

## Stability of blood carbon monoxide and hemoglobins during heating

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### Abstract

The effects of heating on hemoglobin (Hb) and carbon monoxide (CO) levels in human blood were investigated by in vitro experiments. Head-space gas chromatography (HS-GC) using a molecular sieve 5 Å stationary phase and thermal conductivity detection was adopted for the measurement of CO gas, and spectrophotometric methods were used for the measurement of various Hb forms, protein and heme contents. Deteriorated absorbance spectra were observed for heat-treated blood samples, and double wavelength spectrophotometry was proven to give wrong percent saturation of carboxyhemoglobin content (% CO-Hb). The blood sample taken from one fatal fire casualty gave significantly higher % CO-Hb measured spectrophotometrically, compared to that by HS-GC. Control blood or purified Hb solution, which was saturated with CO in designated extent, was heated in a sealed vial. Under the incubation below 54°C, all Hb forms were stable, except for oxyhemoglobin (Hb-O<sub>2</sub>), which was partially oxidized to met-hemoglobin (Met-Hb). In contrast, under the incubation at 65°C, Met-Hb was denatured completely to be insoluble, and Hb-O<sub>2</sub> was partially denatured via Met-Hb formation. CO-Hb was resistant against heating. The difference of heat susceptibility and precipitability among Hb forms resulted in artificial increase of % CO-Hb. During heating, spontaneous CO was produced from blood. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

**Keywords:** Forensic toxicology; Carbon monoxide; Blood; Hemoglobin; Post-mortem alteration; Heating

### 1. Introduction

In fire, toxic gases are produced, and inhalation of such gases makes persons poisoning [1,2]. Carbon monoxide (CO) is the most plausible cause of death, although hydrogen cyanide and oxidizing gases are the other factors. To verify the exposure of CO gas to persons, it is essential to measure percent saturation of carboxyhemoglobin content (% CO-Hb) in blood [3]. However, the possibility arises that persons are burn in fire after death [4], and it is important to consider the post-mortem alteration of toxic gases in the cases of fire gas poisoning [5,6].

The following three post-mortem alterations of toxic substances [6] should be considered in forensic toxicology: phase I, a change which occurs between the time of death and the time of sampling; phase II, a change which occurs

between the time of sampling and the time of analysis, i.e. alteration during sample storage and transfer; and phase III, a change which occurs during analysis. As for phase I change, especially in fire cases, heating is the most serious issue [4]. Forensic toxicologists sometimes encounter blood samples with abnormal appearance. Blood samples drawn from heavily burned fire victims often show signs of heating as evidenced by blood coagulation. Until now, various kinds of methods have been developed for the measurement of blood CO [7,8]. Some methods are proved to be suffered from inaccuracy for heat-denatured or coagulated blood. Spectrophotometric methods, although being simple and convenient, have the disadvantage in the measurement of denatured hemoglobin (Hb) forms. In contrast, head-space gas chromatography (HS-GC) can directly measure CO level. Methods have been improved to accurately measure the levels of CO for such blood samples with abnormal appearance [9,10]. In Japan, double wavelength spectrophotometric method is recommended for the determination of % CO-Hb in forensic chemistry [11] and legal medicine [12].

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There are numerous reports on the post-mortem alteration of blood CO during heating in *in vitro* [13–15] and *in vivo* experiments [16,17], and during storage [18,19]. Post-mortem CO formation has also been reported [20–22]. However, no paper comprehensively revealed the overall alteration of Hb forms and CO in blood. Our laboratory has established HS-GC method for various volatiles [6], and performed researches on post-mortem alteration of toxic gases and volatiles [23]; blood cyanide alteration by heating [24], artifactual cyanide formation from thiocyanate [25], and stability of sulfide and thiosulfate in blood and urine [26]. In this paper, we established HS-GC method for the determination of blood CO, and ascertained the technical limitation of spectrophotometric CO determination for heat-denatured blood. Next, we investigated the effects of heating on Hb forms, CO levels, and also protein and heme levels in blood and Hb solution by *in vitro* experiment.

## 2. Materials and methods

### 2.1. Blood sample

Control human blood was obtained as out-dated transfusion blood from a local police hospital. Blood samples of fatal fire casualties were collected from local forensic science laboratories.

### 2.2. Preparation of oxyhemoglobin, carboxyhemoglobin and met-hemoglobin

Various Hb forms were prepared according to the previous paper [25]. Oxyhemoglobin (Hb-O<sub>2</sub>) was prepared by washing the outdated human blood with phosphate buffered saline followed by hemolysis with two-fold volume of distilled water at 4°C for 1 h, centrifugation at 27 000 rpm for 1 h, and dialysis against distilled water. Carboxyhemoglobin (CO-Hb) was prepared by bubbling CO gas (G-L Science, Tokyo, Japan) through Hb-O<sub>2</sub> solution under a weak vacuum. Met-hemoglobin (Met-Hb) was prepared from Hb-O<sub>2</sub> by addition of a slight molar excess of K<sub>3</sub>Fe(CN)<sub>6</sub> followed by removal of the residual reagent by a Sephadex G-25 (Pharmacia, Uppsala, Sweden) column. Hb solution in 0.1 M sodium phosphate (pH 7) (*p*-Na buffer) was concentrated by ultra centrifugation using Centriflo CF-25 (Amicon, Danvers, MA).

### 2.3. Spectrophotometry

Percent CO-Hb was determined according to the double wavelength method [11]. Blood was diluted about 200-fold with deoxygenated 0.1% sodium carbonate and 0.2% sodium hydrosulfite solution, and allowed to stand at 25°C for 15 min. After centrifugation, absorbances of the resulting supernatant were measured at 538 nm (*A*<sub>538</sub>) and 555 nm (*A*<sub>555</sub>), and % CO-Hb was calculated from the ratio

of *A*<sub>538</sub>/*A*<sub>555</sub> using the calibration curve consisted of 0% CO-Hb using control blood and 100% CO-Hb using CO saturated blood.

Percent Met-Hb content (% Met-Hb) was determined by the method of Sato et al. [27], measuring the absorbance decrease at 630 nm by addition of cyanide. Total Hb concentration was determined by the cyanohemoglobin method [28] using an  $\epsilon = 11.0$  mM per heme at 540 nm. Protein concentration was determined by a biuret reaction [29] in the presence of 1.0% (w/v) sodium deoxycholate in order to remove any turbidity in the sample. Heme concentration was determined by the acid hematin method [30] and the pyridine hemochromogen method [31].

### 2.4. Carbon monoxide determination by head-space gas chromatography

CO concentrations were determined by HS-GC method according to the reference methods [32,33] with some modification. Blood sample or Hb solution was added to a screw-cap septum vial (8.7 ml, Pierce, Rockford, IL), the total volume was adjusted 0.75 ml with distilled water, and the vial was sealed with Tuf-Bond Disc (Pierce). After the addition of 0.25 ml of 20% K<sub>3</sub>Fe(CN)<sub>6</sub> solution (containing 5% saponin for blood) into the sealed vial using a glass tip syringe (0.5 ml, Top Co., Tokyo, Japan) and needle (25 gauge  $\times$  1", 0.50 mm  $\times$  25 mm, Terumo Co., Tokyo, Japan), HS vial was incubated at 30°C for 15 min. Then, 1.0 ml of the gaseous phase in the sealed vial was injected into the following gas chromatograph (GC) using a glass tip syringe (1.0 ml) and needle (25 gauge  $\times$  1"). A 5890 series II GC (Yokogawa Analytical Systems, Tokyo, Japan), equipped with a thermal conductivity detector (TCD) was used. Stationary phase was a molecular sieve 5 Å (2 mm i.d.  $\times$  3 m, 80–100 mesh, G-L Science, Tokyo, Japan) and helium was used as the carrier-gas at a flow-rate of 23 ml/min. The injection port, detector and column oven were maintained at 250, 300 and 100°C, respectively. The peak height of CO was measured, and converted to the relative CO content compared to that in CO saturated blood or Hb solution.

### 2.5. Heating of blood and hemoglobin solution

CO saturated blood was prepared by bubbling CO gas through control blood under a weak vacuum. Control blood and CO saturated blood was mixed to prepare the blood sample with designated CO saturation. Several lots of the mixed blood samples (1.2 ml) in glass tubes were sealed with parafilm, and incubated at 37°C overnight, at 54°C for 3 h, and at 65°C for 1 h. Then, the tubes were immersed into ice, about 0.2 ml aliquot of the sample in one lot tube was transferred to a HS vial, weighed by electric balance, and supplemented with 0.1 ml of 5.0% Triton X-100 solution, the total liquid phase was adjusted 0.75 ml with distilled water, and the vial was sealed. After addition of

0.25 ml of ferricyanide, CO was measured by the HS-GC method. Second lot was subject to the double wavelength spectrophotometry for % CO-Hb. The other lot was diluted 25-fold with 1.0% Triton X-100 solution, and subject to the spectrophotometric determination of % Met-Hb, total Hb and heme contents, after any turbidity was removed by centrifugation. The determination of heme was performed by acid hematin method, because the sample matrix of heated blood was complex. % CO-Hb was also determined by dividing the measured CO gas level by total Hb content.

CO-Hb and Hb-O<sub>2</sub> solution was mixed to prepare the Hb solution with designated CO saturation. The prepared Hb solution or the Met-Hb solution (heme 2  $\mu$ mol) was added to a HS vial, and the total liquid phase was adjusted 0.75 ml with *p*-Na buffer, and the vial was sealed. HS vial was incubated at 50°C or 65°C for 1 h. The vial was immersed into ice, and after the incubation at 30°C for 15 min, 1.0 ml of the gaseous phase in the HS vial was injected into the GC to measure CO gas as 'free form'. Then, 0.25 ml of ferricyanide solution was added to the HS vial, and after further incubation at 30°C for 15 min, 1.0 ml gaseous phase was injected into the GC to measure CO gas as 'free + bound form'. For spectrophotometry, 0.75 ml of the prepared Hb solution (heme, 2  $\mu$ mol) was incubated in the HS vial with sealing at 50°C or 65°C for 1 h. Then, the vial was immersed into ice, diluted 5-fold with *p*-Na buffer, centrifuged, and the resulting supernatant was subject to the measurement of % CO-Hb, % Met-Hb, total Hb, protein and heme (pyridine hemochromogen method) contents. CO gas content was expressed as % relative level compared to that of the CO saturated Hb solution.

### 3. Results and discussion

#### 3.1. Blood carbon monoxide measurement

As shown in Fig. 1A, the control blood consisted of Hb-O<sub>2</sub> as Hb form showed typical one peak absorbance curve within  $\alpha$ - $\beta$ -absorbance region, and the CO saturated blood consisted of CO-Hb as Hb form showed typical two peak absorbance curve. In contrast, the heat-treated control blood (Fig. 1B(a)) showed deteriorated absorbance spectrum, which was different from the control blood. Even after saturation with CO gas, the absorbance spectrum did not give well-shaped two peak curve (Fig. 1B(b)). Denatured Hb forms [34–36] should give abnormal visible absorbance spectra [37], and the inclusion of such denatured Hb forms in the test solution may manifest deteriorated absorbance spectrum.

CO gas liberated from blood or Hb solution was detected by HS-GC using a Molecular sieve 5 Å as stationary phase and TCD as detector. The typical chromatogram is shown in Fig. 2. The detection limit in 1.0 ml HS injection was 2.3 nmol CO gas in liquid phase ( $S/N = 3$ ), which corresponds to about 0.05% CO-Hb in 0.5 ml blood sample. The % CO-Hb levels measured by HS-GC was almost compatible with those measured by double wavelength spectrophotometric method, which is in agreement with Sakata et al. [38]. The % CO-Hb measured by HS-GC, however, gave slightly lower values than those by double wavelength method (Table 2, 4°C). We used the control blood as 0% CO-Hb sample for making the calibration curve, and it might be partially contaminated with CO gas from the environment or tobacco smoke.

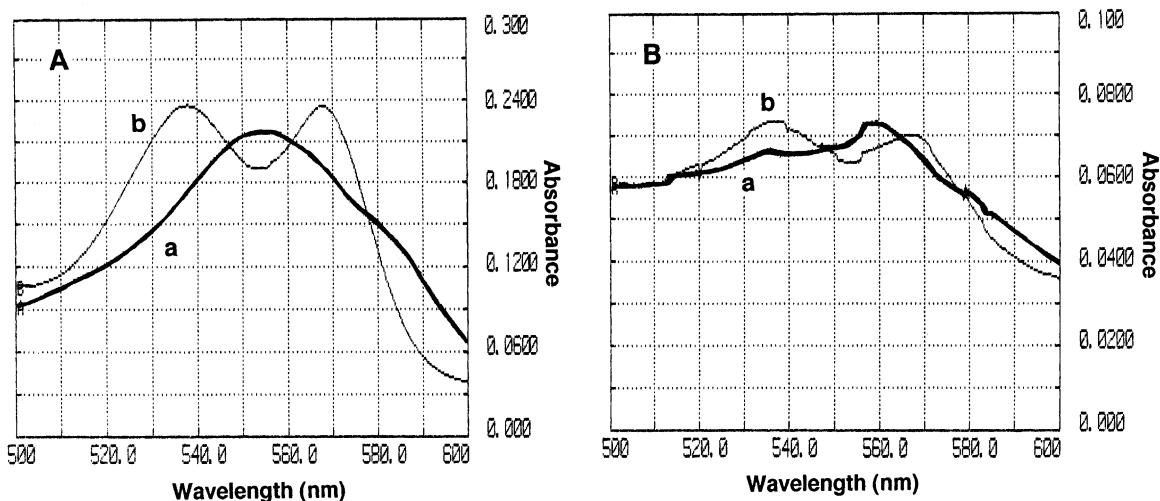


Fig. 1. Visible absorbance spectra of blood samples before and after CO saturation. Control blood (A) and blood sample heated at 65°C for 1 h (B) were diluted 400-fold with 0.1% sodium carbonate and 0.2% sodium hydrosulfite solution, and absorbance spectra were measured by spectrophotometer using 1.0 cm path length ultraviolet cell (a). Then, CO gas was passed through the cell, and the spectra were measured (b).

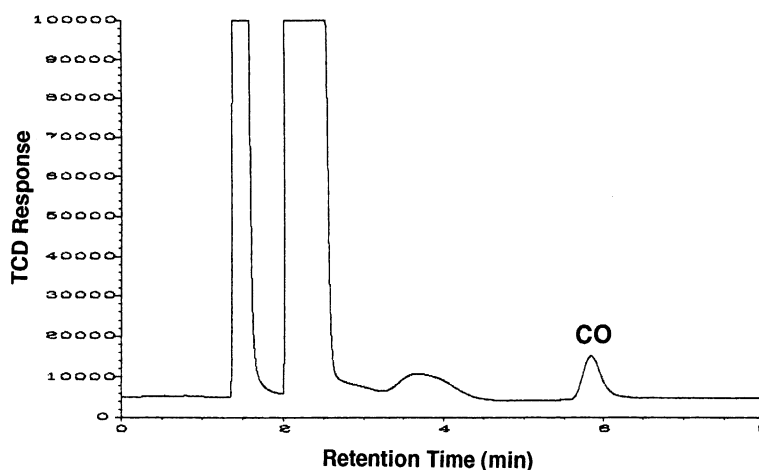


Fig. 2. Headspace gas chromatogram of CO in blood. 0.25 ml of CO saturated human blood and 0.5 ml of distilled water was added to a vial and sealed with disc. After addition of 0.25 ml of 20%  $K_3Fe(CN)_6$  and 5% saponin solution, HS vial was incubated at 30°C for 15 min. 1.0 ml of HS phase was injected into GC (column: molecular sieve 5 Å, 80–100 mesh, 2 mm i.d.  $\times$  3 m, 100°C; carrier gas: He, 23 ml/min; detection: TCD).

We measured % CO-Hb of the blood samples taken from fatal fire casualties. As shown in Table 1, the values measured by double wavelength spectrophotometric and HS-GC methods are almost compatible, except for no. 5 blood. This sample gave high % Met-Hb, indicating the marks of heat denaturation. The visible absorbance spectrum showed distorted pattern deviated from normal Hb spectrum (data not shown), and it is reasonable that artificially raised % CO-Hb was observed by spectrophotometry.

### 3.2. Effect of heat on blood

Control blood with CO saturation in designated extent were heated and subject to the measurement of % CO-Hb, % Met-Hb, Hb and heme contents. The result was shown in Table 2. The incubation at 37°C overnight gave negligible change of the blood appearance and the levels of % CO-Hb, % Met-Hb, total Hb and heme. Considerable Met-Hb formation in the control blood, was only the exception. It is confirmed that at 37°C, Hb is stable, and only autoxidation of Hb-O<sub>2</sub> [39] is the major event.

The incubation at 54°C for 3 h led to the change of the blood color to dark red, except for the CO saturated blood.

The blood color change is the sign of Met-Hb formation. % CO-Hb did not change significantly. CO-Hb may be stable against 54°C heating, because only negligible Met-Hb was formed in the CO saturated blood. The Hb levels were almost similar to the heme levels. Slight spontaneous CO production was observed from all the samples, which resulted in the increase of % CO-Hb measured by HS-GC.

The incubation at 65°C for 1 h dramatically changed the appearance of blood samples. The coagulation and the brown color are typical signs of denaturation of Hb [4]. The precipitable blood components were removed from the samples by centrifugation prior to the spectrophotometric measurement, and so it is reasonable that Hb and heme levels were decreased considerably in the control and the 50% CO saturated blood. Met-Hb was formed significantly in the control blood. In contrast, the change of Hb forms in the CO saturated blood was negligible, and almost all Hb or heme was recovered as soluble. During the heating, Hb-O<sub>2</sub> might be significantly denatured, resulting in decrease of the Hb level. The Hb levels were dissimilar to the heme levels, and it may be attributed to the presence of soluble denatured Hb molecules, or heat-stable serum heme compounds which was in minor level in the untreated blood, but relatively increased after heating. Noticeable phenomenon was spontaneous formation of CO gas. In particular, in the control blood, considerably high % CO-Hb was observed. The % CO-Hb measured by HS-GC were quite deviated from those by spectrophotometry and exceeded 100% for the 50% and the 100% CO saturated blood samples. This may be explained from the possibility that part of CO gas can not be trapped by Met-Hb or denatured Hb produced by heat treatment and it can be measured as free form by HS-GC.

Table 1

Comparison of the % CO-Hb of blood samples taken from fatal fire casualties between double wavelength spectrophotometric method and HS-GC method

Sample number	1	2	3	4	5	6
% Met-Hb	39.4	1.0	3.5	1.8	51.8	1.0
% CO-Hb	58.8	62.3	29.1	69.1	20.5	42.6
spectrophotometry						
% CO-Hb HS-GC	54.0	56.9	23.9	67.5	12.7	37.3

Table 2  
Effect of heating on blood hemoglobin and CO levels

Treatment	% CO-Hb	Appearance	% CO-Hb		% Met-Hb	% Hb	% Heme
			Spectrophotometry	HS-GC			
4°C	0	Fluid, red	3.9	0.4	0.6	100 <sup>a</sup>	100 <sup>b</sup>
	50	Fluid, red	46.8	42.3	0.6	100	100
	100	Fluid, red	96.9	96.9	1.1	100	100
37°C, overnight	0	Fluid, dark red	8.0	1.2	24.8	111.0	115.4
	50	Fluid, red	48.4	42.3	3.6	107.3	99.1
	100	Fluid, red	93.6	100.4	0.9	104.8	99.4
54°C, 3 h	0	Fluid, dark red	4.8	1.5	69.2	101.5	86.5
	50	Fluid, dark red	48.7	50.4	25.7	101.9	108.3
	100	Fluid, red	95.7	103.0	7.1	103.8	123.9
65°C, 1 h	0	Coagulated, brown	35.8	18.9	41.0	11.1	17.4
	50	Coagulated, brown	52.3	112.3	6.5	52.8	80.0
	100	Coagulated, dark red	83.8	143.6	2.2	100.0	104.7

<sup>a</sup> Each hemoglobin concentration was depicted as percentage value compared to those of the Hb solution before heating treatment (148 mg Hb/g sample).

<sup>b</sup> Each heme concentration was depicted as percentage value compared to those of the Hb solution before heating treatment (143 mg Hb/g sample).

### 3.3. Effect of heating on hemoglobin solution

In the toxicological meaning, Hb form is the important index of exposure and poisoning of CO gas and oxidizable gases, although blood contains the other proteinous and nonproteinous components. We examined the effect of heating in simplified system using purified Hb molecules. The result of 50°C heating was shown in Table 3. The Hb, protein and heme contents were almost similar, and their recoveries were almost 100%, indicating that at 50°C Hb is not denatured. Even Met-Hb was not denatured to form precipitates. Small part of CO gas was liberated from the Hb solution, being observed as free form, although % CO-Hb measured by either HS-GC or double wavelength method did not change. Met-Hb formation was observed in all the Hb solution.

The incubation at 65°C led to significant changes. As shown in Table 4, Met-Hb was easily denatured, leading to dramatic decrease of Hb levels, which is compatible with the reference paper [40]. The newly formed Met-Hb from Hb-O<sub>2</sub> might be denatured instantly, for % Met-Hb contents were low for all the Hb solutions. Compared to the blood heated at 65°C (Table 2), the decrease in the Hb levels of the heated Hb solution was remarkable. This can be explained from the possibility that blood ingredients protect Hb molecules from heat denaturation. The Hb, protein and heme levels were almost similar, indicating that the measured heme proteins were spectrophotometrically identical to intact Hb. However, the % CO-Hb levels in the heated Hb solution were strongly deviated from the original levels. Considerably raised % CO-Hb was observed by the spectrophotometric measurement for the Hb solution except for the 100%

Table 3  
Effect of heating at 50°C on hemoglobin and CO levels of various hemoglobin solutions<sup>a</sup>

Hb species	CO gas (%)			% CO-Hb	% Met-Hb	Total Hb	Protein	% Heme
	K <sub>3</sub> Fe(CN) <sub>6</sub> addition							
	Before	After	Total					
100% Met-Hb	0.0	0.0	0.0	0.9	99.9	112	96	116
100% Hb-O <sub>2</sub>	0.0	2.2	2.2	1.2	7.9	104	107	110
20% CO-Hb	0.8	20.1	20.9	19.7	11.4	105	101	102
50% CO-Hb	3.3	49.1	52.4	49.7	13.1	98	104	98
100% CO-Hb	6.1	95.6	101.7	93.3	6.3	97	104	107

<sup>a</sup> Total Hb, protein and heme contents were depicted as percentage value compared to those of the Hb solution before heating treatment (2 μmol heme).

Table 4

Effect of heating at 65°C on hemoglobin and CO levels of various hemoglobin solutions<sup>a</sup>

Hb species	CO gas (%)			% CO-Hb	% Met-Hb	Total Hb	Protein	% Heme
	K <sub>3</sub> Fe(CN) <sub>6</sub> addition							
	Before	After	Total					
100% Met-Hb	0.0	0.3	0.3	31.5	25.9	2.6	1.9	2.1
Hb-O <sub>2</sub>	1.8	4.1	5.9	13.7	4.9	22	25	21
20% CO-Hb	10.9	4.5	15.4	52.8	4.2	27	28	23
50% CO-Hb	14.9	21.0	35.9	82.2	0.8	43	48	41
100% CO-Hb	24.1	125.3	147.4	100.0	0.0	69	73	76

<sup>a</sup> Total Hb, protein and heme contents were depicted as percentage value compared to those of the Hb solution before heating treatment (2  $\mu$ mol heme).

CO-Hb solution. It is probable that the relative content of heat-stable CO-Hb was increased in contrast to the decrease of the contents of heat-labile Hb-O<sub>2</sub> and Met-Hb, which were precipitated and removed centrifugally from the sample solution. Soluble denatured Hb forms possessing distorted absorbance spectra may also contribute to the increase of % CO-Hb, just seen in blood samples of fatal fire casualties (Fig. 1B). Considerable levels of the free CO gas was observed, and it is reasonable that these CO gas could not be trapped by Hb molecules. Spontaneous CO production was observed only in the Hb-O<sub>2</sub> and 100% CO-Hb solutions. These result is in contrast to those in the heated blood (Table 2).

When blood is subject to heating, the first observable event is Met-Hb formation, followed by distorted heme protein formation and protein denaturation. Hb-O<sub>2</sub> is converted to Met-Hb [34], and Met-Hb is readily denatured and precipitates via hemichrome formation [36]. CO-Hb, strongly liganded Hb form, is relatively resistant to heat denaturation. CO was also spontaneously produced. It is not clarified that this CO production from Hb molecules is the same or different from so-called physiologically produced blood CO [41–44]. It is possible that, during heating, superoxide anion radical [25], which is formed through Hb-O<sub>2</sub> denaturation, causes complex oxido-reductive reaction with heme to produce CO. However, it can not be denied that CO is produced from the blood ingredients, because considerable amount of CO was produced spontaneously only from the heated blood (Table 2). Heat-driven decomposition of blood ingredients can be raised as another mechanism of CO production, for the explanation of the spontaneous CO production from the 100% CO saturated blood.

#### 4. Conclusion

Our in vitro experiment demonstrates that heating of blood sample or Hb solution manifest considerable change of Hb forms and CO levels. Even though under mild heating (50–54°C), Met-Hb was formed from Hb-O<sub>2</sub>, which leads to

misjudgment for poisoning from oxidizable gases. CO-Hb was stable against heating, and we could detect toxic level of CO gas even in heat-treated blood. Stability of CO-Hb and severe denaturation of Hb-O<sub>2</sub> and Met-Hb at 65°C heating, resulted in artificial increase of % CO-Hb, which leads to misjudgment for CO poisoning. The absorbance spectra of heat-treated Hb molecules were deteriorated, and this spectral deviation caused wrong spectrophotometric measurement of % CO-Hb, which was ascertained in the blood samples of fatal fire casualties. Spontaneous CO production was observed in the heated samples. Therefore, heat-driven post-mortem alteration of Hb forms and CO in blood should be considered for the elucidation of the cause of death in fire cases.

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