**FORENSIC TOXICOLOGY LABORATORY**
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# CARBOXYHEMOGLOBIN SATURATION by SPECTROPHOTOMETRY

## PRINCIPLE

Carbon monoxide (CO) is a colorless, odorless and tasteless gas produced by incomplete combustion. Hemoglobin binding affinity for CO is ≈ 250 times greater than its affinity for oxygen, meaning that small amounts of CO dramatically reduce hemoglobin's ability to transport oxygen.

Quantitation is based on the difference between two isobestic points obtained with oxidized and reduced hemoglobins. Total hemoglobin is measured, and this value along with the isobestic absorbance is used to calculate the percentage of carboxyhemoglobin. This technique involves the treatment of a dilute hemolysate of blood with sodium hydrosulfite, reducing the methemoglobin and oxyhemoglobin. Carboxyhemoglobin is not affected by this reduction and can be quantitated by measurement of the absorbance at 530 nm, 555 nm and 583 nm.

Elevated amounts of methemoglobin (MetHb) and/or sulfhemoglobin (SulfHb) interfere with quantitation yielding falsely elevated values. However, a true positive can be detected if there is a shift in wavelength in $HbO_2$ peak in the non reduced spectra.



## SAFETY

The handling of all reagents, samples and equipment is performed within the guidelines which are detailed in the safety manual.

## INSTRUMENTATION

1. UV Spectrophotometer, Agilent 8453 or equivalent.

## REAGENTS AND MATERIALS

1. Ammonium hydroxide, 0.4%
    a. To a deionized water rinsed and specially marked 2 liter bottle add deionized water to the fill line. Under a fume hood add 8 mL of concentrated ammonium hydroxide using a 5 mL adjustable pipette with a clean tip. Cap and mix well by inverting. Store at room temperature and make fresh at least every six (6) months.
2. Sodium hydrosulfite (sodium dithionite).

3. Disposable cuvetts, Sarstedt acrylic cuvettes, ref # 67.738 or equivalent.
4. RNA QC 253 bovine based CO-Oximeter Control Solutions Levels 1, 2 and 3 (RNA Medical). Individual controls or a combination control set are supplied as a package of 30 x 1.2mL ampoules. Store refrigerated. Three levels of COHb saturation* are used:

   Example:

| | |
|---|---|
| **Level 1** | **(2.8% - 10.8% COHb)** |
| **Level 2** | **(14.6% - 22.6% COHb)** |
| **Level 3** | **(51.5% - 59.5% COHb)** |

*Refer to manufacturer's insert for current ranges

**INSTRUMENT SET UP**



1. Turn on both lamps
   a. Click on Instrument Online icon if the session is not already open.
   b. On upper taskbar select Instrument(s) □ Lamp(s).
   c. Click the on button for both the deuterium and tungsten lamps.
   d. Click OK.
   e. Allow the instrument to "warm up" for at least an hour.
   f. After warming up for at least one hour, on the Mode drop down box select verification and diagnosis. A warning message "Results changed! Do you want to save your results?" will appear. Select no. Another warning message will appear "The current method has been changed! Do you want to save the changes?" Select yes.
   g. In the upper left window in the task drop down menu select Self-test.
   h. In the lower left window select start. A warning message "Clear sample area for the optical tests and keep it clear during the instrument self test". Clear the sample area and then click OK.
   i. Allow the self test to complete it's run.
   j. All test results should have Passed as their result. If not consult a supervisor before proceeding.
   k. Click the "Save to instrument" icon.
   l. Click the printer icon to print a hard copy of the results.
   m. On the mode drop down menu select Standard.
   n. On the Menu bar select File □Load method □ HBCO.M Click on open.
   o. Verify that the task is Ratio/Equation and that Sampling is manual.
   p. Click on setup and verify that:
      i. Use wavelengths WL1 = 530, WL2 = 555, WL3 = 583.
      ii. Calculation name = "~ HbCO" Equation + 240*((WL1-WL3)/WL2)

iii. Unit = %
iv. Prompt for sample information is checked.
v. Data type is absorbance.
vi. Display spectrum: From 500 To 650.

q. Click OK.

**QUANTITATION PROCEDURE:**

1. Add ~ 75 µL of specimen or control to 4 ml of 0.4% ammonium hydroxide in appropriately labeled 13x100 tubes. Adjust the solution until its $\lambda_{MAX}$ of the alpha and beta bands are both between 1 and 1.5 absorbance units. (NOTE: exact amounts of blood and diluent cannot be given because the total amount of hemoglobin in each specimen varies greatly). Be sure there is no particulate matter, i.e. fat, tissue, or cell clots within the blood solution, as these can interfere with the quantitation and give false positives. Remove these by centrifugation.
2. Mix the solution.
3. Allow the solution to stand at room temperature for 5 minutes.
4. Zero the spectrophotometer utilizing 0.4% $NH_4OH$ as a reference.
5. Transfer 3 mL of specimen(s) and control solutions to respective 1.0 cm cells.
6. Scan the solution from 650 nm to 500 nm. (Unreduced Scan).
7. Note the location of the peak at approximately 576 nm (alpha band), This will help give an approximate idea of how saturated the specimen is. Also observe the size in proportion to the peak at 540 nm (beta band). The 540 peak appears greater in proportion with greater saturation.
8. Look for peaks at 620 and 630 nm. These would indicate the presence of MHb and SHb respectively. Report the possible presence of these when requested
   Note: This scan will allow the detection of other possible hemoglobin derivatives.

| **Derivatives** | **Absorption Bands** |
|---|---|
| Oxyhemoglobin | 540 - 542 nm & 576 - 578 nm |
| Carboxyhemoglobin | 538 - 540 nm & 568 - 572 nm |
| Methemoglobin | 577 nm & 540 nm |
| Cyanomethemoglobin | 540 nm |
| Reduced hemoglobin | 555 nm |

9. Add approximately 40 mg of sodium dithionite to the sample cell and reference cell.
10. Cover the cells with parafilm and invert each cell 5 times.
11. Scan the cells from 650 nm to 500 nm. Note the absorbance at 530nm, 555nm and 583nm (this will be done automatically by the Agilent 8453 Spectrophotometer).

**CALCULATION FOR THE % CARBOXYHEMOGLOBIN**

Calculations are performed by an excel spreadsheet located T:\Carboxyhemoglobin\ OCME Tox HbCO by Spectrophotometry.
The formulas used are:

The approximate % saturation is then multiplied by a correction factor found in the following table:

| % SATURATION | Factor |
|---|---|
| [-]to4 | 0.913 |
| 5-9 | 0.903 |
| 10-14 | 0.893 |
| 15-109 | 0.883 |
| 20-24 | 0.873 |
| 25-29 | 0.863 |
| 30-34 | 0.853 |
| 35-39 | 0.843 |
| 40-70 | 0.833 |
| 71-90 | 0.843 |
| 91 + | 0.853 |

From the **reduced** spectra enter the absorbencies for the wavelengths 530, 555 and 583 into the appropriate cells of the spreadsheet. A factor will be calculated to convert the approximate carboxyhemoglobin to the final percentage saturation.

**ACCEPTANCE CRITERIA**

1. All liquid controls must fall within the expected ranges for the lot numbers currently in use.
2. No to minimal interfering compounds can be present.

**REPORTING**

1. Results less than 5% COHb are reported as “carbon monoxide less than 5% saturation”.
2. Results greater than or equal to 5% COHb are reported as “carbon monoxide *X* % saturation”.

3. If two blood specimen types are analyzed on one case, report them separately. Do not average the two readings.
4. If there is an excess of other heme pigments (i.e. Methemoglobin, sulfhemoglobin etc.) then the specimen is reported as unsuitable for analysis.

**INTERFERING SUBSTANCES**

Met and Sulf hemoglobins can cause elevated %COHb.

**REFERENCE**

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