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Rapid and sensitive method for the analysis of carbon monoxide in blood using gas chromatography with flame ionisation detection

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Abstract

In order to measure changes in physiological CO concentrations in blood with good accuracy, a method was developed using gas chromatography with flame ionisation detection (250°C). A nickel catalyst system was fitted to convert CO to methane at 375°C after separation with a molecular sieve column at 35°C. Helium was used as carrier at 30 ml/min. Porcine or human blood (400 μl) was sampled in gastight tubes and treated with sulfuric acid and saponin (800 μl). Accuracy was 1.4% and 1.5% (RSD), respectively. Precision was 2.8% (porcine blood). Limit of detection was 0.01 nmol/ml gas and limit of quantification 12 nmol/ml blood. Calibration was made in the interval 12–514 nmol/ml blood (corresponding to 0.1–6% COHb). Samples were stable for at least a month at +4°C. This paper describes a method with high sensitivity and good accuracy, suitable for analysis of low CO concentrations.

Keywords: Carbon monoxide

1. Introduction

CO, found to be an endothelial factor of physiological importance [1], is released from the metabolism of haem during its degradation to biliverdin and iron, a reaction catalysed by haem oxygenase. CO has been shown to activate guanylate cyclase which promotes relaxation of blood vessels. In order to study mechanisms elicited by this pathway, an assay of CO characterised by high accuracy and sensitivity is needed since physiological spill-over may be minimal. Previously described methods for CO include microdiffusion, colorimetric, infrared absorption, volumetric or spectrophotometric methods [2]. Other investigators use manometric oxidation, microcalorimetric or indirect methods [3]. However, gas chromatography (GC) has been claimed to be the reference method [4]. Detection of CO is possible using thermal conductivity detection (TCD) [5] or flame ionisation detection (FID) after methane conversion by a nickel catalyst [6]. A release of CO

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from haemoglobin (Hb) can be obtained by various methods. This paper describes and validates a GC method which combines the advantages of the FID-nickel catalyst [7] and the stable release of CO by sulfuric acid and saponin [8].

2. Experimental

2.1. Materials and reagents

All used reagents were of analytical grade or higher purity. Carbon monoxide for calibration was obtained from a standard cylinder containing 5% (certified range: 4.9–5.1%) CO in nitrogen (AGA Gas, Sundbyberg, Sweden). Other gases such as hydrogen and helium were of high purity grade (AGA Gas). Sulfuric acid was diluted from a 95–97% (Merck, Darmstadt, Germany) solution. Other chemicals were saponin from Quillaja Bark (Sigma, MO, USA) and trisodium citrate dihydrate (E. Merck, Darmstadt, Germany). Aqueous solutions were prepared with chromatography-grade water from a Milli-Q water purification system (Millipore, WI, USA). For sample, standard and storage preparations, 5 ml gastight tubes with septum-fitted caps were used (SV 134, Chromacol, CT, USA). A multi-tube vortexer (speed 1.5) was used for mixing of samples (SMI, CA, USA). To obtain thorough stirring, glass beads (KEBO Lab, Spanga, Sweden) with a diameter of 3 mm were added to the test tubes before mixing. Tedlar gas sampling bags (Scantec Lab, Gothenburg, Sweden) with a volume of 10 l were used for preparation of gas standards. Cross validation was made with an OSM3 hemoximeter (Radiometer, Copenhagen, Denmark).

2.2. Gas chromatography

The GC system was a Hewlett-Packard 5790A with a 6 ft. Heysep Q 80/100 mesh column (Hewlett-Packard, Gothenburg, Sweden) (1 ft.=30.4801 cm). After the column, a Hewlett-Packard 5890 series nickel catalyst system (accessory 19205A) was placed. The detector was a flame ionisation detector. The injector (manual) temperature was 40°C, the oven temperature 35°C, the nickel catalyst 375°C and the detector 250°C. The carrier gas was helium at a flow of 30 ml/min. The flow for the gases to the detector were 400 ml/min for air and 30 ml/min for hydrogen. The signals were detected on a computer using Millennium^{32} chromatography software system (Waters, MA, USA).

2.3. Preparation of solutions

Trisodium citrate dihydrate was prepared to a concentration of 0.13 *M* by dissolving 3.82 g in 100 ml purified water. 1 *M* sulfuric acid was obtained by mixing 27 ml sulfuric acid with 473 ml purified water. To this solution, 7.5 g saponin was dissolved to get the CO liberating solution (LS).

2.4. Preparation of standard samples

To gastight tubes, 400 μl water, 40 μl 0.13 *M* trisodium citrate dihydrate, 800 μl LS and a glass bead were added and the tubes were capped. Two hypodermic needles were placed through the septum and helium was flushed for 30 s. Different amounts of CO were added with gastight syringes. The amounts used were 0, 2.5, 5, 10, 20, 50 and 100 μl 5% CO in nitrogen. Duplicate samples were prepared. The zero calibration sample was obtained as a blank sample. The calibration samples were mixed for 40 min and 200 μl of the gas phase was injected manually with a gastight syringe on the GC system. Calibration curves were calculated and concentrations of CO were expressed as nmol/ml liquid using the ambient pressure of 1 atm at 23°C (1 atm= 101 325 Pa). The precision and accuracy for the calibration curve are given in Table 1.

2.5. Sample preparation

To septum fitted gastight tubes, 400 μl blood and a glass bead were added. Helium was flushed through the tubes during 30 s. LS (800 μl) was added, using two hypodermic needles through the septum, and the samples were mixed for 40 min. An aliquot (200 μl) of the gas phase was injected manually with a gastight syringe on the GC column.

Table 1
Precision and accuracy for the whole calibration range

Amount of CO (5% in N_2) added	Concentration (nmol/ml water)	Area (mean, mV)	Precision		Accuracy		
			RSD (%)	*n*	RSD (%)	Range (%)	*n*
0	0	1336	57	12	13	1.2–30	8
2.5	12.86	9494	16	12	6	0.12–15	8
5	25.73	16 657	9	19	4	0.78–21	9
10	51.46	30 781	7	19	3	0.05–7.7	9
20	102.9	57 177	10	15	5	1.7–11	8
50	257.3	144 169	11	11	3	0.44–8.8	9
100	514.6	288 708	11	7	2	0.46–5.1	6

Standard samples prepared with water, LS and different amounts of CO. The measures of variation are calculated from different curves, as analysed during a period of 11 months. The accuracy is given in mean and range for the variation between double samples analysed on the same day.

2.6. Sample preparation development

2.6.1. Volumes of blood and reagent

The influence of blood volume and volume of LS were investigated in some experiments. All samples were prepared using the same procedure as described in sample preparation. Human blood was prepared in triplicate, unless otherwise noted. The different combinations of blood and LS are summarised in Table 2.

2.6.2. Acid concentration

Effects of acidity were investigated by preparing 1, 2 and 4 *M* sulfuric acid containing 1.5% (w/v) saponin. Human blood (200 μl) was mixed with 2 ml 1 *M*, 2 ml 2 *M*, 1 ml 2 *M*, 1 ml 4 *M* or 2 ml 4 *M* sulfuric acid. The samples were prepared as described previously.

Table 2
Different combinations of blood volume and liberating solution volume as tested in sample preparation development (Section 2.6.1), triplicates unless indicated otherwise

Blood volume (μl)	LS volume (μl)
25	25, 50, 100
50	50, 100, 200
100	100, 200, 400, 1000, 1500, 2000
200	200, 400[a], 800[a], 1500, 2000, 3000
250	500, 1000, 2000
400	800[a,b]
500	1000, 2000[a]
750	2000
1000	2000

[a] Ten replicates.
[b] Ten replicates with porcine blood.

2.6.3. Saponin

To 200 μl human blood, 2 ml LS or 2 ml 1 *M* sulfuric acid was added. Thereafter the samples were prepared as described above in sample preparation. Another experiment was performed to investigate the effect of adding saponin solution in water (3%) and sulfuric acid (2 *M*) separately (1+1 ml, respectively) compared with 2 ml LS.

2.6.4. Flushing time with helium

The optimal flushing time for helium was investigated by flushing empty tubes with helium, during 0, 10 s, 30 s, 1 min and 2 min through hypodermic needles. The gas samples (200 μl) were injected onto the GC system.

2.6.5. Mixing time

The effect of different mixing times on the vortexer were tested by analyses of pooled blood using 0, 5, 10, 15, 20, 25, 30, 35 or 40 min mixing time. Otherwise, the samples were prepared as described above.

2.7. Method validation

2.7.1. Validation of analysis

CO was added to a gas sampling bag filled with nitrogen. Gas from this bag was analysed on 135 occasions during 2.5 months.

2.7.2. Validation of preparation

Pools with human or porcine blood were mixed with 10% (v/v) trisodium citrate dihydrate (0.13 *M*). The pooled samples were divided into 10 gastight tubes, 400 μl blood to each tube. Samples were prepared and analysed as described in sample preparation. Relative standard deviations (RSDs) were calculated for both human and porcine blood.

2.7.3. Limit of detection

To determine the limit of detection (LOD) for the chromatography system, gas sampling bags were filled with 10 l of helium and different amounts of CO. Gas samples (200 μl) from these bags were injected onto the GC system. The limit of quantification (LOQ) was determined as the lowest standard calibration point that could be used at an acceptable signal-to-background ratio. This was calculated as the ratio between the calculated response from the calibration point and the actual response from the blank calibration sample. These calculations were performed using seven calibration experiments.

2.7.4. Cross validation

To validate the method, 48 venous, porcine blood samples were analysed with our GC method as well as with a spectrophotometric (OSM3) method. The % COHb in the GC analysis was calculated using the formula:

$$\%\,\mathrm{COHb} = 100 \times [\mathrm{CO\ (mol/ml)} \times M_r\mathrm{Hb\ (g/mol)}]/[\mathrm{Hb\ (g/ml)} \times 4]$$

A molecular mass (M_r) of 64 400 for haemoglobin was used in the calculations. The Hb concentration of the samples were analysed with the hemoximeter (OSM3). The relative differences between the two methods were calculated and plotted against the mean COHb percentage of the methods [4]. The Student's *t*-statistic was calculated to test for a systematic difference, and $p < 0.05$ was considered a statistically significant difference.

2.8. Stability

Porcine blood mixed with 10% (v/v) trisodium citrate dihydrate (0.13 *M*) was divided into 97 tubes, each containing 400 μl blood. Before capping, a glass bead was added to each tube. Thirty-six of these samples were stored at +4°C and 36 were stored at −20°C. The remaining 25 samples were prepared for analysis as described above. After this preparation, 10 of the samples were analysed immediately. The other 15 samples were stored at +4°C, and analysed on days 2, 3, 4, 7 and 8. The remaining samples were prepared and analysed on days 2, 3, 4, 7, 8, 10, 14, 15, 21, 22, 29 and 35. Three replicates of the same sample were analysed on each day. RSDs were calculated for within days, between days and for all samples.

3. Results

3.1. Sample preparation development

The final analytical method was designed using the best conditions as found with each experiment. A sample blood volume of 400 μl was sufficient for good accuracy between samples. Furthermore, this volume did not saturate the gas phase. To obtain total liberation of CO, two times the blood volume of LS was sufficient. The response was better (21%) when samples were treated with saponin than without. It was also found that it does not matter if saponin is added separately or together with sulfuric acid. However, it is easier to add them as one solution because of fewer steps in the preparation procedure. When flushing samples with helium, 30 s was enough for the removal of CO derived from air introduced into the tubes during capping. There was no benefit from flushing the tubes 1 or 2 min. Finally, the optimal mixing time was found to be 40 min. The response after mixing the samples during 5 min was high but there was a large variation between samples. After 40 min of mixing the accuracy was good.

3.2. Separation and specificity

The retention time for CO was 0.5 min. The chromatography for CO on this system was good with no disturbing peaks. Fig. 1 shows the chromato-

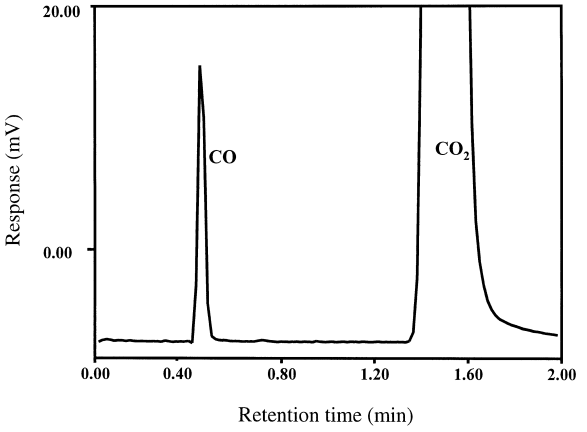

Fig. 1. Chromatogram from a human blood sample, (78.2 nmol CO/ml blood, corresponding to approximately 1.3% COHb).

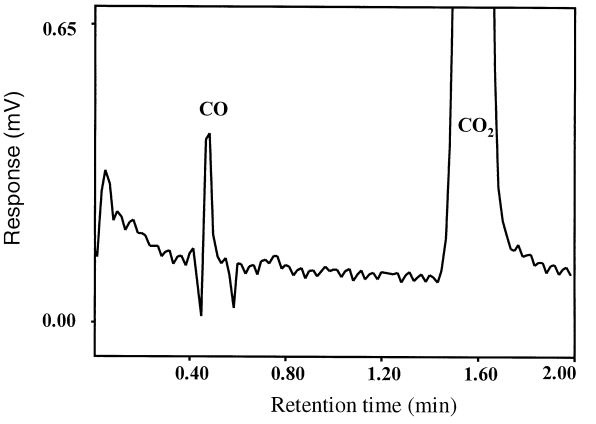

Fig. 2. Chromatogram from a blank sample.

gram for one human blood sample stored at −20°C. The chromatograms were found to contain peaks corresponding only to CO and carbon dioxide. It was found in gas mixture controls (CH_4, CO, CO_2, 10 ppm of each gas in He) that methane was separated easily from these peaks (retention times: CO = 0.5 min; CH_4 = 0.7 min and CO_2 = 1.5 min). It was also found that neither O_2 nor N_2 resulted in any response from the detector using the present chromatographic conditions. Total analysing time was 2 min and after that the system was clean and ready for the next run. A chromatogram from a blank sample can be seen in Fig. 2.

3.3. Calibration, accuracy and precision

Because of the high sensitivity for CO on the nickel catalyst system it was possible to measure low levels with good accuracy. The LOD for the chromatography system was 0.01 nmol/ml gas. Using the described sample preparation, this would correspond to a blood concentration of 0.1 nmol/ml. However the LOQ was 12 nmol/ml water with a signal-to-background ratio of 6.8 (range 3.7–12.2). Blood samples analysed in this assay had CO concentrations of 30–80 nmol/ml blood. The variability of the chromatography system was found to be 2.8% (RSD) over 2.5 months (2.7.1). RSDs between preparations of 10 blood samples were 1.4 and 1.5% for porcine and human blood, respectively (Section 2.7.2). Calibration curves calculated for the standard samples resulted in a linear relationship between concentration and peak area ($y = 561x + 2558$, $r^2 = 0.9995$). The calibration interval was 12–515 nmol/ml and blank samples were also included. Results from the cross validation experiment are shown in Fig. 3. The mean difference between the two methods was 0.30% COHb (RSD = 104%), the GC method giving slightly larger concentrations. This difference indicated a small but significant systematic difference (Student's $t = 6.7$ with 47 degrees of freedom, $p < 0.001$).

3.4. Storage of samples

All storage experiments (Section 2.8) resulted in low variations (Table 3). We found that it is possible to prepare the samples and analyse them on another

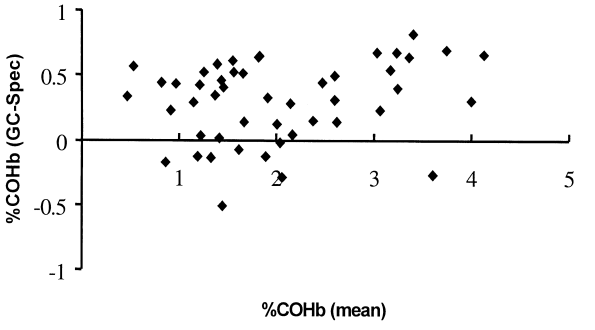

Fig. 3. Cross validation between a spectrophotometric method (Spec) and the GC method described in this article. The results from the GC method have been calculated as % COHb.

Table 3
Variability (RSD) for blood samples stored during different periods of time at +4°C and at −20°C

	Total storage time (days)	Number of storage periods	RSD (%)		
			All samples	Between days	Within days
+4°C	35	13	3.2 ($n=46$)	2.6 ($n=13$)	2.5 ($n=3^a$)
−20°C	35	13	3.9 ($n=45$)	2.1 ($n=13$)	3.9 ($n=3^a$)
Prepared	8	6	3.5 ($n=25$)	2.4 ($n=6$)	3.0 ($n=3^a$)

The variability for samples stored at +4°C after sample preparation is also given.
[a] Except on day 1, where $n=10$.

occasion within 8 days. The variation was lower for samples stored at +4°C compared with −20°C, and samples stored at +4°C were also easier to handle because there was no need to defrost them. The samples could be stored at these temperatures for at least 35 days.

4. Discussion

It has recently been shown that CO acts as a vasodilator, and it is therefore of great interest to further investigate its role in the regulation of cardiovascular homeostasis. CO is liberated intracellularly when haem is oxidised. In this situation, small spill-over amounts of CO is liberated into the regional circulation which may mirror its vasoactive role. The spill-over amount from local synthesis in different vascular beds is expected to be small and the normal level of CO in blood is also small. Therefore, an analytical method with low detection limit and good accuracy is required.

For the analysis of low CO concentrations, there are two main methods described in the literature, spectrophotometry and GC. When the present method was developed GC–FID was used, since previously published spectrophotometric methods describe lower accuracy and higher limits of detection compared with GC [4,9,10]. One of the most sensitive spectrophotometric methods measured CO levels in plasma [11]. This method could measure low levels of CO, but the limit of detection was 290 times higher than our LOQ and the RSD between samples was about seven times larger than in our GC method.

GC analysis can be done with TCD or with FID. Methods utilising TCD often need large blood volumes because of the low sensitivity for CO. However, there are two assays using TCD that have reduced this problem [5,8]. One [5] used a special reaction chamber and the other [8] a micro TCD to obtain better sensitivity. The calibration range in the micro TCD method was 2–80% COHb (normal non-smoking subjects have COHb levels of approximately 0.4–0.8%). Standards for the calibration curve in the micro TCD method were made by mixing 0 and 100% COHb. To achieve 0 and 100% COHb, blood was bubbled with oxygen and CO, respectively. This procedure was also evaluated initially in the present paper. However, when blood was bubbled with oxygen, we did not reach 0% COHb levels even after several hours of bubbling, as determined after head-space analysis of the blood using GC. Therefore, our standards were prepared using purified water. The other TCD method [5], using a reaction chamber, was calibrated in the range 0.5–58% COHb and used standards from a Hb solution saturated with CO. Thus, this method was developed for the analysis of CO in blood in association with intoxication.

GC–FID methods have previously been described in the literature. A reference method was developed by Collison et al. [7], using $K_3Fe(CN)_6$ to liberate CO from blood. The method worked properly with an RSD of 1.8%. Another paper using GC–FID [6] evaluated different reagents for liberating CO from Hb and concluded that phosphoric acid gave the lowest variability (9%) [6]. However, the combination of sulfuric acid and saponin was not tested. Using this combination as a liberating solution a

RSD of 1.5% was obtained in our method. The reaction between formic acid and sulfuric acid can be used for calibration of samples [6]. With this technique it was possible to detect fairly low levels of CO (LOD = 0.5 nmol/ml). Nevertheless, the method was developed to measure blood from patients suffering from CO poisoning. Our calibration method worked properly with low variations between double samples. Calibration curves from the standards showed good linearity. For laboratory safety reasons, it was also good that there was no need to handle pure CO or $K_3Fe(CN)_6$. Our calibration curve was in the interval between 12 and 514 nmol/ml (about 0.1–6% COHb). The sensitivity of our method (LOD = 0.01 nmol/ml gas, LOQ 12 nmol/ml water) permits the detection of very low CO amounts. A high sensitivity is also useful if small sample volumes are to be analysed.

Since the amount of CO depends on the amount of Hb or iron present in the sample, it is necessary to analyse Hb or iron at the same time as CO is analysed. Using a hemoximeter, Hb concentrations may be analysed and the CO concentration may then be expressed as nmol/g Hb. When the % COHb is calculated, it is important to understand that this measure is not exact, since the molecular mass of haemoglobin is not exact due to genetic differences. In the cross validation experiment, the small systematic difference and large variability between the two methods depends on limitations in the spectrophotometric method, as previously described by Johansson and Wollmer [4]. They concluded that the GC method is more stable and reliable. Furthermore, they conclude that the spectrophotometric method underestimates the COHb concentration relative to the GC method. This was also demonstrated in the present study.

The storage of samples has been studied in some of the above publications. Many assays used heparin or EDTA when the blood was sampled [7,10,11]. We found that when using heparin the samples coagulated after some time at +4°C. When using sodium citrate dihydrate instead, this problem did not occur, and our samples were stable for at least 35 days. One article studying the stability of CO in post-mortem blood [12] concluded that if stored in gastight containers, samples were stable for at least 4 months in both room temperature and at +4°C.

5. Conclusion

We have developed a method with very high sensitivity (LOD = 0.01 nmol/ml gas and LOQ = 12 nmol/ml blood) and good accuracy (RSD = 1.5%), making it suitable for the measurement of CO produced endogenously. The method should also be possible to use for assays of CO in different tissues. To calibrate these low levels we have developed a method for standard samples with excellent linearity ($r^2 = 0.9995$). Storage of the samples can be done for at least 35 days at +4°C if they are collected in sodium citrate dihydrate.

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