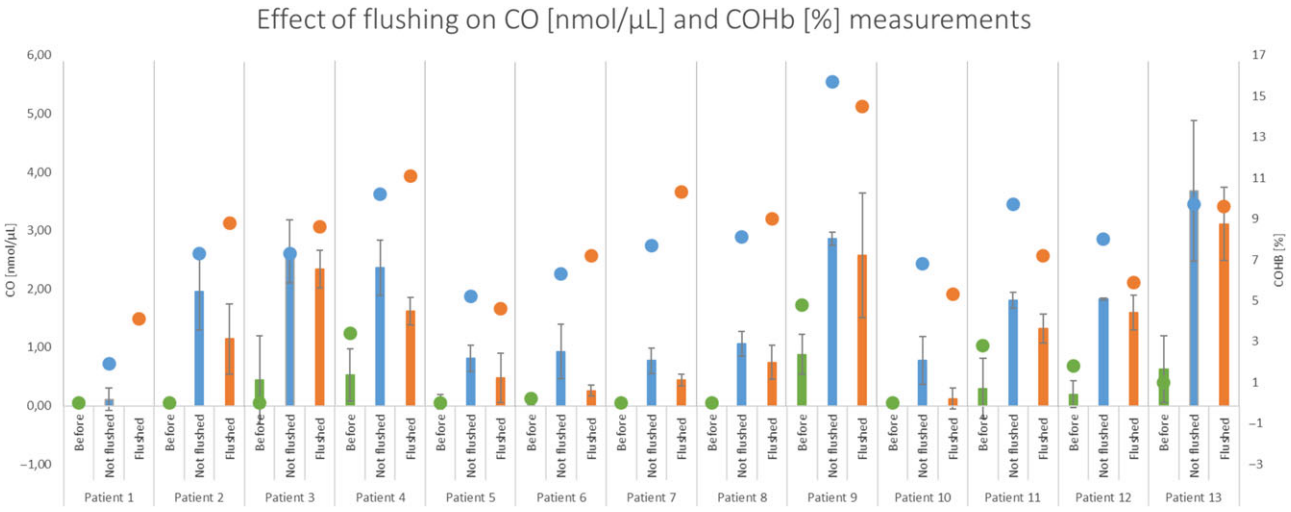

Figure 2. Results for the total CO concentration in blood (TBCO) in μmol/mL (bars, left axis) measured by AGS–GC–MS and the COHb saturation in % (dots, right axis) measured through CO-oximetry in blood for 13 patients before (green) and after (blue) CO exposure and after a flushing step (orange).

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

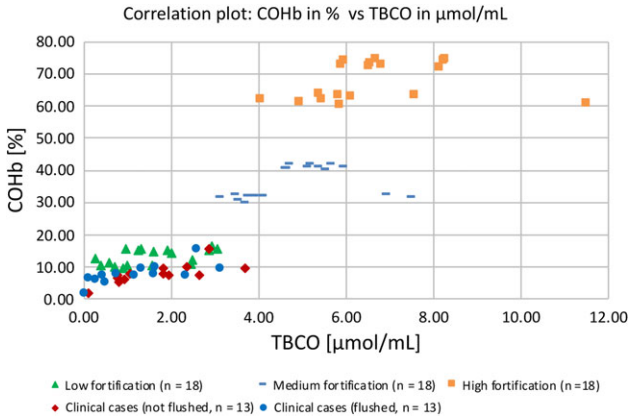

Figure 3. Correlation plot between TBCO in µmol/mL measured by AGS–GC–MS vs. the COHb saturation in % measured through CO-oximetry for two groups of samples: stored blood samples fortified with CO (squares for high fortification level 60–70%, lines for medium fortification level 30–40% and triangles 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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REVIEW ARTICLE

What are the limitations of methods to measure carbon monoxide in biological samples?

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Abstract

Purpose Carbon monoxide (CO) is one of the most important toxic gases in the atmosphere. Its high affinity for hemoglobin has made carboxyhemoglobin (COHb) the most appropriate biomarker for CO poisoning. COHb is measured using spectrophotometric (ultraviolet-spectrophotometry, CO-oximetry) or gas chromatographic (GC) methods combined with flame ionization or mass spectrometry (MS) detectors. However, inconsistencies in many cases have been reported between measured values and reported symptoms, raising doubts as to the suitability of COHb as a biomarker and the accuracy and reliability of its measurement methods. Therefore, we aimed to review the accuracy of current methods used to measure CO and to determine their sources of error and their effects on the interpretation process.

Methods A detailed search of PubMed was performed in November 2018 using relevant keywords. After exclusion criteria were applied, 46 articles out of 191 initial hits were carefully reviewed.

Results While optical methods are highly influenced by changes in blood quality due to degradation of samples during storage, GC methods are less affected. However, measurement of COHb does not quantify free CO, which is mainly responsible for toxicity mechanisms other than hypoxia, such as inhibition of hemoproteins, thus underestimating the true CO burden. Therefore, measurement of COHb is not sufficiently accurate for diagnosis of CO poisoning.

Conclusions An alternative biomarker is needed, such as determining the total amount of CO in blood. Although further research is required, we recommend that toxicologists consider all sources of error that can alter COHb concentrations, and in more challenging cases, they should use GC–MS methods to confirm the results obtained by spectrophotometry.

Keywords Carbon monoxide poisoning · Carboxyhemoglobin · CO-oximetry · UV-spectrophotometry · GC–MS · Source of error

Introduction

Carbon monoxide (CO) concentrations may be measured in exhaled breath, ambient air or blood. Because of the high affinity of CO for hemoglobin (Hb), it has been assumed that the majority, if not all, of CO binds with Hb when introduced into the blood circulation. As a result, carboxyhemoglobin (COHb) has traditionally been considered the most appropriate clinical marker of exposure in CO poisoning [1]. However, COHb does not represent the only reservoir of CO in the human body; CO may be found in a free state dissolved in blood and can bind to other heme-containing respiratory globins, such as myoglobin in muscle, neuroglobin in the nervous system and, to a lesser extent, cytoglobin [2]. Although CO dissolved in blood in free form is acknowledged to have a role in the pathophysiology of CO poisoning [3, 4], its influence may be more substantial than what has been revealed in studies thus far. This would result in under- or overestimation of the true level of CO present in the analyzed blood sample, potentially elucidating some of the cases where inconsistencies between measured COHb levels and reported symptoms were found. However, there is currently little data available on free CO.

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COHb in blood is measured directly or indirectly using either optical methods, such as CO-oximetry, ultraviolet (UV)-spectrophotometry and pulse oximetry, or gas chromatography (GC) in combination with a variety of detectors (flame ionization detector, mass spectrometer). In clinical cases, the "gold standard" for the measurement of COHb in blood is by CO-oximetry (or pulse oximetry), either as a separate instrument or integrated in what is commonly known as a blood gas analyzer (BGA) or radiometer [5]. Although UV-spectrophotometry remains the most frequently used method in forensic cases, CO-oximetry and GC methods are also widely employed in this field.

Like any biomarker, the quantitative measurement of COHb is subject to a variety of factors that influence the measurement. Measurement error in analytical studies is defined as "uncertainty" or "bias". Uncertainty originates when several predictable, but not always controllable factors affect the measured values and potentially alter the values obtained, resulting in a deviation from the true value. In medical practice, and especially for toxicologists, the correct and accurate determination of a biomarker is crucial in order to make the correct diagnosis and initiate the proper treatment in clinical cases, and to determine the correct cause of death in forensic cases. Failure to do so can have severe clinical and legal consequences. Therefore, in this paper, we aim to review the accuracy of current methods for measuring CO and to determine their potential sources of error and effects on the interpretation process.

Method of literature search

PubMed was searched in November 2018 using the keywords ("carbon monoxide" or "carboxyhemoglobin") and ("poisoning") and ("measurement" or "determination" or "quantification" or "analysis" or "breath" or "blood" or "oximet*" or "spectro*" or "gas chromatography" or "storage"); this produced 191 hits. Systematic reviews, meta-analyses, general review articles, and retrospective, prospective, observational and clinical cohort studies were excluded as well as case reports, limiting articles included in those which were focused specifically on describing a method for analysis of CO or COHb in various tissues and those describing issues related to analysis of samples (storage, sample pretreatment, etc.). This left 49 relevant articles on measurement methods and sources of errors.

Measurement of CO in breath

Analytical techniques

Analysis of CO in exhaled breath was evaluated as a measurement method for clinical cases, because a good correlation between alveolar breath CO and COHb was found by several research groups [6–9]. Portable devices, called MicroCOmeters or CO monitors, are often used in smoking cessation programs [8, 10] and may be useful when a rapid on-site assessment in multiple casualties is necessary, enabling the most severe cases to be identified [11]. This measurement is based on an electrochemical fuel cell sensor, which works through the reaction of CO with an electrolyte on one electrode and oxygen (from ambient air) on the other. This reaction generates an electrical current proportional to a CO concentration. The output from the sensor is monitored by a microprocessor, which detects a peak at expired concentrations of CO in the alveolar gas [12]. These are then converted to COHb% using the mathematical relationships described by Jarvis et al. [8] for concentrations below 90 parts per million (ppm) and by Stewart et al. [13] for higher levels.

Sources of error

Measurement of CO in breath cannot account for the total CO concentration present in the blood at the time of exposure. The method is very susceptible to the influence of a variety of factors that can easily alter the result, leading to under- or overestimation of the true concentration (Table 1). One major factor is the variability in subjects' breath-holding ability. To obtain the alveolar gas, it was found that the breath needs to be held for 20 s, and then only the end-tidal expired air is used for CO measurement. Given the individual differences in pulmonary function, capillary diffusion surface, and inspiration and expiration rates, coupled with the inability to fully control whether a subject is properly holding their breath, the portion of expired alveolar gas sampled and the results obtained can have a high degree of variability [6, 8, 13]. This can also pose an issue in susceptible groups of the population, such as the elderly, children or those with respiratory diseases. Furthermore, because they were initially designed for smoking cessation programs, the accuracy of CO monitors is better in lower CO concentrations and might therefore not be sufficiently accurate for acute intoxication [14]. Nevertheless, CO monitors are highly useful on sites of mass casualties or for first responders. They are portable and can provide an indication of the gravity of the case, enabling both the appropriate treatment of the patient and proper precautions to be taken by first responders.

Table 1 Overview of methods used for carboxyhemoglobin/carbon monoxide analysis, their main characteristics and limitations, and reference examples

Specimen/method	Technique	Main characteristics	Pitfalls	References
Breath	Electrochemical sensor	Easy to use Noninvasive Rapid (multiple determinations in short time period—useful in mass accidents) Low-cost Portable Alveolar breath CO correlated to COHb Used in smoking cessation programs and to detect hemolytic diseases	Only fraction of CO exhaled is measured Not able to determine total amount of CO in blood circulation No correlation to CO in tissues Not sufficiently sensitive for low-level CO exposure Only approximate diagnosis can be made Correlation between exhaled CO and COHb still debatable Not suitable for all patients (elderly, diseased)—requires sufficient exhaled air flow	Ogilvie et al. [6] Jarvis et al. [7, 8] Vreman et al. [14] Middleton and Morice [9] MacIntyre et al. [10] Penney [11]
Blood	Double-wavelength (DW)/automated differential/ultraviolet (UV) spectrophotometry	Use of multiple wavelengths Rapid Easy to use Fairly accurate Small sample size	DW: not precise, accurate and specific Sensitive to alteration of sample quality Not optimal especially for PM samples with long/unknown PMI and/or storage conditions Risk of misdiagnosis due to artifacts Not able to determine total amount of CO in blood circulation No correlation to CO in tissues Focused only on COHb Time-consuming sample preparation (COHb reduction) Frequently observed inconsistency between measured levels and reported symptoms	Ramieri et al. [16] Winek and Prex [17] Fukui et al. [19] Vreman et al. [46] Lewis et al. [55] Luchini et al. [56] Olson et al. [22] Varlet et al. [36, 52] Hao et al. [37]
	CO-oximetry	Easy to use Rapid Low-cost Accurate Precise COHb saturation correlated to severity of poisoning and symptoms reported by patients	Limit of accuracy: > 5% COHb Not applicable to low-level CO exposure Invasive Only CO bound to Hb taken into account Frequently observed inconsistency between measured levels and reported symptoms Susceptible to alterations due to sample quality Difficult interpretation for PM samples with long/unknown PMI and/or storage conditions	Dubowski and Lu [57] Costantino et al. [58] Mahoney et al. [24] Oritani et al. [65] Levine et al. [59] Bailey et al. [21] Widdop [23] Lee et al. [60, 61] Brehmer and Iten [62] Boumba and Vougiouklakis [18] Penney [11] Piatkowski et al. [5] Olson et al. [22] Fujihara et al. [20]

Table 1 (continued)

Specimen/method	Technique	Main characteristics	Pitfalls	References
Attachment to the finger	Pulse CO-oximetry	Measurement of COHb% saturation in circulation Continuous measurement Noninvasive Rapid Cheap Applicable in clinical setting No laborious calibration needed	Not applicable in PM setting Only CO bound to Hb taken into account No correlation to CO in tissues No blood sample available for confirmation/counter-expertise Low precision and accuracy for COHb > 10% Scarce information on device maintenance	Piatkowski et al. [5] Zaouter and Zavorsky [39] Feiner et al. [40] Weaver et al. [41] Wilcox and Richards [42] Kulcke et al. [43]
Blood, tissue	GC–RGA	Measurement of CO in tissues No dependence on blood quality Automation possible	Use of highly toxic mercury vapors Time-consuming sample preparation Invasive	Coburn et al. [44] Vreman et al. [46] Mahoney et al. [24] Marks et al. [63] Vreman et al. [30]
Blood	GC–TCD	Accuracy for low COHb% Precise Specific Measurement of CO released into HS of tube possible No dependence on blood quality Automation possible	Time-consuming sample preparation High cost Invasive	Ayres et al. [45] Dubowski and Lu [57] Fukui et al. [19] Van Dam and Daenens [64] Oritani et al. [65] Lewis et al. [55] Brehmer and Iten [62]
Blood, tissue	GC–FID	Rapid Best sensitivity for CO Specific Lowest LOD and LOQ Assessment of different sample preparation and storage conditions (liberating agent, heating time, heating temperature, etc.) Application to CO in tissues (PM) Automation possible Measurement of CO in tissues No dependence on blood quality	Instrument specific for CO due to need for methanizer Not applicable to analysis of other substances Time-consuming sample preparation Invasive Back-calculation of COHb from measured CO Flushing of calibrators → removal of dissolved CO	Collison et al. [51] Rodkey and Collison [48] Guillot et al. [66] Vreman et al. [46] Costantino et al. [58] Cardeal et al. [49] Levine et al. [59] Penney [69] Sundin and Larsson [53] Czogala and Goniewicz [67] Boumba and Vougiouklakis [18] Vreman et al. [30] Walch et al. [47]

Table 1 (continued)

Specimen/method	Technique	Main characteristics	Pitfalls	References
Blood, tissue	GC–MS	Versatile Simple Rapid Accurate Reproducible High power of identification (retention time + mass spectrum) Automation possible Application to clinical and PM samples No dependence on blood quality Use of isotopically labeled formic acid for calibration and internal standard Measurement of total amount of CO	Back-calculation of COHb from measured CO Debatable correlation between CO and COHb% → often inconsistency between COHb% and reported symptoms Flushing of calibrators → removal of dissolved CO Invasive Time-consuming sample preparation	Middleberg et al. [31] Oritani et al. [50] Marks et al. [63] Varlet et al. [36, 52] Hao et al. [37] Oliverio and Varlet [4, 70]

CO carbon monoxide, *COHb* carboxyhemoglobin, *PM* postmortem, *PMI* postmortem interval, *GC–RGA* gas chromatography–reduction gas analyzer, *GC–TCD* gas chromatography–thermal conductivity detector, *GC–FID* gas chromatography–flame ionization detection, *LOD* limit of detection, *LOQ* limit of quantification, *GC–MS* gas chromatography–mass spectrometry

Measurement of CO in blood: optical techniques CO-oximetry and spectrophotometry

Analytical techniques

Spectrophotometric or optical methods measure the concentration of COHb based on the quantity of light absorbed when the compound is exposed to light of different wavelengths. Early methods involved single-beam UV or double-wavelength spectrophotometry, and were developed for use due to the spectral absorbance of the Hb structures and to the distinct spectral differences between oxyhemoglobin (O_2Hb) and COHb [15–17]. A similar method measures differences in absorbance in the visible spectra between reduced Hb (HHb) and COHb, where a reducing agent is added to the blood sample that reduces O_2Hb but not COHb [18, 19].

However, double-wavelength spectrophotometry was not a very accurate and specific method [16], because results were based on the measurement of only two wavelengths. Automated differential spectrophotometry was later developed, which uses double-laser beams to determine the difference in absorbance of a sample compared to a negative sample; thus with this method, matrix effects are accounted for, resulting in better accuracy.

CO-oximetry is a measurement technique based on multiple-wavelength spectrophotometry, which uses up to the full range of wavelengths for analysis, allowing for more accurate measurement of COHb [20–22]. They are currently the standard analytical technique used for measurement of COHb, either with a separate instrument or, for hospital cases, integrated into a BGA [18, 23, 24].

Despite the advantages of CO-oximetry, for the sake of cost-efficiency, UV and double-wavelength spectrophotometers are currently still used in many developing countries and are also listed in the International Organization for Standardization (ISO) standard 27368:2008, "Analysis of blood for asphyxiant toxicants—carbon monoxide and hydrogen cyanide" [25].

Sources of error

Several issues can alter the measurement results from optical methods, mainly due to the susceptibility of these methods to changes in sample quality as a result of poor sample handling techniques and storage conditions (e.g., temperature, preservative) and biochemical alterations that occur over time [26]. Some of the most important potential errors for COHb determination are as follows:

1. Type of preservative: the type of preservative used in the blood tube used to store the sample can alter the results

due to biochemical reactions that can take place, which can either increase or decrease the concentration of CO [27, 28].

2. Storage temperature: the use of different storage temperatures was shown to alter the results; storage over prolonged periods of time can lead to degradation of the sample, which can lead to in vitro CO production, resulting in overestimation of the concentration; storage at room or hot temperatures leads to faster degradation as compared to storage in the fridge or freezer [26, 28, 29].
3. Dead volume: the different amounts of headspace (HS) volume in the sampling tube (which is known as dead volume) can alter the results because of the reversibility of the bond between CO and Hb; the more dead volume in the tube, the more likely the dissociation of CO from Hb and release into the HS [30].
4. Freeze-and-thaw cycles: whether a sample has been frozen and then thawed one or more times can also alter the resulting measurement, due to the breakdown of the erythrocytes [28].
5. Reopening of the sampling tubes: the repeated opening of the tube can lead to substance loss (in gaseous state when CO is not bound to Hb) with increasing number and time of reopening as well as increased exposure of the sample to oxygen [23, 28].
6. Postmortem (PM) changes: thermocoagulation, putrefaction and PM CO production are all known sources of error, but they cannot be quantified due to their biologically unpredictable nature [27, 31, 32].
7. Instrument and personal error: errors due to the instrument or the operator are random, but they can be corrected by using an internal standard when possible, which minimizes the error [33].

These factors are applicable to both optical measurements of COHb and GC measurements of CO. Specifically for spectrophotometric methods, several of the factors listed in Fig. 1 have been investigated and are described in more detail as follows.

Studies performed earlier by Chace et al. [28] and later by Kunsman et al. [27] evaluated a number of storage conditions, including the amount of air present in the sampling tube (known as dead volume, which can alter the results because of the reversibility of the bond between CO and Hb and potential dissociation of the gas into the HS of the tube), storage temperatures, preservatives and initial COHb saturation levels. They observed that decreased COHb levels were related to the ratio of the exposed surface area to the volume of blood (the higher the exposed surface area, the greater the loss), the storage temperature (the higher the temperature, the greater the loss) and the initial COHb% saturation level (the higher the COHb level, the greater the loss). A hypothesis was proposed whereby the influence of the HS volume in the sampling tube was explained by the formation of an equilibrium between CO in the blood and the air above the blood sample in the tube [28]. Storage of blood at room temperature or higher leads to faster degradation and lower sample stability, affecting spectrophotometric measurement of CO, which was also confirmed by other research groups [26, 34]. Additionally, they found no effect from the preservative used; however, testing was performed with an insufficient number of preservatives [only two, namely sodium fluoride (NaF) and ethylenediaminetetraacetic acid (EDTA)], which were compared to samples with no preservative, and only on samples stored frozen immediately after sampling over a period of 2 years. Analysis of the samples on only two significantly distant time points might fail to detect changes in short-term storage due to the use of preservatives, which is more relevant than long-term storage, since in the majority of cases samples are analyzed within a few hours to days. Nevertheless, these findings are especially relevant for forensic or legal cases, where retrospective analyses can still provide sufficiently reliable information. The resulting lack of impact from the preservative might however be biased because the measurements were performed with optical methods only, which are known to be influenced by the blood state. Therefore, smaller changes due to the preservatives might not have been detected by this less sensitive measurement method. However, Vreman et al. [35] were able to determine that the use of EDTA as preservative led to falsely increased COHb values when measured by CO-oximetry. Nevertheless, these findings would have been more significant with confirmation by another measurement method, such as GC.

Furthermore, these conditions may influence not only the CO levels present in the blood, but also the blood quality [28]. For samples that cannot be readily analyzed and are not stored under optimal conditions, degradation of the sample occurs, which was confirmed to hamper optical measurement methods used to determine COHb levels [36]. This can be a major issue for many laboratories where optical techniques are routinely used for sample analysis.

Additional factors influencing the measurement of COHb-levels that have been reported in the literature include the presence and amount of oxygen in air [23] and, in PM samples, thermocoagulation in fire victims [34], putrefaction during a prolonged PM interval (PMI) [37], contamination due to hemolysis, high lipid concentrations or thrombocytosis, all of which result in turbidity of the sample, hampering measurements performed with optical techniques. Another frequent and significant phenomenon to consider during evaluation of the results is the PM production of CO in the organism [32, 38]. CO was found to be produced in significant quantities in cases that were not related to fire or CO exposure. However, the cases in which this occurs are

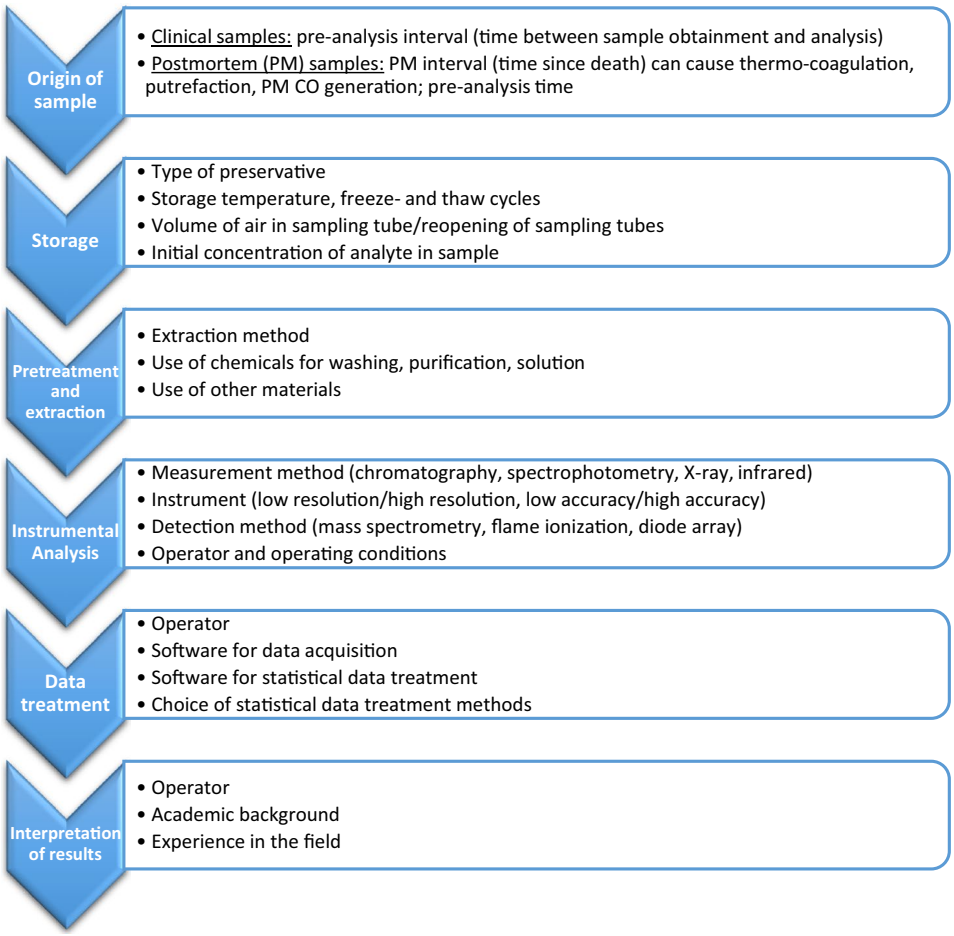

Fig. 1 General steps for a quantitative laboratory analysis and respective potential sources of error for carbon monoxide (CO) determination

mostly cases of putrefied bodies. It was confirmed that CO is formed due to the decomposition of various substances present in the body, such as erythrocyte catabolism, a phenomenon that also occurs in living organisms [32]. Therefore, it is important to differentiate those cases from the real CO intoxication cases, which can be done with the help of autopsy-determined cause of death, even though it is not always a simple task to completely exclude the possibility of the role played by CO in these cases [23]. As a result, PM decomposition currently constitutes a field with open questions that require further investigation.

Antemortem COHb measurement by pulse CO-oximetry

Analytical techniques

In clinical settings and generally for living patients, a noninvasive alternative to venous or arterial blood COHb measurement by BGA or CO-oximetry that has been widely investigated is pulse CO-oximetry [39–43]. Similarly to standard CO-oximetry, pulse CO-oximetry is a spectrophotometric method that quantifies multiple types of hemoglobin, including COHb, based on the absorbance of light after exposure to different wavelengths [43]. As opposed to regular CO-oximeters, pulse CO-oximeters have the ability to measure COHb continuously and without the need for blood sampling, thus allowing the monitoring of COHb levels in real time and simultaneously with the administration of treatment.

Sources of error

Noninvasiveness and cost- and time-efficiency are some evident advantages of using pulse CO-oximeters. However, for CO poisoning diagnosis, there are more important factors from a medical perspective, such as accuracy, precision and reliability. The ability to diagnose a CO poisoning case quickly is necessary, but if the results obtained over- or underestimate the true COHb levels, this can have severe and potentially fatal consequences. Several studies have reported low precision and accuracy as well as elevated false-positive and false-negative rates in comparison with regular blood measurements [5, 39–42]. Especially for COHb levels above

10%, pulse CO-oximeters significantly underestimated the COHb levels [39].

Furthermore, factors such as blood pressure, oxygen saturation and body temperature also appear to affect the accuracy of pulse CO-oximeters [42]. Feiner et al. [40] reported that the pulse CO-oximeter always gave low signal quality errors and did not report COHb levels when oxygen saturation decreased below 85%, which is indicative of hypoxia. Considering that hypoxia is one of the main effects of CO poisoning, it is a severe disadvantage not to be able to accurately measure COHb in hypoxic states. However, a more recent study by Kulcke et al. [43] reported good accuracy in measuring COHb even during hypoxemia by use of an upgraded/revised version of the pulse CO-oximeter, although slightly greater underestimation of COHb levels was reported for COHb concentrations above 10%. This confirms that pulse CO-oximeters can be useful for monitoring exposure to low CO levels, but accuracy and precision are not guaranteed for more severe poisoning or for smokers, who generally have baseline COHb levels that range from 3 to 8% in normal smokers but can easily reach 10–15% in heavy smokers [1, 2].

In contrast to postmortem CO-oximetry, antemortem COHb measurement by pulse CO-oximetry is not affected by storage or sampling parameters, thus reducing the potential sources of error. Additionally, no laborious and time-consuming calibration of the device seems to be needed based on what is reported in the literature, leading to a more simplified routine analysis, although there is scarce information regarding device maintenance. Similar to general CO-oximetry, and despite good accuracy and precision, measurement of only CO bound to Hb can lead to underestimation of the total CO burden and thus to misdiagnosis. Another relevant point from a juristic perspective is that pulse CO-oximetry does not provide samples that can be used for confirmation or counter-expertise in legal disputes.

Measurement of CO in blood: gas chromatography

Analytical techniques

The principle behind GC CO detection is based on the measurement of the released CO dissolved in blood as well as the one bound to Hb through a liberating agent (after red cell lysis). Therefore, the sample is firstly treated with a hemolytic agent, such as saponin, Triton X-100 or other detergents, and subsequently acidified to liberate the CO in blood [34, 44–47]. The reaction of COHb with a powerful acid/oxidizing agent was found to efficiently release CO and water as products. The releasing agents commonly used are sulfuric acid (H_2SO_4), hydrochloric acid (HCl) and potassium ferricyanide ($K_3Fe(CN)_6$). Other acids including lactic acid [48], citric acid [48, 49] and phosphoric acid [49] have also been tested.

In the studies performed in earlier years (1970s, 1980s and 1990s), potassium ferricyanide was introduced for the release of CO and became very popular due to its easy availability, since it was already used in spectrophotometric methods as hemolytic agent. It was also found to be efficient in liberating the CO, and the extent of its reaction was not influenced by the presence of O_2 or O_2Hb over a wide pH range, as compared to other acids tested [30, 46, 48, 50, 51]. However, in more recent studies, sulfuric acid has been preferred, mostly because it is more readily available and cheaper than other acids of the same efficiency, and allows the simultaneous liberation of CO and production of ^{13}CO from formic acid-^{13}C used as internal standard [4, 30, 31, 47, 49, 52–54]. After successful liberation, CO is analyzed by GC and then detected with one of the below-mentioned detectors.

For the GC separation, a capillary column with a 5 Å molecular sieve has been found to be specific for the separation of CO from other interfering gases such as nitrogen (N_2), oxygen (O_2) and methane (CH_4) [51]. Various packed columns have been used previously, but have been replaced by capillary columns because of the significantly reduced size.

To enhance sensitivity and accuracy and increase the range of analysis, GC methods have been studied with various types of detection, such as thermal conductivity detection (TCD), flame ionization detection (FID), mass spectrometry (MS) and reduction gas analyzers (RGA) [55–66]. The most commonly used and investigated detector was FID, first reported in relation to CO determination in 1968 [51]. After GC separation, the CO was chemically reduced to methane (CH_4) with a methanizer and subsequently analyzed via FID.

Sources of error

The most important sources of error for GC techniques are found in the process of calibration before analysis and the methods for correlating measured CO concentrations to COHb levels that have previously been linked to the symptomatology. Generally, calibration of the instrument is performed either with pure CO gas, which is diluted to obtain the desired CO concentrations, or with fortification of blood with CO to reach different COHb% saturation levels. Additionally, excess CO has been removed by performing a "flushing" step, in which the calibrators are flushed with a stream of inert gas (usually N_2). This step enabled the removal of unbound CO from the sample, thus leaving only CO bound to Hb to be analyzed, but thereby deliberately neglecting the potential toxicity of free CO.

The first changes in the calibration method were made in 1993, when Cardeal et al. [49] took advantage of the reaction of formic acid with sulfuric acid to form CO for calibration. However, no details were given on how the analyzed blood was saturated with CO, nor was it explained how the formula was created for back-calculation of the measured CO concentration to a COHb level.

Czogala and Goniewicz [67] proposed a GC–FID-based method which directly correlated the CO levels in air to COHb in blood through back-calculation and extrapolated it to the other factors assessed (exposure time, smoking frequency, number of smoked cigarettes and ventilation conditions). The technique was designed to ensure complete release of CO from the blood samples by performing the reaction and subsequent analysis in an airtight reactor. Similarly, the air samples were directly transferred from the room to the analysis instrument, thus avoiding time delays and possible loss of CO, and allowing for direct correlation of the results to the other measurements. However, no details about the procedure for obtaining 100% CO-saturated blood used for calibration were described, which is necessary to assess whether the method is reliable and reproducible. Furthermore, the formula used to back-calculate the COHb saturation levels from the measured CO concentrations contained a Hüfner factor of 1.51, which differs from the factor reported by other studies [30, 46]. The Hüfner factor expresses the maximum amount of CO that can be bound to 1 g of Hb [68, 69]. A detailed list of additional pitfalls of GC methods is found in Table 1.

Measurement of CO in blood: GC–MS and HS–GC–MS

Analytical techniques

MS is the method of choice for detecting CO because identification is based on both the retention time and the mass spectrum. Middleberg et al. [31] developed a method which combined GC–MS with flame atomic absorption spectroscopy (FAAS). CO was determined by GC–MS after release with sulfuric acid and heating, while FAAS was used to determine the total iron content of the blood, which was used to calculate a more precise total amount of available Hb. It should be mentioned that with this assay, it was assumed that all the iron present in blood was part of the heme protein and was capable of binding to CO; however, this is not completely true, as it depends on the state of the organs, tissues and possible diseases present. Therefore, the obtained values may not accurately reflect the real CO levels.

Sources of error

Similar to other GC methods, the main errors in MS also derive from calibration of the methods, the subsequent back-calculation of COHb from CO, and extrapolation of already existing COHb% saturation–symptom correlation (Table 1).

Hao et al. [37] published an approach built on an HS–GC–MS method for analysis of CO in putrefied PM blood. The standard curve was constructed from putrefied blood, which was saturated by CO-bubbling to reach 100% COHb and then flushed to remove excess CO. COHb% levels were then calculated from the ratio of saturated to untreated blood. In PM cases, to prevent the variation in Hb levels from affecting the results, direct blood saturation was performed. The authors reported that 30 min of pure CO exposure was necessary to fully saturate blood, although the procedures used to assess complete saturation, putrefied blood state and PMI were not described [37]. Furthermore, according to the results for the storage condition tests (possible loss of sealing parts of the HS vial, water bath temperature, stability, interval and temperature), the storage temperature did not affect COHb% levels. This appears to contradict findings in the majority of previously published studies, although they were obtained using other approaches, such as optical methods and other GC detections.

Varlet et al. [52] developed and validated a new method which used isotopically labeled formic acid ($H^{13}COOH$) to produce ^{13}CO as internal standard for HS–GC–MS. This was very advantageous, because formic acid (HCOOH) was already used for the calibration, and sulfuric acid could be used to react with both types of formic acid, forming a mixture of CO and ^{13}CO, from which the CO concentration could be derived mathematically and correlated to the COHb levels using previously published formulae [46, 49]. However, these formulae describing back-calculation of COHb from CO concentrations measured by GC could be debatable due to the random finding of a good correlation between the spectrophotometrically measured COHb levels and the CO levels measured by GC–MS [52]. Varlet et al. [36] improved their method and compared it with results obtained through the CO-oximeter. They were able to obtain cutoff values for different categories of back-calculated COHb% levels as compared to those directly measured by the CO-oximeter. However, while this approach seems to show reliability for both clinical and forensic cases, only a limited number were tested. Oliverio and Varlet [4, 70] further developed this approach by validating both clinical and PM settings for the measurement of the total amount of CO in blood (TBCO) by GC–MS with the use of an airtight gas syringe for sampling, which minimized any potential loss that could occur with a normal syringe or HS sampler. Application to PM samples showed relevant differences between the content of CO and COHb when applying formulae in the literature for

back-calculation. Significant differences were also observed between flushed and non-flushed samples from a clinical cohort exposed to CO [70]. This demonstrates the presence of free CO and confirms the weaknesses of COHb for accurate CO poisoning determination, even though the number of subjects in the cohort was limited. Thus, the measurement of TBCO should be performed as an alternative to COHb and the current routinely used spectrophotometric methods for the determination of CO.

Interpretation of results and choice of biomarker

After analysis of the samples, an important and challenging aspect in CO determination is the interpretation of the results. There is no consensus on cutoff values for the different levels of exposure and severity of poisoning. According to the World Health Organization (WHO), COHb levels in blood of the healthy non-smoking population should not exceed 2.5–3%, while for smokers, levels above 10% are considered abnormal [11, 71–73]. Values of 30–35% COHb are the upper extreme reportedly found in clinical poisoning cases. Above this limit, irreversible damage to the organs is expected, thus initiating a cascade of events eventually leading to death.

However, these values are interpreted differently according to different cases. Various parameters can affect perimortem COHb% levels and in the agonal period before death, which include the presence of oxidative smoke or other gases that can interfere and/or compete with the CO absorption mechanism, such as nitrogen dioxide (NO_2) (increased methemoglobin), or the formation of other toxic gases such as hydrogen cyanide (HCN) [74]. Pre-existing cardiovascular, hemolytic and respiratory diseases also can alter the mechanism and magnitude of CO absorption, with the potential to both decrease and increase the resulting COHb% levels [11, 23]. Therefore, each case must be analyzed and interpreted individually, based on all relevant information available. For example, a COHb level of 25% in a PM case may be considered a contributing factor to the cause of death, but should not be considered exclusively as cause of death. Similarly, in clinical cases, 15% COHb can be considered a poisoning case, but in heavy smokers, levels up to 18% have been found [72] in individuals who showed no symptoms of CO poisoning. Overall, there seem to be some significant discrepancies between COHb values and reported symptoms, which makes the correct diagnosis of CO poisoning in clinical cases and the determination of the cause of death in forensic cases challenging.

A possible explanation for these phenomena is that a diagnosis of CO poisoning based only on COHb% levels might actually underestimate the real CO burden. There may be an unknown amount of CO that on the one hand dissociates back from COHb, and on the other hand is dissolved in the blood without being bound to Hb, resulting in higher total CO content than that determined by CO-oximetry. The conventional assumption that the part of CO bound to Hb causes the most significant adverse health effects has been repeatedly debated [3, 4, 75–78]. Free CO in blood could constitute a toxic reservoir of CO for the organism and could also have major implications for the central nervous system (CNS) by the known binding to other globins such as myoglobin, neuroglobin and cytoglobin [79, 80]. The ratio of COHb to dissolved and dissociated CO is also probably subject to interpersonal variability, which includes factors such as metabolic rate and age [11], and needs to be taken into account when interpreting the results obtained by CO-oximetry.

Another issue is that GC assays, with the exception of Varlet et al. [36, 52] and Oliverio and Varlet [4, 70], include the "flushing" step in their sample preparation procedure. The excess CO which is not bound to Hb is flushed away with inert gas, allowing the determination of only CO bound to Hb. This procedure is performed under the assumption that only CO bound to Hb is relevant and responsible for the adverse effects of CO poisoning. However, this point has been widely debated, raising the possibility that additional CO found in the blood and not bound to Hb could have an effect on an intoxicated individual. Furthermore, in routine clinical COHb analysis, blood samples are not flushed, because it is usually considered not to comply with the pathophysiology of CO poisoning. In general, the use of formulae to back-calculate GC-measured CO to COHb may be prone to additional errors and could lead to misestimation of the true amount of CO present in the blood of an individual.

All these issues raise doubt as to whether the measurement of COHb is the most appropriate method for determination of CO poisoning. It seems plausible that a more accurate biomarker of CO poisoning may be found. Several alternative biomarkers have been proposed, including lactate [81–83], bilirubin [84], S100β [85] and troponin concentrations in blood. Some of these demonstrated positive and good correlations with COHb and were reported to be potentially helpful for diagnosing CO poisoning. However, none of these biomarkers is specific to CO poisoning; rather they are indirect biomarkers derived from toxicity caused by CO in the cardiovascular system, nervous system and cellular levels, which can also be attributable to other diseases.

The development of an alternative biomarker specific to CO should be directed toward finding a novel measurement approach that not only focuses on the CO bound to Hb, but also takes into consideration the role and toxicity of CO at the cellular level, by measuring the total amount of CO present in the sample, such as TBCO. Mainly because of the

dependence of spectrophotometric methods on good-quality samples, which in forensic cases in particular is not always available, it seems that GC methods are currently the most suitable techniques to be further explored. With regard to detectors, the MS is the most versatile, accurate and user-friendly, and is nowadays routinely present in the majority of laboratories. The ability to determine the true CO exposure and to correlate this with the symptoms reported by patients would allow for more conclusive and comprehensive CO poisoning determination, reducing the number of misdiagnosed cases and falsely determined causes of death.

Conclusions

Although COHb is routinely measured by spectrophotometric methods, several issues concerning sample stability and the dependence of optical methods on sample quality have led to the search for alternative ways to measure CO, such as GC. In addition, there is evidence that a significant amount of CO present in blood is in free form. Free CO has major toxic effects at a cellular level, affecting not only the respiratory system, but also especially the CNS. However, it is not quantified with current methods, which focus only on COHb; hence the back-calculation of COHb from CO leads to misestimation. Therefore, an alternative approach for quantifying the total amount of CO in blood directly instead of using CO in breath or COHb in blood should be used for determination of CO poisoning, such as the proposed TBCO measurement by GC–MS. Although blood CO concentration cutoffs and their correlation with symptomatology are not yet available, and GC–MS is more time-consuming, we recommend that toxicologists use GC–MS methods to verify the results obtained by CO-oximetry or spectrophotometry, especially for doubtful or very challenging cases. This leads to results closer to the true CO burden, reducing the underestimation caused by COHb measurement and thus the risk and number of misdiagnoses. Especially if the analysis is delayed from sampling requiring storage, we further recommend that toxicologists document information about sampling time, analysis time and storage conditions, as these factors can significantly influence the final interpretation.

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Compliance with ethical standards

Conflict of interest There are no financial or other relations that could lead to a conflict of interest.

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New strategy for carbon monoxide poisoning diagnosis: Carboxyhemoglobin (COHb) vs Total Blood Carbon Monoxide (TBCO)

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ABSTRACT

Diagnosis of carbon monoxide (CO) poisonings has always been a challenging task due to the susceptibility to alterations of the optical state and degradation of blood samples during sampling, transport and storage, which highly affects the analysis with spectrophotometric methods. Methodological improvements are then required urgently because of increased reports of cases with discrepancies between results of the measured biomarker carboxyhemoglobin (COHb) and reported symptoms. Total blood CO (TBCO) measured chromatographically was thus proposed in a previous study as alternative biomarker to COHb. This approach was investigated in this study by comparing the two biomarkers and assessing the effects of various storage parameters (temperature, preservative, time, tube headspace (HS) volume, initial saturation level, freeze- and thaw- and reopening-cycles) over a period of one month. Results show that while for TBCO, concentrations are relatively stable over the observation period regardless of parameters such as temperature, time and HS volume, for COHb, concentrations are altered significantly during storage. Therefore, the use of TBCO as alternative biomarker for CO poisonings has been proposed, since it provides more valid results and is more stable even under non-optimal storage conditions. Additionally, it can be used to predict COHb in cases where sample degradation hinders optical measurement. Furthermore, a correction formula for COHb and TBCO is provided to be used in laboratories or circumstances where optimal storage or analysis is not possible, to obtain more accurate results.

1. Introduction

Carbon monoxide (CO) is colloquially named *'silent killer'* because despite it being a tasteless, odorless and colorless gas, its toxicity and occurrence in the atmosphere are important [1]. Hundreds of people in many western countries and thousands in less developed countries are killed because of CO poisonings each year [2–6]. However, due to the non-specific symptomatology and low medical awareness, CO poisonings are often misdiagnosed, leading to a potentially higher number of deaths that should be attributed to CO [7,8]. Recent studies have shown an increasing number of long-term neurological sequelae attributed to CO exposure *a posteriori*, raising questions and doubts about the accuracy of our current knowledge on CO, its pathophysiological effects and methods for quantification [9–12].

Procedures for sample collection and storage are one of the main aspects in the practice of clinical and forensic analyses of biological specimens. Given the medical and legal implications of these types of analyses, it is crucial that standardized protocols are in place to allow for correct and accurate interpretation of the results obtained, which help to provide adequate diagnoses and treatment strategies in clinical cases and hold up in court in forensic cases [13–15]. The fundamentals of these protocols are driven by the biochemical, physical and toxicological processes that occur when a substance of interest in a biological matrix is extracted, transported and stored prior to analysis. Storage after analysis is also of interest, since in many cases, especially forensic, reanalysis of a sample after several days or weeks is a necessary step in the investigation [14].

In cases of CO poisonings, currently the most common biomarker for diagnosis is carboxyhaemoglobin (COHb), due to

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the known high affinity of CO for the oxygen-carrying protein in erythrocytes, which displaces oxygen and, thus, reduces the oxygen transport to tissues, leading to hypoxia [1,16]. Methods for COHb quantification have been developed in blood [17–19] as main matrix, since it is readily available in both clinical and forensic cases. Biochemical alterations are known to occur in blood samples over time due to catabolism of proteins, which can be exacerbated by exposure to elevated temperatures, light, air or other compounds that might have contaminated the sample [20]. Common practice is to store blood samples possibly frozen or at least refrigerated, with addition of an anticoagulant and in a place not exposed to light [13]. Samples are usually obtained by laboratories in standard volume tubes, therefore the volume of the headspace (HS) or air volume above the sample is determined based on the volume of the sample, not the selected sampling tube. Furthermore, in a laboratory routine a blood sample is often employed for multiple analyses, including drug screenings and alcohol testing [21,22].

Since CO is a gaseous compound, samples obtained from individuals suspected of CO poisoning have the potential of additionally being very susceptible to exposure to air and frequent reopening. Even though the bond between CO and Hb is very strong, it is also a reversible reaction, which, over long periods, can lead to dissociation, releasing CO into the HS of the sampling tube [10,23]. Frequent reopening can hence lead to analyte loss. Another consequence of COHb dissociation includes the potential influence of the ratio of sample volume to HS in the sampling tube on the amount of CO dissociating into the HS. The formation of an equilibrium between CO in blood and HS was proposed by the study group of Kunsman et al. [24], who observed loss of COHb in samples with a higher volume of air in the sampling tube. They also showed a decrease in COHb levels over time for samples that had a higher initial saturation level [24]. Other storage parameters that were investigated in previous studies include different temperatures and preservatives, which often were contradictory: some studies showed no or little change with storage over long periods of time and at elevated temperatures [25–27], while others showed decreased COHb levels for different preservatives [24,28].

These differences can be explained by the fact that the majority of these studies were mainly performed using spectrophotometric methods for analysis, which are known to be susceptible to optical changes in the blood quality [29].

To be able to avoid erroneous results derived from poor sample quality due to inadequate sample collection and storage conditions, gas chromatographic (GC) methods can be alternatively employed. GC methods lead to results that are independent of optical changes to the specimen and enable the measurement of the total amount of CO in blood (TBCO) and in the HS of the sampling tube, as an alternative to COHb [19,30,31]. In addition, the measurement of TBCO is in conformity with the pathophysiological mechanisms of a CO poisoning, which recent developments have shown to be related not only to COHb, but also free CO [32–34].

Therefore, with this study, we aim to evaluate the effects of storage parameters such as temperature, preservative, HS volume, reopening cycles, freeze- and thaw-cycles and the level of initial COHb saturation over a storage period of one month on the quantification of both COHb and TBCO, in order to determine the most appropriate practices for sample collection and storage in CO poisoning cases with delayed analyses or storage in non-optimal conditions. Furthermore, we compare the spectrophotometric technique of CO-oximetry to a gas chromatography-mass spectrometry-based one, introducing the concept of TBCO measurements as a necessary addition to COHb measurements, which are more sensitive to the quality of the matrix and storage conditions.

2. Materials and methods

2.1. 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). For the *in vitro* study of storage parameters, bovine blood was obtained from a local butcher and collected in 1 L polypropylene bottles, which were previously fixed with the four investigated preservatives to obtain concentrations equivalent to the respective concentrations in the blood collection tubes (Monovettes). Ethylenediaminetetraacetic acid (EDTA) salt dehydrate was purchased from Sigma-Aldrich (St Louis, USA), sodium fluoride (NaF) was provided by Fluka (Buchs, Switzerland), lithium heparin (LiH) was from Fresenius Medical Care (Bad Homburg, Germany) and sodium citrate (NaCit) was obtained from Merck (Darmstadt, Germany).

2.2. Materials

The AVOXimeter 4000 Whole Blood CO-Oximeter and cuvettes were obtained from International Technidyne Corporation – ITC (Edison, USA). S-Monovettes of following types: 2.6 mL K3E (EDTA, 1.6 mg/mL), 3 mL 9NC (NaCit, 0.106 mol/L), 2.7 mL FE (NaF, 1 mg/mL, + EDTA, 1.2 mg/mL), 2.6 mL LH (LiH, 16 IU/mL), 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 HS vials from Agilent Technologies (Santa Clara, CA, USA).

2.3. Instruments and GC–MS conditions

AVOXimeter 4000 Whole Blood CO-Oximeter from ITC was used for all COHb analyses. Manufacturer guidelines were followed to obtain COHb concentrations.

Agilent 6890N 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 carrier gas, at a flow rate of 40 mL/min.

An Agilent 5973 mass spectrometer (Palo Alto, USA) was used for detection, operating in electron ionization (EI) mode at 70 eV. Selected Ion Monitoring (SIM) mode was used to acquire the signal for CO at *m/z* 28 and ^{13}CO at *m/z* 29.

2.4. Sample preparation

2.4.1. CO-fortification of blood

Blank bovine blood, which was obtained freshly from a local butcher for each study period, was added with the respective preservative immediately after collection and subsequently fortified with pure CO gas through bubbling for a certain amount of time. COHb saturation levels were checked before bubbling and after bubbling with a 10-minute-interval until the desired initial saturation level was reached. To ensure homogenization, the blood-containing bottles containers were agitated for 20 min, after which the final COHb concentration was determined by CO-Oximetry.

2.4.2. Calibration standards

An aliquot of fresh bovine blood, which was previously controlled by CO-oximetry and found at 0 % COHb, is used as matrix for GC calibration. Calibration standard working solutions of formic acid (43 μmol/mL) and working solutions of the internal standard isotopically labelled formic acid (84 μmol/mL) were prepared daily *de novo* in order to prevent degradation. Calibration points were set in a working range between 0−208 nmol/mL HS, with points at 6.5, 13, 26, 52, 104, 156 and 208 nmol/mL HS (equivalent to 1.3, 2.6, 5.2, 10.4, 20.8, 31.2 and 41.6 μmol/mL in blood). Matrix effects were evaluated by preparing a blank sample with the matrix without any reagent. 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.

2.4.3. Extraction procedure

2.4.3.1. CO in blood. 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 sulfuric acid, which is used as both releasing agent for CO from COHb and reagent with formic acid/isotopically labelled formic acid to generate the CO/^{13}CO needed for calibration/quantification, 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.

2.4.3.2. CO in HS. To determine the CO in the HS of the sampling tubes, the same procedure as for the CO in blood samples was used. 250 μL of HS were directly sampled from the sampling tube with a 500 μL airtight gas syringe and injected into a HS-vial containing the internal standard that had previously been generated.

2.5. Analysis procedure

2.5.1. 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.

2.5.2. CO in blood determination via HS sampling and GC–MS analysis

1 mL HS was sampled from the 20 mL HS-vial containing the extract and injected in the GC–MS for analysis, which was performed following a previously validated method [19].

To ensure that no contamination from CO contained in the air affected the measurements, a 1 mL aliquot of air in the analysis-room was additionally analyzed prior to sample analyses.

2.6. In vitro *storage study*

The *in vitro* study to evaluate several storage parameters was carried out over a period of one month, with samples analyzed on days 0, 1, 2, 4, 7, 14, 21 and 28.

Blood specimens were generated on day 0 to investigate following parameters at various levels:

- Temperature: room temperature (RT), refrigeration at +4 °C, freezing at −20 °C
- Preservative: EDTA, NaF, LiH, NaCit
- HS volume: <25 %, 25–50 %, >50 % of the total tube volume
- Saturation levels: 10–20 %, 30–40 %, 50–70 %
- Reopening cycles
- Freeze- and thaw-cycles

One set of samples used to investigate the reopening cycles were reanalyzed on each day of analysis, while another set of samples used to investigate all other parameters were analyzed once on day of analysis. To assess the freeze- and thaw-cycles, the samples for investigating the reopening cycles stored in the freezer were used. A total of 2376 blood samples were analyzed, which were distributed for each parameter and day of analysis as follows: 108 samples for each saturation level per day, of which 27 per preservative, 36 per temperature and 36 per HS volume. Analyses were carried out in triplicates.

2.7. Back calculation of COHb from CO

Various research groups have previously proposed formulae to back calculate COHb from CO measured through GC approaches [35–38]. We compare the CO concentrations measured with the AGS-GC–MS method and back calculated to COHb through the formula proposed by Cardeal et al. [37] with the COHb measured by CO-oximetry to establish statistical significance.

2.8. Statistical analyses

Since saturation level is expected to have the most significant effect on the data and to simplify the data analysis, the dataset was split into the three categories (high, medium and low saturation level) and used for modeling. Data was then checked for normal distribution and transformed accordingly (Table 1). Due to the upper detection limit of the employed CO-oximeter of 75 %, a large portion of the samples analyzed with high saturation level was found at 75 %, despite potentially being higher. This is not an issue from a pathophysiological point of view, since the value is clearly in the toxic range of COHb concentrations. However, from a statistical perspective, this generated a severely left-skewed distribution, which could not be corrected for through transformations. Therefore, censored regression was considered for statistical analyses of the data. “censReg” is a package in the statistical software R, which can be useful when faced with censored data. The way the software deals with the values in case of a right-sided censoring is that it estimates the values above the censored limit based on maximum likelihood with the data available [39]. This might be, however, problematic, since the software returns estimated values that can exceed 100, which is the physiological limit for COHb saturation.

Non-parametric tests were used for assessment of single storage parameters in high saturation levels, but no assessment was possible with multiple storage parameters. Missing values in cases of instrument malfunctioning or due to advanced stage of sample degradation were completely excluded for statistical analyses. Kruskal-Wallis test for high saturation COHb levels,

Table 1
List of transformations employed for data according to analyte of interest and saturation level, *: for high saturation level COHb, no normal distribution was obtained, thus non-parametric tests were employed.

Saturation Level	COHb	TBCO
High* (60–70 %)	–	Log_{10}
Medium (30–40 %)	–	Log_{10}
Low (10–20 %)	–	Cube root

multiway analysis of variance (ANOVA) for the other saturation and response variables (COHb and TBCO) as well as multiple linear regression (MLR) and comparisons via Student *t*-test were used to assess effect of the investigated parameters and generate correction and prediction models. All statistical analyses were performed with R (version 3.3.1, 2016-06-21).

3. Results

3.1. Correlation between COHb and TBCO

Fig. 1 shows the plot of results obtained for COHb vs results of the same samples for TBCO. A linear regression was applied to the data and the obtained linear regression line is depicted in red. A correlation factor (R^2) of 0.68 with a p-value well below the significance limit of 0.05 (p-value <2.22e-16) represents a moderate positive correlation between the two measures.

To determine whether the formula proposed by the study group of Cardeal et al. [37] is applicable to our method, we have used it to backcalculate the values obtained from the AGS-GC–MS measurements and compared the measured COHb with calculated COHb from TBCO values. A paired Student *t*-test was performed to statistically compare the two groups. With a p-value of <0.05 (p-value <2.2e-16), it was determined that the groups are significantly different.

3.2. Influence of storage parameters

3.2.1. Relevance of CO in HS

To determine whether there was a significant amount of CO released into the HS of the sampling tube, the results for CO in blood were added to the results of CO in HS and compared to the results of CO in blood with a paired Student *t*-test. With a p-value of <2.2e-16, the two groups were found to be significantly different. Average relative differences in values were found to be between ±0.01 % (histogram of relative differences distribution in Fig. 2).

3.2.2. Reopening and freeze- and thaw-cycles

3.2.2.1. Reopening. To determine the influence of reopening the sampling tubes on the results obtained from the measurements of COHb and TBCO, the unpaired Student *t*-test was used to compare the samples that were reopened during the study period to samples that were not reopened. Results for both COHb and TBCO gave p-values >0.05, thus indicating that there is no statistically significant difference in results for samples that were reopened for reanalysis.

3.2.2.2. Freeze- and thaw-cycles. The effect of freezing and thawing a sample multiple times on the obtained measurement results for COHb and TBCO was determined by comparing results obtained from samples, which underwent multiple freeze- and thaw-cycles, to samples, which underwent only one freeze- and thaw cycle. The unpaired Student *t*-tests for both COHb and TBCO lead to p-values >0.05, resulting in no statistically significant difference between the comparison groups. To further test whether the first freezing cycle had a major effect on the concentrations, we compared the results of samples that underwent one freeze- and thaw-cycle with the samples that did not. The Student *t*-test for both COHb and TBCO gave p-values >0.05, thus affirming no statistical difference.

3.2.3. Multiway-ANOVA

To determine which and if any of the investigated parameters has a significant effect on the measures COHb and TBCO, an ANOVA was first carried out for each parameter and saturation level in relation to COHb and TBCO, respectively, with exception of high saturation COHb levels. Due to the inability of the data to reach normal distribution despite transformation attempts, the non–parametric Kruskal-Wallis test was employed to assess the different storage parameters one by one. Subsequently, an additive model selection process was performed, which consisted in the generation of several models through addition of one parameter in

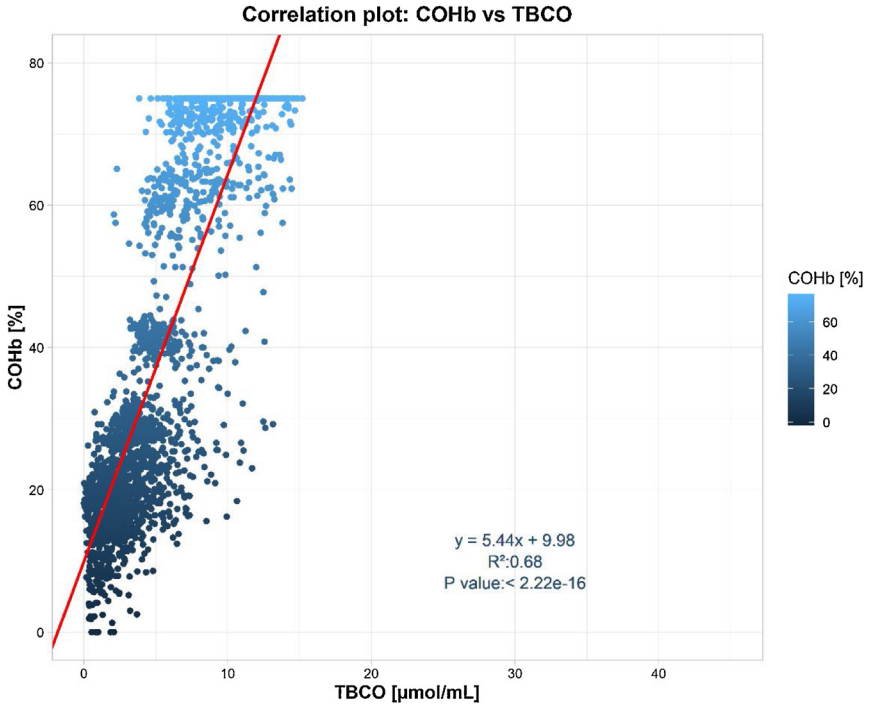

Fig. 1. Correlation plot for COHb [%] vs TBCO [µmol/mL] from the storage study results, with correlation formula, correlation factor (R^2) and p-value.

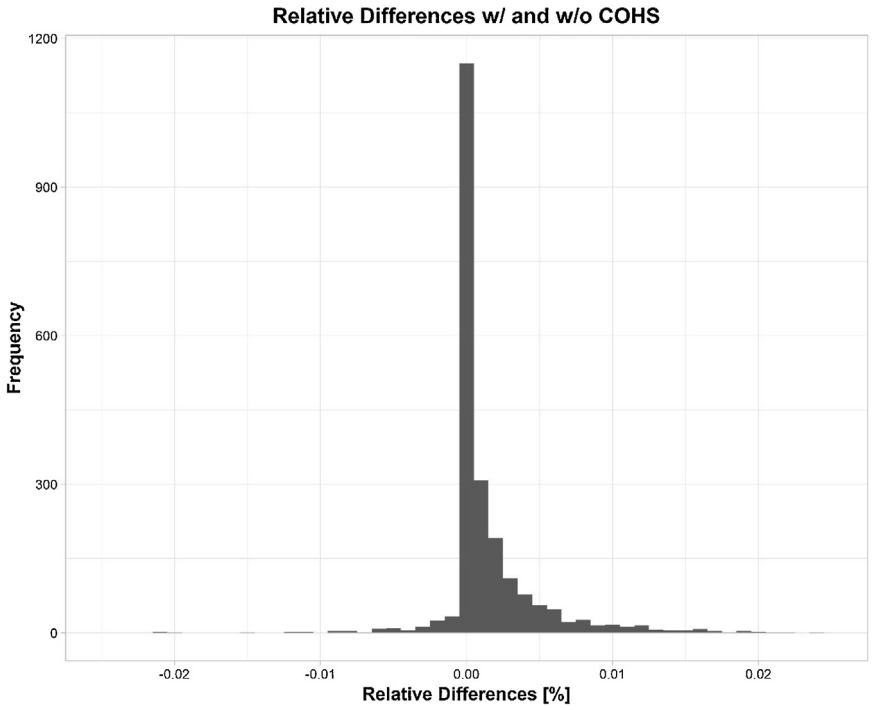

Fig. 2. Histogram of relative differences in % of total blood CO (TBCO) concentrations with and without the CO in the headspace (HS).

each new model, which were, in order, temperature, preservative, time (day of analysis) and HS volume. We were not able to investigate interactions between the parameters, since the number of outputs remaining after cleaning of data was not sufficient to the number required to obtain enough study power. ANOVA was then used to determine the significance of the parameters in the models (significance was obtained with a p-value < 0.05). Results are summarized in Table 2.

3.3. Correction model

To be able to generate a correction model applicable to COHb or TBCO measurements based on this dataset, first, the behavior of the response variables over time for each saturation level needed to be identified. Therefore, time plots for COHb and TBCO were produced (Fig. 3a–f), with a black line going through the means of the COHb/TBCO concentrations for each day of analysis. The graphs show a general weak linear trend for all saturation levels and response variable. For high and medium COHb levels (Fig. 3a and b), a weakly decreasing trend can be observed, whereas for low COHb saturation (Fig. 3c) there is a slight decrease in the initial phase, followed by a plateauing towards second half of the month. For high TBCO levels (Fig. 3d), there is a sudden drop after the first day, followed by a stabilization and weak decrease along the monitoring period. A similar drop can be seen for medium TBCO concentrations (Fig. 3e) on day 7, which is again followed by a stabilization and generally a weak decrease towards the end of the storage period. TBCO in low concentrations (Fig. 3f) shows a slightly increasing tendency. Generally, increasing variation can be observed the higher the saturation level and the higher the number of storage days from t0.

Due to the general linear behavior, MLR analysis was selected and used for each response variable and each saturation level to determine the coefficient estimate for each parameter and their

Table 2
Results of ANOVA for single parameters and combination of parameters for high, medium and low saturation level; COHb: carboxyhemoglobin, TBCO: total blood carbon monoxide; **: $p < 0.01$, *: $0.05 \leq p \leq 0.01$, –: not significant parameter ($p > 0.05$), #: for high saturation COHb levels, non parametric Kruskal-Wallis test was performed for single variables only.

Saturation level		High (60–70 %)		Medium (30–40 %)		Low (10–20 %)	
Model Number	Variables	COHb#	TBCO	COHb	TBCO	COHb	TBCO
1	Temperature	**	**	–	**	–	*
2	Preservative	**	**	**	**	**	**
3	Time	–	–	*	–	–	–
4	HS volume	–	–	–	–	–	–
5	Temperature	#	**	**	**	**	**
	Preservative	#	**	**	**	**	**
6	Temperature	#	**	**	**	**	**
	Preservative	#	**	**	**	**	**
	Time	#	–	**	*	*	*
7	Temperature	#	**	**	**	**	**
	Preservative	#	**	**	**	**	**
	Time	#	–	**	–	*	*
	HS volume	#	–	**	–	–	–

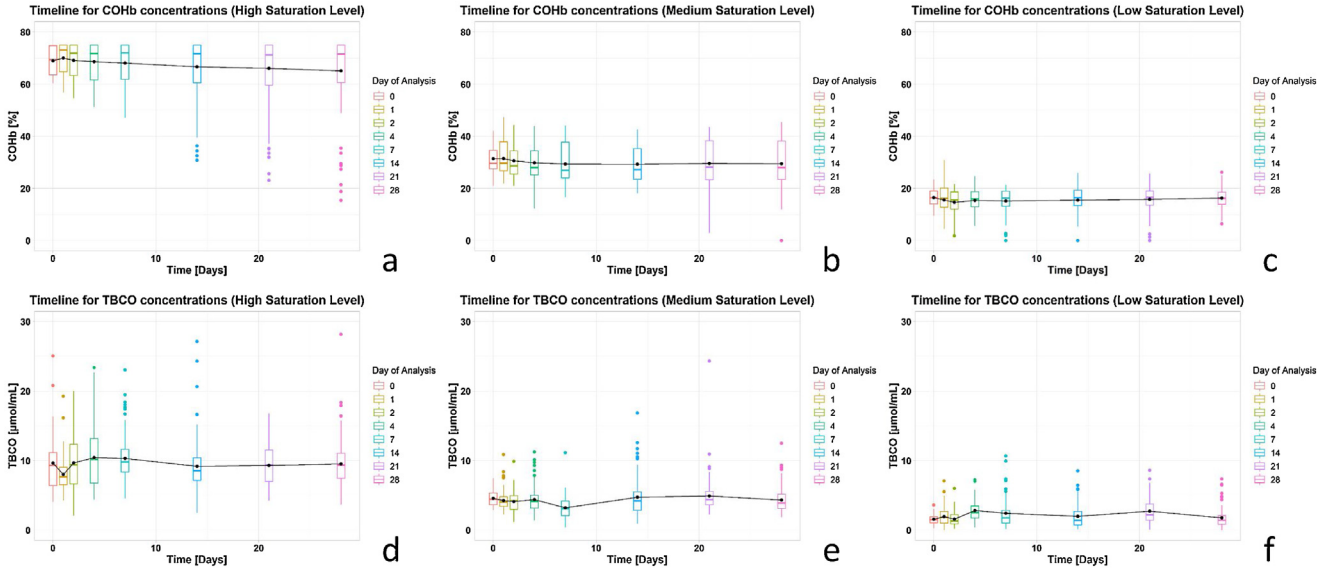

Fig. 3. a–f: Boxplots with error bars for COHb concentrations in % (a–c) and TBCO concentrations in µmol/mL (normalized) (d–f) over time for high, medium and low saturation levels, black dot: mean COHb/TBCO concentration for day of analysis, line in box: median.

Table 3
Coefficient estimates (β) and 95 % confidence intervals (CI) from Multiple Linear Regression (MLR) with storage parameters preservative, temperature, time and HS volume for measurement of COHb and TBCO for high, medium and low saturation levels. In **bold** the significant parameters ($p < 0.05$). MLR was performed with normalized data for TBCO (see Table 1).

Saturation level	High (60–70 %)		Medium (30–40 %)		Low (10–20 %)	
Parameter (Reference)	COHb ($R^2 = 0.67$) Coefficient estimate β (95 % CI)	TBCO ($R^2 = 0.39$) Coefficient estimate β (95 % CI)	COHb ($R^2 = 0.81$) Coefficient estimate β (95 % CI)	TBCO ($R^2 = 0.22$) Coefficient estimate β (95 % CI)	COHb ($R^2 = 0.76$) Coefficient estimate β (95 % CI)	TBCO ($R^2 = 0.22$) Coefficient estimate β (95 % CI)
Preservative (EDTA)						
NaF	**−16.35 (-17.47, -15.24)**	**−0.24 (-0.29, -0.19)**	**−13.92 (-14.58, -13.26)**	**−0.34 (-0.41, -0.26)**	**−7.01 (-7.54, -6.48)**	**−0.20 (-0.26, -0.13)**
LiH	**2.39 (1.25, 3.53)**	**0.27 (0.21, 0.32)**	**−13.49 (-14.14, -12.83)**	**−0.51 (-0.59, -0.44)**	**5.30 (4.83, 5.77)**	**−0.10 (-0.15, -0.04)**
NaCit	**−5.96 (-7.20, -4.72)**	**0.18 (0.12, 0.23)**	**−17.63 (-18.30, -16.96)**	**−0.27 (-0.35, -0.19)**	**3.00 (2.53, 3.47)**	**0.21 (0.16, 0.27)**
Temperature (-20 °C)						
+ 20 °C	**5.63 (4.63, 6.64)**	**0.06 (0.01, 0.10)**	**−0.71 (-1.28, -0.13)**	**−0.20 (-0.26, -0.13)**	0.07 (-0.37, 0.51)	**−0.09 (-0.14, -0.04)**
+ 4 °C	**2.60 (1.57, 3.64)**	−0.02 (-0.07, 0.02)	0.12 (-0.46, 0.69)	−0.04 (-0.10, 0.03)	**0.75 (0.33, 1.17)**	0.02 (-0.03, 0.07)
Time (Day 0)						
Day x	**−0.83 (-1.02, -0.63)**	0.01 (0.00, 0.02)	**−0.44 (-0.55, -0.33)**	0.00 (-0.01, 0.01)	**−0.16 (-0.24, -0.08)**	0.00 (-0.01, 0.01)
HS volume (<25 %)						
25 %	−0.23 (-1.25, 0.78)	0.00 (-0.05, 0.04)	**−0.72 (-1.30, -0.14)**	0.05 (-0.02, 0.12)	0.04 (-0.39, 0.46)	0.00 (-0.05, 0.05)
>50 %	**−1.32 (-2.33, -0.31)**	−0.03 (-0.07, 0.02)	**−1.14 (-1.72, -0.57)**	−0.04 (-0.11, 0.03)	−0.35 (-0.78, 0.07)	−0.03 (-0.08,0.02)

significance, based on following equation:

$$c = c_M - x_t\beta_t - \beta_P - \beta_T - \beta_V \quad (1)$$

with c: corrected concentration of analyte of interest (here COHb in % or TBCO in µmol/mL), c_M: measured concentration of analyte of interest; x_t: number of days since sampling of specimen, β_t: coefficient estimate for time, β_P: coefficient estimate for selected preservative, β_T: coefficient estimate for selected storage temperature, β_V: coefficient estimate for selected HS volume.

Reference level for each parameter was selected based on common guidelines for sample collection and storage in toxicological analyses (if specified), with EDTA as reference for preservative, freezing as reference temperature, low HS volume (<25 %) and day 0 as reference for time. Results of the MLR are summarized in Table 3. To be noted here that all results for TBCO are based on normalized data and, thus, coefficients need to be transformed back to be able to obtain the actual TBCO concentrations (e.g., for high saturation TBCO, log transformation was applied, therefore the back transformation involves application of the exponential function to the coefficient estimates).

3.3.1. Saturation level

For COHb, all parameters show statistical significance except HS volume 25–50 % for high saturation levels, while all parameters are significant except storage in the fridge (+4 °C) for medium saturation levels and storage in the fridge, preservatives NaF, LiH and NaCit and time are significant for low saturation levels.

For TBCO, across all saturation levels, all preservatives are significant as well as storage at room temperature (+20 °C), while no statistical significance was found for the other investigated parameters.

3.4. Prediction model

To be able to predict the COHb concentrations based on a measured TBCO value and the given storage conditions, the dataset

Table 4
Coefficient estimates (β) and standard error (SE) from Multiple Linear Regression (MLR) for prediction model (R^2 = 0.94), with storage parameters preservative, temperature, time and HS volume for measurement. In **bold** the significant parameters.

	COHb	
Parameter (Reference)	Coefficient estimate (β)	Standard error (SE)
Saturation level (Low, 10–20 %)		
Medium (30–40 %)	**13.75**	0.57
High (60–70 %)	**48.79**	0.87
Preservative (EDTA)		
NaF	**−12.00**	0.63
LiH	**−2.04**	0.59
NaCit	**−7.53**	0.62
Temperature (-20 °C)		
+ 4 °C	**2.16**	0.53
+ 20 °C	**2.87**	0.53
Time (Day 0)		
Day x	**−0.10**	0.02
HS volume (*<25 %*)		
25 %	−0.43	0.53
>50 %	−0.71	0.53

was split into a modeling set and a testing set. The testing set was obtained by extracting the data of one repetition for each analysis. The modeling set was then employed to generate a prediction model based on the linear function of

$$c_{COHb} = c_{TBCO} - x_t\beta_t - \beta_P - \beta_T - \beta_V \quad (2)$$

with c_{COHb}: concentration of COHb in [%], c_{TBCO}: concentration of TBCO in μmol/mL, x_t: number of days since sampling of specimen, β_t: coefficient estimate for time, β_P: coefficient estimate for selected preservative, β_T: coefficient estimate for selected storage temperature, β_V: coefficient estimate for selected HS volume.

Coefficients and standard errors of the model are found in Table 4. This model was then used to predict the COHb concentrations based on the TBCO values and storage parameters form the training set.

To evaluate efficiency of the prediction model, predicted values were compared with measured values with a Student *t*-test, which resulted in a p-value above 0.05, thus indicating that the measured and predicted values are not statistically different.

Prediction efficiency was further confirmed by a linear regression of predicted and measured COHb concentrations, which resulted in a good correlation (R^2 = 0.87) and is shown in Fig. 4.

4. Discussion

4.1. Correlation between COHb and TBCO

Before assessing each storage parameter and their potential impact on the measurement results, we first needed to determine the direction and magnitude of correlation between the employed methods, namely CO-oximetry and GC–MS. Previous studies have determined a strong positive correlation between COHb determined via CO-oximetry and CO measured by GC, with R^2 found generally above 0.9 for detection via flame ionization detector (FID) or reduction gas analyser (RGA) [36,37,40,41] and 0.85 for detection via MS [31,38,42,43]. Additionally, Cardeal, Vreman and others have proposed formulae to back calculate COHb from the CO measured through these GC methods, which are based on the correlation they obtained by comparing the two measurement methods [36–38,44].

Results from this study, however, do not confirm the results of these research groups. A weaker correlation between COHb measured by CO-oximetry and CO measured by GC—MS (R^2 = 0.68, see Fig. 1) was determined. Furthermore, the statistically significant difference found between the measured values and the ones back calculated through the applied formula from Cardeal et al. (see Section 3.2.1) does not confirm results previously obtained [43]. Therefore, the formula seems to be unsuitable. One possible explanation for this discrepancy in results can be found in the different analytical approaches used by each research group. While Vreman uses GC-RGA for detection [35,36], Cardeal uses GC-FID [37] and Hao [38] and Varlet [43] GC—MS. The advantages and disadvantages of each detection method have been discussed

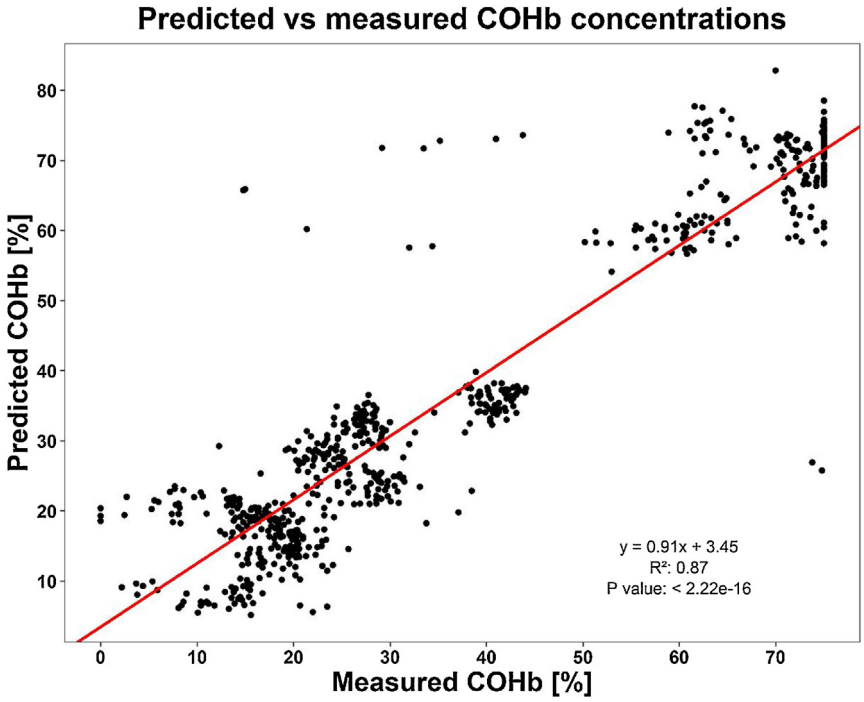

Fig. 4. Correlation plot for measured vs predicted COHb concentrations in %.

thoroughly in the past [17,45,46]. Generally, it is determined that GC-FID is the most sensitive method for CO analysis, but time-consuming and impractical due to the additional need of a methanizer, which makes the instrument limited to only a specific analysis, while GC—MS is the most versatile, accurate, rapid and reproducible method for CO determination in blood [17,45]. In addition to a different detection method, the research groups also use different calibration and sample preparation approaches. Various acids and oxidizing agents have been employed as 'liberating agents' to release CO for analysis via GC, which can result in different recoveries and efficiencies in CO release, hence altering the final CO concentrations obtained [19,31]. Furthermore, the calibration solutions were prepared differently. All previous studies have performed a *flushing* step of the calibrators prepared from CO-bubbled blood, with the aim of removing the 'excess' CO and, thus, recover only the CO bound to Hb. However, this does not comply with the pathophysiology of CO poisonings: both bound and free CO are responsible for the toxicity mechanisms of CO in the human body [1,7,16,34]. Consequently, removing and not analyzing free CO can underestimate the true CO burden, potentially resulting in fatal misdiagnoses. Therefore, in this study we do not determine only the bound CO fraction, but the TBCO, which includes both free and bound CO. The amount of free CO was already found to be significant and may be one of the reasons for the discrepancy between our results and those from previous researchers regarding both the correlation of COHb and (TB)CO and the back calculation of COHb through formulae [30].

4.2. Influence of storage parameters

4.2.1. CO in HS

The bond between CO and Hb is very strong, due to the high affinity of CO for the hemoprotein, which leads to COHb as being considered the sole biomarker of CO exposure. It is often reported as constituting the major form CO acquires when crossing the lung-blood barrier, making up more than 90 % of inspired CO [47]. However, recent studies have also acknowledged the incongruence between symptoms and measured COHb and the possibility of a higher percentage of CO not bound to Hb than previously assumed [7,8,16,30,34,48]. This can partially be explained by the reversible reaction between CO and Hb: despite the high affinity, there is still a part of CO that can go back to its unbound form, even though it most likely constitutes only a small fraction. This equilibrium can however be shifted towards free CO by an increased HS volume: since CO is a gas, it behaves according to the ideal gas law, and according to Le Chatelier's principle and the entropy laws, an increase in volume drives the gas molecules to shift and distribute towards the additional space, where the gas concentration is lower. An increased HS volume can, thus, increase the concentrations of CO in the HS significantly.

Based on the results of the measurements of CO in the HS of the blood tubes after statistical analysis, CO in HS is determined to be significant. However, statistical significance does not always reflect a significance from a biochemical point of view, and, thus, needs to be put into the right context. As represented in the histogram in Fig. 2, relative differences are generally below 0.01 % COHb, which from a pathophysiological perspective do not have an impact on the severity of the poisoning. Therefore, we conclude that there is not a significant amount of CO that is released into the HS of the blood tube during storage.

4.2.2. Reopening and freeze- and thaw-cycles

Exposure to air through repeated reopenings of the samples was reported to decrease COHb values, which is mainly due to a loss of CO through an increase in the available volume. This can cause a shift of the equilibrium of free CO driven by entropy [49]. Similarly, blood samples stored below freezing temperature that had to undergo multiple freezing and thawing cycles due to repeated measurements required showed reduced COHb values, even though at a lesser extent [28,38]. In this study, however, results showed that both reopening and freeze- and thaw-cycles did not have a significant impact on the measurement values for neither COHb nor TBCO. Previous research into alterations to COHb values due to storage of blood samples showed mild reductions when observed for periods varying between 45 days and 2 years [24,28,38]. Considering that in the current study the observation period was of 28 days, this could explain the lack of significant alterations observed, reopening and freeze- and thaw-cycles may affect the COHb and TBCO values only at a later storage period. Furthermore, in the study performed by Chace et al. [28], samples were allowed free air exchange during the whole period of storage, whereas the samples in this study were reopened only on the days of analysis. Kunsman et al. stored the samples for a period of 2 years and reopened the tubes only for the second analysis, thus only accounting for one reopening and one freezing- and thawing-cycle [24]. Therefore, no substantial loss of CO could have occurred due to exposure to air or the freezing- and thawing-cycle, which is in accordance with the observed results. Hao et al. describe a substantial loss of COHb during storage over 45 days when measured with UV-spectrophotometry, while COHb back calculated from CO measured by HS-GC–MS is shown to be stable over the course of the storage period [38]. A similar behavior is confirmed in this study.

4.2.3. Storage parameters: temperature, preservative, time and HS volume

Various storage conditions have previously been investigated by multiple research groups, with results usually showing either increased or decreased COHb concentrations based on the storage temperature chosen, the preservative used to prevent blood clotting or exposure to different amounts of air over the course of prolonged storage time. However, there is also the possibility that these parameters affect the alteration of measurement differently based on whether the CO level in blood is high or low. While a parameter might have significant effects when low CO levels are present in the blood specimen, the variation in higher saturation levels might not be as significant, thus making that parameter to be considered for certain types of poisoning cases. Therefore, the different storage parameters were evaluated for each saturation level separately, which was not investigated previously by other research groups.

The Multiway-ANOVA (Table 2, **model 7**) shows that all investigated parameters significantly affect the COHb concentrations for all saturation levels (except HS volume for low COHb concentrations), which is in congruence with previous studies reporting CO changes due to these parameters [24,25,28,38]. This behavior is, however, different for TBCO, where HS volume does not influence the measurement results in any saturation level and time only affects these at low concentrations. Hence, TBCO seems to be less affected by storage conditions compared to COHb, especially at medium to high ranges, which are of particular relevance for forensic cases.

This is further confirmed by the MLR analysis results (Table 3), where a higher number of parameters are found to be significant for COHb than TBCO. A closer look at the variables shows that all preservatives are influential for both COHb and TBCO measurements (the higher the magnitude of the coefficient estimates (in either positive or negative direction), the more significant their effect).

For TBCO the effects of the different preservatives are of similar magnitude and direction in each saturation level, except for NaF in high saturations, which shows a decrease in TBCO (−0.24), as

opposed to the other two preservatives showing an increase (0.27 for LiH, 0.18 for NaCit). Additionally, TBCO in low saturation levels shows a decreasing trend (−0.20 for NaF, −0.10 for LiH), with only NaCit resulting increased levels (0.21).

For COHb, a clear trend can be observed with NaF, where a decrease is observed in all saturation levels and the magnitude decreasing from higher to lower saturation (−16.35, −13.92, −7.01). Since NaF is a weak anticoagulant, its effect might diminish during prolonged storage, therefore increasing the chance of blood clotting, which reduces the blood quality. Therefore, it is not surprising that a decrease in COHb is observed and that the effect is more evident with higher COHb concentrations. However, for LiH and NaCit, no consistency can be observed between saturation levels and the effect of the preservative. With LiH as preservative, the highest effect shows at medium COHb levels, with an average decrease of 13.49, whereas for the other saturation levels, increased COHb concentrations are observed (2.39 in high COHb levels, 5.30 in low COHb levels). Heparin is a widely used anticoagulant, especially in clinical toxicology and biochemistry, despite its high cost and short lasting action. It belongs to the family of glycosaminoglycans. The anticoagulant feature of this complex chemical structure are the sulfated pentasaccharide units, which have a high binding affinity for antithrombin III, a plasma protein that inhibits blood clotting [50,51]. Considering that Hb has a relatively high binding affinity for oxygen and that the CO—Hb bond is reversible, it is possible that at certain concentrations the sulfated pentasaccharide units of heparin interact with Hb, thus altering the measured COHb concentrations. Furthermore, LiH is employed as a liquid solution, rather than a salt as all other preservatives. This increases the potential for blood dilution, therefore leading to decreased COHb measurement results [52]. These explanations for the observed behavior are all hypothetical, no specific study was conducted in the past assessing the effect of storage with heparin for blood samples used in CO poisoning determinations.

When using NaCit as preservative, the highest decrease in COHb is shown, similarly to LiH, in medium COHb ranges (−17.63), while a less significant decrease is reported in high saturations (−5.96) and an increase in low saturations (3.00). NaCit is, similarly to NaF, a weak anticoagulant, used primarily in blood transfusions and generally clinical blood samples, mainly due to its low cost and reversibility of the anticoagulant mechanism (chelation of calcium ions) [53,54]. Therefore, it is possible that at certain COHb concentrations, driven by a concentration gradient or chemical interactions, either the chelation of calcium or the bond between Hb and CO is reversed (which is a coordinated bond and not a covalent bond), leading to decreases in COHb. In addition, it has been previously reported in several studies that citrate alters the measurements of other compounds, such as gamma-hydroxybutyrate (GHB), leading to false positives. While the mechanism has not yet been elucidated, it was recommended that citrate as preservative should not be used for forensic drug analyses [55–57]. Therefore, we hypothesize that a similar reaction might take place for COHb measurements, even though further investigation is needed to confirm this.

Regarding HS volume and temperature, these are shown to be more influential at higher saturation levels for COHb concentrations, which is in accordance with results reported by Hao et al. [38], who also showed a more marked change in COHb concentrations with increased COHb saturation level. Storage at room temperature, as opposed to storage in the fridge or freezer, shows more prominent increases of high COHb concentrations. This is in accordance with biochemically- and bacterially-induced blood degradation, which is increased with higher temperatures. Results reported by Kunsman et al. [24] showing reduced COHb levels with increased exposure to air is also confirmed by the MLR results, with a negative and more significant coefficient estimate (β) reported for COHb levels (Table 3). However, this behavior is not shown with TBCO, for which HS volume, time and storage in the fridge or freezer do not play an influential role. Only the choice of the preservative and storage at RT has a significant impact on TBCO measurements.

This supports the hypothesis that TBCO appears as more stable and less prone to significant changes due to temperature, time and air exposure, as opposed to COHb. COHb measurement by spectrophotometry is affected by changes to the optical blood quality, which are mainly due to blood constituents catabolism occurring with time and also temperature changes, making the measurement more challenging and in some cases even impossible (the instrument returns an error message). Furthermore, measurement by CO-oximetry is also affected by the amount of Hb present in the blood sample, with a range of 5–25 g/L limiting the measurements, which is especially relevant for forensic cases, where with long PMI, the blood quality is often altered, potentially leading Hb levels higher or lower to the instrument's limits [29]. The majority of these factors are, however, not relevant for TBCO measurements. Optical blood changes, blood component catabolism, shifts of CO from bound to free, redistribution or increases of Hb in the blood compartments – neither of these factors influence measurement of CO via GC–MS. The pre-analytical reaction that takes place does not differentiate free or bound CO, all CO is transferred to the gas phase and then analyzed with a GC-column specific for gaseous molecules, thus reducing the potential interference of compounds present in the sample. However, TBCO measurements are impacted by PM generation of CO, similarly to COHb measurements, which is more likely to occur when samples are stored at higher temperatures. This explains why TBCO is shown to be influenced by storage at room temperature. Nevertheless, TBCO measurement may constitute a more reliable method for quantification of CO in non-optimal sampling and storage circumstances.

4.3. Correction model

In this study, several storage conditions have been investigated over a prolonged period, with parameters influencing the measurement results differently based on the chosen conditions and saturation level. Therefore, the selection of appropriate storage conditions is essential in guaranteeing accurate and reliable results, which can determine whether a case is attributed to CO poisoning as cause of death, contributing factor or unrelated to death, with significant legal consequences. However, optimal conditions cannot always be guaranteed. Based on the laboratory equipment, resources, location and collaboration with local law enforcement and emergency departments, conditions of sampling and storage may vary. To be able to obtain consistent and accurate results across laboratories, we have used our data and MLR analysis to generate a correction model for both COHb and TBCO with parameters temperature, time, preservative and HS volume as input variables.

Eq. (1) can be adapted to the case at hand: depending on whether COHb or TBCO is being measured, the coefficient estimates for the selected storage conditions (if they vary from the reference conditions, otherwise the variable is equal to 0) that are significant for the relevant saturation level are back-transformed (if necessary), input into Eq. (1) and the corrected concentration is obtained.

As an example, if there is a blood sample that was stored with NaCit and >50 % HS volume in the freezer for 28 days and obtained a COHb concentration of 35 %, the corrected concentration would be:

$$c_{COHb} = 35.00\% - 28*(-0.08) - (-17.37) - 0 - (-1.24) = 55.85\%$$

For a sample stored with the same conditions and with a measured TBCO concentration of 5.00 μmol/mL, the coefficients need to be back transformed for use with the correction formula. In this case (medium saturation level), log transformation was performed, therefore the exponential function needs to be applied to the coefficients, giving us following corrected concentration:

$$c_{TBCO} = \ 5.00 \ \mu\frac{mol}{mL} - 0 - \left(e^{(-0.21)}\right) - \ 0 - 0 = 4.19 \ \mu\text{mol/mL}$$

This provides an important tool to be employed by laboratories and emergency departments that do not have the financial or logistical capacity to guarantee the best conditions for sampling and storage of specimens, such as in less developed countries where samples might need to be mailed to a laboratory with the appropriate equipment. It will enable them to obtain accurate and reliable determinations in CO poisoning cases, despite non optimal storage conditions. However, this formula cannot be applied if temperatures during transport exceed 20 °C, as temperatures above were not investigated here. Generally, laboratory guidelines and best practice regulations may vary across countries, even though a lot of effort is being put into reaching a global consensus on clinical and forensic laboratory standards. However, differences in storage and sampling practice are still common and therefore a consensus should at least be reached regarding the accuracy of results, which is the main goal and, finally, achievement of this study. With this model, not only can correct diagnoses in suspected CO poisonings be obtained regardless of the sampling and storage conditions, but results can also be compared across laboratories and countries, allowing the creation and expansion of a collaboration network, which can be fruitful under other aspects as well.

4.4. Prediction model

Going a step further to obtain the most accurate and reliable CO poisoning determinations possible, we have integrated the storage conditions with the proposed alternative biomarker TBCO to be able to obtain COHb values that reflect with higher accuracy the levels present in blood specimen, even in cases where COHb cannot be measured due to degradation. By measuring TBCO and inputting the coefficient estimates (Table 4) into Eq. (2), COHb concentrations can be predicted. Efficiency of the prediction model was confirmed by testing it on a set of data with known COHb and TBCO concentrations and storage conditions, which gave a satisfactory correlation coefficient of 0.87. Therefore, this prediction model together with TBCO measurement can be employed by laboratories for cases where measurement with CO-oximetry is not possible, allowing CO poisoning determinations in all possible conditions. However, a limitation of this prediction model is that it can only be applied to samples with a short postmortem interval (PMI). PM degradation affects the concentrations of CO in ways that go beyond storage, such as PM CO production through bacterial activity in the body. This was not a factor investigated in this study, but would be an important aspect to research in order to further expand the potential application range of the proposed prediction model.

5. Limitations

In forensic cases, samples are usually stored for periods longer than 1 month, often for more than 1 year, since the timeline of court cases is very long and samples might be reanalyzed for cross-examination. Therefore, it is reasonable that the effects of time on COHb and TBCO are not very significant. Even though they are arithmetically significant for COHb, the differences over one month of less than 1 % COHb will not affect the interpretation of toxicological findings. Studies with prolonged storage time should be carried out to examine the long-term effects. Another aspect that needs to be taken into consideration is that these tests were performed on non-human blood. Despite the similarities in blood density and Hb concentrations between bovine and human blood, it is possible that results might differ when using human blood. Nevertheless, we believe that these differences would not be very significant. Furthermore, this study focused on investigating storage parameters, not considering PM changes occurring when dealing with forensic cases. Therefore, the models generated here are applicable to clinical cases, but when dealing with forensic cases, PM changes need to be taken into consideration for interpretation of the results. Nevertheless, we believe that the models can be used to assess the storage conditions and are to be added to the interpretation of potential PM changes. An additional aspect that might limit this study is the instrument's limit of 75 % on COHb measurements. However, considering that from a toxicological perspective, the findings will not change based on whether the COHb concentration is at 75 % or above (CO at 75 % or above is considered as cause of death), this is not a significant limitation.

6. Conclusion

In this study, we have not only compared two biomarkers and detection methods (COHb measured via CO-oximetry and TBCO measured via GC–MS) for the application in CO poisoning determinations, but also investigated the nature and magnitude of effects caused by different storage conditions on the accuracy of the obtained measurement results by both biomarkers.

The significant discrepancy between TBCO and COHb is shown by the weaker correlation found between the two measures, as opposed to correlations of previously reported studies, who used to flush the calibrators prior to analysis. This affirms the importance of the measurement of free CO in addition to bound CO to obtain results that more closely correspond to the true pathophysiological levels.

Furthermore, TBCO appears to be more stable during storage for prolonged time intervals, with no significant alterations observed due to different HS volumes, storage in the fridge or freezer and several preservatives during this period. On the contrary, COHb is affected by all investigated parameters, even though at different extents. This confirms that optical measurement methods are more prone to deliver inaccurate results due to storage conditions. Conversely, TBCO measurement should be promoted, especially in forensic investigations, where trials can be delayed and last for long periods and often require reanalysis of supportive evidence. Therefore, we recommend the use of TBCO as alternative biomarker to COHb for CO poisoning determinations. Moreover, unlike general storage guidelines for clinical and forensic toxicology (e.g. TIAFT, UKIAFT, etc.), who suggest NaF as the preservative of choice [15,22,58], based on our results, we generally recommend collection of samples for CO analysis in EDTA tubes for short storage periods (up to one month), stored possibly in the freezer or fridge. When COHb is analysed, it is also important to fill the collection tube at more than 50 % of its volume and to analyse the sample as soon as possible.

However, in laboratories or institutions where optimal storage is not possible, the use of the proposed correction formula provides an important tool to obtain more accurate measurements, even in non-optimal conditions. Additionally, in cases where spectrophotometric measurements are not possible due to degradation of the sample during storage, the provided prediction formula can be used to estimate the corresponding COHb concentration by measuring TBCO.

CRediT authorship contribution statement

Stefania Oliverio: Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Visualization. **Vincent Varlet:** Conceptualization, Methodology, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare no conflict of interest.

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"*The silent killer*": exploring new approaches for quantification of carbon monoxide exposures

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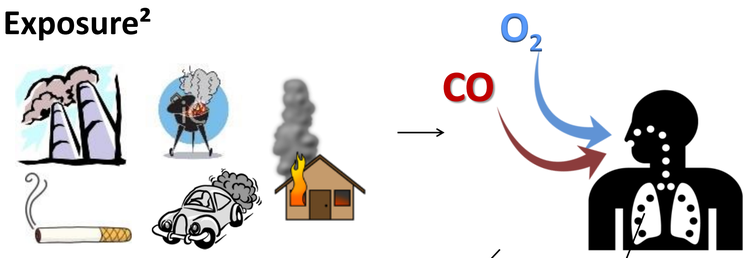
1 Introduction

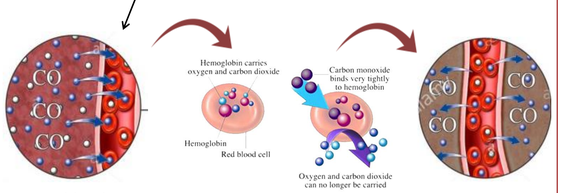
Carbon monoxide (CO) is one of the leading causes of domestic deaths and one of the most abundant **toxic air pollutants** in the atmosphere[1].

1) Exposure[2]

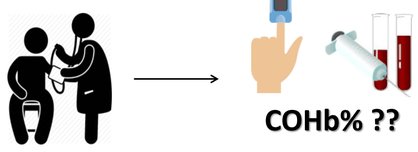


2) Symptoms[3,4]

→ **Not specific** for CO!



3) Diagnosis



→ Often **missed** due to non-specific symptoms and low index of suspicion[5]

4) Testing

→ Tests specific for CO only done when CO suspected

→ **COHb%** results often **inconsistent** with symptoms → **problem** with biomarker? **New biomarker** needed?

→ **Factors** affecting measurement → **measurement error**

2 Aims

To **develop** a **novel method** for **CO poisoning detection**, which is not based on the measurement of COHb, but the **total CO** present in the sample (**TBCO**).

To determine the **measurement error** deriving from the **storage conditions** of blood samples (*in vitro* study) and evaluate their relevance.

3 Methods and Materials

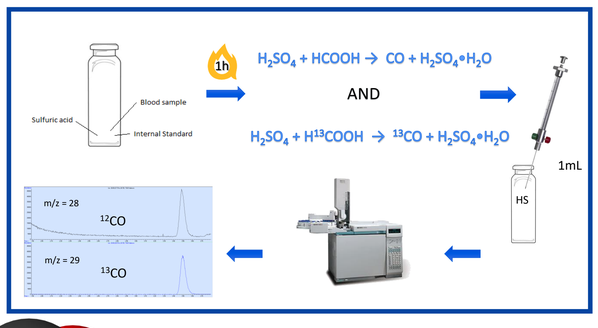
Bovine blood samples are fortified with pure CO gas in different initial COHb% **saturation levels**: **≈10/20%, ≈30/40%, ≈60/70%.**

Monitoring is performed over a period of one month, with measurements on following days:

Day 0 → Day 1 → Day 2 → Day 4 → Day 7 → Day 14 → Day 21 → Day 28

Analyses are also performed on **postmortem samples** obtained from routine work.

CO-oximetry **VS** GC-MS



PARAMETERS

- **Preservative**: EDTA, NaF, LiH, NaCit
- **Temperature**: -20°C, +4°C, +20°C
- **Tube Dead Volume**: <25%, 25%<x<50%, >50%
- **Freeze- and thaw-cycle**
- **Reopening**

MEASUREMENTS

- **CO in headspace (HS)** by AGS-GC-MS
- **CO in blood** by AGS-GC-MS
- **CO in blood** by CO-oximetry

AGS: Airtight Gas Syringe

4 Results

Completed **accuracy profile validation** according to the "French Society of Pharmaceutical Sciences and Techniques" (SFSTP) for a calibration range between **10-200 nmol/mL HS** (2-40µmol/mL blood).

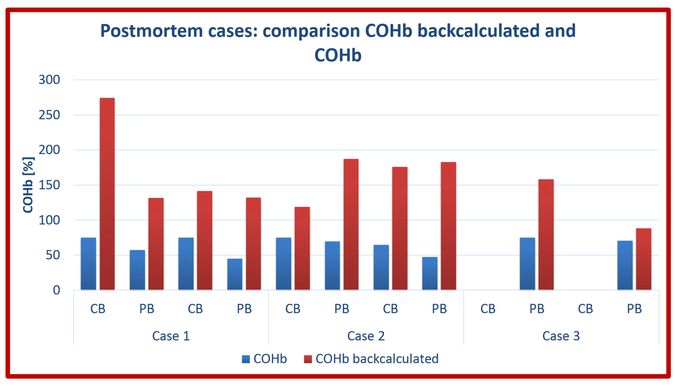


Chart 1. COHb measurement by CO-oximetry (blue bars) and the COHb backcalculated (red bars) by application of formulas found in the literature from the CO in blood measurements by AGS-GC-MS on real postmortem cases; CB: cardiac blood; PB: peripheral blood.

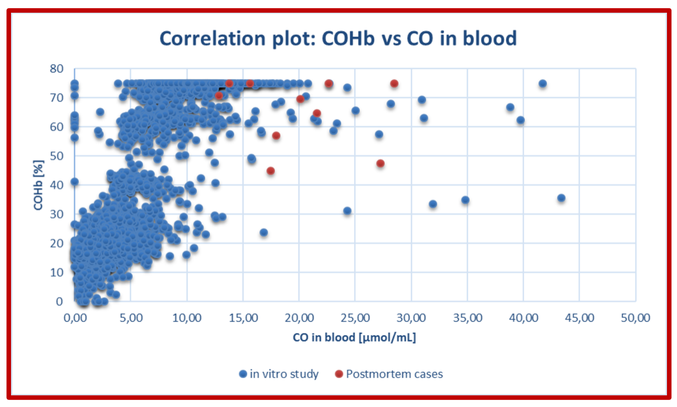


Chart 2. Scatterplot of measured COHb [%] by CO-Oximetry vs. CO measured in blood by AGS-GC-MS [µmol/mL]; in red the postmortem cases.

Parameter (Reference)	Coefficient estimate (β)	95% CI
Intercept		
-	22.16	20.84; 23.48
TBCO		
-	0.42	0.34; 0.50
Preservative (EDTA)		
NaF	-11.91	-12.60; -11.21
LiH	-2.06	-2.72; -1.39
NaCit	-7.26	-7.94; -6.57
Temperature (-20°C)		
+ 20°C	-2.54; -1.36	-2.54; -1.36
+ 4°C	-1.27; -0.08	-1.27; -0.08
Tube dead volume (<25°C)		
25%<x<50%	-0.98; 0.19	-0.98; 0.19
>50%	-1.49; -0.31	-1.49; -0.31
COHb% initial saturation (10%/20%)		
30%/40%	13.39; 14.64	13.39; 14.64
60%/70%	48.60; 50.33	48.60; 50.33
Time (Day 0)		
Day 1	-0.94; 1.59	-0.94; 1.59
Day 2	-2.00; 0.53	-2.00; 0.53
Day 4	-2.69; -0.15	-2.69; -0.15
Day 7	-2.92; -0.37	-2.92; -0.37
Day 14	-3.52; -0.96	-3.52; -0.96
Day 21	-3.95; -1.38	-3.95; -1.38
Day 28	-4.11; -1.54	-4.11; -1.54

Table 1. Results of a multiple linear regression analysis on the storage study results with 95% confidence interval (CI) for the coefficients.

Based on the measurements of the *in vitro* study, a multiple linear regression analysis was performed on the data (**Table 1**), leading to following formula:

$$y = x_0 + x_1\beta_1 + \beta_2 + \beta_3 + \beta_4 + \beta_5 + \beta_6$$

Where: y = **COHb**[%]; x_0 = intercept; x_1 = **TBCO** [µmol/mL]; β_1 = coefficient for TBCO; β_2 = coefficient for **preservative**; β_3 = coefficient for **temperature**; β_4 = coefficient for **tube dead volume**; β_5 = **COHb% initial saturation**; β_6 = coefficient for **time**.

6% (140/2376) of the dataset were missing observations and not included.

All storage **conditions** are **significant**, **except** for following levels: tube dead volume "25%<x<50%", time "Day 1" and "Day 2".

5 Discussion

All backcalculated COHb values are significantly above the ones measured by CO-oximetry, suggesting that there is a significant quantity of **CO** that is not bound to Hb (since it is not measured optically), but presumably **dissolved** in blood in free form. Potentially, it can **explain** the **discrepancy** between **measured COHb%** and reported **symptoms**, leading to a decrease of misdiagnoses (**Chart 1**).

The correlation between COHb and TBCO is not linear, but presents a more cone-shaped pattern, with increasing scattering the higher the saturation level (**Chart 2**).

A multiple linear regression analysis of the results obtained from the storage study shows a very good correlation coefficient (R^2 = 0,938, p < 2,2e-16) with significant **influences** of **all parameters** investigated (Table 1). **No significant** amount of CO released into the **HS** during storage was detected (**p-Value = 0.9873**).

6 Conclusions

- CO levels based on optically measured COHb may lead to **underestimation** of CO exposure. **CO not linked to Hb** might be of pathophysiological relevance.
- The use of **TBCO** as more reliable biomarker of CO exposure in the clinical field requires further testing.
- Furthermore, this approach allows the analysis of blood samples under controlled conditions, even if the samples are optically altered (CO-oximetry is infeasible) .
- **Best storage conditions** are: EDTA tube, filled as much as possible, stored frozen up to 2-4 days.

Contact

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References

1. Brunekreef, B. and S. T. Holgate (2002). "Air pollution and health." The lancet **360**(9341): 1233-1242.
2. Weaver, L. K. (2009). "Carbon monoxide poisoning." New England Journal of Medicine **360**(12): 1217-1225.
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5. Raub, J. A., et al. (2000). "Carbon monoxide poisoning—a public health perspective." Toxicology **145**(1): 1-14.

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"The silent killer"

Demonstrating the importance of controlled storage conditions in CO poisonings

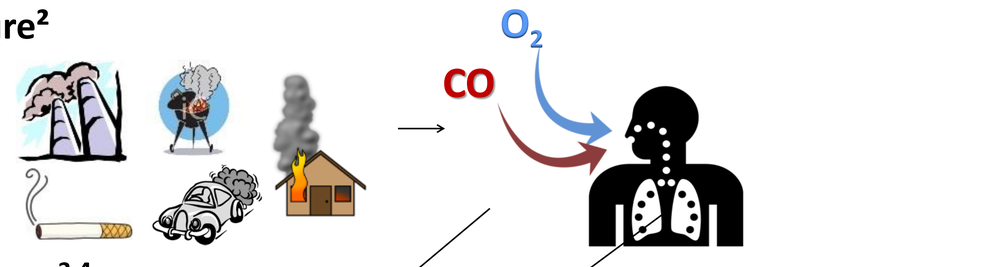
Oliverio, S.[1,2]; Zeka, A.[2]; Leonardi, G.[3], Varlet, V.[1]

[1]University Center of Legal Medicine Lausanne-Geneva, Switzerland,
[2]Brunel University London, United Kingdom, [3]Public Health England

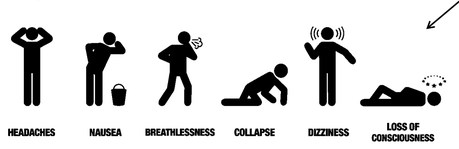
1 Introduction

Carbon monoxide (CO) is one of the most abundant **toxic air pollutants** and one of the leading causes of accidental poisoning and suicide deaths[1].

1) Exposure[2]



2) Symptoms[3,4]



→ **Not specific for CO!**

CO into bloodstream

CO to tissues

3) Diagnosis and Testing

- Tests specific for CO only done when CO suspected
- **COHb%** results often **inconsistent** with symptoms → **problem** with biomarker? **New biomarker** needed?
- **Factors** affecting measurement → **sample preparation and storage**

2 Aims

- Develop a **novel method** for **CO poisoning determination**, based on the total CO amount in blood (**TBCO**).
- Determine impact and magnitude of **storage conditions** on blood samples (*in vitro* study) and evaluate their relevance.

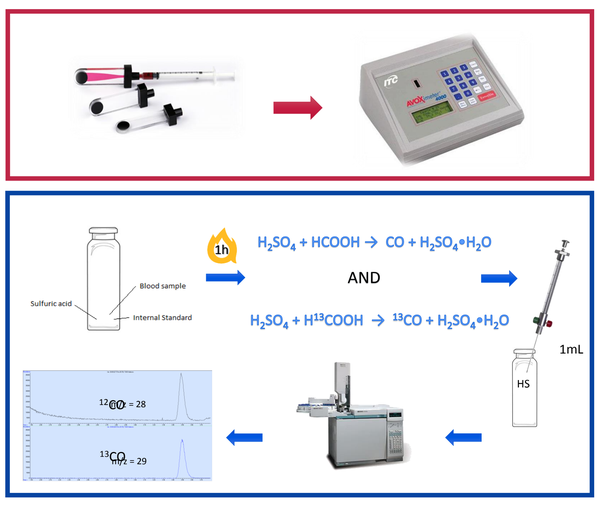
3 Methods and Materials

Bovine blood samples are fortified with pure CO gas in different initial COHb% **saturation levels**: **[10/20%], [30/40%], [60/70%].**
Monitoring is performed over a period of one month, with measurements on following days:

Day 0 → Day 1 → Day 2 → Day 4 → Day 7 → Day 14 → Day 21 → Day 28

Analyses are also performed on **postmortem samples** obtained from routine work.

CO-oximetry VS GC-MS



PARAMETERS

- **Preservative**: EDTA, NaF, LiH, NaCit
- **Temperature**: -20°C, +4°C, +20°C
- **Tube Headspace (HS)**: <25%, 25%<x<50%, >50%
- **Freeze- and thaw-cycle**
- **Reopening**

MEASUREMENTS

- **CO in HS** by AGS-GC-MS
- **CO in blood** by AGS-GC-MS
- **CO in blood** by CO-oximetry

AGS: Airtight Gas Syringe

4 Results

Completed **accuracy profile validation** according to the "French Society of Pharmaceutical Sciences and Techniques" (SFSTP) for a calibration range between **10-200 nmol/mL HS** (2-40µmol/mL blood).

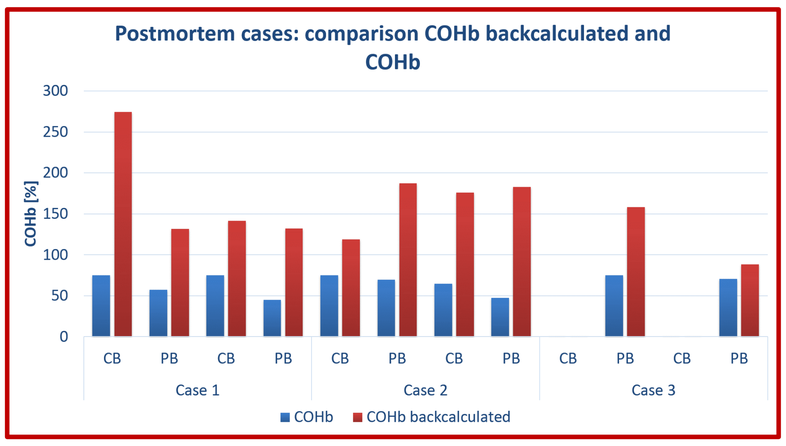
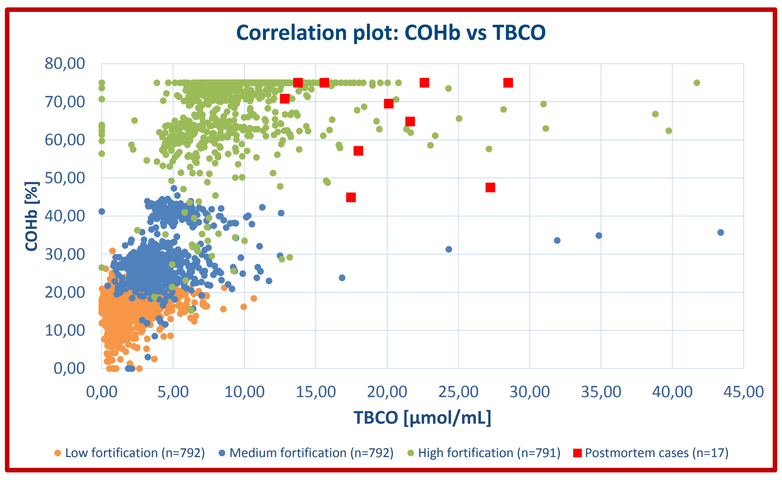


Chart 1. COHb measurement by CO-oximetry (blue bars) and COHb backcalculated (red bars) by application of formulae found in the literature, from the CO in blood measurements by AGS-GC-MS on real postmortem cases; CB: cardiac blood; PB: peripheral blood.

Chart 2. Scatterplot of measured COHb [%] by CO-Oximetry vs. CO measured in blood by AGS-GC-MS [µmol/mL]; in red the postmortem cases, in orange, blue and green the low, medium and high fortifications respectively.



Parameter (Reference)	Coefficient estimate (β)	95% CI
Preservative (EDTA)		
NaF	-12,52	-13,22; -11,82
LiH	-1,91	-2,6; -1,23
NaCit	-6,74	-7,43; -6,04
Temperature (-20°C)		
+ 20°C	-1,6	-2,29; -1,08
+ 4°C	-0,47	-1,07; 0,14
Tube dead volume (<25%)		
25%-50%	-0,39	-0,99; 0,22
>50%	-1,02	-1,63; -0,42
COHb% initial saturation (10%/20%)		
30%/40%	15,06	14,45; 15,66
60%/70%	52,69	52,08; 53,3
Time (Day 0)		
Day 1	0,1	-1,2; 1,39
Day 2	-0,83	-2,12; 0,46
Day 4	-1,2	-2,5; 0,1
Day 7	-1,62	-2,92; -0,32
Day 14	-2,23	-3,53; -0,92
Day 21	-2,49	-3,81; -1,18
Day 28	-2,76	-4,08; -1,45

Table 1. Results of a multiple linear regression analysis on the storage study results, with 95% confidence interval (CI) for the coefficients.

6% (140/2376) of the dataset were missing observations and not included.
Following storage conditions are **not statistically significant**: temperature "4°C", tube dead volume "25%-50%", time "Day 1", "Day 2" and "Day 4", reopening and freeze- and thaw-cycles.

5 Discussion

All backcalculated COHb values are significantly above the ones measured by CO-oximetry, suggesting that there is a significant quantity of CO that is not bound to Hb, but presumably **dissolved** in blood in **free form**. Potentially, it can help **explain** the **discrepancy** between **measured COHb%** and reported **symptoms**, leading to a decrease of misdiagnoses (**Chart 1**).

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A multiple linear regression analysis of the results obtained from the storage study shows a very good correlation coefficient (R^2 = 0,938, p < 2,2e-16) with significant influences of most parameters investigated (Table 1). **No significant** amount of CO released into the **HS** during storage was detected (**p-Value = 0.9873**).

6 Conclusions

- CO levels based on optically measured COHb may lead to **underestimation** of CO exposure. **CO not linked to Hb** might be of pathophysiological relevance.
- Use of **TBCO** as more reliable biomarker of CO exposure requires further testing.
- This approach allows the analysis of blood samples under controlled conditions, even if optical measurement is infeasible.
- **Best storage conditions**: EDTA tube, filled as much as possible, stored frozen up to 4-7 days.

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"THE SILENT KILLER"

New approaches to minimize errors in carbon monoxide poisoning diagnosis

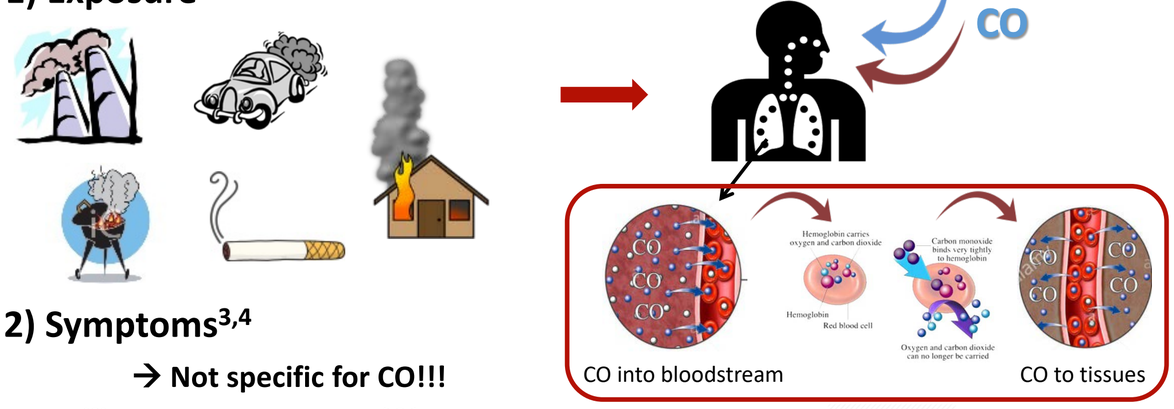
Oliverio S.[1,2], Varlet V.[2], Leonardi G.[3], Zeka A.[1]

[1]Department of Environmental Sciences, CHLS, Brunel University London, United Kingdom, [2]UTCF, University Center of Legal Medicine Lausanne-Geneva, Switzerland, [3]Public Health England

1 Introduction and Aim of Study

Carbon monoxide (CO) is one of the most abundant **toxic air pollutants** and one of the leading causes of accidental poisoning and suicide deaths[1].

1) Exposure[2]



2) Symptoms[3,4]

→ **Not specific for CO!!!**

3) Diagnosis & Analysis

- Low index of suspicion → **misdiagnosis**
- Results of biomarker COHb often inconsistent with symptoms → **misdiagnosis**
- Errors in sample preparation, storage and analysis → **misdiagnosis**

Develop a **novel biomarker** for CO poisoning determination

Determine **magnitude** and **impact** of **errors** in sample preparation, storage, analysis and interpretation → **decrease misdiagnoses**

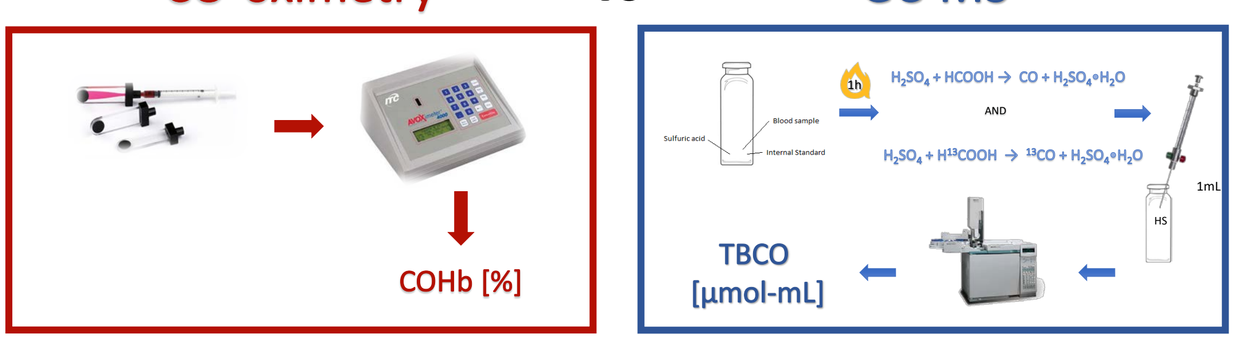
2 Materials and Methods

Development and optimization of novel analysis method for CO determination is based on measurement of **total CO in blood** (**TBCO**) as alternative biomarker to **COHb**.

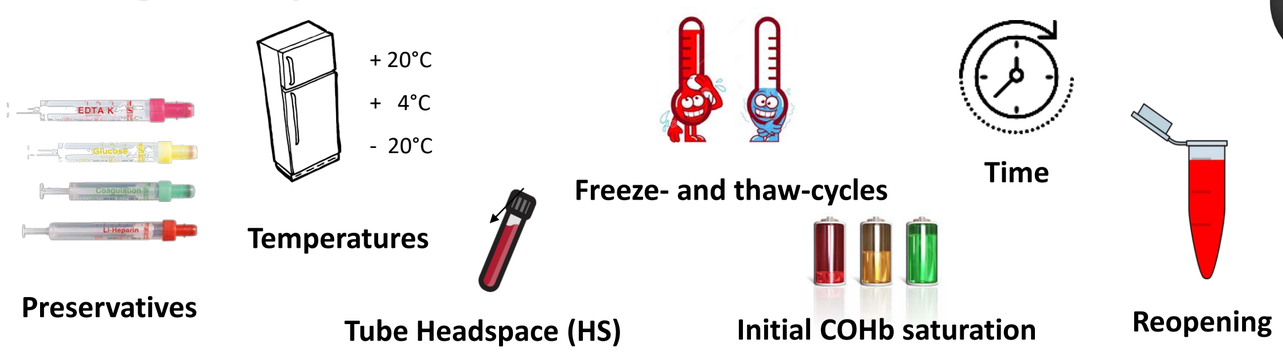
Hypothesis: measurement of COHb only measures **CO bound to Hb**, while TBCO measures also amount of **free CO** → potentially responsible for discrepancy in results and misdiagnoses!

Comparison of **standard** method (COHb by CO-oximetry) and **new** method (TBCO by GC-MS) through application on **real cases** (13 clinical and 3 postmortem).

CO-oximetry VS GC-MS



Investigation of potential sources of error:



3 Results

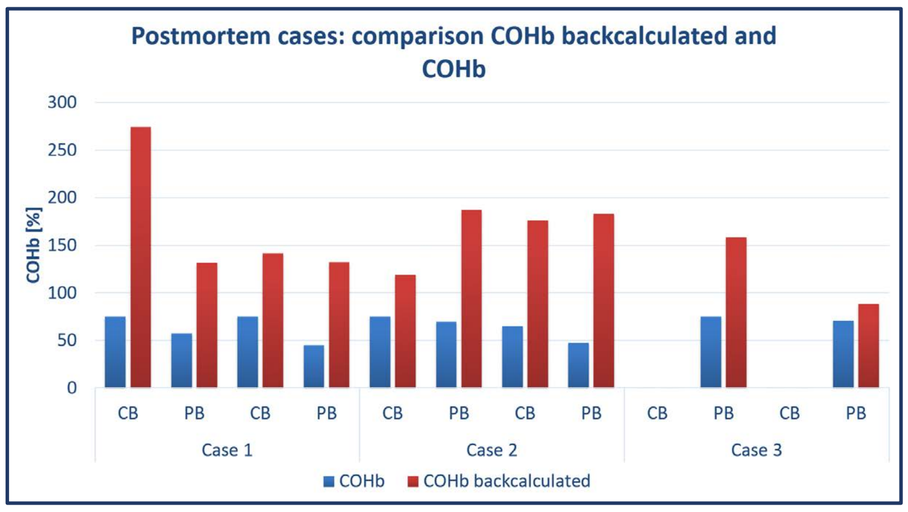


Figure 1: Results for COHb measurement by CO-oximetry (blue bars) and COHb backcalculated (red bars) by application of formula from the literature to the TBCO measurements by GC-MS on real postmortem cases; CB: cardiac blood; PB: peripheral blood.

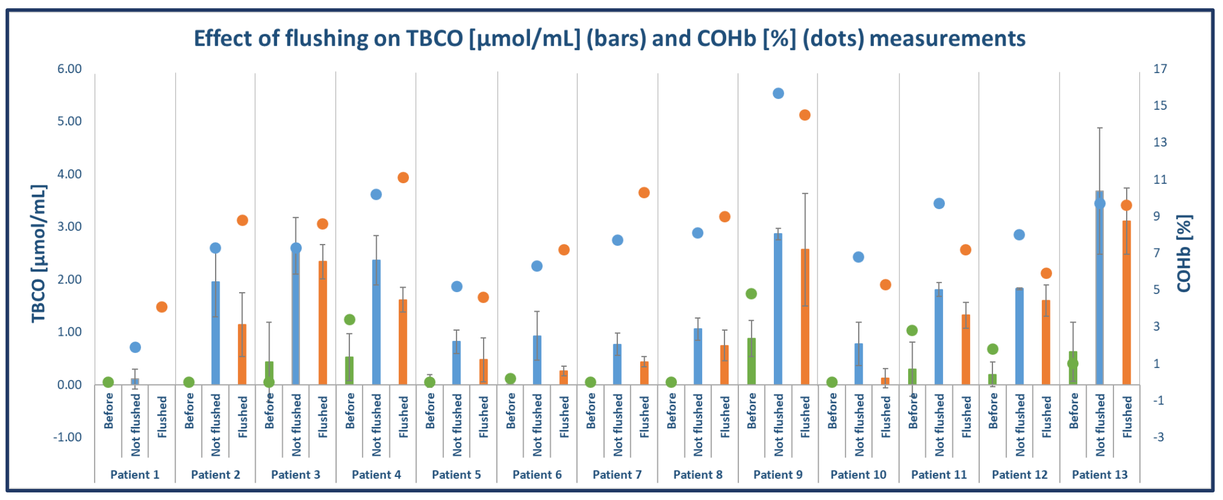


Figure 2: Results for TBCO (bars, left axis) measured by GC-MS and COHb% (dots, right axis) measured by CO-oximetry in 13 patients before (green) and after (blue) CO exposure and after a flushing step (orange).

- New method was successfully validated according to toxicological guidelines.
- Multiple Linear Regression (MLR) Analysis - Parameters that are **not statistically significant** (p-value > 0.05): temperatures -20°C and +4°C, tube HS >50%, days of analysis 0, 1, 2 and 4, reopening and freeze- and thaw-cycles. All others are statistically significant.

4 Discussion

All **TBCO** and **back calculated COHb** values are significantly above **COHb** measured by CO-oximetry (Figures 1 and 2). Not flushed samples have more TBCO than flushed samples (10% - 80%). This suggests that there is a significant quantity of CO not bound to Hb, but in blood in **free form**. This can help **explain** the **discrepancy** between measured COHb% and reported symptoms, leading to a **decrease** of **misdiagnoses**.

MLR analysis of the results obtained from the storage study shows significant influences of most parameters, thus appropriate sample preparation and storage is required to avoid errors. **No significant** amount of CO released into the **HS** or lost during **reopening** was detected (**p-Values > 0.05**).

5 Conclusions

- Using COHb as only biomarker may lead to **underestimation** of CO exposure. **Free CO** is of pathophysiological relevance and is found in **significant** quantities.
- **TBCO** as alternative biomarker of CO exposure requires optimization.
- **Best storage conditions**: EDTA tube, filled as much as possible, stored frozen up to 4-7 days.
- Assessment of impact of TBCO on **global CO burden** needs to be finalized.

Contact

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References

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"THE SILENT KILLER"

Sources of error in carbon monoxide exposure measurement: a systematic review

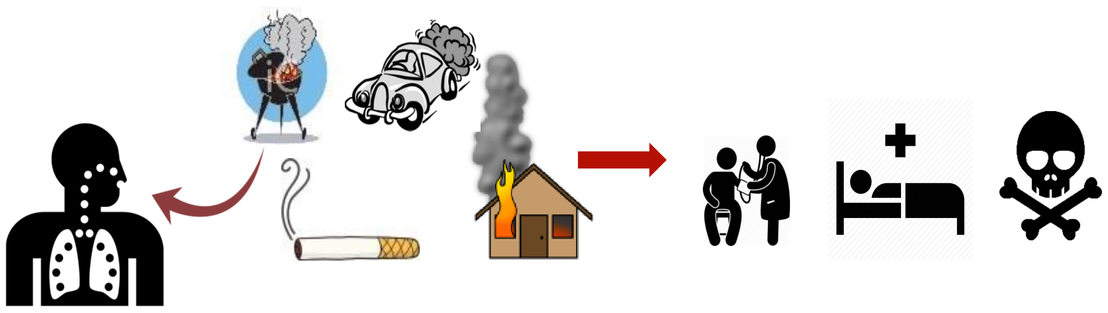
Oliverio S.[1,2], Leonardi G.[3,4], Varlet V.[2], Zeka A.[1]

[1]Department of Environmental Sciences, CHLS, Brunel University London, United Kingdom, [2]University Center of Legal Medicine Lausanne-Geneva, Lausanne, Switzerland, [3]Public Health England, Didcot, United Kingdom, [4]London School of Hygiene and Tropical Medicine, London, United Kingdom

CONCLUSION

Improved CO exposure assessment methods leading to reduced misclassification and more valid estimates of CO effects, even at low CO exposure levels, are obtained by consideration of the limitations of current CO exposure assessment methods and integration of expected sources of error (esp. biomarker validation) in the study design.

Introduction



Diagnosis and Analysis

- Low index of suspicion → **misclassification**
- Insufficient data on exposure assessment methods (EAM) for low-level indoor CO exposures → **misclassification**
- Surrogate exposure measure → **measurement error**

- Identify and evaluate available CO exposure assessment methods
- Determine **sources of error** in CO EAM → **decrease** exposure misclassification and measurement error

Methods

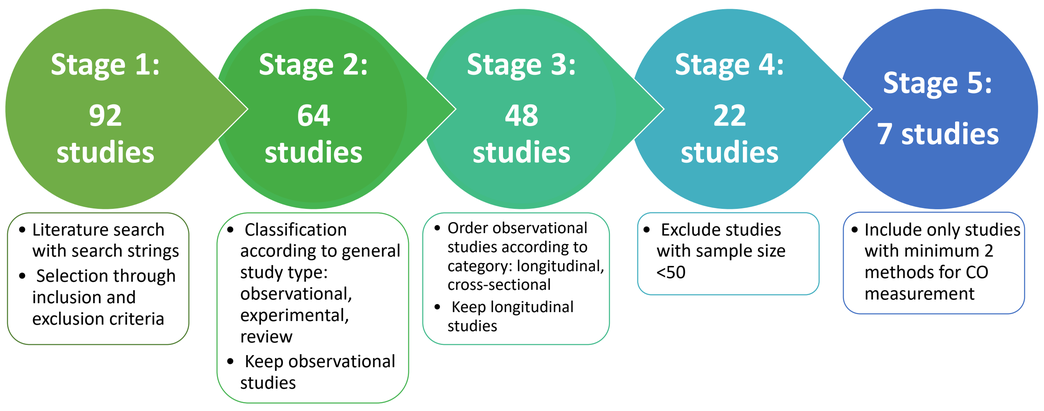


Figure 1: Overview of literature search and study selection process, step-by-step.

Results

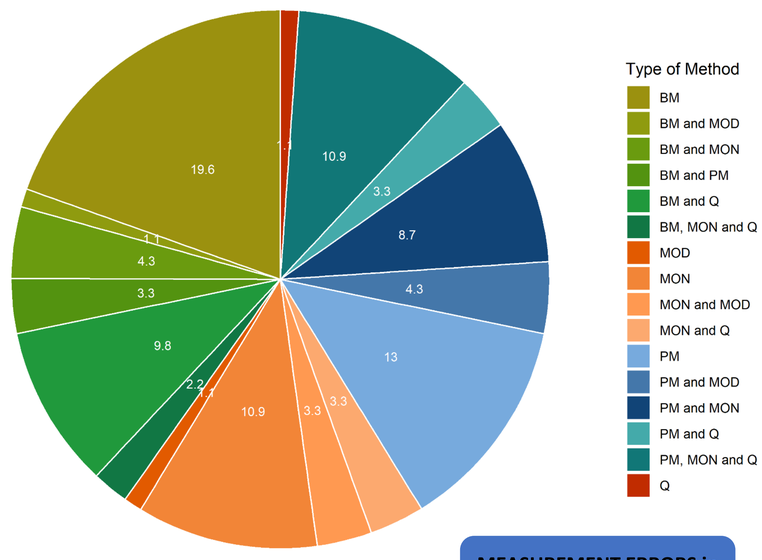


Figure 2: Pie-chart of frequencies for the different exposure assessment methods used in the selected CO exposure studies; BM: biological monitoring; PM: personal monitoring; MON: microenvironment monitoring; MOD: microenvironment modeling; Q: questionnaire

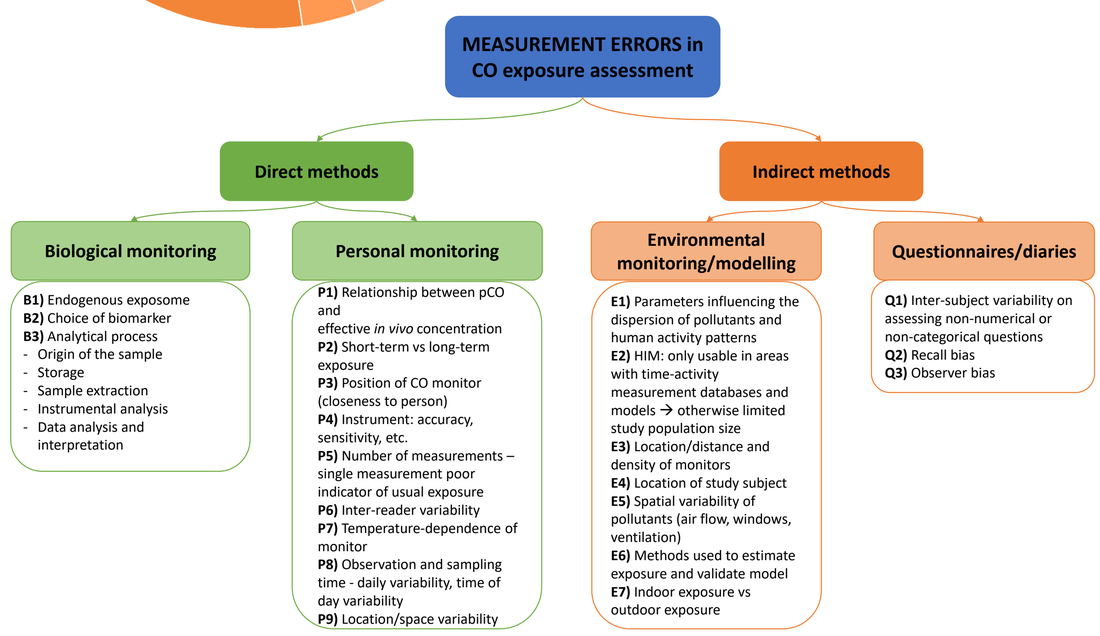


Figure 3: Overview of identified sources of measurement error in CO exposure assessment studies, classified according to the exposure assessment method.

Discussion

- Low # of indoor CO EA studies, with even lower # of studies using >1 measurement method and 0 performing a validation or reliability study
- Current EAM: prone to significant measurement error (both classical and Berkson-type) → due to inaccuracy of current biomarkers and CO measurement devices
- Not accounting for: intra- and inter-individual variability, errors from laboratory sampling, storage and analysis conditions → significant impact on low CO-levels
- Best results obtained from combination of more than one EAM

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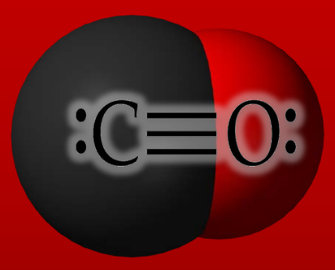
For more details

"THE SILENT KILLER"



Carbon Monoxide Poisoning: comparison of spectrophotometric and gas chromatographic methods for quantification under controlled storage conditions

Oliverio S.[1,2], Zeka A.[2], Leonardi G.[3], Varlet V.[4]

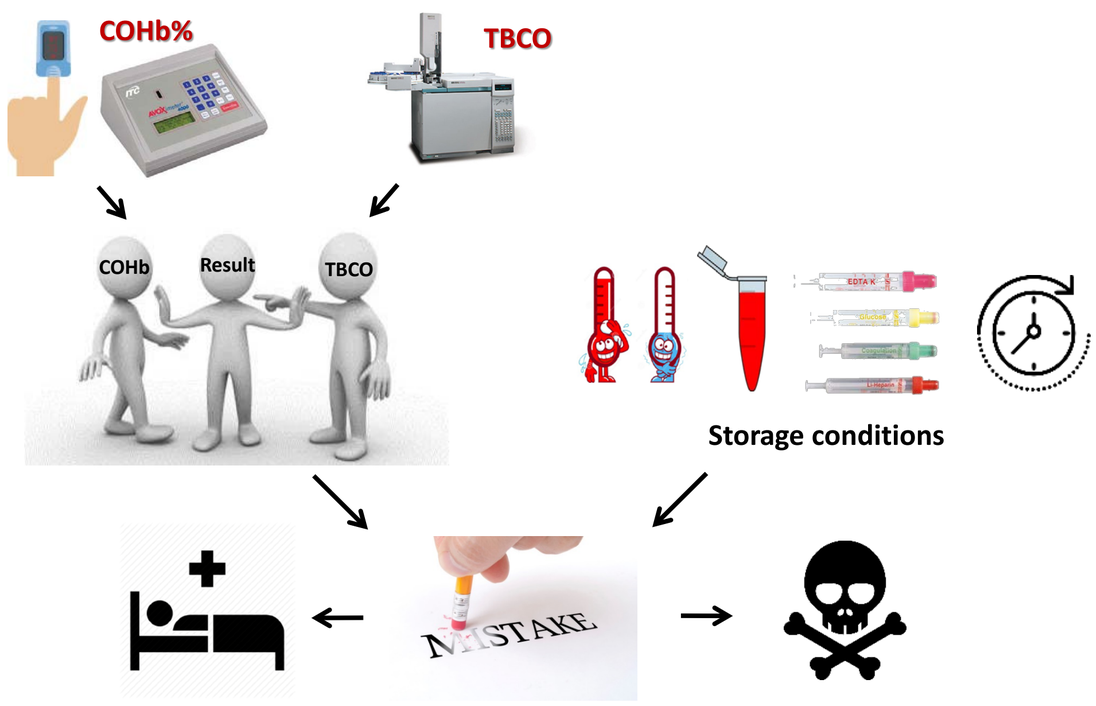
[1]Forensic Toxicology and Chemistry Unit, University Center of Legal Medicine Lausanne-Geneva, Switzerland, [2]Department of Environmental Sciences, College of Health and Life Sciences, Brunel University London, United Kingdom, [3]Public Health England, Didcot, United Kingdom, [4]Swiss Human Institute of Forensic Taphonomy, University Center of Legal Medicine Lausanne-Geneva, Switzerland

CONCLUSION

Total Blood CO (TBCO) as biomarker for CO poisonings provides more valid results and is more stable even under different storage conditions compared to carboxyhemoglobin (COHb). It can be used to predict COHb even when sample degradation hinders optical measurements.

A correction formula is provided to be used in laboratories or circumstances where optimal storage is not possible, for more accurate determinations.

1 Introduction



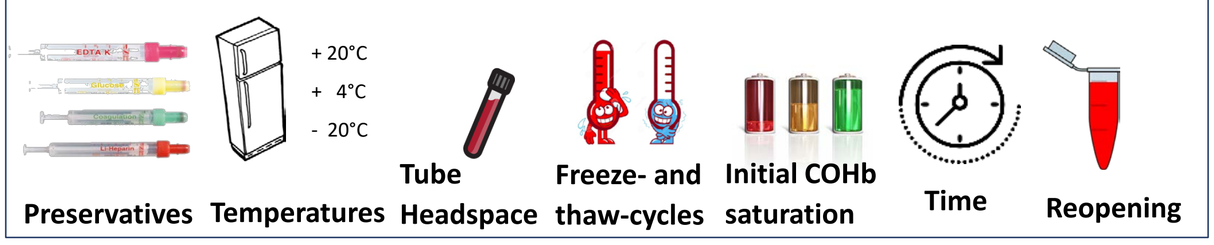
- Evaluate effects of **storage conditions** on COHb and TBCO
- Provide **correction model** for different storage conditions
- Generate model to **predict COHb** from TBCO and storage conditions

2 Methods

- ~2500 bovine blood samples are fortified with pure CO gas in different initial COHb% **saturation levels**: **[10/20%], [30/40%], [50/70%].**
- Monitoring is performed over a period of one month, with measurements on following days: 0, 1, 2, 4, 7, 14, 21, 28.

Measurements: COHb via **CO-oximetry**, **TBCO** via **GC-MS[1].**

Investigated parameters:



3 Results

SATURATION LEVEL	HIGH		MEDIUM		LOW	
Analyte / Parameter (Reference)	COHb	TBCO	COHb	TBCO	COHb	TBCO
Preservative (EDTA)						
NaF	-16.34	0.77	-13.92	1.44	-6.96	-0.01
LiH	2.47	1.33	-13.49	0.60	5.31	0.00
NaCit	-6.27	1.33	-17.37	0.81	3.01	0.01
Temperature (-20°C)						
+ 20°C	5.65	1.01	-0.88	0.79	0.07	0.00
+ 4°C	2.47	0.99	-0.05	0.93	0.73	0.00
Time (Day 0)						
Day x	-0.18	1.00	-0.08	1.00	-0.02	0.00
HS volume (<25%)						
25%-50%	-0.47	1.01	-0.74	1.05	0.04	0.00
>50%	-1.23	0.98	-1.24	0.95	-0.35	0.00

Table 1: Coefficient estimates (β) from Multiple Linear Regression with storage parameters preservative, temperature, time and HS volume for measurement of COHb and TBCO for high, medium and low saturation levels. In red the significant parameters.

Correction formula

$$c = c_M - x_t\beta_t - \beta_P - \beta_T - \beta_V$$

with c: corrected concentration of analyte of interest (here COHb in % or TBCO in µmol/mL), c_M: measured concentration of analyte of interest; c_{COHb}: concentration of COHb in [%], c_{TBCO}: concentration of TBCO in µmol/mL; x_t: number of days since sampling of specimen, β_t: coefficient estimate for time, β_P: coefficient estimate for selected preservative, β_T: coefficient estimate for selected storage temperature, β_V: coefficient estimate for selected HS volume.

Prediction formula

$$c_{COHb} = c_{TBCO} - x_t\beta_t - \beta_P - \beta_T - \beta_V$$

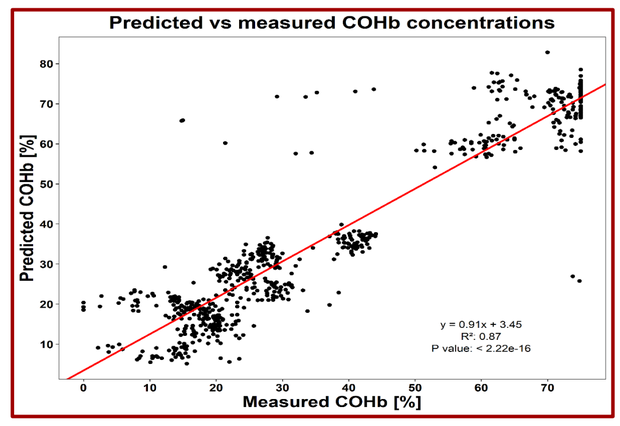


Figure 1: Correlation plot for measured vs predicted COHb concentrations in %.

4 Discussion

- No substantial loss of CO observed during storage due to reopening, freeze- and thaw-cycles; no significant amount of CO released in tube headspace.
- Storage conditions have different impact on COHb/TBCO behavior over time based on initial saturation level; all parameters are significant to some extent.
- COHb is more affected by storage conditions as opposed to TBCO.
- Correction formula can be used to obtain more valid results despite non-optimal storage conditions
- Prediction formula was successfully tested and, thus, can be applied to samples where COHb cannot be measured due to sample degradation during storage.

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[1]For more details