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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 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 heasdoace (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.

Keywords: carbon monoxide; carboxyhaemoglobin; analytical measurement; airtight gas syringe; GC-MS

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 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 to 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 5Å 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: 2.6mL K3E, 3mL 9NC, 2.7mL FE, 2.6mL KH, were obtained from Sarstedt (Nürnbrecht, Germany). Precision sampling gas syringes equipped with a press button valve with capacity of 500µL for dilutions and 2mL for injections were from VICI (Baton Rouge, LA, USA). Aluminium caps from Milian (Vernier, Switzerland). All headspace extractions were carried out in 20mL 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 x 0.32 mm x 30µm) obtained from Restek (Bellefonte, USA) was used. Following temperature programme was used: 50°C, held for 4 minutes; 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 minutes was used.

For detection, Agilent 5973 mass spectrometer (Palo Alto, USA) was used, operating in electron ionization (EI) mode at 70eV. 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 minutes 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 0-208 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

100µL of blood was introduced in a 20mL HS-vial, followed by aliquots of the various formic acid solutions for the calibrators and 10µL internal standard solution. Subsequently, an aluminium cap of 11mm (i.d.) was first filled with 100µL 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 minutes.

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 50-100µL 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

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

2.5.3 CO in blood

1mL HS was sampled from the 20mL 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 $TI = X \pm k \times \sqrt{(S^2_r + S^2_R)}$, 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, $k = t\square \times \sqrt{(1 + [1 / (I \times J \times B^2)])}$, 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 $R = S^2_r / S^2_R$. $t\square$ is Student's coefficient with degrees of freedom v defined as $v = (R + 1)^2 / \{[(R + 1 / J)^2 / (I – 1)] + [(1 - 1/J) / (I \times J)]\}$.

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 autopsy were all with a postmortem-interval (PMI) of less than 40 hours, 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 to 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 Figure **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).

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 Figure **B.2**, expresses the method's ability of providing analytical results by using both systematic and random errors, with a risk set at $\alpha = 5\%$ 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 (0.86 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 **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 (Figure **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 142.5nmol/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 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, a 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, with the need for use also 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.

Figure **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 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. There are several reasons that can be exploited as to be accountable for the unexpected high backcalculated COHb results with the different approaches:

(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 a reasonable doubt in whether there is more CO present in blood than just COHb and that the CO dissolved plays a more important role than expected. This can ultimately result in a highly relevant role from a physiological perspective, with pathophysiological effects caused by the amount of CO dissolved in blood, which have not yet been investigated, but might be relevant for both clinical and forensic cases. This can possibly also explain the disagreement

between the symptoms reported and COHb% levels measured in individuals, with the need for a newfound correlation between total CO in blood and reported symptoms, an area that requires further research.

The hypothesis of the presence of CO dissolved in postmortem blood is also valid. If CO production from decomposition processes (in body or tube) were already discussed above, CO might also originate from dissociations from Hb, Mb or cytochromes because their bindings are reversible. 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 [31]. 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 values obtained for the measurements with different preservatives do not show significant differences, thus suggesting that the preservative is not a relevant factor of influence on the CO measurement. However, 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, 31-33]. 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.

Finally, the hypothesis of CO dissolved in blood is strengthened by the fact that some of the COHb values from unflushed CO-fortified samples (COHb between 60% and 80%, measured by CO-oximetry) matched with the backcalculated COHb obtained from the total CO content measured by GC-MS. Therefore, the flushing of samples can be interpreted as being useful to obtain exclusively the amount of COHb present in the sample at time of analysis, even though it is not in conformity with the pathophysiology of CO. In fact, this interpretation gives only a partial view of the CO intoxication, since it does not take into account the CO dissolved. If the use of COHb% after flushing can be sufficient in a forensic domain (COHb >50% is defined as significant to be diagnosed as a cause of death), it appears to be incomplete for the interpretation and diagnosis of the true role played by CO if COHb >50% is measured, which is clearly not sufficient in clinical cases because the CO dissolved that is not measured by CO-oximetry (or optical methods in general) might be crucial in explaining post-intoxication pathologies.

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 10-200 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, concluding that the use of COHb as a biomarker might not be the appropriate choice for CO exposures, underestimating the true role played by CO in such an intoxication.

Additionally, this is of high importance due to numerous cases in which the reported COHb% levels did not correlate to the symptoms shown by intoxicated patients.

The hereby presented method allows a more appropriate approach, which can be of high significance from a pathophysiological point of view, 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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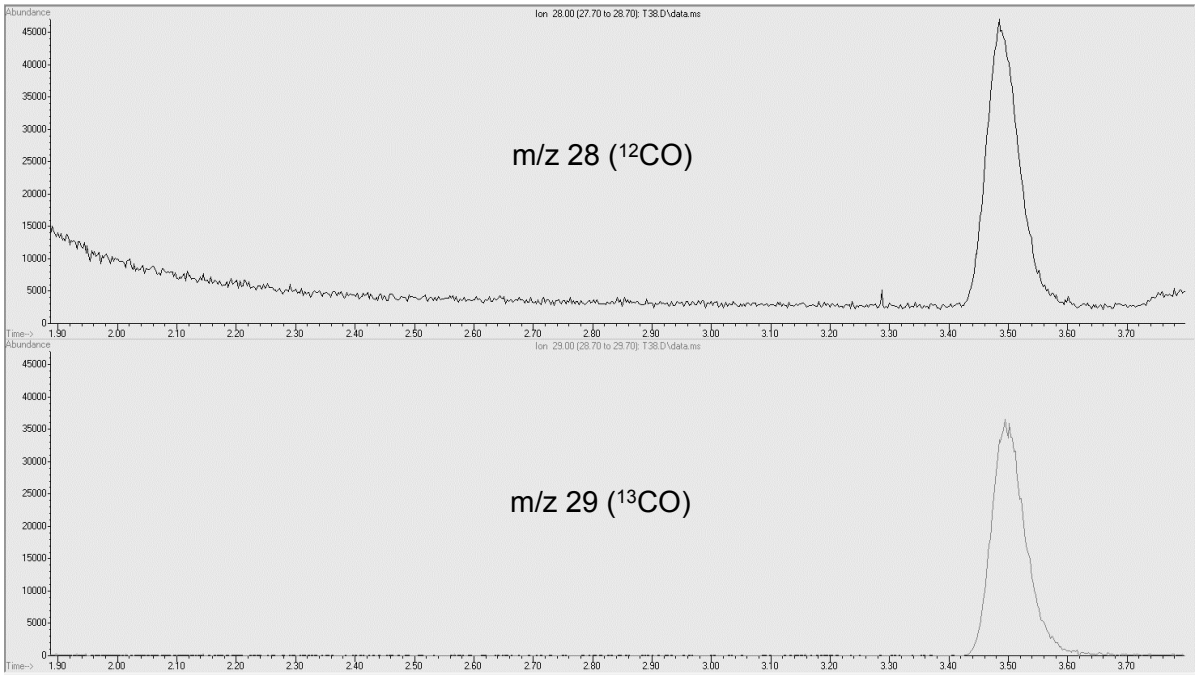

Figure B.1: Extracted ion chromatograms for m/z ratios 28 (upper) and 29 (lower), corresponding to CO and ^{13}CO respectively, for selectivity tests.

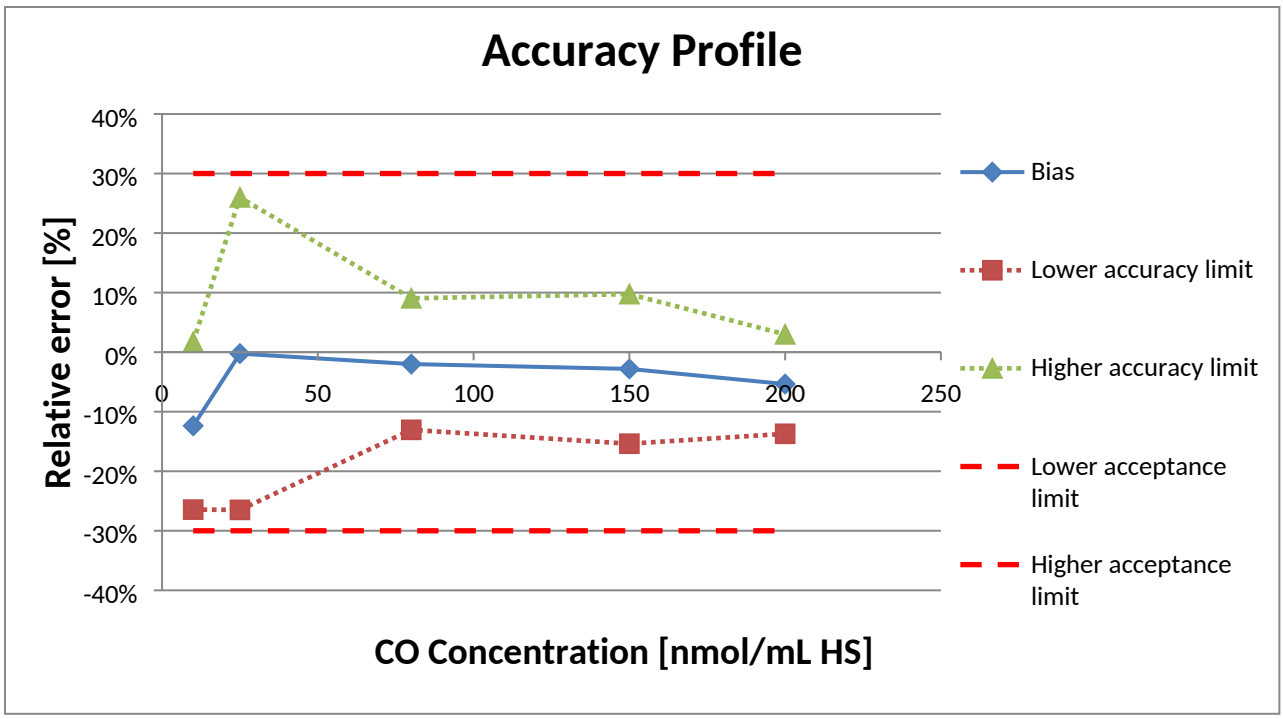

Figure B.2: Accuracy profile for CO determination using a simple linear regression model within the range of 10-200 nmol/mL HS (2-40 µmol/mL blood). The continuous line represents the trueness (bias), the dashed lines represent the acceptance limits set at ± 30% and the dotted lines are the relative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

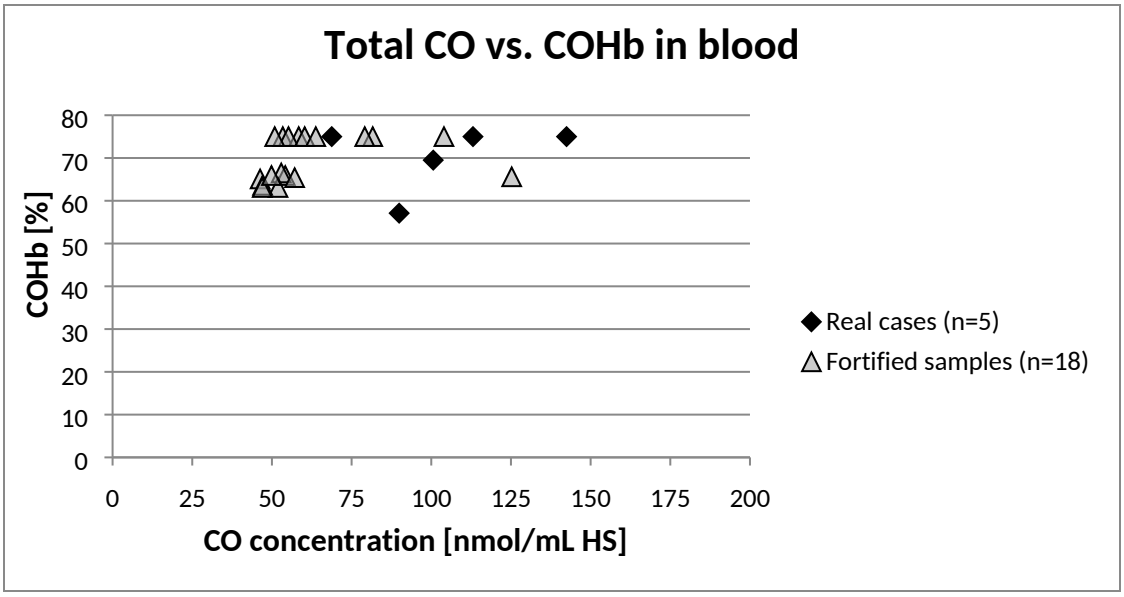

Figure B.3: Correlation plot between the total CO concentration in nmol/mL HS (measured by AGS-GC-MS) vs. the COHb saturation in % (measured through CO-oximetry) measured in blood for two groups of samples: real case samples (n=5) and samples fortified with CO stored in blood (n=18).

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

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

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 CB	EDTA	>75	>75	142.45 (28.49)	80.00 (16.00)
	NaF		>75		78.04 (15.61)
	LiH		>75		93.15 (18.63)
	Cit		>75		77.73 (15.55)
1 PB	EDTA	57,1	49.7	89.92 (17.98)	68.80 (13.76)
	NaF		44.9		87.33 (17.47)
	LiH		61.8		62.38 (12.48)
	Cit		55.3		47.57 (9.51)
2 CB	EDTA	>75	70.7	68.77 (13.75)	95.55 (19.11)
	NaF		64.8		108.10 (21.62)
	LiH		70.5		79.75 (15.95)
	Cit		71		57.10 (11.42)
2 PB	EDTA	69.5	65.1	100.62 (20.12)	119.50 (23.90)
	NaF		47.5		136.21 (27.24)
	LiH		64.7		81.94 (16.39)
	Cit		64.8		52.15 (10.43)
3* CB	NaF	NA	NA	50.48 (10.10)	47.60 (9.52)
3* PB	NaF	>75	70.8	113.09 (22.62)	64.15 (12.83)

*for Sample 3, second analyses were carried out after 52 days;
Samples: cardiac blood (CB), peripheral blood (PB); preservatives: ethylenediaminetetraacetic acid (EDTA), sodium fluoride (NaF), lithium heparin (LiH), trisodium citrate (Cit)