DETERMINATION OF CARBON MONOXIDE IN BLOOD BY HEAD SPACE ANALYSIS

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Abstract.....A method for the determining the amount of carbon monoxide (CO) in blood is described. CO bound to hemoglobin is released with potassium ferricyanide into a known volume of head space of a disposable syringe. Vapor in the head space is withdrawn with a gastight microclyringe and injected into the gas chromatograph.

CO dissolved in saturated blood is also measured by head space analysis to ascertain that dissolved CO is able to be removed with only one shaking of blood with nitrogen (N₂).

N₂ put into the head space previously is used as internal standard. The advantages of this method lie in the ease and speed of operation with a simple and widely available instrument.

Key words: carbon monoxide in blood, dissolved carbon monoxide, head space gas analysis, gas chromatography

INTRODUCTION

The need for rapid and accurate measurement of CO in blood in forensic and clinical toxicology has led to development of a wide variety of techniques.

The methods for determination of CO in blood by gas chromatography are divided into two groups according to elution methods of the CO released from the blood.

One group is the method in which CO released into a known volume of space is transferred to the gas sampler and eluted to the gas chromatograph by switching a valve (Ayres et al., 1966; Hessel and Modglin, 1967; McCredie and Jose, 1967; Blackmore, 1970). The other group is the method which the reaction vessel is designed to be installed in the carrier gas line upstream from the separating column and all the released CO is made to flow with helium through the 4-way valve (Kakutani et al., 1965; Collison et al., 1968; Miyauchi and Sakaki, 1974; Dahms and Horvath, 1974; Hishida and Mizoi, 1976).

Neither these methods is very general because special apparatus such as a gas sampler, Van Slyke apparatus or reaction vessel are necessary. On the other hand, head space analysis is an important method for analysis of volatile substances in biological samples in which a known volume of the head-space gas, in equilibrium with its liquid phase (blood or
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urine), is directly injected into the gas chromatograph with a gas tight microsyringe. Usually hypovials sealed with butyl septa and aluminum seals are used for equilibrium bottles. The advantage of this method is the simplicity of instruments such as the microsyringe and hypovial. The application of the method for analysis of CO in blood was first reported by Kupfelschmidt (1974, 1977).

This paper describes an application of a modified head space method, using plastic disposable syringes for equilibrium bottles. Usually the CO saturation percent of the blood is calculated by the ratio of CO amounts from untreated blood and saturated blood. When blood is saturated with CO, it is also dissolved physically in blood other than chemically bound with hemoglobin. Amount of dissolved CO and the method of its removal are also described.

**MATERIALS AND METHODS**

*Reagent.* Preserved blood was used in this experiment and pure CO was made from formic acid and sulfuric acid. Saponin-ferricyanide solution was made from 2 g of saponin and 8 g of potassium ferricyanide dissolved in 40 ml of distilled water. One drop of octyl alcohol was used for antiform agent.

*Apparatus.* Shimadzu GC-6APTF gas chromatograph with thermal conductivity detector, equipped with 3 mm × 1 m glass column containing molecular sieve 5A, 80/100 mesh was used. Helium was used as the carrier gas at a flow rate of 40 ml/min. The temperature of the column, injector and detector were set at 90°C, 120°C and 120°C respectively. Detector current was 120 mA.

Shimadzu ITG-4A desital integrator which gives an integrated value (1 μV × sec) up to eight digits with 1.0% of C.V. (coefficient of variation) in linearity and reproducibility was used for quantitative analysis.

Dissociation syringes were made with 20 ml plastic disposable syringe (Terumo SS-20ES) into which the needle attaching part was cut and injection hole was extended to fit it with a small silicon-rubber plug for gas chromatograph injection. (Fig. 1).

*Analytical procedure.* One drop of octyl alcohol was added in a dissociation syringe followed by sealing with a rubber stopper. One end of a double ended Venoject single needle (Terumo VN-2238M) used for vacuum intravenous blood collection syringes was inserted through the stopper of the dissociation syringe, then the plunger was pushed down completely to check whether the plunger position matched the zero point of the scale on the barrel of the syringe. The other end of the needle was then inserted into the rubber tubing in which nitrogen gas was flowing. The plunger was moved back and forth five times and then the plunger position was aligned at 10 ml in order to put exactly 10 ml of N2 into the syringe. Then the end of the needle in the N2 tubing was pulled out followed by the syringe end. At this point, there was 10 ml of N2 at atmospheric pressure in the dissociation syringe. One ml of blood and 0.5 ml of saponin-ferricyanide solution were added in the syringe with a microsyringe and the plunger of the syringe was pulled 1.5 ml to maintain
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Analytical procedure.
Step 1. 10 ml of N₂ is introduced into the syringe.
Step 2. Blood and saponin-ferricyanide solution are added and plunger is pulled 1.5 ml.
Step 3. After shaking, plunger is extended by 1 ml and 0.5 ml of head space gas is withdrawn for injection into the gas chromatograph.

atmospheric pressure in the syringe. The syringe was shaken for 4 min by hand or shaker to release CO from blood. Before sampling, the plunger of the syringe was extended by 1 ml and 0.5 ml of the gas in the head space was withdrawn with a gastight microsyringe to be injected into the gas chromatograph. At this point, the pressure in the head space was slightly more than the atmosphere. After analyzing a second time, the plunger was extended by 0.5 ml and the gas in the head space withdrawn. This procedure prevented the contamination of air in the syringe. The complete procedure is shown in Fig. 1.

Preparation of CO saturated blood. One ml of blood and 1 drop of octyl alcohol were added in a dissociation syringe and CO gas was introduced into the syringe by the same manipulation of the plunger as described above. After shaking for 4 min, the gas in the head space was displaced with N₂ by moving the plunger of the syringe back and forth five times. The syringe was shaken for 4 min to equilibrate dissolved CO between liquid and the head space. Then the vapor in the head space was displaced again with N₂ and finally the plunger was set at the 11 ml position followed by analysis by the procedure described above.

Calculation. The per cent of CO saturation of the blood was calculated by the ratio of integrated digits, peak heights or CO/N₂ values between untreated sample and saturated sample.
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Determination of physically dissolved CO in blood and plasma. Five ml of blood and 5 ml of plasma separated from the same blood were each put in a dissociation syringe and the gas in the head space was displaced with pure CO and the syringe shaken for 5 min. Then the vapor in the head space was displaced with 10 ml of pure CO and this procedure was repeated three times. After the small bubbles had disappeared in the liquid, 1 ml each of the sample were taken with microsyringe to put into the dissociation syringe previously filled with 10 ml of N₂. The syringes were shaken for 4 min to equilibrate the dissolved CO in the sample and the head space. 0.5 ml of the gas in the head space was injected to the gas chromatograph. CO concentration in the head space was estimated from the calibration curve which was obtained by plotting the integrated value of the known serially diluted concentration of pure CO in a glass syringe calibrated up to 100 ml. After triple analysis, the gas in the head space was replaced by 10 ml of fresh N₂ by moving the plunger several times in the manner described above. Then a second analysis followed. This procedure was repeated several times.

Linearity of the method. The linearity of the method was checked by making triple measurements on samples of 100%, 75%, 50%, 25%, 10%, 4% and 2% of CO saturated blood which had been prepared by diluting CO saturated blood (100%) with calculated amounts of untreated preserved blood.

RESULTS

Accuracy of the volume of the dissociation syringe. Distilled water was introduced into the stoppered syringe and all air expelled setting the plunger at 10 ml on the scale. The volume of the head space of the syringe was measured from the weight of water occupying the space. The mean weights of the water from ten syringes set at 10 ml was 9.997 g (S. D. \( \pm 0.037 \) g, C. V. 0.37%).

CO saturation and liberation time in blood. In order to decide optimum saturation time, 1 ml of blood was saturated with pure CO and CO saturation was checked for time. Liberated carbon monoxide from the saturated blood in a head space was measured at various intervals. The results indicated that complete saturation or liberation from 1 ml of blood were achieved within 4 min.

<table>
<thead>
<tr>
<th>Table 1. Reproducibility of peak height, peak area and CO/N₂ ratio from CO liberated from 1 ml of saturated blood.</th>
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<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>Peak height</td>
</tr>
<tr>
<td>(cm)</td>
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<tr>
<td>Peak area</td>
</tr>
<tr>
<td>(Integrated value)</td>
</tr>
<tr>
<td>CO/N₂ calculated from</td>
</tr>
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<td>integrated value</td>
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Accuracy and reproducibility of the analysis. Blood was saturated by the method described above in six different syringes. Six consecutive head space gas sampling from a single syringe were performed to determine the reproducibility of multiple analysis with a single syringe. Table 1 shows the reproducibility of peak height, peak area (integrated value) and CO/N₂ ratio calculated from integrated value of CO liberated from 1 ml of saturated blood.

<table>
<thead>
<tr>
<th>Range of full scale</th>
<th>Injection volume</th>
<th>CO/N₂ Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Peak height</td>
</tr>
<tr>
<td>512mV + 2mV</td>
<td>0.45 ml</td>
<td>1.28</td>
</tr>
<tr>
<td>512mV + 2mV</td>
<td>0.5 ml</td>
<td>1.27</td>
</tr>
<tr>
<td>512mV + 2mV</td>
<td>0.55 ml</td>
<td>1.23</td>
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Fig. 2. Gas chromatogram of the analysis with different injection volume and CO/N₂ ratio. Range of the gas chromatograph was changed at ↓ mark.

To confirm the validity of using N₂ as internal standard, the samples of 0.5 ml, 0.45 ml and 0.55 ml from the same head space were injected into the gas chromatograph and CO/N₂ ratio calculated from peak height and peak area. The variations of CO/N₂, especially from peak area, were less than that of injection volume (±10%). (Fig 2).

Measurement of physically dissolved CO and CO liberated from hemoglobin. CO equilibrated between 10 ml of head space and 1 ml of plasma was easily removed by only one 4
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minute shaking. However, in blood, while liberated CO was almost same as in plasma at first analysis, from the second analysis 1.7—2.3 μl of CO was liberated from blood at every time.

<table>
<thead>
<tr>
<th>Number of analysis</th>
<th>Blood</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO amounts (μl)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood</td>
<td>22</td>
<td>2.3</td>
<td>1.7</td>
<td>1.7</td>
<td>2.3</td>
<td>1.7</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Plasma</td>
<td>21</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Linearity of the method.* The linearity of the method is shown in Fig. 3 which was obtained by plotting CO/N₂ ratio against CO saturation (%). The results indicate satisfactory linearity at every point and reproducibility at every point has less than a 1% C. V. (S. D. /mean×100) at 4% saturation or above, and 2—3% C. V. at under 2% saturation.

![Fig. 3. Linearity of the method. CO/N₂ ratio was calculated with peak area (integrated value)](image-url)
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DISCUSSION

Head space analysis is an important method for analysis of volatile substances in biological samples such as inhalation anaesthetics (Yamamura et al., 1966; Molloy et al., 1973), ethanol (Wilkinson, 1975), acetone (Stekelenburg and Bruyn, 1970), propane (Nagata et al., 1971) and halogenated hydrocarbon (Breimer et al., 1974). When a dissolved substance is sufficiently volatile, the determination of its concentration in the vapor phase can be used as a measure of the concentration in the liquid phase, provided that an equilibrium between the vapor and liquid phases has been reached. The head space gas is the vapor in equilibrium with its liquid phase. With a gas-tight microsyringe, part of the head-space vapor is injected directly into the gas chromatograph.

Usually the sampling bottles were used with hypovials sealed with silicon rubber caps and aluminum seals or volumetric flasks cut at the calibration mark and fitted with self-sealing silicon rubber caps (Breimer, 1974). If there is some variation in bottle volume, head space volume has to be measured by the weight of water replaced in the space. In this study, 20 ml of disposable syringe was used for degassing the sample bottle because a definite head space volume was easily withdrawn. For accurate measurement of the space, the plunger position set on 0 ml was checked with the plunger extended completely. Then N₂ was drawn in as the plunger was adjusted to 10 ml. This procedure was performed on each syringe because of possible discrepancies in the scale on the barrel. This way a volume accurate to a C. V. of 0.37% could be taken in.

There are two advantages in using a syringe: 1) with repeated moving of the plunger exchanges of the head space vapor with appropriate gas was easily achieved, and 2) the head space was able to be used for analysis without contamination with air because the head space could be interchanged by plunger manipulation.

In the chromatographic analysis of gaseous substances, the internal standard technique cannot be used, so precise amounts of sample must be injected. Usually, a gas sampler is used which has a loop of known volume in the carrier gas line upstream from the separating column, and after introduction into the sampler of the gas for analysis, the valve position is changed to introduce the sample to the gas chromatograph.

In the case where the sample is injected with a gas tight microsyringe, the leakage between barrel and plunger or attaching part of the exchangeable needle of the microsyringe may cause a variation in the injection volume and produce inaccurate analytical data. In this study, nitrogen put into the syringe previously is used for internal standard. As shown in Fig. 2, CO/N₂ ratio is relatively constant between different injection volumes though the range would necessarily change if peak height was used.

Since CO dissolved in saturated blood causes analytical error, it is necessary to remove the excess CO. Dissolved CO equilibrated from 1.0 ml of plasma and whole blood were almost same at first analysis i.e. 21 µl/ml and 22 µl/ml which agrees well with the theoretical value of 22 µl/ml for solubility of CO in water (Dahms and Horvath, 1974). A
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detectable amount of CO can not be observed in the plasma on second analysis, meaning
that dissolved CO is removed in only one equilibration. CO detection in the blood after
second analysis which was about 1% of the saturation volume of CO shows dissociation of
CO from carboxy hemoglobin.

This method can be applied to smaller samples with a smaller head space such as 0.5
ml of blood with 5 ml of head space of a 10 ml disposable syringe, but accuracy falls
slightly. The advantages of this method lie in the ease and speed of operation with a
simple and widely available instrument.

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