Carboxyhemoglobin stability evaluation in stored and heat-treated biological samples

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Abstract: The aim of our study was to evaluate the carboxyhemoglobin (COHb) stability in biological samples stored for different periods of time and heated at different temperatures. The variations of the COHb concentration depending on the length of samples storage was investigated on blood samples collected from the medico-legal cases in which the cause of death was carbon monoxide (CO) poisoning. Six and nine months after the first COHb determination a new measurement was made. The differences between the COHb values measured initially and those measured at six and nine months ranged between 3% and 30% and were influenced by the blood quantity in the storage vials. In order to establish the possibility of COHb determination in blood heated at various temperatures, biological samples with 0.03%, 65% and 100% COHb were heated at 60°C, 80°C and 100°C for time intervals ranging between 2 and 20 minutes. Spectrophotometric measurements of COHb can be performed safely from blood samples heated at a temperature of maxim 60°C. Above this temperature, the absorption spectra undergo alterations that provide misleading results. Regarding the samples heated at a temperature higher than 80°C, it was noticed that the alteration of blood is influenced not only by the temperature, but also by the length of the heat treatment.

Key Words: carboxyhemoglobin, stability, storage period, heating temperature.

The measurement of the carboxyhemoglobin (COHb) concentration is considered to be the only established marker for the proper diagnosis of carbon monoxide (CO) poisoning [1, 2]. In addition, COHb value is important for the elucidation of the cause of death in cases of fatal fire casualties [3, 4]. During a fire, depending on the burning materials, CO, hydrocyanic acid [5], and other toxic gases can be produced. In order to verify a person's exposure to CO it is essential to determine the COHb concentration [6] which can provide clues concerning the time interval between fire ignition and death onset. Values of COHb below 10% could suggest that death occurred before the fire ignition, while values between 10% and 50% COHb could indicate that the person was alive when the fire started [7]. For these reasons the measurement of COHb concentrations in biological samples collected from cadavers is often determined in forensic toxicology laboratories [8]. However, the possibility of post-mortem deterioration of the biological materials should be taken into account: a) changes that can occur between the time of death and the time of sample collection. In case of a fire, the rise of the body temperature is the most important

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issue. Blood samples drawn from heavily burned fire victims often show signs of exposure to elevated temperatures, represented by blood coagulation [6]. Situations in which the biological sampling is performed after various post-mortem intervals can be encountered (e.g. in the cases of exhumation).

b) changes that may occur between the time of sampling and the time of the analysis (e.g. during sample storage) [6]. In practice are encountered situations when a new analysis of the COHb value is required at a certain time interval after the first determination.

One of the objectives of this study was to evaluate the COHb concentration in biological samples stored for different periods of time. Another objective was to assess the possibility to determine the COHb concentration in biological samples heated at different temperatures and for different time intervals.

MATERIALS AND METHODS

**Variations of the COHb concentration depending on the length of samples storage.** This issue was investigated on blood samples collected from the medico-legal cases in which the cause of death was CO poisoning. The COHb levels measured immediately after the samples collection ranged between 65% and 100%. The blood samples were collected in 6 ml tubes, sealed and analyzed 6 and 9 months after the first determination. Between determinations the samples were refrigerated at minus 18°C. Not all the collected biological samples filled the storage containers completely.

**Evaluation of the thermal stability of COHb.** Three types of samples have been subjected to heat treatment: samples with 0.03% COHb, collected from a non-smoker, samples with 65% COHb, collected from a corpse and samples containing 100% COHb, obtained by bubbling CO gas through a blood sample collected from a non-smoker. In order to evaluate the thermal stability of COHb, the blood samples were heated at 60°C, 80°C and 100°C for 2 and 10 minutes. The absorption spectrum of unreduced and reduced samples treated this way was recorded, in order to assess the spectral changes of the COHb after heat treatment. The effects of 100°C heat treatment, depending on time, at intervals between 2 and 20 minutes, were studied. Method: 2 ml of blood were placed in 2 ml glass tubes and sealed with screwed-on caps with teflon armature, so that the air above the sample had not exceeded 200 μl (required for changes in the volume of the liquid caused by temperature). The glass tubes were placed in thermostatic water bath at preset temperature, removed and cooled at room temperature at various time intervals.

Because the heat-treated samples precipitated the recording of the spectrum in the 450-600 nm field was done after suspension of the samples in 0.4% ammonia solution and centrifugation. The samples reduction was made with 40% ammonium sulphide solution. Determination of COHb concentration was done according to a standard curve designed previously on the field 0-100% COHb, using a classical method, by measuring the absorption of the unreduced and reduced sample at 480 and 555 nm [9] and an original method, by determining the absorption in the reduced samples alone, at 540 and 579 nm. The results were verified by the overlapping of the reduced samples spectrum with the standard spectrum corresponding to the calculated value. The calculations for the determination of COHb and overlapping spectrum were carried out with an original Excel program.

RESULTS AND DISCUSSION

**Variations of the COHb concentration depending on the length of samples storage.** The results of the determinations are presented in Table 1. In all cases the COHb values are decreasing, in variable percentages, between 3% and 30%. The decrease of the COHb concentration is accentuated by the amount of air above the sample.

### Table 1. The levels of COHb in stored blood samples measured at different time intervals

<table>
<thead>
<tr>
<th>Sample number</th>
<th>COHb% at different storage duration</th>
<th>COHb% decrease in the period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0-6 months</td>
<td>9 months</td>
</tr>
<tr>
<td>1</td>
<td>86</td>
<td>81</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
<td>84</td>
</tr>
<tr>
<td>3</td>
<td>90</td>
<td>80</td>
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<td>4</td>
<td>100</td>
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<td>5</td>
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<td>81</td>
<td>79</td>
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<td>8</td>
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<tr>
<td>9</td>
<td>86</td>
<td>80</td>
</tr>
<tr>
<td>10</td>
<td>68</td>
<td>60</td>
</tr>
</tbody>
</table>

Evaluation of the thermal stability of COHb. The absorption spectrum of a reduced blood sample with 100% COHb, heated at 60°C for 10 minutes is represented in Fig. 1. A complete overlay with the spectrum of a standard reduced sample containing 100% COHb can be noticed.

The absorption spectrum of a reduced blood sample with 100% COHb, heated at 80°C for 10 minutes, is represented in Fig. 2. A slight shift of the absorption spectrum of the analyzed sample in comparison with the
The absorption spectrum of the standard sample curve can be noticed. The calculated value for COHb exceeded 100%, which indicates a spectral alteration of the analyzed samples.

The absorption spectrum of a reduced blood sample with 100% COHb, heated at 100°C for 10 minutes is represented in Fig. 3. An important modification of the absorption spectrum of the reduced sample in comparison with the absorption spectrum of the standard sample curve is noticed. COHb concentration could no longer be determined through spectrophotometric method.

The effects of the length of samples heating at 100°C for 2 to 20 minutes on the absorption spectrum of reduced samples containing 0.03%, 65% and 100% COHb are presented in Fig. 4, 6 and 8. In these cases, the time-dependant spectral changes were quantified by mathematic processing of the spectral data. As a measure of the time-dependant spectral changes, we used the method of Euclidean geometrical distance between the spectra, that were each assimilated with a n-dimensional vector, after normalization in relation to the sub spectral area [10]. In the 480-600 nm wavelength field, there are 121 points for each spectrum, at 1 nm intervals.
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The Euclidean distance between the initial sample spectrum (not subjected to heat treatment) and the absorption spectra of the samples heated at 100°C for 2 to 20 minutes was determined.

Fig. 5 shows the evolution of the Euclidean geometric distance of unreduced and reduced blood samples with 0.03% COHb depending on the length of heating, from 2 to 20 minutes at 100°C. The time dependence is described by a second degree function, with a determination coefficient over 0.999. The same data are presented in Fig. 7 and 9 for blood samples with 65% and 100% COHb.

All variation curves of the Euclidean distance between the initial spectrum and the spectra recorded for samples heated for 2 to 20 minutes are described by the monotonous second degree function with coefficients of determination over 0.98, with the exception of the reduced samples with 100% COHb for which the coefficient of determination is 0.926.

The speed of the spectral change between 2 and 20 minutes can be determined using these functions. In all cases we noticed that the highest difference appears in the first 2 minutes, indicating that in this time frame a major structural change takes place; it continues with moderate speed between 2 to 20 minute. The examination of the absorption spectra at the unreduced and at the reduced samples, led to the following observations:

- initial samples spectra (unheated) are different from all of the heated samples, both at the unreduced and at the reduced samples. Spectral changes, in terms of Euclidean distance, have the highest value in the first 2 minutes from the beginning of the heat treatment.

- the presence of two isosbestic points, around 522 nm and 581 nm, both at the unreduced and at the reduced samples.

Regarding the spectral changes occurred after the exposure to high temperature, the question whether they influence the quantitative determination of COHb by spectrophotometric method arises. There are several ways of determining the COHb concentration through this method. The classic method [10] consists in the measurement of the absorption of the sample diluted with a solution of ammonia 0.4% at 2 wavelengths (480 and 555 nm), after which the oxyhemoglobin is reduced to hemoglobin and the measurements are repeated. The ratio \( \frac{A_{555}^{n}}{A_{480}^{n}} \left/ \frac{A_{555}^{r}}{A_{480}^{r}} \right. \) is calculated and the concentration of COHb in relation to a standard curve, previously built up with samples containing known concentrations of COHb, is calculated. The measurement can be also made by determining the absorption ratio at 2 wavelengths (in this case 540 nm and 579 nm) only at the reduced samples [11].

In the present study we examined the effect of the samples exposure at 100°C for 2, 5, 10 and 20 minutes upon the COHb determination by the calculation of the ratio \( \frac{A_{555}^{n}}{A_{480}^{n}} \left/ \frac{A_{555}^{r}}{A_{480}^{r}} \right. \) and \( \frac{A_{540}^{r}}{A_{579}^{r}} \). 

Figure 7. Euclidean distances. Unreduced and reduced samples with 65% COHb, heated at 100°C for 2, 5, 10 and 20 minutes.

Figure 8. Overlapping absorption spectra of reduced blood samples with 100% COHb, heated at 100°C, for 2, 5, 10 and 20 minutes.

Figure 9. Euclidean distances. Unreduced and reduced samples with 100% COHb, heated at 100°C for 2, 5, 10 and 20 minutes.
Table 2. Variation of R1 and R2 ratio from the initial value depending on the length of heating

<table>
<thead>
<tr>
<th>Heating duration (minutes)</th>
<th>Sample with 0,03 % COHb</th>
<th>Sample with 65 % COHb</th>
<th>Sample with 100 % COHb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R1</td>
<td>A1</td>
<td>R2</td>
</tr>
<tr>
<td>0</td>
<td>0.438517</td>
<td>0.00</td>
<td>1.133273</td>
</tr>
<tr>
<td>2</td>
<td>0.592542</td>
<td>35.12</td>
<td>1.325674</td>
</tr>
<tr>
<td>5</td>
<td>0.617321</td>
<td>40.77</td>
<td>1.329116</td>
</tr>
<tr>
<td>10</td>
<td>0.618641</td>
<td>41.08</td>
<td>1.320749</td>
</tr>
<tr>
<td>20</td>
<td>0.620640</td>
<td>41.53</td>
<td>1.316977</td>
</tr>
</tbody>
</table>

R1 = (A555/A480)/(A555/A480);
R2 = A540/A579;
A% = % modification from the initial value.

CORRELATIONS

COHb is relatively stable; no significant concentration changes within the mentioned period were noticed. The differences between COHb values measured initially and those measured at 6 and 9 months, were influenced by the blood quantity in the storage vials. The smallest differences between the COHb values were recorded in the blood samples from full or nearly full vials.

Spectrophotometric measurements of COHb can be performed safely from blood samples heated at a temperature of maxim 60°C. Above this temperature, the absorption spectra for both the unreduced and the reduced samples undergo alterations that provide misleading results. Regarding the samples heated at a temperature higher than 80°C, we found that the alteration of blood is influenced not only by the temperature, but is also time-dependant - the alterations are more significant as the exposure time of the samples at that temperature is longer. Spectral alteration speed in the range of 2 to 20 minutes can be described with a high correlation coefficient by determining the Euclidean distance between the initial spectrum and the spectra obtained from the soluble fraction of heat-treated samples.

Acknowledgements

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