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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 made carboxyhemoglobin (COHb) the most appropriate biomarker for CO poisonings. COHb is measured using spectrophotometric (UV-spectrophotometry, CO-oximetry) or gas chromatographic (GC) methods hyphenated with flame ionization or mass spectrometry detectors. However, inconsistencies in many cases were reported between measured values and reported symptoms, raising doubts over the suitability of COHb as biomarker together with the accuracy and reliability of its measurement methods. Therefore, we aimed to review the accuracy of current methods 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, 46 articles out of 191 initial hits were carefully reviewed.

Results

While optical methods are highly affected by changes of blood quality due to degradation of the 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 insufficiently accurate for CO poisoning diagnosis.

Conclusions

An alternative biomarker is required, such as determining the total amount of carbon monoxide in blood. Even though further research is required, we recommend toxicologists to 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 in blood. Due to the high affinity of CO to hemoglobin (Hb), it has been assumed that the majority, if not all, CO binds with Hb when introduced into the blood circulation. This has resulted in carboxyhemoglobin (COHb) being considered the most appropriate clinical marker of exposure for CO poisoning [1]. However, COHb does not represent the only reservoir of CO in the human body because CO may be found dissolved in blood at free state 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]. CO dissolved in blood in free form is known to have a role in the pathophysiology of CO poisonings [3, 4], but might be more substantial than what studies revealed so far. This would result in under- or misestimation of the true CO level present in the analyzed blood sample, potentially elucidating some of the cases where inconsistencies between measured COHb level and reported symptoms were found. However, currently there is not much data on free CO available.

COHb in blood is measured directly or indirectly by using either optical methods (CO-oximetry, UV-spectrophotometry or pulse-oximetry) or gas chromatographic methods in combination with a variety of detectors (flame ionization detector, mass spectrometer). In clinical cases, the “gold standard” is 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 ultraviolet (UV)-spectrophotometry remains the most frequently used method in forensic cases, CO-oximetry and gas chromatographic 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 may potentially alter the obtained value, resulting in a deviation from the true value due to these factors. In medical practice and especially for toxicologists, it is crucial to correctly and accurately determine a biomarker, 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. Shortcomings in doing so can have severe clinical and legal consequences. Therefore, in this paper, we aim to review the accuracy of current methods to measure CO and to determine their potential sources of error and their effects in the interpretation process.

Methods

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 gave 191 hits. Systematic reviews, meta-analyses and general review articles, retrospective, prospective, observational and clinical cohort studies were excluded as well as case reports, limiting included articles to 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, since 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 errors

Measurement of CO in breath cannot account for the total CO concentration present in the blood at the time of exposure. It is a very susceptible method and affected by a variety of factors that can easily alter the result into under- or overestimating the true concentration (Table 1). A major aspect is the variation among the subjects' abilities of breath-holding. 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 interpersonal differences in pulmonary function, capillary diffusion surface and inspiration and expiration rates, as well as the inability to fully control whether a subject is properly holding the 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 elderly, children or subjects with respiratory diseases. Furthermore, since they were initially designed for smoking cessation programs, accuracy of CO monitors is better in lower CO concentrations and might therefore not be sufficiently accurate for acute intoxications [14]. Nevertheless, CO monitors have high usefulness on sites of mass casualties or for first responders. They are portable and can give an indication of the gravity of the case, which can allow the appropriate treatment of the patient as well as proper precautions to be taken by first responders.

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 absorbance of light when the compound is exposed to light of different wavelengths. In the past, single-beam UV-spectrophotometry or double wavelength spectrophotometry was first developed due to the spectral absorbance of the Hb structures and due to the distinct spectral differences between oxyhemoglobin (O_2Hb) and COHb [15–17]. A similar method involves the measurement in the visible spectra of the differences in absorbance 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], since 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 the multiple wavelengths 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, due to 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) 27368:2008 'Analysis of blood for asphyxiant toxicants – carbon monoxide and hydrogen cyanide' standards [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 in the light of poor choice of sample handling techniques and storage conditions (e.g., temperature, preservative, etc.) as well as biochemical alterations that occur overtime [26]. Some of the most important potential errors for COHb determinations include:

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 volume of headspace (HS) 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 there is 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: thermo-coagulation, putrefaction and PM CO production are all known sources of error, but they cannot be quantified due to their biological 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 altering factors are applicable not only to optical measurements of COHb, but also to gas chromatographic 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.

Storage 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 decreased COHb levels that were related to the ratio of 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) as well as initial COHb% saturation levels (the higher the COHb levels, the greater the loss). The hypothesis of the formation of an equilibrium between CO in blood and air above the blood sample in the tube was proposed to explain the influence of the HS in the sampling tube [28]. Storage at room or hot temperatures of blood 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, even though it was tested on 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 right after sampling over a period of two years [39]. Analysis of the samples on only two significantly distant time points might fail to notice changes in short-term storage due to preservative use, which is more relevant than long-term storage, since samples are in the majority of cases 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 picked up by this less sensitive measurement methods. However, Vreman et al. [35] were able to find that using EDTA as preservative led to falsely increased COHb values when measured by CO-oximetry. Nevertheless, stronger significance of these findings would have been achieved with confirmation by another measurement method, such as gas chromatography (GC).

Furthermore, these conditions may not only influence 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, a degradation of the sample occurs, which was confirmed to trouble the 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, thermo-coagulation 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 troubling the measurement performed with optical techniques. Another recurring and significant phenomenon to be considered 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 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 erythrocytes catabolism, a phenomenon that occurs also 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 the cause of death determined with an autopsy, 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 requires 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 a 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 of blood sampling, thus allowing the monitoring of COHb levels in real time and simultaneously to 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 factors of higher importance from a medical perspective, such as accuracy, precision and reliability. Being able 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 an elevated false positive and negative rate, as opposed to 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 seem to affect the accuracy of pulse CO-oximeters [42]. Feiner et al. [40] reported low signal quality or no report of CO saturation levels when the oxygen saturation decreased below 85%, which is indicative of hypoxia. Considering that hypoxia is one of the main effects of a CO poisoning, it is a severe disadvantage not to be able to measure COHb accurately in hypoxic states. However, a more recent study by Kulcke et al. [43] found good accuracy levels in measuring COHb during hypoxemia, even though a slightly higher underestimation of COHb levels is reported for COHb concentrations above 10%. This confirms that pulse CO-oximeters can be useful for monitoring exposures to low CO levels, but accuracy and precision are not guaranteed for more severe poisonings as well as for smokers, who generally have baseline COHb levels that can range from 3-8% in normal smokers but can easily reach 10-15% in heavy smokers [1, 2].

In comparison to postmortem CO-oximetry, antemortem COHb measurement by pulse CO-oximetry is not affected by storage or sampling parameters, which reduces the 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, even though there is scarce information regarding device

maintenance. Similarly 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 lead to misdiagnosis. Another relevant point from a judicial 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 gas chromatographic 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 such as lactic acid [48], citric acid [48, 49] or phosphoric acid [49] have also been tested.

In the studies performed in earlier years (70s, 80s and 90s), potassium ferricyanide was introduced for the release of CO and became very popular due to the availability, since it was already used in spectrophotometric methods as hemolytic agent. It was also found to be efficient in liberating the CO and its extent of reaction was not influenced by the presence of O_2 or O_2Hb at 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, as compared to other acids of same efficiency, it is more readily available, cheaper and allows the simultaneous liberation of CO and production of ^{13}CO 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 above mentioned detectors.

For the GC separation, a capillary column with a 5Å 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 were used previously, but have been substituted by the capillary columns due to their significantly reduced size.

To enhance sensitivity and accuracy and increase the range of analysis, GC methods were studied with various types of detection, such as thermal conductivity detection (TCD), flame ionization detection (FID), mass spectrometry (MS) and reduction gas analyzer (RGA) [55-66]. The most commonly used and investigated detector was FID, firstly reported in relation to CO determination in 1968 [51]. After GC separation, the CO is 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 of 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 was diluted to obtain the desired CO concentrations, or with fortification of blood with CO to reach different COHb% saturation levels. Additionally, excess CO was removed through the performing of a “flushing” step, in which the calibrators were 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.

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

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, which avoided time delays and possible losses of CO and allowed for direct correlation of the results to the other measurements. However, the details about the procedure to obtain 100% CO saturated blood used for calibration were not 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 the Hüfner factor of 1.51, which differed 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: mass spectrometry, GC-MS and HS-GC-MS

Analytical techniques

MS is the method of choice to detect CO because the identification is not only based on the retention time, but also the mass spectrum. Middleberg et al. [31] developed a method, which combined GC–MS with flame atom absorption spectroscopy (FAAS). Hereby 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 is used to calculate a more precise total amount of available Hb. By using this assay, it was assumed that all the iron present in blood was part of the heme protein and was capable of binding CO, even though it needs to be taken into account that this is not completely true and depends on the state of the organs, tissues and possible present diseases. Therefore, the obtained values might not accurately reflect the real CO levels.

Sources of error

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

Hao et al. [37] published an approach built on a HS-GC–MS method for analysis of CO in putrefied PM blood. Hereby, 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 of Hb levels to affect the results, direct blood saturation was performed. It was stated that 30 min of pure CO exposure of the blood was necessary to fully

saturate blood, even though the procedure applied to assess complete saturation, putrefied blood state and PMI were not described [37]. Furthermore, according to their results of 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 the COHb% levels. However, this appears in contradiction with the majority of previously published studies, even though they were obtained with the use of other approaches, such as optical methods and other GC-detectors.

Varlet et al. [52] were able to develop and validate a new method, which used isotopically labelled formic acid ($H^{13}COOH$) to produce ^{13}CO as internal standard for a HS-GC–MS method. This is very advantageous, since 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 through the use of formulae previously published by other authors [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 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 CO-oximeter. They were able to obtain cut-off values for different categories of back-calculated COHb% levels as compared to the ones directly measured by the CO-oximeter. However, even if this approach seems to show reliability for both clinical and forensic cases, only a limited number was tested. Oliverio and Varlet [4, 70] further developed this approach by validating both clinical and PM settings for the measurements of total amount of CO in blood (TBCO) by GC–MS with use of airtight gas syringe for samplings, which minimized any potential loss that could occur with a normal syringe or HS sampler. Application to PM samples showed relevant differences between the contents of CO and COHb when applying formulae in the literature for back-calculation. Significant differences were also observed between flushed and not 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 done as an alternative to COHb and the currently 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 for CO determination is the interpretation of the results. There is not a consensual agreement on the cutoff values for the different levels of exposure and severity of poisonings. 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 to be abnormal [11, 71–73]. Values of 30-35% COHb are the upper extreme values reportedly found in clinical poisoning cases. Above this limit, irreversible damages to the organs are expected, thus resulting in a cascade of events that eventually leads to death.

However, these values are interpreted differently according to the cases. Various parameters can affect the perimortem COHb% levels and in the agonal period before death, which include the presence of oxidative smokes or other gases that can interfere and/or compete with the absorption mechanism of CO such as nitrogen dioxide (NO_2) (increased MetHb), or the formation of other toxic gases like 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 needs to be analyzed and interpreted individually, based on all the relevant information available. For example, a COHb level of 25% in a PM cases may be considered as 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 as a poisoning case, but in heavy smokers, levels up to 18% have been found [72] in individuals that did not show any symptom of CO poisoning. Overall, there seem to be some significant discrepancies between COHb values and reported symptoms, which make the correct diagnosis of CO poisonings in clinical cases and the determination of the cause of death in forensic cases challenging.

A possible explanation for these phenomena is that basing the diagnosis of a CO poisoning only on COHb% levels might actually underestimate the real CO burden. There might 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 a higher total CO content than the one determined by CO-oximetry. The conventional assumption that the part of CO bound to Hb causes the most significant adverse health effects was repeatedly debated [3, 4, 75–78]. Free CO in blood could constitute a toxic reservoir of CO for the organism and additionally fuel the major implications on 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 CO dissolved and dissociated probably is also subject to interpersonal variability, which includes all factors such as e.g., metabolic rate and age [11] and needs to be taken into account when interpreting the results obtained by CO-oximetry.

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

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

The development of an alternative biomarker specific to CO should be derived from the investigation of a novel measurement approach that does not only focus on the CO bound to Hb, but also takes into consideration the role and toxicity of CO at cellular level, by measuring the total amount of CO present in the analyzed sample, such as TBCO. Mainly due to the dependency of spectrophotometric methods from the good quality of the sample,

which especially in forensic cases is not always available, it seems that GC methods are currently the most suitable techniques to be further explored. As detector, the mass spectrometer is the most versatile, accurate, user-friendly and nowadays routinely present in the majority of laboratories. Being able to determining the true CO exposure and correlating this to the symptoms reported by patients would allow a more conclusive and comprehensive CO poisoning determination, diminishing the number of misdiagnosed cases and falsely determined causes of death.

Conclusions

Even though COHb is routinely measured by spectrophotometric methods, several issues concerning sample stability and the dependency of optical methods from the sample quality have led to the search for an alternative way for measuring CO, such as GC. In addition, there is evidence showing a significant amount of CO present in blood 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 focusing only on COHb; hence the back-calculation of COHb from CO leads to misestimations. Therefore, an alternative approach to quantify the total amount of CO in blood directly instead of using CO in breath or COHb in blood should be used for CO poisoning determinations, such as the proposed TBCO measurement by GC–MS. Even though blood CO concentration cut-offs and their correlation with symptomatology are not yet available and GC–MS is more time-consuming, we recommend toxicologists especially for doubtful or very challenging cases to use GC–MS methods to verify the results obtained by CO-oximetry or spectrophotometry. 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 toxicologists to document and indicate information about sampling time, analysis time and storage conditions because they 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.

Ethical approval

No ethical approval was required for the preparation of this review article.

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Table 1 Overview of analytical methods used for carboxyhaemoglobin/carbon monoxide analysis, their main properties and limitations and reference examples

Specimen/method	Technique	Main characteristics	Pitfalls	References
Breath	**Electrochemical sensor**	• Easy to use • Non-invasive • 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 exposures • 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. 1957 [6] Jarvis et al. 1980 [7] Jarvis et al. 1986 [8] Vreman et al. 1994 [14] Middleton et al. 2000 [9] Macintyre et al. 2005 [10] Penney 2007 [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 • Focus only on COHb • Time consuming sample preparation (COHb reduction) • Often observed inconsistency between measured levels and reported symptoms	Ramieri et al. 1974 [16] Winek et al. 1981 [17] Fukui et al. 1984 [19] Vreman et al. 1984 [46] Lewis et al. 2004 [55] Luchini et al. 2009 [56] Olson et al. 2010 [22] Varlet et al. 2012 [52] Hao et al. 2013 [37] Varlet et al. 2013 [36]
	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 exposures • Invasive • Only CO bound to Hb taken into account • Often observed inconsistency between measured levels and reported symptoms • Susceptible to alterations due to sample quality	Dubowski and Lu 1973 [57] Costantino etal. 1986 [58] Mahoney et al.1993 [24] Oritani et al. 1996 [65] Levine et al. 1997 [59] Bailey et al. 1997 [21] Widdop 2002 [23]

			• Difficult interpretation for PM samples with long/unknown PMI and/or storage conditions	Lee et al. 2002 [60] Lee et al. 2003 [61] Brehmer and Iten 2003 [62] Boumba and Vougiouklakis 2005 [18] Penney 2008 [11] Piatkowski et al. 2009 [5] Olson et al. 2010 [22] Fujihara et al. 2013 [20]
Attachment to the finger	**Pulse CO-oximetry**	• Measurement of COHb% saturation in circulation • Continuous measurement • Non-invasive • 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. 2009 [5] Zaouter and Zavorsky 2012 [39] Feiner et al. 2013 [40] Weaver et al. 2013 [41] Wilcox and Richards 2013 [42] Kulcke et al. 2016 [43]
Blood, tissue	**GC–RGA**	• Measurement of CO in tissues • No dependency on blood quality • Automation possible	• Use of highly toxic mercury vapors • Time consuming sample preparation • Invasive	Coburn et al. 1964 [44] Vreman et al. 1984 [46] Mahoney et al. 1993 [24] Marks et al. 2002 [63] Vreman et al. 2006 [30]
Blood	**GC–TCD**	• Accuracy for low COHb% • Precise • Specificity • Measurement of CO released into HS of tube possible • No dependency on blood quality • Automation possible	• Time consuming sample preparation • High cost • Invasive	Ayres 1966 [40] Dubowski and Lu 1973 [57] Fukui et al. 1984 [19] Van Dam and Daenens 1994 [66] Oritani et al. 1996 [57] Lewis et al. 2002 [55] Brehmer and Iten 2003 [64]
Blood, tissue	**GC–FID**	• Rapid • Best sensitivity for CO • Specificity • Lowest LOD and LOQ	• Instrument specific for CO due to necessity of methanizer • Not applicable to analysis of other substances • Time consuming sample preparation	Collison et al. 1968 [51] Rodkey and Collison 1970 [48] Guillot et al. 1981 [58]

		• 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 dependency on blood quality	• Invasive • Backcalculations of COHb from measured CO • Flushing of calibrators → removal of dissolved CO	Vreman et al. 1984 [46] Costantino et al. 1986 [60] Cardeal et al. 1993 [49] Levine et al. 1997 [61] Penney 2000 [69] Sundin and Larsson 2002 [53] Czogala and Goniewicz 2005 [67] Boumba and Vougiouklakis 2005 [18] Vreman et al. 2006 [30] Walch et al. 2010 [47]
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 dependency on blood quality • Use of isotopically labelled formic acid for calibration and internal standard • Measurement of total amount of CO	• Back calculations 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. 1993 [31] Oritani et al. 2000 [50] Marks et al. 2002 [63] Varlet et al. 2012 [52] Hao et al. 2013 [37] Varlet et al. 2013 [36] Oliverio and Varlet 2018 [4] Oliverio and Varlet 2019 [70]

CO carbon monoxide, *COHb* carboxyhemoglobin, *PM* post-mortem, *PMI* post-mortem interval, *GC–RGA* gas chromatography–reduction gas analyser, *GC-TCD* gas chromatography–thermal conductivity detector, *GC–FID* gas chromatography–flame ionization detection, *GC–MS* gas chromatography–mass spectrometry

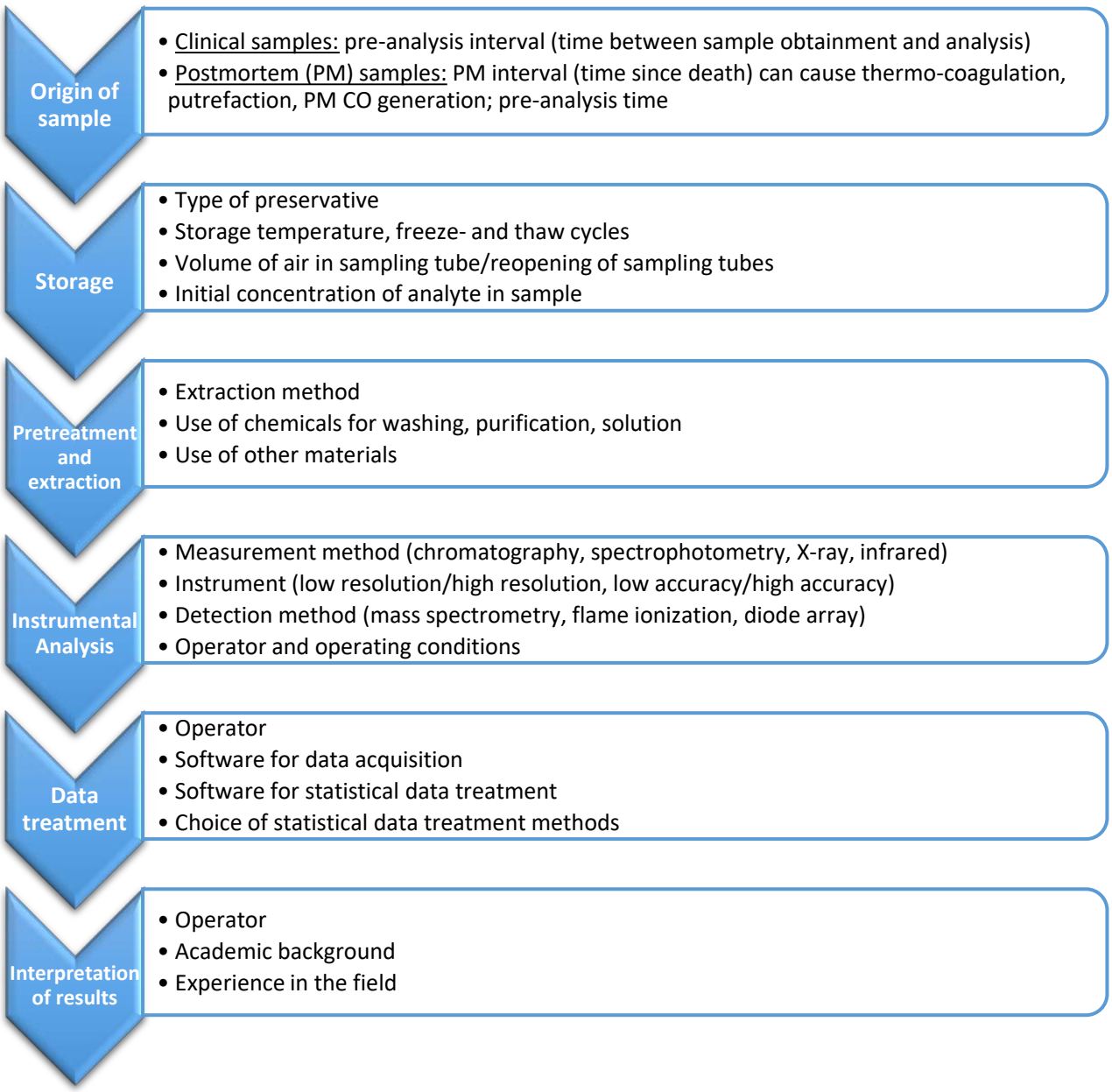

Fig. 1 General steps for a quantitative laboratory analysis and their respective potential sources of error for carbon monoxide (CO) determinations