Rapid Determination of Carboxyhemoglobin in Postmortem Blood using Fully-Automated Headspace Gas Chromatography with Methaniser and FID

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Abstract: A new method, combining fully-automated headspace gas chromatography with methaniser and flame ionisation detector (FID) is introduced to determine carboxyhemoglobin (COHb) in postmortem blood samples. This highly automated procedure utilises a robot-like autosampler for reproducible mixing and thermostating (30 min at 50°C) during carbon monoxide (CO) liberation of COHb. Apart from transferring the blood sample and CO liberating solution (saponin (15 g/L) in 1 M sulphuric acid), all steps are performed without manual intervention. After headspace injection and gas chromatographic separation, the CO is reduced by a nickel catalyst to methane, which is then detected by using FID. The COHb saturation of the sample is calculated as percentage of a 100% carboxylated sample as follows: COHb [%] = Area (Original Sample) * 100 / Area (100% carboxylated sample).

The method was shown to be precise with coefficients of variation between 1.2 and 5.0%. Linearity was obtained from 0.5-100% COHb with excellent correlation (R=0.998). The applicability of the procedure was proven by analysis of postmortem blood samples and the results were compared to those of the standard photometric procedure. The method is especially applicable when postmortem blood samples have decomposed or their hemoglobin composition has been changed by thermal stress.

Keywords: Carboxyhemoglobin (COHb), postmortem blood, headspace gas chromatography, methaniser, flame ionisation detector.

INTRODUCTION

Endogenous carbon monoxide is produced by the catabolism of hem and results in a background carboxy-hemoglobin (COHb) saturation of 0.4-0.7% in resting healthy subjects [1]. The gas is a ubiquitous product of incomplete combustion of materials containing carbon [2], and many accidental poisonings are due to faulty domestic heating appliances [3,4]. Car exhaust fumes can contain up to 10% of carbon monoxide and deliberate self-exposure to these in an enclosed space remains a common and very effective form of suicide [1,5,6].

Carbon monoxide has an affinity for hemoglobin that is approximately 220 times that of oxygen. It displaces oxygen from oxyhemoglobin and at the same time changes the allosteric structure of hemoglobin in such a way that the affinity of the remaining sites for bound oxygen increases. As a consequence, the oxygen-carrying capacity of the blood is reduced and the release of oxygen (and therefore its delivery) to the tissue is inhibited, leading to progressive asphyxia.

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It is a routine procedure for forensic toxicologists to examine blood samples from fire victims for COHb content. Whereas a saturation level of >50% indicates carbon monoxide poisoning as the primary cause of death, levels of 10-50% show that smoke was inhaled, carbon monoxide could have been a contributing factor to death and prove beyond doubt that the deceased was alive when the fire started. If the value is below 10%, either the individual was dead before the fire began or died shortly afterwards. This information can be quite crucial in cases of civil litigation and in criminal investigations [7].

Standard procedures for the determination of COHb have concentrated on spectrophotometric methods designed to estimate the percentage of hemoglobin that has combined with carbon monoxide to produce COHb [8,9]. These measurements can be unreliable in old or postmortem blood samples due to the spontaneous production of methemoglobin and sulphemoglobin. Blood samples that have been subjected to great heat can show gross changes, notably thermocoagulation resulting in a significant decrease in total soluble hemoglobin and the appearance of methemoglobin [10,11].

For these reasons, it is theoretically preferable to use techniques that release carbon monoxide from the blood sample and allow the concentration of the gas itself to be measured. In this work, the CO liberating procedure of *Sundin and Larsson* [12] using sulphuric acid and saponin (a

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detergent that causes hemolysis even in low dosages) is applied, for the first time fully automated with a robot-like autosampler. The subsequent analysis uses gas chromatography with a FID-nickel catalyst.

MATERIALS AND METHODS

Sample Preparation

The sample preparation was based on the method of *Sundin and Larsson* [12]. The liberating solution was prepared by adding 7.5 g of saponin, taken from quillaja bark, to 473 mL of water and 27 mL of sulphuric acid. 400 µL of blood were transferred into a 20 mL headspace vial, which was rapidly closed with a silicon/teflon septum and a magnetic cap. Two syringe needles were used to purge the sample with nitrogen for 5 minutes. Afterwards 800 µL of the liberating solution were added.

For the calculation of the COHb saturation a 100% saturated sample had to be prepared. Therefore another sample aliquot of 400 µL was placed in a 20 mL headspace vial and closed similar to the samples. Again two syringe needles were applied and the blood was saturated with CO for 30 minutes followed by purging with nitrogen for 5 minutes. Again 800 µL of liberating solution were added with a syringe needle through the septum.

For the calculation of the COHb saturation the method of *van Dam and Daenens* [13] was used:

COHb [%] = Area (Original Sample) · 100 / Area (100% carboxylated sample)

GC/FID-Method

For the analysis an Agilent 6890 gas chromatograph (Agilent, Waldbronn, Germany) was used. The system was equipped with a CTC-CombiPAL auto sampler. The samples were mixed (at 450 rpm) for 30 minutes by an agitator prior to the analysis to achieve a complete CO liberation. 400 µL of each sample were injected with a headspace syringe (heated at 50°C) applying a split/split less injector with closed split (injector temperature 210°C). The gas chromatographic separation was performed with a 50 meter PLOT fused-silica column with an inner diameter of 0.53 mm and a molecular sieve coating of 5Å (Varian). Helium was used as a carrier gas, using a constant flow of 12 mL/min. The temperature program consisted of the following steps: 80°C for 5 min followed by 300°C for 15 min achieved by a heating rate of 20°C/min. The post-column nickel catalyst was operated with a hydrogen addition of 5 mL/min at a temperature of 375°C. The catalyst converts CO to CH_4, which is detected using a flame-ionisation-detector at a temperature of 320°C.

Spectrophotometric Method

For the validation of the gas chromatographic method all samples were examined using the photometric standard method. The two wavelength method for spectrophotometers without isobestic wavelength in accordance to *Hüfner, Heilmeyer, Schwerd and Schwemmer* [14] was applied. For comparison, we used 20 samples of authentic forensic cases of which, due to suspicion of CO intoxication due to fires, blood samples were stored for the COHb determination. These samples were stored at 4°C and actually analysed using the spectrophotometric method before applying the gas chromatographic procedure.

RESULTS AND DISCUSSION

The earlier mentioned CO liberating conditions were determined by optimisation of the agitator temperature and

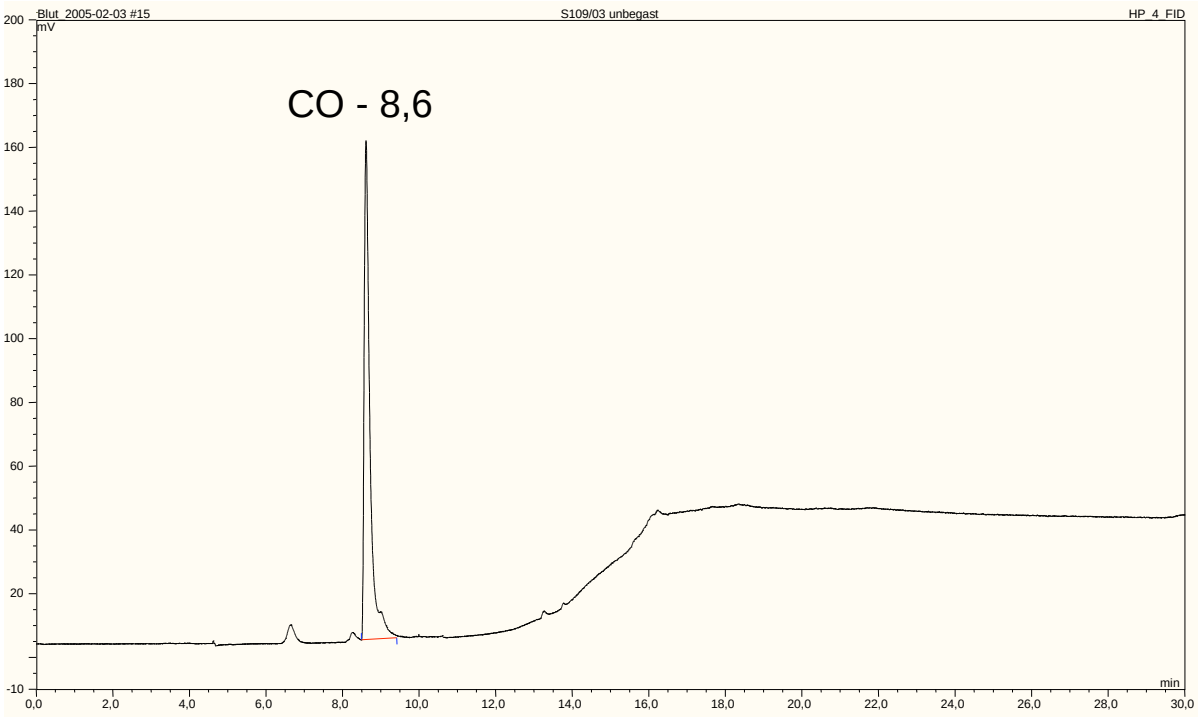

Fig. (1). Chromatogram of an authentic postmortem blood sample with a COHB content of 2.0%.

the time of liberation. The time of liberation was varied between 5 and 80 minutes. As a matter of fact neither a significant difference in the CO yield could be found, nor could a trend be seen.

Therefore, it can be assumed that already a short time of exposure to the saponin/sulphuric acid leads to a complete liberation of CO. Furthermore, the temperature of liberation was examined between 40 and 70°C. No significant influence on the CO yield was observed. *Sundin and Larsson* proposed no heating at all (i.e. measurement at room temperature). However, this approach leads to longer times of liberation [12]. To achieve an assured and reproducible release, a liberation time of 30 min and a temperature of 50°C were chosen.

Figs. (**1** and **2**) show chromatograms of authentic post mortem blood samples typically obtained by the described method. The CO peak is clearly visible and no peaks of impurities were observed during the analysis. It was found that methane was separated easily from the CO peak. It was also found that neither O_2 nor N_2 resulted in any response from the detector using the present chromatographic conditions. The peak purity and selectivity are therefore secured by the chromatographic separation of all possible interfering compounds.

Because of the high sensitivity for CO on the nickel catalyst system it was possible to measure even low levels with good accuracy. The method was shown to be precise with coefficients of variation between 1.2-5.0% (Intraday) and 3.4-11% (Interday) (Table **1**). Linearity was checked by the analysis of blood dilutions of varying COHb concentrations in the range of 0.5-100% COHb. An excellent correlation (Pearson correlation coefficient R=0.998) was obtained. The validation data prove the selectivity, sensitivity and reproducibility of the method.

Table 1. Results of Validation

COHb Saturation	2.5%	10%	40%	100%
Intraday precision [%][a]	1.2	4.5	5.0	2.3
Interday precision [%][a]	3.4	7.9	7.0	11.0

[a] Precisions are calculated as coefficient of variation (n=6 on one day or on six different days).

The applicability of the procedure was proven by analysis of post mortem blood samples and the results were compared to those of the standard photometric procedure. A very good correlation of R=0.917 was achieved (Fig. **3**). Especially in the analysis of highly decomposed samples where no results could be obtained with the photometric method the gas chromatographic procedure provided results. In these samples COHb contents of 0.4 and 4.5% were detected. In this respect, we confirm the headspace GC/MS results of *Oritani et al.* [15].

However, our results must be read in the light of two possible methodological limitations to our study. First, we have not conducted a reduction of methemoglobin as proposed in some studies [16-18], but have complied to the protocol of *Sundin and Larsson* [12], who did not use a reduction. As the good correlation with the reference procedure demonstrates, the approach without reduction has proved to be suitable for our sample collective of postmortem cases. Reduction agents such as sodium dithionite may present problems like interferences with the binding of CO during saturation if used at higher concentrations [16]. Therefore, we think that this approach should only be used in special cases (e.g. aviation accident victims) but not in standard forensic cases. Second, the method relies on full saturation of the sample with CO. While there is a lack of

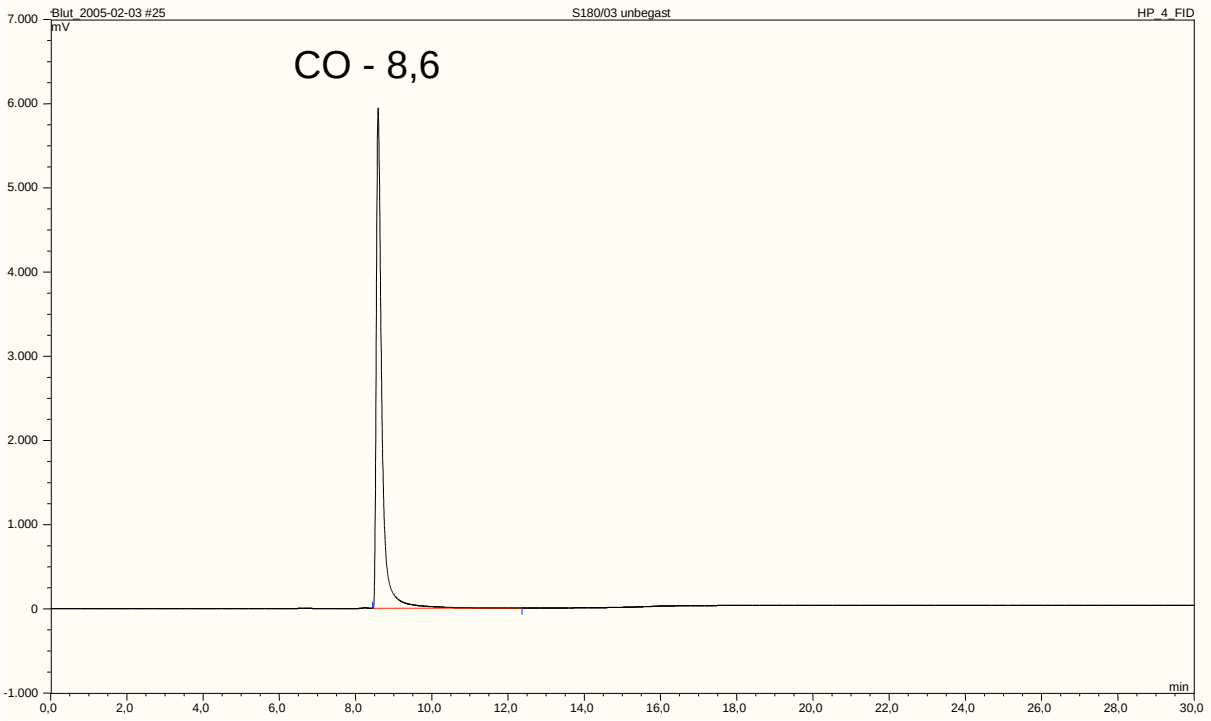

Fig. (2). Chromatogram of an authentic postmortem blood sample with a COHB content of 90.6%.

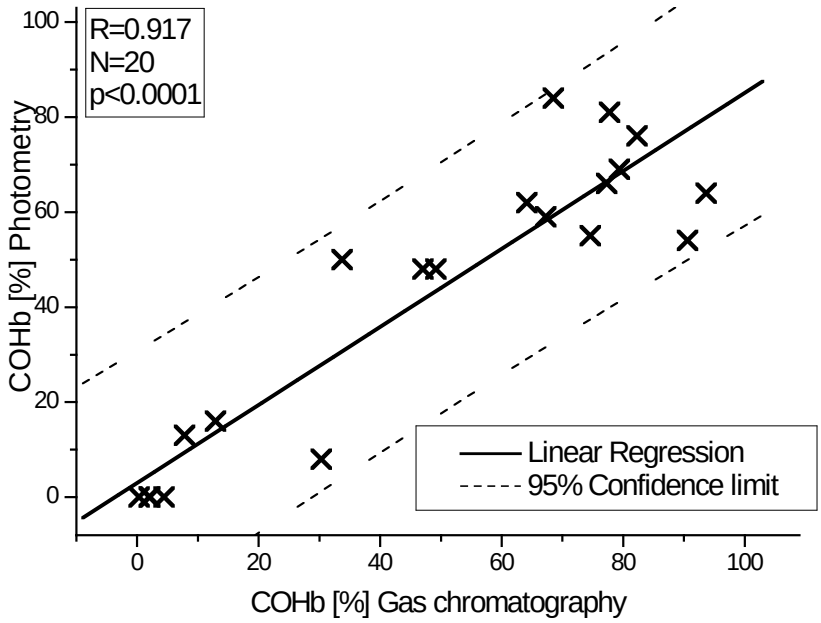

Fig. (3). Correlation between the gas chromatographic and spectral photometric results of 20 postmortem blood samples.

literature on this question, it may be that highly degraded samples do not retain their full capacity to bind CO leading to lower saturated values concurrently influencing the result. However, the comparison with the photometric procedure shows that this influence appears as being negligible, at least in our sample collective. Clearly, the observation of *Levine et al.* [19] that COHb can be influenced by the analytical methods used is still valid and must be critically considered in every expert opinion on CO intoxication.

Besides post-mortem cases, the method might also be usable for other purposes, e.g. to study the CO binding kinetics during smoking as preliminary results show (Fig. **4**). In conclusion, the developed method presents an easy alternative to spectrophotometric techniques. The theoretical advantages arise when postmortem blood samples have decomposed or their hemoglobin composition has been changes by thermal stress.

REFERENCES

[1] Widdop B. Analysis of carbon monoxide. Ann Clin Biochem 2002; 39: 378-91.

[2] Goldsmith JR, Landaw SA. Carbon monoxide and human health. Science 1968; 162: 1352-9.

[3] Thomassen O, Brattebo G, Rostrup M. Carbon monoxide poisoning while using a small cooking stove in a tent. Am J Emerg Med 2004; 22: 204-6.

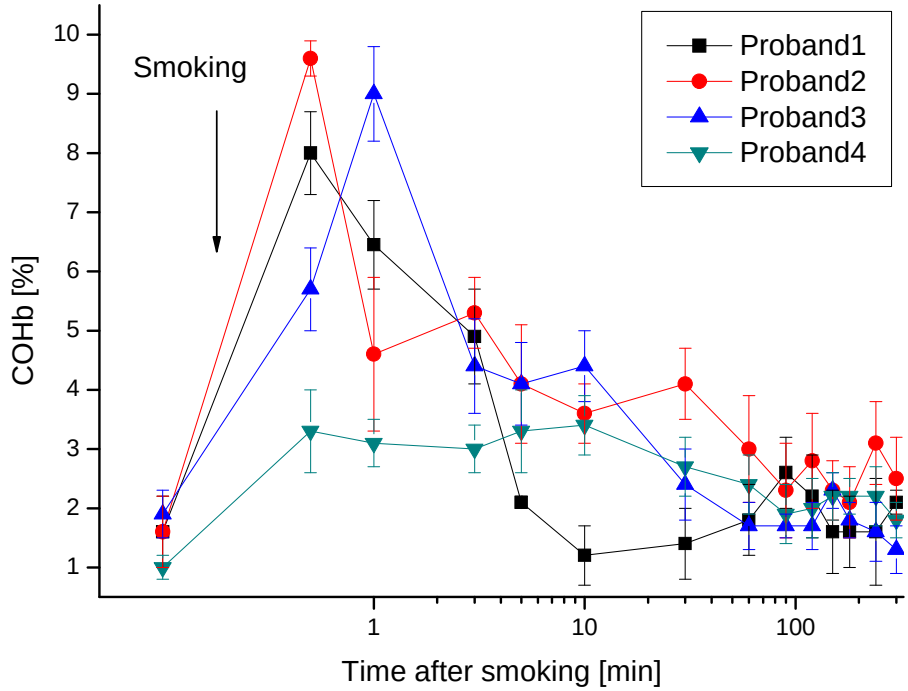

Fig. (4). COHb values after smoking. Blood was taken before smoking 5 cigarettes and in intervals up to 300 min after the last cigarette.

[4] Demirci S, Dogan KH, Erkol Z, Gunaydin G. Two death cases originating from supplementary heater in the cabins of parked trucks. J Forensic Leg Med 2009; 16: 97-100.

[5] Griffin SM, Ward MK, Terrell AR, Stewart D. Diesel fumes do kill: a case of fatal carbon monoxide poisoning directly attributed to diesel fuel exhaust with a 10-year retrospective case and literature review. J Forensic Sci 2008; 53: 1206-11.

[6] Thomsen AH, Gregersen M. Suicide by carbon monoxide from car exhaust-gas in Denmark 1995-1999. Forensic Sci Int 2006; 161: 41-6.

[7] Madea B, Dettmeyer R, Musshoff F. Basiswissen Rechtsmedizin. Heidelberg: Springer 2007.

[8] Boumba VA, Vougiouklakis T. Evaluation of the methods used for carboxyhemoglobin analysis in postmortem blood. Int J Toxicol 2005; 24: 275-81.

[9] Luchini PD, Leyton JF, Strombech ML, *et al*. Validation of a spectrophotometric method for quantification of carboxyhemoglobin. J Anal Toxicol 2009; 33: 540-4.

[10] Klöppel A, Weiler G. Fäulnisbedingte Konzentrationsänderungen und zeitliche Nachweisbarkeit von Kohlenmonoxid in Leichenblutproben. Z Rechtsmed 1986; 97: 75-81.

[11] Iffland R, Madea B, Balling P. Diagnose Kohlemonoxidvergiftung nach Einbalsamierung und Exhumierung. Arch Kriminol 1988; 182: 101-6.

[12] Sundin AM, Larsson JE. Rapid and sensitive method for the analysis of carbon monoxide in blood using gas chromatography with flame ionisation detection. J Chromatogr B Anal Technol Biomed Life Sci 2002; 766: 115-21.

[13] Van Dam J, Daenens P. Microanalysis of carbon monoxide in blood by head-space capillary gas chromatography. J Forensic Sci 1994; 39: 473-8.

[14] Schütz H, Machbert G. Photometrische Bestimmung von Carboxy-Hämoglobin (CO-Hb) im Blut. Mitteilung VIII der Senatskommission der Deutschen Forschungsgemeinschaft für Klinisch-toxikologische Analytik Weinheim: VCH-Verlagsgesellschaft 1988.

[15] Oritani S, Zhu BL, Ishida K, *et al.* Automated determination of carboxyhemoglobin contents in autopsy materials using head-space gas chromatography/mass spectrometry. Forensic Sci Int 2000; 113: 375-9.

[16] Lewis RJ, Johnson RD, Canfield DV. An accurate method for the determination of carboxyhemoglobin in postmortem blood using GC-TCD. J Anal Toxicol 2004; 28: 59-62.

[17] Dubowski KM, Luke JL. Measurement of carboxyhemoglobin and carbon monoxide in blood. Ann Clin Lab Sci 1973; 3: 53-65.

[18] Dominguez AM, Halstead JR, Domanski TJ. The effect of postmortem changes on carboxyhemoglobin results. J Forensic Sci 1964; 9: 330-41.

[19] Levine B, D'Nicuola J, Kunsman G, Smith M, Stahl C. Methodologic considerations in the interpretation of postmortem carboxyhemoglobin concentrations. Toxicology 1996; 115: 129-34.

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