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Headspace GC-MS Detection of Carbon Monoxide in Decomposed Blood and Hepatic Tissues

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Abstract

Here in a systematic, accurate and reliable method, Head-Space Gas Chromatography-Mass Spectrometry (HS-GC/MS) was developed to determine blood carboxyhemoglobin (COHb%), in order to investigate deaths related to CO exposure especially involving blood and hepatic tissues. Using a column packed with molecular sieve, COHb levels were quantified down to 0.2% in small blood samples quickly and showed good reproducibility with RSD of the COHb <1%. COHb% in hepatic samples stored at different temperatures (-20 °C for 1- 2 years, 0 °C, and 18 °C for two months) can be determined even when the samples are decomposed. The 3-min procedure requires only 0.25 mL of blood sample or 1.0 g of hepatic tissue each time. The technique has a clear advantage over other methods such as UV spectrophotometry.

Keywords: Carbon monoxide poisoning; COHb; Decomposed hepatic tissues; Headspace gas chromatography/mass spectrometry (HS-GC /MS)

Introduction

Carbon monoxide (CO) exposure in humans is impossible to detect as it is colorless, tasteless, odorless, and nonirritating. When inhaled, CO is readily absorbed from the lungs into the bloodstream, where it forms a tight but slowly reversible complex with hemoglobin (Hb) known as carboxyhemoglobin (COHb). The presence of COHb in the blood decreases its oxygen-carrying capacity, reducing the availability of oxygen to body tissues, resulting in tissue hypoxia. A reduced oxygen delivery associated with elevated COHb level, exacerbated by impaired perfusion resulting from hypoxic cardiac dysfunction, potentially will impair cellular oxidative metabolism. In China, death due to CO poisoning was identified by elevated COHb levels (> 40%) in postmortem blood.

During the past twenty years, several methods for the determination of COHb levels in postmortem specimens have been published. These methods include UV and FTIR spectrophotometry [1-3], CO-oximetry [2,4], and Capillary Electrophoresis (CE) [5,6]. Although gas chromatographic techniques are more suitable for forensic materials [3,7,8], they are complicated, time-consuming and require larger samples when compared with CO-oximetry and GC/MS. However, CO-oximeters cannot detect less than 10% COHb content in putrid blood [4]. In China, several postmortem samples collected in suspected CO poisoning cases are decomposed and devoid of blood, as the deceased individuals were discovered days or months following death. In such cases, the quantification of COHb may be difficult for a toxicologist. In the present paper, the HS-GC/MS method is described for the determination of COHb in decomposed blood and to examine the CO content of the hepatic tissues extracted from cadavers in order to provide an experimental basis for the analysis of CO poisoning among deceased humans.

A study of 22 deaths resulting from unvented gas heaters revealed a mean COHb level of 49.5% in the victims' blood with a minimum value of about 30% and a maximum of 75% for COHb [9]. Another study examined the distribution of COHb among survivors (mean = 28.1%, n = 159) and fatalities (mean 62.3%, n = 101); the 50% survival probability was associated with approximately 50% of COHb [10]. The liver is the largest gland in human body and plays an important role in metabolism. It has complex functions including manufacturing bile, glucose metabolism, protein metabolism, fat metabolism, biotransformation, etc. Moreover, the liver stores blood and regulates blood volume. At rest or in emotional stability, massive blood is stored in the liver. At work or when excited, the hematic quantity rises, and massive blood supplies are released

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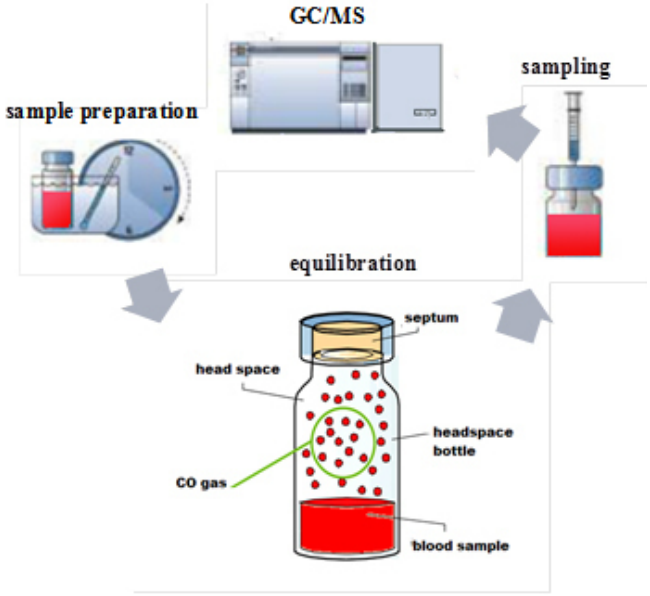

Figure 1: Experimental design and CO detection process

Materials and Methods

The protocols in this study were approved by Institutional Review Board and the Animal Care and Use Committee of China University of Political Science and Law, Ministry of Education (Beijing, China).

Experimental design

The static head-space technique is an indirect method for the analysis of volatile compounds in liquid or solid samples. Measurement of CO levels in the present study relied upon addition of lactic acid to blood samples in order to release CO. Samples were prepared as follows: blood (0.25 mL per vial) was transferred into head-space vials and 0.25 mL of lactic acid (25%) was added to each vial to release the CO. The vials were then heated on a heating block for 30 min at 50 ˚C, after which 200 µL of head-space gas (the vapor phase, in thermodynamic equilibrium with the sample) was extracted with a syringe for rapid analysis using gas chromatography-mass spectrometry (GC/MS). The combination of GC with MS provides a powerful analytical tool for the separation and identification of individual components of an organic solution and determination of the individual quantities of each of these

When the vapor pressure of a sample is relatively low, the chromatographic peak area (A_i) of a volatile component changes proportionally with its vapor pressure (P_i):

$$A_i = S_i P_i$$

where S_i is the sensitivity constant of component i. The partial vapor pressure may be expressed as:

$$P_i = P_i^0 X_i$$

where Pi0 is the vapor pressure of component i and X_i the mole fraction of the component in the sample. The chromatographic peak area A_i can then be rewritten as:

$$A_i = S_i P_i^0 X_i = P X_i$$

Then the percentage saturation of blood with CO was calculated by the ratio of the peak areas obtained from untreated blood (A_c) and CO saturated blood (A_s):

$$A_c/A_s \times 100\% = \text{COHb\%} \quad [8]$$

where A_c = Area of CO peak from untreated blood, and As = Area of CO peak from CO-saturated blood

$$\text{CO in liver}(\mu L) = \frac{\text{Peak Area of Hepatic Tissues}}{\text{Addictive Peak Area of Blank Liver}} \times 100$$

As the blood volume in liver is constant, it may be inferred that CO content in the liver and blood are correlated.

When dealing with the putrid blood in CO poisoning cases, in order to saturate hemoglobin CO was bubbled through the same blood from the deceased. This excludes the effects of variable Hb concentration when dealing with putrid samples which maybe showing low or high Hb concentrations due to unequal setting of blood [13].

Materials

10 rabbits were purchased from Shanghai Slack Corporation (Shanghai, China). Blood was obtained from the Blood Center of the Beijing Red Cross Society. Formic acid, CO gas (99.9%), sulfuric acid, and sodium hydroxide (to absorb gases produced from secondary reactions, such as SO2 and SO3) were purchased from Sinopharm Chemical Reagent Corporation (Shanghai, China).

Blood sample preparation and determination

Saturated blood preparation: Fresh blood (150 mL) was transferred into a conical flask (de-bubbling by n-butanol), and CO gas was gently bubbled through the blood for varying periods of time in order to saturate hemoglobin with CO. The minimum time required to achieve saturation was determined to be 30 min. Following the study of optimal N2 aeration time, a stream of nitrogen gas was passed through the blood for 5 min at a rate of 6-10 mL/min to remove the free CO in blood. The flask was then covered with a tight-fitting lid and shaken slowly for 30 min, after which the sample was transferred and preserved at 4˚C in an airtight container. Blood samples prepared as described above were considered to have a COHb saturation of 100% [7,8].

Blank blood preparation: Air was bubbled through 100 mL of fresh blood for 30 min followed by a stream of nitrogen gas for 5 min. The blank blood was then preserved at 4˚C in an airtight container.

Preparation of a concentration series of COHb blood: Appropriate volumes of COHb-saturated blood and blank blood were mixed to prepare of a series blood samples containing various COHb concentrations: 0% (blank blood), 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100%. These samples were preserved in sealed syringes and stored in airtight containers at 4°C, 17°C, and -20°C.

Headspace sample preparation: A 0.25 mL aliquot of blood was placed into a 10-mL head-space vial. To this was added 0.5 mL of distilled water and 0.25 mL of 25% lactic acid and the vial was covered immediately with an aluminum cap containing a rubber septum. The vial was then heated on a heating block for 30 min at 50˚C and 200 μL of gas was extracted for GC/MS analysis.

Determination the COHb linear range of blood: Head-space samples were prepared as above using 0.25 mL of blood from each of the following standard concentrations of COHb in blood: 0% (blank blood), 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100%. GC/MS analysis of each of these samples enabled the construction of a standard curve for COHb%.

Determination of CO in hepatic tissues

Experimental animal hepatic tissue: Healthy male rabbits (10) with similar weight were taken, No.1 and No10 rabbits were sacrificed by intravenous injection of air into ear margin; another 8 died of CO poisoning. The experiment was so designed that male rabbits inhaled CO (CO >1%, lasting for 30 min) in an exposure apparatus, i.e. a homemade glass box with CO gas inlet and outlet. Immediately after death, the blood and hepatic tissue samples were collected and analyzed for CO and COHb with HS-GC/MS. Finally, hepatic tissue was extracted (Figure 2).

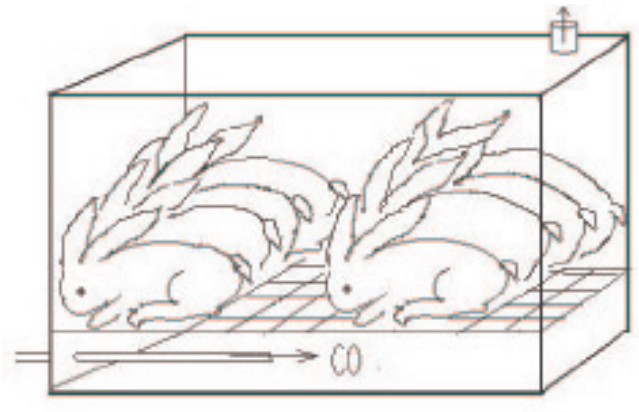

Figure 2: Testing equipment for CO poisoning of animals

It was observed that exposure to CO at 4600 to 5000 ppm and COHb levels greater than 60% for 30 min causes death of rabbits within minutes of exposure, the rabbits' eyes began twitching and their lips turned red. After 10 minutes later, the rabbits began to scream and 30 min later, the rabbits died one by one, with their lips and mouth open. After the dissection, 10-15 mL of the cherry-red cardiac blood was transferred into an airtight cuvette. Remaining organs were transferred into bags and sealed. Livers, which were used in the present study, and other organs stored as samples for use in later experiments.

Determination of CO in hepatic tissues: About 1.0 g of hepatic tissues was isolated, and subjected to similar analysis as described above. The determination of CO content in putrid liver tissues was as follows: the hepatic tissues were prepared as above and stored in sealed plastic bags at room temperature (15~17°C). On the 8th day blood on the liver surface darkened and the 35th day when the liver turned into mud with a terrible smell. Sample preparation and CO analysis was similar as described in the previous section. Preparation of saturated hepatic tissue entailed transferring 1.0 g blank liver into the hepatic tissue as described previously. About 100 μL sterling CO.

Stability of bloods COHb%: Three 0.25 mL portions of four different COHb blood concentrations (n = 3 for each temperature) were stored under airtight conditions at 4°C, 17°C and-20°C (Table 1).

GC/MS analysis

A Shimadzu GC/MS System Model QP 5000 was used as follows: (1) GC parameters: fused silica capillary column with Hp-plot, 30 m × 0.25 mm × 12 μm 5A Molecular sieve (Shimadzu); column temperature heated from 50 – 250 °C; injection temperature, 250 °C; carrier gas, He flow, 1.5 L /min, splitting ratio 10: 1; head-space injection. (2) MS parameters: mass range 10~60; scanning interval 0.5 s; acquisition time, 0.5~3 min; source temperature 200 °C. multiplier voltage, 1.35 kV; electronic energy, 70 eV.

Samples were prepared as follows: Blood (0.25 mL each) and hepatic tissues (1.0 g each) were transferred into headspace vials separately which released CO after addition of 25% lactic acid. The vials were then heated for 30 min at 50 °C, from which 200 μL headspace gas was extracted for accurate and rapid GC/MS analysis. The samples were then stored in test tubes with plugs at different temperatures (-20 °C, 0 °C, 18 °C) and COHb was determined after 7 days, 14 days, 30 days and 40 days. In each experiment, three samples were prepared in parallel (n = 3) and each sample was analyzed 5 times. Extra care was taken to ensure that each 200 μL extraction of headspace gas was carried out accurately and used rapidly for GC/MS analysis.

Comparison with UV method

To perform UV-VIS analysis, one drop of blood samples was added to a test tube containing 5.0 mL sodium hyposulfate solution and gently shaken (Table 2).

Results and Discussion

In this paper, a protocol was established for the determination of CO content in liver tissues. The stability of CO in blood was investigated and the experimental basis for these cases of CO poisoning was determined.

CO stability in blood

In 2000, Kunsman [11] reported that COHb levels in blood were stable for two years. In reality, blood in CO poisoning

Temperature	Time	Measured values of COHb in blood (mean)							
		10%		30%		50%		70%	
		HS-GC/MS	UV	HS-GC/MS	UV	HS-GC/MS	UV	HS-GC/MS	UV
4°C	1	10.0	10.0	30.1	30.3	49.7	50.7	70.0	70.0
	4	10.5	9.6	29.6	28.1	49.8	46.6	70.1	62.8
	14	9.6	---	28.1	28.8	48.6	48.1	68.8	33.6
	45	9.6	--	28.9	---	47.7	---	68.9	32.7
17°C	1	10.0	8.4	31.2	25.8	50.7	49.9	69.4	66.3
	4	10.7	---	29.9	---	49.5	35.5	68.4	28.6
	14	9.4	--	29.8	10.8	48.9	22.3	69.7	--
	45	9.8	--	28.5	--	49.1	--	69.5	--
-20°C	1	10.1	10.0	31.1	30.0	52.6	50.7	71.4	72.9
	4	10.4	10.0	30.9	30.3	51.1	50.7	69.2	60.0
	14	10.0	9.6	29.7	28.1	49.7	51.7	68.3	62.8
	45	9.1	---	32.5	---	54.6	56.6	72.9	33.6

*-- not detectable

Table 1: Stability of COHb under different storage conditions

Rabbits	COHb% (HS-GC/MS)			COHb% (UV method)
	0d	8d	35d	35d
1 (blank)	0.2	0.2	2.3	--
2	62.1	61.2	57.0	--
3	68.8	64.0	64.3	28
4	63.4	63.4	64.6	--
5	68.3	62.4	60.0	--
6	73.2	70.7	69.1	29
7	67.5	62.0	65.7	--
8	64.5	64.5	64.5	--
9	64.6	65.4	63.6	--
10(blank)	0.6	0.6	0.5	--

*-- not detectable, d = day

Table 2: Comparison of the saturation levels at room temperature

cases is left outside for extended periods of time before it is sent to the laboratory for analysis. Our research emphasizes on the stability of COHb under ambient conditions or low temperature (for instance, 0~4 °C or -20 °C). Standard CO blood was prepared at different concentrations (10%, 30%, 50%, and 70%), stored at room temperature (17~19 °C), refrigerated at 0~4 °C, or in cold storage at -20 °C. Table 1 shows the experimental results.

Based on the data shown in Table 1, it is concluded that both time and temperature affect the determination of COHb, with 4 °C being the most suitable temperature for storage. Under prolonged conditions of freezing, COHb in the blood sample appears to rise, possibly due to unstable or low temperatures.

At freezing temperatures below -30 °C, the level of blood methemoglobin (MetHb) increases. When it does not combine with CO the high levels of MetHb diminish the ability of CO complexation resulting in elevated levels of COHb [12].

The HS-GC/MS method offers a clear advantage over the UV method as seen from the data in Table 1. When the specimens were stored at room temperature, the COHb levels were reduced by about half: the original 70% COHb levels fall to 45.1%, which implies that it was not a case of CO-poisoning death, contrary to the facts.

Determination of CO content in liver tissues

The No.1 and No.10 rabbits died of intravenous injection of air into ear margin. Results from animal experiments of CO-poisoning death are shown in chart 2 revealing that the content of CO is sharply reduced with increased storage time of liver tissue. The reduced ratio needs further study.

The hepatic tissues were isolated on the day of the rabbit death. The samples from rabbit No.1-5 were placed at room temperature (15-17 °C); No.6, 7, 10 under freezing temperature; and those from No.8, 9 cryopreserved. All blood samples were in good condition at the baseline. They were cherry-red, and delaminated after stratification. After storage at room temperature for 2 days, the blood darkened and the sample hemolyzed. The blood turned into dark red after 45 days of preservation. When the blood was stored at 4 °C, no obvious change occurred in the first 8 days. After one month the color turned dark after which the sample became moderately hemolyzed 35 days later. When the blood was preserved at – 20 °C, no obvious change was seen within one month, however, it turned dark after one month and the sample was hemolyzed after 35 days. When the hepatic tissue was stored at normal temperature, the blood agglutinated to the surface, the tissue started to decompose, and the color became garnet. The liver became highly decomposed after 35 days turning into a greyish-green and malodorous mush (Figure 3, Table 2).

Comparison of HS/GC/MS and UV techniques for measurement of CO levels

The bodies of two people were found 4 days after death by CO poisoning. The blood samples were stored at room temperature for 15 days, and then transferred to our lab. The blood turned black-red and no results were available when they were analyzed by the UV method. However, using the HS/GC/MS method we developed, the COHb was detected as showed in Table 3 and Figure 4.

	Area of blood	Area of CO-saturated blood	Area of hepatic issues	Area of CO-saturated hepatic issues	Volume of CO in hepatic issues (μL)	COHb%	COHb%
A	249518	366275	219258	478256	49.8	65.1%	UV method
B	337065	513052	428105	956339	58.0	68.7%	--

*-- not detectable
Table 3: Specimen analysis

Area of blood	Area of CO- saturated blood	Area of hepatic issues	Area of CO-saturated hepatic issues	COHb%	Volume of CO in hepatic issues (μL)	COHb% (UV method)
310603	586305	996706	4010199	52.9%	33.1	28.1%

*-- not detectable
Table 4: Results of CO poisoning specimens after 2 years of cryopreservation.

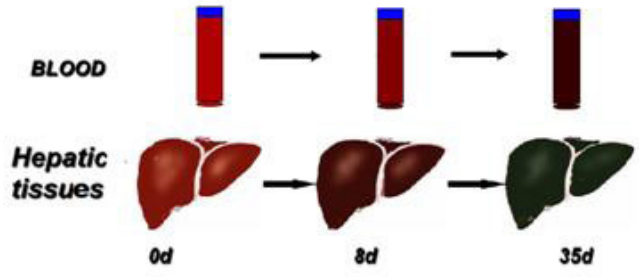

Figure 3: Change in samples at room temperature

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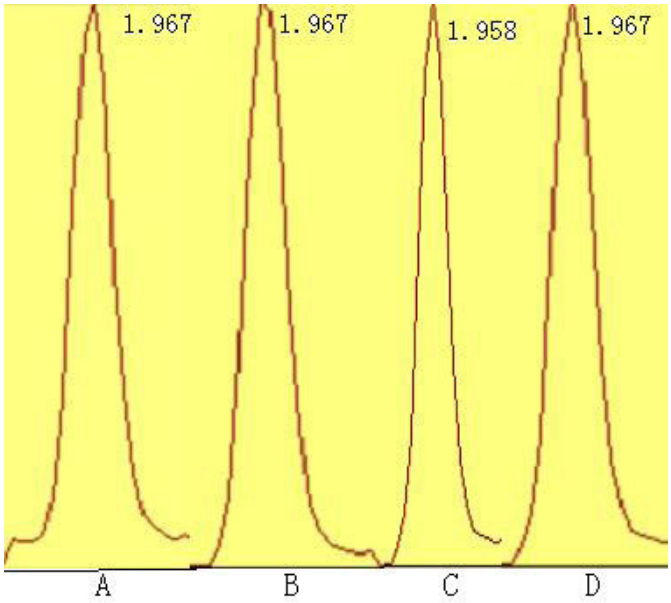

Figure 4: Mass chromatograms of the blood and hepatic tissue specimens

The blood samples from CO poisoning death (COHb% of 54%) were cryopreserved for two years and analyzed again by HS-GC/MS method as showed in Table 4.

In contrast, use of UV spectrometry provided values of COHb% that were well below 30%, and in the first case not detectable, which would not have supported CO poisoning as the cause of death in all individuals. The UV method is often used to detect CO concentrations in fresh blood samples, but may not be suitable for decomposed blood, where it may give values lower than the true levels. Thus, the HS/GC/MS technique may have significant advantages over the UV method, particularly when blood samples are not fresh.

Conclusion

HS/GC/MS is associated with high sensitivity regardless of the sample condition— fresh or highly decomposed. Using a packed molecular sieve column, CO levels were able to be quantitating down to 0.01% and COHb levels down to 0.2% in small blood (0.25 mL) and liver (1.0 g) samples. Our method showed that we can disregard the strong effect of storage temperature on the COHb% determination. The equipment is easily available since most labs already have gas chromatography and mass spectrometry instrumentation. In fact, manual injection may substitute for the absence of a headspace automatic sampler. The method has a much higher sensitivity than the commonly used techniques such as UV method.

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