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Accuracy profile validation of a new method for carbon monoxide measurement in the human blood using headspace-gas chromatography–mass spectrometry (HS-GC–MS)

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ABSTRACT

The aim of our study was to provide an innovative headspace-gas chromatography–mass spectrometry (HS-GC-MS) method applicable for the routine determination of blood CO concentration in forensic toxicology laboratories. The main drawback of the GC/MS methods discussed in literature for CO measurement is the absence of a specific CO internal standard necessary for performing quantification. Even if stable isotope of CO is commercially available in the gaseous state, it is essential to develop a safer method to limit the manipulation of gaseous CO and to precisely control the injected amount of CO for spiking and calibration. To avoid the manipulation of a stable isotope-labeled gas, we have chosen to generate in a vial in situ, an internal labeled standard gas (^{13}CO) formed by the reaction of labeled formic acid formic acid ($H^{13}COOH$) with sulfuric acid. As sulfuric acid can also be employed to liberate the CO reagent from whole blood, the procedure allows for the liberation of CO simultaneously with the generation of ^{13}CO. This method allows for precise measurement of blood CO concentrations from a small amount of blood (10 μL). Finally, this method was applied to measure the CO concentration of intoxicated human blood samples from autopsies.

1. Introduction

Carbon monoxide (CO) is one of the most toxic gases, responsible for a large number of deaths in the cases of smoke and fire exposure. CO combines reversibly with hemoglobin to form carboxyhemoglobin (HbCO), which decreases the oxygen-carrying capacity of blood. Consequently, the determination of CO concentration and the degree of HbCO saturation in blood are frequently required in toxicology laboratories. In the past, numerous methods were developed for this purpose; for example, the simple comparative color test, Hartridge reversion spectroscope, and microdiffusion, as well as volumetric and colorimetric methods [1]. Among the current methods for HbCO estimation in blood, spectrophotometry (including automated systems called CO-oximeters) and gas chromatography (GC) constitute the methods of choice. CO-oximeters are convenient for clinical diagnosis of acute and chronic exposure, as well as for fresh cadaver blood samples. Moreover, CO-oximeters are applicable to levels of HbCO of >5%. However, a loss of accuracy is observed when this method is applied to putrefied postmortem samples. Indeed, many postmortem interferences, such as thermo-coagulation, putrefaction or contamination, can disturb the spectrophotometric HbCO measurements due to errors associated with the turbidity of blood samples containing lipids or microcoagulates [2]. In this case, GC methods appear to be the best tool for forensic investigations, even for low levels of CO. GC techniques are also useful because they are provide a direct measurement of CO concentration. Nevertheless, even if GC methods are very precise, those developed before 2000 are complex and time consuming, and thus cannot be applied in cases of emergency clinical work. Thus, the CO-oximeter method is typically chosen for clinical purposes, and GC for forensic purposes.

With the GC methods, a preparation of the blood sample is required. First, precautions must be taken for sampling and conditioning. If possible, blood should be collected in tubes with an anticoagulant such as heparin [3–5]. Several studies have been conducted on the effect of collection tubes and storage conditions [6,7] and one of them focused on the fact that EDTA Vacutainer tubes must be avoided because studies have shown an increase of HbCO content when blood is stored in these tubes [8]. The problem seems to derive from the Vacutainers, and not EDTA, which would allow a better anti-coagulation of stored blood at 4 °C [9].

Numerous methods have been described to study the effect of the added reagent to whole blood on the liberation of CO. Potassium ferricyanide was the most commonly used substance as the liberating agent [4,5,10–15] and resulted in better CO release than potassium ferrocyanide, especially when mixing was applied

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[16,17]. Acids, such as sulfuric acid [9,13,18–21], lactic acid [13], citric acid [13,16], and phosphoric acid [16] have also been studied, and have provided good results. Mixtures of potassium ferricyanide and acids such as lactic acid [22,23] have also been developed to improve the liberation of CO. Different buffers were also used with potassium ferricyanide to investigate the pH effect on the CO release, although acidic conditions were more frequently employed [13,24]. Heating is often performed to enhance the liberating action of the reagent. However, a too hightamperature applied during more than one hour can lead to the spontaneous generation of CO from blood and an artificial increase of the HbCO content, leading to an improper determination of CO poisoning [12].

Once in the gaseous state, CO must be transferred into the GC injector. Earlier works described complex glass systems that have been improved upon over the years [8,23,25–28]. Today, a headspace extraction system coupled with GC offers improved safety and an ease of use [2]. To facilitate and guarantee complete CO liberation from HbCO, a hemolysis agent is added to ensure complete lysis of the red cell membrane [29]. Among the various hemolytic agents, distilled water [14], but especially detergents such as Triton X-100 [30], Sterox [26] and saponin [12,25] are often used. However, saponin is preferred because detergents can generate volatile compounds that interfere with the analysis [8]. For methods involving important sample amounts (>mL), an antifoaming agent is also added to avoid foaming from the blood protein degradation [21]. Octanol is often employed [6,16,28,31,32], but a silicone antifoam [22] is preferred because the solubility of O_2 and N_2 are the same in water, whereas octanol dissolves approximately six times the amount of O_2 as N_2 [23].

Concerning the GC separation, a stainless steel packed column containing 60–80 mesh molecular sieve (5 Å) with helium as the carrier gas has been the method of choice for CO separation from other gases such as oxygen and nitrogen [26]. Today, capillary molecular sieve columns are available and improve the sensitivity of CO measurement [14]. Moreover, the split/splitless injector used with capillary columns allows for the injection a very small amount of sample, and prevents the degradation of the column. For the detection of CO in blood, four GC detectors are available.

Initially, Thermal Conductivity Detector (TCD) was widely used [6,21,23,25,27], but this detector operates at high temperatures, which caused degradation of instable analytes. Moreover, this type of detector requires the complete removal of the oxygen gas from the sample to avoid the deterioration of the filaments. The efficiency of the TCD is apparent in the cases of a high rate of CO in the blood. This detector has been improved upon to produce the micro TCD, which is a more convenient [18,19,33] method. An alternative to the TCD is Flame Ionization Detector (FID) that is coupled to a nickel catalyst placed before the FID [3,10,11,15]. The use of the FID provides the specificity and sensitivity required at low CO levels through the conversion of CO to methane by a nickel-hydrogen reduction [17,34]. The main inconveniences of this technique include both the requirement of a quantitative reduction of CO to methane [22], and on the fact that a single instrument must be dedicated to this procedure. In the beginning, the catalyst had to be prepared in laboratory, but it has now been commercialized, allowing this to be a cheap and accurate method for CO monitoring [9,22]. Another GC-coupled detector is the Reduced Gas Analyzer (RGA), which has been found very specific to CO measurements, even at low levels [4,5,8]. This method utilizes the chemical reduction involving CO and mercuric oxide (HgO) at high temperatures (>200 °C). Elemental Hg is therefore released and spectrophotometrically detected in the gas phase at 254 nm [35,36]. This method also requires a dedicated GC for CO analysis because the detector is very specific. Finally, mass spectrometry (MS) can also be used to monitor the CO gas released from blood [24]. The universality of this detector is particularly useful because the GC/MS system can also be employed for other analyses without changing the configuration. CO can be detected even at low levels [20], but only a few studies have described how the HS-GC/MS method can be used to investigate the CO content in human blood.

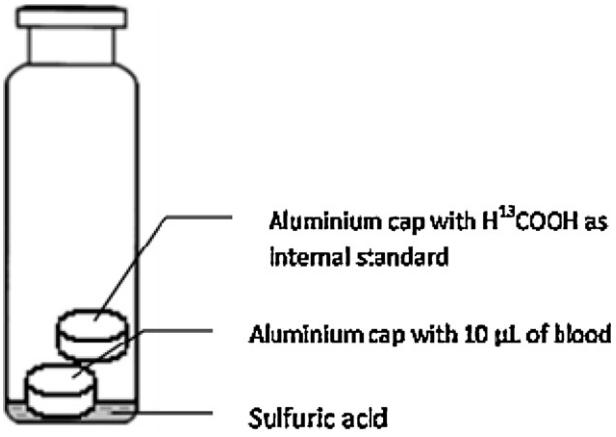

Fig. 1. CO in-vial generation from HCOOH and $H^{13}COOH$ in aluminum caps.

HS-GC/MS appears to be the method of choice for measuring CO levels in blood. Nevertheless, the main drawback is the absence of a liquid internal standard necessary for performing a quantitative measurement, as well as safe calibration standards. An isotopically labeled CO internal standard has been already used in the gaseous state [37], but the gaseous state did not allow a precise control of the added standard [38,39]. Moreover, it is neither easy nor safe to manipulate gaseous CO for sampling. The stoichiometric formation of the labeled carbon monoxide internal standard, by the reaction of formic acid with hot sulfuric acid, allowed us to generate CO under safe conditions [40,41]. Using liquid $H^{13}COOH$, it is possible to precisely generate ^{13}CO as the internal standard.

The aim of our study was to develop an innovative HS-GC/MS method applicable to routine forensic work in toxicology laboratories for the determination of CO blood concentration. A complete protocol is described, which is subsequently applied to measure the CO content of contaminated human blood from recent autopsies.

2. Materials and methods

2.1. Materials and reagents

All aqueous dilutions of formic acid were performed with distilled water. Formic acid (HCOOH) with a purity of 98% was purchased from Fluka (Buchs, Switzerland). $H^{13}COOH$ with a purity of 99% was obtained from Cambridge Isotope Laboratories CIL Inc. (Andover, USA). All formic acid solutions were prepared daily to prevent degradation. Sulfuric acid with a purity between 95 and 97% came from Merck (Darmstadt, Germany). All headspace extractions were carried out in headspace vials of 20 mL. Bovine blood was used as a blank for the calibrations.

2.2. Extraction method

An excess amount of sulfuric acid (400 µL) was carefully introduced to a 20 mL headspace vial. Next, an aluminum cap of 11 mm (i.d.) without septa or holes was introduced and 10 µL of blood was transferred into this cap. Then, a second aluminum cap of 11 mm (i.d.) without septa or holes was introduced in the headspace vial, above the first cap. Formic acid solutions (diluted HCOOH and $H^{13}COOH$ in water) were directly added into this second cap (Fig. 1), according to the calibration standards and control required quantities. The headspace vial was then hermetically sealed with

magnetic PTFE/silicone septum caps of 20 mm (i.d.) and vigorously shaken and vortexed to allow for intimate contact between sulfuric acid, blood and formic acid solutions. The effect of temperature on the CO liberation from formic acid and HbCO was investigated by setting the vials in the oven at 90 °C for 60 min.

2.3. GC/MS analysis

An Agilent 6890 N GC (Agilent Technologies, Palo Alto, CA) combined with a headspace gas autosampler and equipped with a HP Molecular sieve 5 Å PLOT capillary column (30 m × 0.32 mm, 30 μm) from Restek was used. The temperature program was as follows: 40 °C, held for 5 min; the injector set to 100 °C and the interface MS temperature to 230 °C. Helium was employed as the carrier gas at a flow rate of 1.9 mL/min. A solvent delay of 3 min was used and a split (1:5) was applied at the injector.

The detection was performed with an Agilent 5973 mass spectrometer (Agilent Technologies, Palo Alto, CA), operating in the electron ionization mode (EI) at 70 eV. The selected ion monitoring (SIM) mode was used to acquire the CO signal at *m/z* 28 and 29 for ^{13}CO.

2.4. Calibration standards and controls

Four working calibration standards of formic acid at concentrations corresponding to 0.25, 0.5, 1 and 2.5 μmol of CO/mL were prepared daily by diluting the formic acid with water. A blank of bovine blood was analyzed to guarantee the absence of CO before performing the validation step.

Intermediate quality control samples were also prepared daily from formic acid diluted in water at concentrations of 0.25, 1 and 2.5 μmol of CO/mL. The working internal standard solution (H^{13}COOH) was diluted with water (1:1000). Ten microliters of the working internal standard was added to each sample prior to extraction, resulting in a final concentration of 0.26 μmol of ^{13}CO/mL. Standard solutions were stored at +4 °C when not in use.

2.5. Validation procedure

The validation procedure was performed according to the guidelines of the "French Society of Pharmaceutical Sciences and Techniques" (SFSTP) based on the following criteria: selectivity, response function (calibration curve), linearity, trueness, precision (repeatability and intermediate precision), accuracy, limit of detection (LOD) and limit of quantification (LOQ). Linearity was achieved with a minimal coefficient of determination above 0.994. The stability of extracted headspace (CO released in the vial) was examined over one month by placing three sets of controls (0.25, 1 and 2.5 μmol/mL) in the freezer (−20 °C), fridge (+4 °C) and ambient temperature with daylight.

The validation experiments were performed with calibration standards and control samples over 3 non-consecutive days ($p = 3$) and were not analyzed in the same week.

2.6. Postmortem specimens

All samples used for additional toxicological analyses were stored at −20 °C until GC analysis. Blood samples were used from various cases in which CO could have played a direct role in the cause of death. Samples were further diluted in water (1:10). The HbCO saturation ($HbCO_{(CO\ back\ calculated)}$) was estimated according the following three references:

1. $HbCO\ (\%) = [CO]_{(\mu mol/mL\ blood)} \times 100/0.06 \times [Hb]_{(g/L\ blood)}$ from [16]
2. $HbCO\ (\%) = ([CO]_{(\mu L/100\,mL\ blood)}/2.4) - 177)/82.5$ from [46]
3. $HbCO\ (\%) = [CO]_{(mL/100\,mL\ blood)} \times 100/1.42 \times [Hb]_{(g/100\,mL\ blood)}$, i.e., using a normal Hb concentration comprised of 12–16 g/100 mL blood:
   $HbCO\ (\%) = [CO]_{(mL/100\,mL\ blood)} \times 5$ from [4,8,42–45]

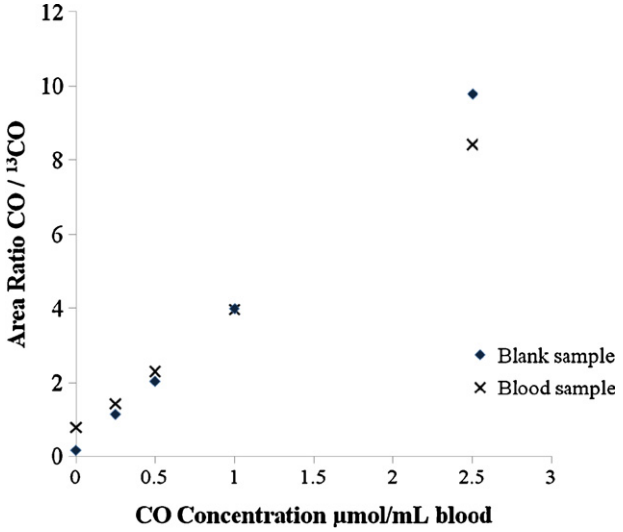

Fig. 2. Study of matrix effect on CO generation performed with unheated samples.

To evaluate the feasibility of this method, the CO content was measured in 7 postmortem blood samples: 2011 (1 case, two different samples: A1 and A2), 2010 (1 case, identified as B), 2009 (4 cases, identified with letters from C to F) and 2007 (1 case, identified as G).

3. Results and discussion

3.1. Matrix effect

The possibility of a matrix effect was investigated by comparing the results obtained in the presence and absence of blank bovine blood. The results presented in Fig. 2 show a matrix effect between two sets of unheated samples. An initial CO concentration in bovine blood is also evaluated to be an HbCO saturation of 1.35%. This result is consistent with the results obtained from animal blank blood (sheep and cow). Part of reason for this concentration could be attributed to an endogenous blood contamination (related to animal metabolism and/or conditions of cattle rearing) and blood storage. However, this initial CO concentration could also come from the extraction conditions. The use of sulfuric acid at ambient temperature could introduce a low CO generation even if no formic acid was added. The strong dehydration of biological material under acidic conditions could produce a small amount of CO, in addition to the initial endogenous CO concentration. This result has been already reported in extractions involving acidic and high-temperature conditions. In the presented protocol, we did not use heat during the preparation of the calibration curve, and the results demonstrate that the use of sulfuric acid seems to have a greater influence than the use of heat in CO generation.

To avoid quantification problems, a validation step with the blood matrix was chosen.

The blood volume sampling was set to be as low as possible. Indeed, in postmortem cases, a large amount of blood to sample is not always available, and priorities must be established in the analyses. The method developed herein has integrated this requirement so that only 10 μL of blood is needed to perform a CO measurement.

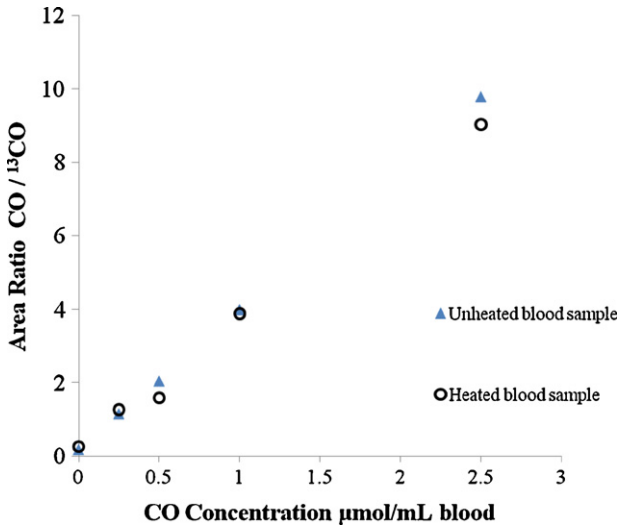

Fig. 3. Effect of vial heating on CO generation on blood sample.

Table 1
Validation parameters.

Calibration curve (0.25–2.5 µmol/mL blood] (k = 5, n = 3, p = 3)			
	Day 1	Day 2	Day 3
Slope	1.93	1.83	1.92
Intercept	0.23	0.40	0.36
r^2	0.99718	0.99291	0.99874

Linearity (0.25–2.5 µmol/mL blood] (k = 3, n = 3, p = 3)	
Slope	1.0018
Intercept	−0.0018
r^2	0.998

Trueness (relative bias %) (k = 3, n = 3, p = 3)	
Levels (µmol/mL blood)	Trueness (%)
0.25	4
1	−1
2.5	−2

Precision (RSD %) (k = 3, n = 3, p = 3)		
Levels (µmol/mL blood)	Repeatability	Intermediate precision
0.25	1.174	1.27
1	0.068	0.07
2.5	0.083	0.1

3.2. Heating effect

The CO generation from formic acid used for the calibration must be differentiated from the CO liberation from HbCO, both of which are formed because of the presence of sulfuric acid.

In this study, due to the CO generation from formic acid, no CO concentration differences were noticed when blood was treated with sulfuric acid during 60 min at 90 °C or at ambient temperature (Fig. 3). The average CO/^{13}CO ratios of heated samples comprise the confidence interval of unheated samples average ratios for each calibration point (Student's t-test, α = 5%, n = 15). Therefore, it was decided early on not to heat the calibration standards to reduce the analysis time. The reaction between sulfuric and formic acid appears to be quantitative under these conditions. The results presented in Fig. 3 illustrate an insignificant heating effect on the calibration standards prepared using undiluted blood samples.

Considering the CO liberation from HbCO, some authors have previously reported the influence of temperature and the amount of heating time. A thermal spontaneous generation of CO was observed even without a releasing agent (sulfuric acid or other) and even at mild temperatures (50–60 °C) [12]. However, this low spontaneous thermal CO generation is negligible in comparison to the CO released from intoxicated samples (Fig. 4). Indeed, the heating of the headspace vials containing blood samples is required. Unheated, diluted (1:10) CO-positive blood samples have led to underestimation of the $HbCO_{(CO\ back\ calculated)}$ content compared with the $HbCO_{(spectrophotometry)}$ levels for heated samples (60 min at 90 °C). This underestimation has been observed to be greater than 50% in some cases. When heated, the $HbCO_{(CO\ back\ calculated)}$ value obtained was in complete agreement with the $HbCO_{(spectrophotometry)}$ levels.

Even if blood sample vials must be heated, the use of diluted blood samples enables the minimization of spontaneous thermal CO generation. Moreover, the GC technique does not require the transformation of hemoglobin forms (such as methemoglobin) to obtain the total hemoglobin content as needed in the spectrophotometric method. The use of sulfuric acid to release the CO from blood sample was sufficient to enable a CO release from all CO blood sources (hemoproteins). If the heating step is essential to guarantee a complete CO generation from HbCO, this step is not required to produce the calibration curve.

3.3. Selectivity of the method

The selectivity of the method was investigated by measuring the CO concentrations for 6 postmortem blood samples that contained normal CO content according to the cause of death, and 15 blood samples from living drivers. These 21 analyses were evaluated for co-eluting chromatographic peaks that might interfere with the detection of CO or ^{13}CO. No interference peak was observed at the CO retention time and for the m/z of 28, indicating that the method provides satisfactory selectivity for CO determination.

3.4. Calibration curve for the method

Each point on the calibration curve using bovine blood was defined as the area ratio of CO to ^{13}CO within a concentration range. Three assay calibration curves were performed for CO determination, prepared on 3 non-consecutive days (p = 3), over two weeks. Calibration standards were prepared at 5 (k = 5) concentration levels: 0, 0.25, 0.5, 1 and 2.5 µmol/mL, each in triplicate (n = 3). Calculated concentrations of each calibrator were compared to target values and were found to be within ±20%. A linear relationship was established between the spiked CO concentration from HCOOH and the measured response in the calibration range. To increase the statistical weight of the lowest concentrations, a linear regression model based on weighted least squares was tested (weighting factor used was $1/x^2$). The best model was a simple linear regression not based on weighted least squares. The validation results for the calibration curves are compiled in Table 1.

3.5. Linearity of the method

The linearity was assessed by fitting back-calculated concentrations of the control samples against the theoretical concentrations by applying the linear regression model based on the least squares method. Each non-consecutive day, control samples were measured at 3 concentration levels (k = 3) in triplicate (n = 3). The control sample concentrations were calculated using a calibration curve determined for each measurement day. As presented in Table 1, good linearity was obtained with a slope value of 1.0018 and a

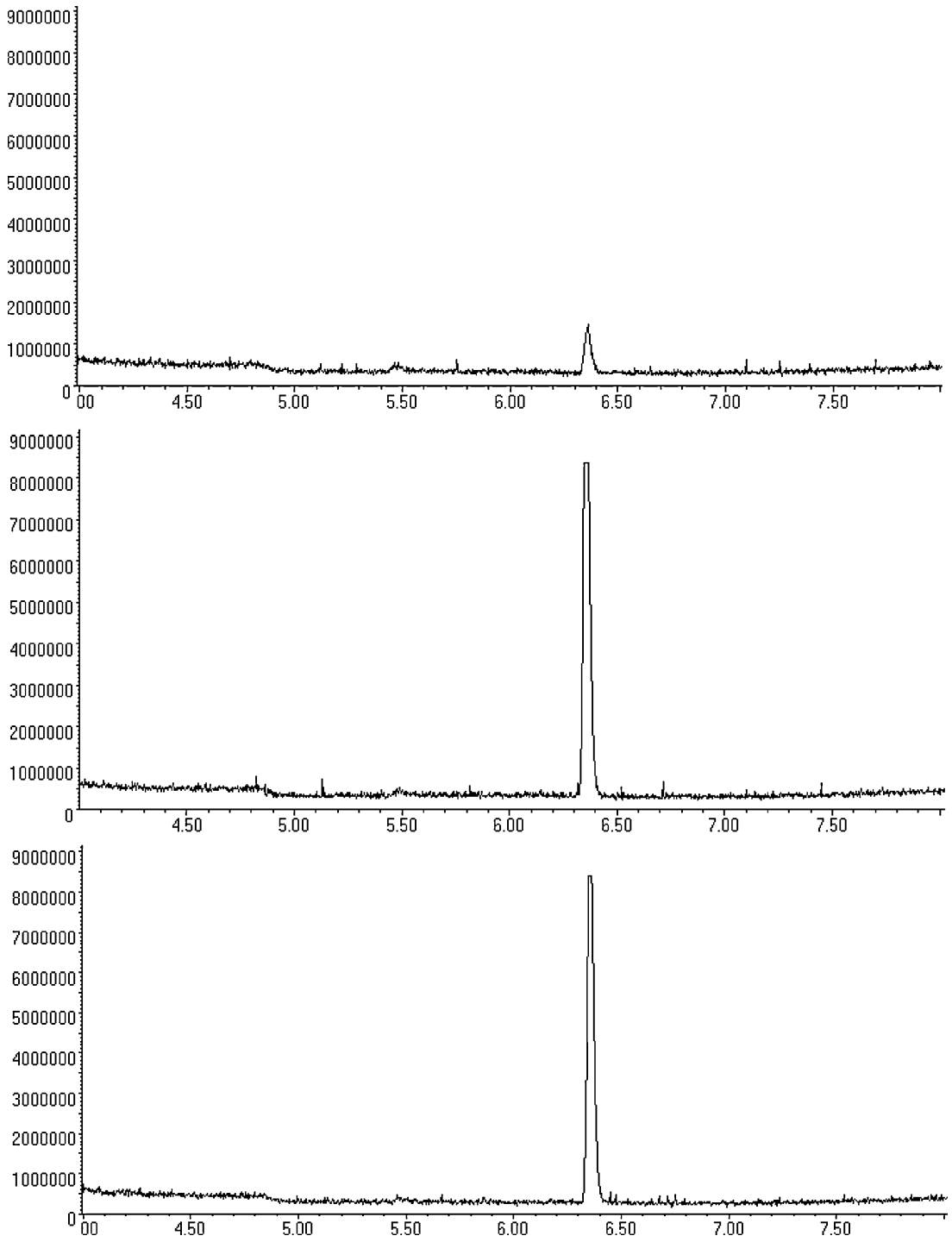

Fig. 4. Chromatograms of carbon monoxide (SIM acquisition *m*/*z* 28) in bovine blood sample (A), in calibrator at 0.25 μmol/mL of blood (B) and in a real ten times diluted intoxicated blood sample (C), measured at 0.29 μmol/mL.

coefficient of determination above 0.998 in the range from 0.25 to 2.5 μmol/mL blood.

3.6. Trueness of the method

Also called the bias, the trueness test expresses the closeness between the experimental average value and the accepted reference value. This test detects systematic errors and is expressed as a percent deviation from the accepted reference value. Several daily repetitions of control samples were analyzed over several months at their respective concentrations, which were used to establish a true value for each concentration. As shown in Table 1, trueness was found to be lower than the acceptance criteria (within ±15% of the accepted reference value and within 20% at LLOQ, 0.25 μmol CO/mL blood). In fact, trueness was measured within ±5% of the accepted reference value in the considered range (0–2.5 μmol/mL) and was consequently satisfactory for the CO analysis.

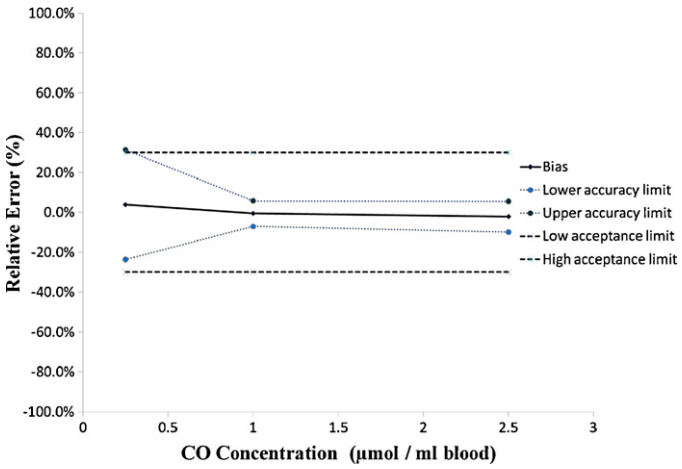

Fig. 5. CO accuracy profile using a simple linear regression model within a range of 0.25–2.5 μmol/mL blood (continuous line: trueness, bold dashed lines: acceptance limits set at ±30%, dashed lines: lower and upper accuracy limits in relative values).

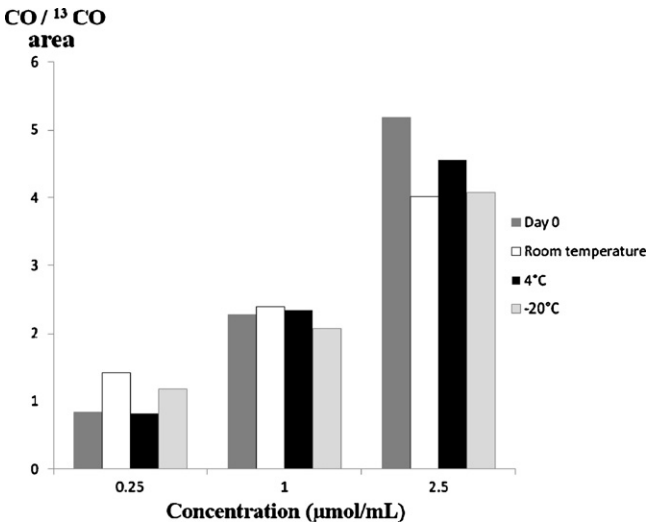

Fig. 6. In-vial generated CO stability after one month of different storages (room temperature, 4 °C and −20 °C).

3.7. Precision (repeatability and intermediate precision) of the method

The precision test detects random errors. Precision was assessed by calculating the repeatability (intra-day precision) and intermediate precision (inter-day precision) for each control sample concentration. The repeatability variance was estimated by calculating the intra-days variance (S^2_r) and the intermediate precision variance was estimated by adding the intra- and inter-day variances (S^2_{IP}). As shown in Table 1, the relative standard deviation values for repeatability and intermediate precision were between 0.07 and 1.27%.

3.8. Accuracy and LOQ of the method

The accuracy expresses the total error defined by the sum of trueness (systematic error) and precision (random error). The accuracy profile given in Fig. 5 shows the ability of the method to provide an analytical result using systematic and random errors with a risk of $\alpha = 5\%$ at each concentration level. The mean bias (%) confidence interval limits for the control samples were within the ±30% acceptability limits typically allowed by Swiss forensic laboratories.

With a threshold of 30% as the acceptability limit, the lower limit of quantification (LLOQ) was set to 0.25 μmol CO/mL of blood.

3.9. Limit of detection (LOD) of the method

The LOD was determined by headspace extraction of negative blood samples containing sulfuric acid and decreasing amounts of formic acid. The LOD was assessed using a signal-to-noise ratio of S/N > 3. The noise was estimated by measuring more than 15 blank samples. As a result, using 10 μL of blood, the LOD for CO quantification was estimated to be 0.25 nmol/mL, a value two orders of magnitude lower than the LOD reported in the literature (16).

3.10. Stability of CO in the vial

Studies were performed to determine the stability of CO in the HS vials stored under different conditions. Temperature stability studies were performed on three sets of control samples containing 0.25, 1 and 2.5 μmol CO/mL blood. The first set of control samples ($n = 3$) was stored at −20 °C, a second set of controls ($n = 3$) was stored at +4 °C and a third set of controls ($n = 3$) was stored at room temperature under natural light. Concentrations of CO were calculated and compared with the results obtained one month before with the same control samples. Mean comparison was assessed by Student's t-test ($\alpha = 5\%$). The results are presented in Fig. 6 and show no significant differences between the different control sets. Therefore, CO generated in the vial is stable regardless of the storage conditions for more than one month.

3.11. Analyses of postmortem specimens

Seven postmortem blood samples were evaluated to study the performance of the CO method. Due to the differences in the blood viscosity of the samples, all the postmortem blood specimens were diluted (1:10). For 2011 case, the dilutions (A1 and A2) were performed directly after sampling from the bodies. In most of the cases, peripheral blood samples from fresh cadavers were used. Alternatively, cardiac blood samples were also used in putrefaction and drowning cases, or in fire cases, where the peripheral parts were too damaged by the fire.

All the results are presented in Table 2. The CO concentrations range from 0.1 to 3.8 μmol/mL blood, corresponding to a HbCO saturation comprised between 2% (blood B) and 45% (blood A2). An agreement between the $HbCO_{(CO\ back\ calculated)}$ and the $HbCO_{(spectrophotometry)}$ is observed for most of the cases. However, it is important to note that in some cases, the $HbCO_{(CO\ back\ calculated)}$ is more important than the $HbCO_{(spectrophotometry)}$. Indeed, the HbCO content measured by spectrophotometry has already been reported not to be the best for post mortem analysis, not only from the point of view of turbidity and physical sample quality, but also from the point of view of stability. A decrease in the HbCO content has already been reported as a function of sampling time [47]. After three hours, a decrease in the $HbCO_{(spectrophotometry)}$ content to 40% has been observed. Consequently, to express the oxycarbonemia in postmortem blood, the GC approach is more reliable because a direct CO concentration is measured and therefore expressed in the $HbCO_{(CO\ back\ calculated)}$. Cardiac blood sample A2 illustrates this observation, wherein the $HbCO_{(spectrophotometry)}$ can lead to an underestimation of the real CO blood concentration. For cases A1 and A2, the $HbCO_{(CO\ back\ calculated)}$ and the $HbCO_{(spectrophotometry)}$ contents were measured less than 30 hours after death. Correct sampling and storage have led to similar results on peripheral blood (HbCO 35–36%). However, considering that cardiac blood exhibited an $HbCO_{(CO\ back\ calculated)}$ (46%) that was higher than the $HbCO_{(spectrophotometry)}$ (38%) illustrates the necessity to

Table 2
Application of HS-GC–MS for CO and HbCO measurements on postmortem real cases.

Year	Death circumstances	Sample	Concentration blood (μmol/mL blood)	Concentration blood (μL/100 mL blood)	HbCO[a]	HbCO[b]	HbCO[c]	HbCO[d]
2011	Suicide – Exposure to fire	Peripheral blood (NaF) (A1)	3.0	7200	36	34	36	35
		Cardiac blood (NaF) (A2)	3.8	9120	45	44	46	38
2010	Dead after a go-kart race	Blood (B)	0.1	312	2	0	2	0
2009	Exposure to fire	Peripheral blood (NaF) (C)	0.4	888	4	2	4	0
2009	Unknown	Peripheral blood (EDTA) (D)	3.4	8160	40	39	41	40
2009	Exposure to fire	Peripheral blood (NaF) (E)	0.7	1632	8	6	8	5
2009	Exposure to fire	Cardiac blood (NaF) (F)	0.2	552	3	1	3	3
2007	Car accident	Blood of pleural cavity (G)	3.0	7200	36	34	36	38

[a] According to Cardeal et al. [16]: HbCO % = CO (μmol/mL) × 100/0.06 × Hb (g/L).
[b] Adapted from Vreman et al. [46]: HbCO % = [(CO (μL/100 mL blood)/2.4) − 177]/82.5.
[c] According to [4,8,42–45]: HbCO % = CO (mL/100 mL blood) × 5.
[d] HbCO obtained by spectrophotometry.

measure CO and estimate the $HbCO_{(CO\ back\ calculated)}$ to confirm the $HbCO_{(spectrophotometry)}$ content.

The method described herein was evaluated to be satisfactory for providing reliable, accurate and repeatable CO results in a short time and from a very small amount of blood sample.

4. Conclusion

A selective and sensitive method for the identification and quantification of CO in postmortem blood samples was presented. This method offers a new opportunity for the CO measurement in forensic sciences, particularly for postmortem cases where the samples are often of low quality. Turbidity, coagulates and other interfering phenomena can be avoided by the use of HS-GC–MS. The technique was validated according to the guidelines of the French Society of Pharmaceutical Sciences and Techniques (SFSTP). This method allows for an accurate and reliable measurement (±30%) of CO concentrations in a range from 0.25 to 2.5 μmol/mL blood. The method is not time-consuming and is safe because the generation of CO takes place in a hermetically closed headspace vial. The method also provides for precise quantification because ^{13}CO is used as the internal standard from $H^{13}COOH$. Finally, only a small amount of blood sample is required for the CO measurement, which is especially useful in cases where only a small amount of blood is available. The applicability of this method has been tested on real postmortem cases with a known history involving fires or CO generation and has provided satisfactory results.

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