Carboxyhemoglobin

Application	Determination in blood
Analytical principle	Gas chromatography
Completed in	June 1975
Revised in	May 1983

Summary

This method is well-suited for the determination of carboxyhemoglobin concentrations that occur ecologically as well as those caused by the work environment. For the determination of carboxyhemoglobin concentrations below 5%, it is superior to all methods based on other analytical principles [3] and should, therefore, be the method of choice for ensuring that the BAT value (Biologischer Arbeitsstoff-Toleranzwert, Biological Tolerance Value for Working Materials) is not exceeded.

The hemoglobin-bound carbon monoxide is released with potassium ferricyanide(III). It is separated from the other constituents of the blood sample on a gas chromatograph, catalytically converted to methane and measured sensitively using a flame ionization detector. The analytical determination is based on calibration curves from blood with known carboxyhemoglobin content. The detection limit of the method is about 0.17 % carboxyhemoglobin. At a carboxyhemoglobin concentration of 5 % (BAT value) the between-day imprecision of 8.8 % satisfies all requirements.

Within-series imprecision:	Standard deviation (rel.)	s = 2.5 %
	Prognostic range	u = 5.6 %
	At a concentration of 5.07 % carboxyhemoglobin and where n = 10 determinations	
Between-day imprecision:	Standard deviation (rel.)	s = 8.8 %
	Prognostic range	u = 18.4 %
	At a concentration of 5% carboxyhemoglobin and where n = 20 days and with the use of an external standard (see Sect. 10)	
Inaccuracy:	Recovery rate	r = 101%
Detection limit:	0.17 % Carboxyhemoglobin	

Carbon monoxide, CO

Carbon monoxide (molar mass 28 g · mol^{-1}) is a colorless, tasteless, odorless and extremely toxic gas. It is lighter than air and forms an explosive mixture with air. Carbon monoxide is produced by the incomplete combustion of organic substances, i.e., carbon compounds such as wood, coal, petroleum and petroleum products like gasoline. Because of the widespread use of carbon compounds as fuel, carbon monoxide is a ubiquitous harmful substance in industrialized countries.

Industrially it is produced in vast quantities by the coking of coal to produce coal gas in a blast furnace process or the like. Carbon monoxide finds special application, for example, in the manufacture of nickel or metal carbonyls.

Due to its high toxicity carbon monoxide holds special significance for environmental and occupational health. The toxicity of carbon monoxide is based on the fact that its affinity for hemoglobin in the blood is 200–300 times higher than that of oxygen. Therefore, the hemoglobin binds preferentially to the carbon monoxide to form carboxyhemoglobin and is no longer available for the transport of oxygen. Carbon monoxide concentrations in the air of 1000–10 000 mL/m^3 lead to loss of consciousness and death due to oxygen starvation. An atmospheric carbon monoxide concentration of 50 mL/m^3 produces an average of 7 % carboxyhemoglobin in the blood [1] and is sufficient to cause mild headaches. The blood of nonsmokers contains about 1 % carboxyhemoglobin, whereas that of smokers contains between 2 % and 18 %.

In 1981, the MAK value (Maximum Concentration Value at the Workplace) for carbon monoxide was set at 30 mL/m^3. No injury to the health of exposed individuals is expected, as long as the carboxyhemoglobin concentration in the blood does not exceed the BAT value (Biological Tolerance Value for Working Materials) of 5 % [2].

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Contents

1 General principles

The carbon monoxide is released from the hemoglobin by potassium ferricyanide(III), separated from other sample constituents on a molecular sieve column, reduced to methane by hydrogen with a nickel catalyst and determined in a flame ionization detector. Blood samples containing the same amount of hemoglobin but different known amounts of carboxyhemoglobin are analyzed to set up a calibration curve. The values from such a calibration curve for the carboxyhemoglobin concentration of different blood samples are adjusted according to the hemoglobin content of the sample. The hemoglobin content of each blood sample must be determined at the same time.

2 Equipment, chemicals and solutions

2.1 Equipment

Gas chromatograph with flame ionization detector
10-mV Chart recorder
Heating jacket
Transformer
Gas chromatographic columns (see Sect. 5)
Photometer with filter Hg 546 nm or spectrophotometer
10 mm Cuvettes
250 µL Hamilton syringe, gas-tight
20 mL "Head-space sample flasks" and special forceps for putting on and taking off the PTFE-coated caps (e.g., from Perkin-Elmer)
Magnetic stirrer with holder for the sample flasks
PTFE-coated bar magnets
10 mL Tonometer flask (e.g., from L. Eschweiler, Kiel)
Electric motor
Roller mixer (e.g., from Denley, Sussex, U. K.)
1 mL Graduated pipettes
0.5, 1, and 5 mL Volumetric pipettes
Set of automatic pipettes, adjustable in the ranges 20–200 µL and 200–1000 µL (e.g., Pipet-man from Abimed)
Rotary evaporator
Desiccator
Sand bath
Shaker
Waterbath

2.2 Chemicals

All chemicals must be of analytical grade.
Ammonium oxalate
Potassium ferricyanide(III)
Nickel(II) nitrate
Potassium hydroxide
Methanol
Potassium cyanide
Sodium bicarbonate
Linde molecular sieve 5 Å, 30–80 mesh
Chromosorb G/AW, 80–100 mesh
0.01 % Carbon monoxide in nitrogen (e.g., Linde Plastigas)
Carbon monoxide (> 98.6 %) in a steel cylinder with a fine-control valve
Synthetic air (80 % purified nitrogen, 20 % oxygen)
Nitrogen (99.999 %) in a steel cylinder with a fine-control valve
Hydrogen (99.90 %)
Double-distilled water

2.3 Solutions

About 10 mL whole blood for the preparation of calibration standards
30 % Aqueous potassium ferricyanide(III) solution (Can be kept for 14 days at room temperature in a dark bottle.)

Cyanohemiglobin reagent solution according to *Drabkin* [4]: An aqueous solution of 0.607 mmol/L potassium ferricyanide(III) p.a., 0.767 mmol/L potassium cyanide p.a., and 11.9 mmol/L sodium bicarbonate p.a. (e.g., in Testpack Asid)

Cyanohemiglobin standard solution:
79.6 mg Hemiglobin cyanide in 100 mL water, corresponds to 200 g/L hemoglobin in blood (e.g., in Testpack Asid)

2.4 Calibration standards

The preparation of the stock solution and the calibration standards requires about 10 mL venous blood to which 50 mg ammonium oxalate has been added as an anticoagulant. Blood of an unexposed nonsmoker should be preferred. The hemoglobin content of this blood sample (ρ_c) is determined as described in Sect. 6.
Stock solution:
The stock solution is blood, 100 % saturated with carbon monoxide. It is prepared by swirling 4 mL blood in a tonometer flask (10 mL volume) with an electric motor for 30 min under a stream of carbon monoxide (0.8 L/min). A stream of nitrogen (1 L/min) is then passed over this blood for 5 min in a 37°C waterbath to remove the excess, physically dissolved carbon monoxide.

Our studies show that the carboxyhemoglobin content of blood prepared in this way attains a constant level.
A round-bottomed flask of 10 ml volume may be substituted for the tonometer flask for the CO-saturation of blood if the contents are stirred with a magnetic stirrer.
Calibration standards:
Calibration standards in concentrations ranging from 0% to 40% carboxyhemoglobin are prepared by diluting the CO-saturated blood with appropriate amounts of untreated whole blood. Immediately after the treated and untreated blood is pipetted into sample flasks that have been flushed with nitrogen gas, the flasks are sealed and the blood is mixed thoroughly on a roller mixer for at least 30 min. The pipetting schedule is as follows:

Stock solution (100 % CO-Hb) Volume	Untreated whole blood Volume	Added carboxy-hemoglobin content of calibration standard
µL	µL	%
50	1000	4.8
100	1000	9.1
200	1000	16.7
300	1000	23.1
700	1000	41.2

3 Specimen collection and sample treatment

A venous blood specimen (about 1.2–1.5 mL) is taken with a disposable syringe and mixed well with 20 mg of the anticoagulant ammonium oxalate in a sample vial that has been flushed with nitrogen gas. Blood specimens can be kept at least 5 days at room temperature but should be deep-frozen if longer storage is necessary. In any case the hemoglobin content of the blood should be determined before storage (see Sect. 6).

For the carboxyhemoglobin analysis 0.5 mL of the blood sample is pipetted into a second sample vial that has also been flushed with nitrogen gas. To this is added 0.3 mL 30% aqueous potassium ferricyanide(III). After a PTFE-coated bar magnet has been placed in the sample flask, it is sealed and the sample is mixed for 1 h at room temperature with a magnetic stirrer.

4 Operational parameters for gas chromatography

Figure 2 is a schematic representation of the gas chromatographic determination of carbon monoxide. The operational parameters as well as the instrumental settings can be summarized as follows:

Gas chromatograph

Detector:	Flame ionization detector (FID)	
Temperatures:	Injection block	100 °C
	Detector	300 °C
Carrier gas:	Purified nitrogen (25 mL/min)	
Detector gases:	Synthetic air (250 mL/min)	
	Hydrogen (30 mL/min)	
Sample volume:	150 µL	

Gas chromatographic columns

Material:		Stainless steel
Inner diameter:		4.0 mm (1/4 in)
Pre-column:	Temperature	100 °C
	Packing	KOH on Chromosorb G/AW, 80–100 mesh
	Length	20 cm
Separation column:	Temperature	100 °C
	Stationary phase	Linde Molecular Sieve 5 Å, 30–80 mesh
	Length	2 m
Catalytic column:	Temperature	300 °C
	Packing	Ni on Chromosorb G/AW, 80–100 mesh
	Length	20 cm

The pre-column contains potassium hydroxide on Chromosorb G/AW. It is made by mixing the Chromosorb and a saturated methanolic solution of potassium hydroxide in a 2 to 3 ratio and removing the solvent using a rotary evaporator. The material is then dried in a desiccator.

To make the catalytic column, Chromosorb G/AW is placed in an aqueous solution of nickel nitrate and the mixture is shaken for 60 min. The material is then separated from the aqueous phase by filtration under reduced pressure and dried for 15 h at 120 °C and for 6 h at 400 °C in a sand bath.

5 Analytical determination

A 150 µL sample is drawn into a gas-tight syringe from the "head space" of the sample flask containing the prepared blood sample and injected into the gas chromatograph. After the carbon monoxide is separated from the other constituents, e.g., carbon dioxide and water, it is reduced with hydrogen to methane on the catalytic column and determined with the flame ionization detector.

6 Hemoglobin determination

The hemoglobin content of each blood sample, which is necessary to calculate the carboxy-hemoglobin content, is determined photometrically after the hemoglobin has been converted to cyanohemiglobin. This method is now used generally, primarily because of the high stability of the pigment.

A 0.02 mL blood sample is mixed well with 5 mL cyanohemiglobin reagent according to *Drabkin* [4] (see Sect. 2.3) and is left at least 20 min at room temperature before its extinction (E_S) is measured at 540 nm. The hemoglobin content of the sample (ρ_S) is calculated in g/L in relation to the extinction (E_{St}) of the cyanohemiglobin standard solution according to the following equation:

$$\rho_S = \rho_{St} \frac{E_S}{E_{St}}$$

where ρ_s = the hemoglobin mass concentration of the blood sample in g/L
ρ_{St} = the corresponding hemoglobin mass concentration of the cyanohemiglobin standard solution in g/L
E_s = the extinction of the blood sample at 540 nm
E_{St} = the extinction of the cyanohemiglobin standard solution (see Sect. 2.3) at 540 nm

Alternatively the hemoglobin content of the sample can be read directly from a calibration curve composed of the extinction values for different hemiglobin concentrations. Such a curve can be obtained by diluting the cyanohemiglobin standard solution with different amounts of cyanohemiglobin reagent solution according to *Drabkin.*

7 Calibration

The calibration standards (see 2.4), which have been subjected to the same treatment as the blood samples, are analyzed gas chromatographically in parallel with several samples of the same blood that has not been treated with carbon monoxide. The peak heights,

corresponding to the different carboxyhemoglobin concentrations, are measured and the mean peak height for the untreated whole blood is deducted from these values. The difference is plotted as a function of the added carboxyhemoglobin concentration (%) to give the calibration curve. An example is shown in Fig. 1.

8 Calculation of the analytical result

The carboxyhemoglobin content in percent that corresponds to the measured peak height of the blood sample is read from the calibration curve that gives peak height as a function of carboxyhemoglobin content.

In order to take into account the hemoglobin concentrations of both the blood used for the calibration standards (ρ_c) and the sample blood (ρ_s), the values given by the calibration curve for the carboxyhemoglobin content of the sample blood are corrected according to the following equation:

$$x_{corr} = x \frac{\rho_s}{\rho_c}$$

where x = the value given by the calibration curve for the carboxyhemoglobin content of the sample blood in %
x_{corr} = the corrected value for carboxyhemoglobin content of the sample blood in %
ρ_s = the hemoglobin mass concentration of the sample blood in g/L
ρ_c = the hemoglobin mass concentration of the blood used for calibration in g/L

This gives the actual percentage of carboxyhemoglobin in the sample blood.

9 Standardization and quality control

To compensate for calibration errors that could occur in routine operation and to avoid having to repeatedly set up calibration curves, a gas standard (100 mL/m^3 carbon monoxide in nitrogen; Linde Plastigas) is measured with every analytical series as well as when a calibration curve is measured. For this gas standard a virtual "carboxyhemoglobin content" is set on the basis of the calibration curve. If the results of subsequent analyses deviate from the expected value, the results for the blood samples in that series are corrected accordingly. This correction has proved very useful for operations carried out over a longer period of time.

To maintain a check on quality as required, for example, by the guidelines of the Bundesàrz-tekammer (German Medical Association) [5], a blood sample with known carboxyhemoglobin content is analyzed with every analytical series as a precision control.

The accuracy of the method is checked by analyzing a blood sample with known carboxyhemoglobin content with every fourth analytical series and keeping a record of these results. A larger supply of blood samples with known carboxyhemoglobin content to be used for quality control can be prepared and stored in the deep freeze.

10 Reliability of the method

10.1 Precision

To determine the within-series imprecision blood with a mean carboxyhemoglobin content of 5.07% was analyzed ten times to give a relative standard deviation *(s)* of 2.5 % and a corresponding prognostic range *(u)* of 5.6 %.

To determine the between-day imprecision, a blood sample containing 5 % carboxyhemoglobin was analyzed on 20 days over a period of 3 months. Using the standardization described in Sect. 9, it was possible to achieve a relative standard deviation of 8.8 % and a prognostic range of 18.4 %. Without this standardization the corresponding values were s = 18.5 % and u = 38.7 %.

10.2 Accuracy

To determine the inaccuracy of the method, recovery experiments were carried out on venous blood containing 5 % carboxyhemoglobin, which had been prepared as described above. The carboxyhemoglobin determination was performed ten times on this blood to give a mean recovery rate of r = 101 %.

10.3 Detection limit

Under the given analytical conditions the detection limit was 0.17 % carboxyhemoglobin, calculated as the mean reagent blank value plus three times its standard deviation.

10.4 Sources of error

If the molecular sieve used for separation is overloaded with water or carbon dioxide, the carbon dioxide causes a rise in the baseline. Should this happen, the gas chromatographic separation column must be regenerated overnight by heating. In contrast to *Grieder* and *Buser* [6] in our investigations on blood samples we have not found it necessary to regenerate column packing even with continuous use over several months. This may be due to our use of a pre-column containing potassium hydroxide.

Conceivably an error in measurement could be introduced by contamination of the sample with atmospheric carbon monoxide. This error is avoided by flushing the sample flasks with nitrogen. Furthermore, since the carbon monoxide concentration normally

found in the environmental air (up to 20 mL/m^3) is five to ten times less than the carbon monoxide concentration in the sample flasks due to the carboxyhemoglobin content of the blood, this error should be negligible.

11 Discussion of the method

The method described here using gas chromatography to determine the carboxyhemoglobin content of blood [7] is also applicable for measuring concentrations in the ecological range due to its high sensitivity and low detection limit as well as its selectivity. These advantages make it superior to all methods that are based on other analytical principles [3]. We modified and optimized this method, which is based on specifications given in the literature [8–10], primarily with regard to its practicability for epidemiological investigations and for its analytical reliability. We were, thus, able to eliminate the drawbacks described in the literature for other gas chromatographic analyses of carbon monoxide [10–12]. Moreover, the calibration procedures given in the literature do not always satisfy today's qualitative requirements. The analysis is not very complicated, and up to 50 carboxyhemoglobin determinations can be performed daily. The relatively time-consuming step of releasing the carbon monoxide from the blood, as well as the long analysis duration caused by the retention time of 5.3 min, do not require individual attention and, thus, scarcely interfere with the course of routine operation.

In the evaluation of the chromatograms, peak height can be used instead of peak area without any loss of precision as long as the retention time is constant.

Instruments used:
Gas chromatograph 420 from Becker Delft

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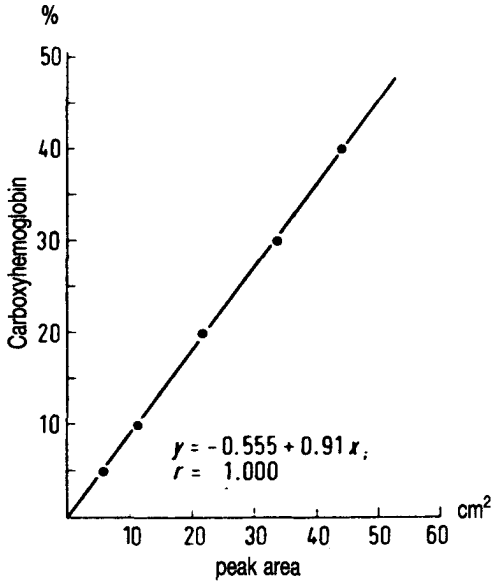

Fig. 1. Calibration curve for the determination of carbon monoxide in blood.

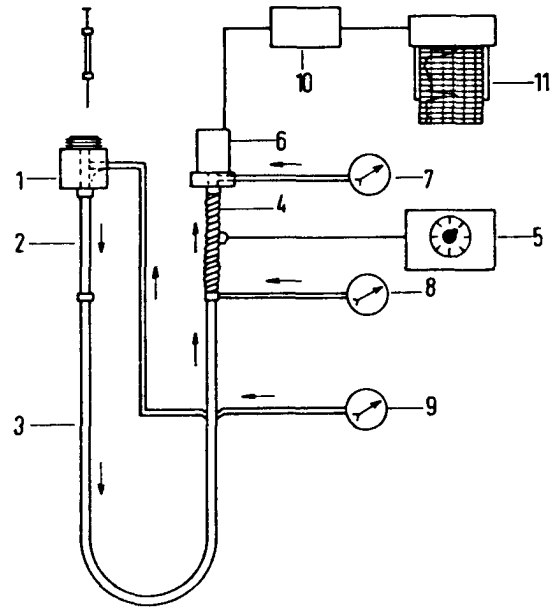

Fig. 2. Schematic representation of the gas chromatographic determination of carbon monoxide (1 injector, 2 pre-column, 3 separation column, 4 catalytic column with heating jacket, 5 transformer, 6 FID, 7 synthetic air, 8 hydrogen, 9 nitrogen, 10 amplifier, 11 recorder).