

SIMULATION OF MEASUREMENT OF HEMOGLOBIN IN BLOOD BY SPECTROPHOTOMETRY

Spectronic 20 Genesys Version

INTRODUCTION

Hemoglobin is the portion of the red blood cell that is responsible for transporting oxygen from the lungs, gills, or skin of an animal to its capillaries for use in the respiration process. Detecting levels of hemoglobin in the blood is important to the medical profession to assist in diagnosing and treating patients.

Instrumental techniques are continually replacing "wet" methods of chemical analysis. Quality control in manufacturing, routine medical tests, and basic research rely on a variety of instruments for qualitative and quantitative determinations. An instrument called a **spectrophotometer** will be used in this experiment.

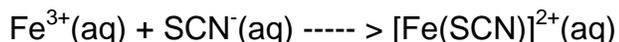
A spectrophotometer is an instrument, which generates a light beam of a particular color and measures the amount of that light absorbed by a substance in a solution. The most commonly used instrument for clinical use in the U.S. is the Bausch & Lomb Spectronic 20 or its newer version, the Spectronic 20 Genesys.

The color that we perceive is due to the specific wavelengths of light reflected or transmitted by an object. If white light (a combination of all the wavelengths in the visible region) shines on an object and all the wavelengths except red are absorbed, the red is reflected, and we see the object as red. Likewise, a red solution absorbs all colors except red, which it transmits.

The amount of light absorbed at an appropriate wavelength by a substance in solution is proportional to the concentration of a substance. Thus, by using a spectrophotometer to determine the absorbance of light by a solution, one can determine the concentration of the solution. Concentration refers to the relative amount of solute dissolved in the solvent to make the solution.

THEORY

The thiocyanate method of spectrophotometric analysis employs the thiocyanate ion (SCN^-), called a chromogenic reagent, which reacts with the Fe^{3+} ion in a sample to form an orange-red complex, $[\text{Fe}(\text{SCN})]^{2+}$. The chemical reaction, which illustrates this, is shown in Equation 1 below.



If proper control over the acid and thiocyanate concentrations are exercised, the appearance of the red color of this solution in room light (white light containing all colors of the visible light spectrum) is due to the transmission of wavelengths of light corresponding to the red region of the spectrum. The other primary colors in white light are absorbed by the solution.

The Spectronic 20 spectrophotometer is an instrument which allows a specific wavelength of light to be focused upon an absorbing solution and then measures the percentage of this light that is not absorbed (i.e. transmitted) by the solution. The transmitted light is related to the concentration of the colored substance in the solution. The greater the concentration of the absorbing substance, the smaller the amount of light transmitted by the solution.

Measuring the concentration of Fe^{3+} by spectrophotometry involves a method of comparative analysis using solutions of known Fe^{3+} concentration, treated identically to the sample of interest. The light absorbed by all samples (standards and unknown samples) is measured with the Spectronic 20.

The known solutions of iron are used to create a calibration curve, which is a graph relating the absorbance of a solution (on the y-axis) to the concentration of iron in that solution (on the x-axis). The plotted results generate a scatter graph through which a straight line can be drawn. When the absorbance of an unknown solution is determined with the Spectronic 20, we can determine the concentration of Fe^{3+} that corresponds to that absorbance.

In this experiment, a Spectronic 20 will be used to demonstrate how the concentration of hemoglobin (Hb) is measured in blood samples. Instead of actually using blood, we will use a solution containing $[\text{Fe}(\text{SCN})]^{2+}$. Both blood and the test solution owe their red color to the covalent bonding of Fe in their respective complexes.

TECHNIQUES

Using a Volumetric Flask

The solutions prepared in this lab will require the use of a volumetric flask. Volumetric glassware is relatively easy to use once you have had some practice.

1. Add the desired quantity of each chemical to the volumetric flask.
2. Add distilled water CAREFULLY until the water level approaches the dilution mark on the flask (see figure below). DO NOT go past the mark!
3. Use a water bottle or an eye dropper to add the last few milliliters to the flask such that the bottom of the meniscus is level with the dilution mark on the flask (see "Reading a Meniscus" on page of 12 if you need help).

Use of Spectrophotometers

Essentially, a spectrophotometer sends a beam of light through your sample, and measures how much of it gets through (is transmitted). This is a function of how much light is absorbed by the specific chemicals contained in the sample. Spectrophotometers can read either "%T" (=percentage of light that is transmitted through the sample) or "Absorbance" (=the amount of light absorbed, expressed in arbitrary units called absorbance units, or optical density). Several types of spectrophotometers are routinely used in biological laboratories. This first is a relatively simple one also called a colorimeter, that measures only the visible range of light waves. For many years the standard colorimeter used in teaching laboratories has been the Spectronic 20. A newer version is the Spec 20 Genesys, described below; its improvements include digital instead of analog readout.



Using a Spectronic 20 Genesys Spectrophotometer™

When you turn on the Spectronic 20 Genesys™ instrument, it performs its automatic power-on sequence (check to be sure the cell holder is empty and its cover closed before turning on the instrument). This includes a self-check of the software, and initializing the wavelength filter mechanism. The sequence takes about 2 minutes to complete; do not interrupt during this sequence. Allow the instrument to warm up for about 15-20 minutes before you are ready to use it. When your samples and blanks (or calibration tubes) are ready, follow the steps below to operate the instrument.

1. Press 'A/T/C' to select the absorbance or % transmittance mode. The selected mode will appear on the display.
2. Press 'nm^' or 'nm"' to select the wavelength specified in your exercise. Note: holding either key will cause the wavelength to change more rapidly than pressing many times.
3. Insert your blank into the cell holder and close the sample door. Be sure to position the cuvette so that the light passes through the clear walls from front to back.
4. Press '0 ABS/100%T' to set the blank to 0 absorbance, or to 100% transmittance, depending upon which mode was selected in #1 above.
5. Remove your blank and insert your sample into the cell holder. The sample measurement appears on the LCD display. Repeat as often as necessary to read the values for all of your samples, periodically returning to the blank (steps 3-4) to check that the calibration of the instrument remains stable.
6. The Spectronic 20 Genesys™ may also be used to directly determine concentration using either a known factor or a freshly prepared standard curve. See your instructor or the manufacturer's Operator's Manual for directions. The "Print" option is only functional

when the instrument is connected directly to an external printer. Do not press buttons to access other functions of the spectrophotometer unless instructed to do so, including the Utilities option.

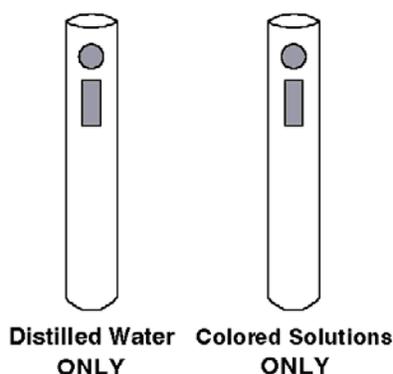
Note: there are two arrow keys and two unlabeled keys below the LCD display. These keys are used to select or access alternate functions available on certain menus. Do not select these keys unless instructed to do so.

7. When you have completed all of your sample measurements, remove all samples from the spectrophotometer. Rinse your cuvettes and leave to dry. Always turn off and cover the instrument before leaving the laboratory.

Using Spec 20 Tubes

The Spectronic 20 Spectrophotometer uses special tubes called cuvettes (see diagram below). You will be provided with (2) Spec 20 tubes.

1. In one of the Spec 20 tubes, fill the tube with distilled water until it is three-quarter full. This tube will be your “blank” and it is to contain ONLY distilled water.
2. The second Spec 20 tube will be used for all of your colored solutions. Rinse two or three times with small portions of the solution to be measured. Fill three-quarters full before measuring the absorbance of the colored solution.



SAFETY AND DISPOSAL

- The Hb Stock will stain your skin and clothes. Handle with care.
- All solutions may be disposed of down the drain with water.

EXPERIMENTAL PROCEDURE



will appear to indicate helpful hints, additional information, or interesting facts.

I. Establishing a calibration curve

- A. Prepare the solutions below one at a time in a 100 mL volumetric flask using the stock solutions on the center bench. The stock solutions are in repipettors and dispense the same amount of solution each time if the plunger is pushed straight

down and allowed to rise completely. **Watch out for bubbles/gaps in the repipetter tube.** The Hb Stock repipetter is set to dispense 4 mL and the NH_4SCN repipetter is set to dispense 3 mL. Once the appropriate quantity of Hb Stock, NH_4SCN , and 10% HNO_3 have been added to the 100 mL volumetric flask, dilute to 100 mL as described in the TECHNIQUES section. Put the stopper into the volumetric flask and invert several times to mix the solution. Measure and record the absorbance of the solution on the Spectronic 20 immediately after preparation. Save a portion of the solution in a test tube, rinse the volumetric flask thoroughly with distilled water, and proceed to the next solution. Do not discard your solutions until your calibration curve has been completed and approved.

1. Solution 1 -- 4 g Hb/100 mL
Add 1 pump from the Hb Stock repipetter, 2 drops of 10% HNO_3 , and 1 pump from the NH_4SCN repipetter to the 100 mL volumetric flask. Dilute to the mark with distilled water.
 2. Solution 2 -- 8 g Hb/100 mL
Add 2 pumps from the Hb Stock repipetter, 4 drops of 10% HNO_3 , and 2 pumps from the NH_4SCN repipetter to the 100 mL volumetric flask. Dilute to the mark with distilled water.
 3. Solution 3 -- 12 g Hb/100 mL
Add 3 pumps from the Hb Stock repipetter, 6 drops of 10% HNO_3 , and 3 pumps from the NH_4SCN repipetter to the 100 mL volumetric flask. Dilute to the mark with distilled water.
 4. Solution 4 -- 16 g Hb/100 mL
Add 4 pumps from the Hb Stock repipetter, 8 drops of 10% HNO_3 , and 4 pumps from the NH_4SCN repipetter to the 100 mL volumetric flask. Dilute to the mark with distilled water.
 5. Solution 5 -- 20 g Hb/100 mL
Add 5 pumps from the Hb Stock repipetter, 10 drops of 10% HNO_3 , and 5 pumps from the NH_4SCN repipetter to the 100 mL volumetric flask. Dilute to the mark with distilled water.
- B. Prepare graph using computer (option 1)
1. An Excel computer program will be set up on a computer at the back of the lab. Input your 5 absorbances on the worksheet.
 2. Double click inside the graph to highlight the graph. Before you print, check print preview to ensure that the graph is set to print.
 3. An equation will print on your graph. The equation is derived from the data you entered for the graph. Show the graph to your instructor before discarding your solutions or attempting to go on to part II.
- C. Prepare graph using graph paper (option 2)
1. Turn the page sideways to set up the graph so that the absorbance is plotted on the vertical (y) axis.

2. The horizontal (x) axis will be concentration in g Hb/100 mL and should run from 0 to 20 g Hb/100 mL or so. Try to develop a scale that will utilize most of the graph paper and also have convenient units.
3. Be sure to label your axes and to give the graph appropriate titles.
4. Plot the five data pairs and draw a single, straight, light line about the points. DO NOT CONNECT THE DOTS. See example below.
5. Show your plot to your instructor before you discard your solutions or attempt to go on to part II.

II. Determination of unknown concentration of blood

- A. Prepare sample
 1. Obtain 5.0 mL of an unknown sample of "blood".
 2. Dilute your sample carefully with 5.00 mL of distilled water. This should be done in a small clean and dry Erlenmeyer flask or beaker where the sample can be well mixed by swirling the flask or beaker.
- B. Read the absorbance of the sample. Using the equation or your calibration graph, find the Hb concentration in the diluted "blood".
- C. Calculation. The true content in the whole blood will be twice that value because of the 1:1 dilution. This dilution is necessary since the intense color saturates out at high absorbencies.
- D. Repeat your analysis of the "blood" unknown by diluting a fresh 5.0 mL sample, measuring the absorbance, etc., to obtain a second value. The two analyses will usually result in slightly different values. This should give you an idea of the reproducibility of the method. Be sure you have cleaned up all your glassware.
- E. Normal human levels of Hb in Blood (The Merck Manual, 16th ed., 1992)
 1. Male 13-18 g Hb/100 mL
 2. Female 12-16 g Hb/100 mL

