MEASUREMENT OF "HEMOGLOBIN" IN BLOOD BY SPECTROPHOTOMETRY

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 light that is absorbed by a substance in a solution.

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, $[Fe(SCN)]^{2+}$. The chemical reaction, which illustrates this, is shown below.

$$Fe^{3+}(aq) + SCN^-(aq) \rightarrow [Fe(SCN)]^{2+}(aq)$$

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 $[Fe(SCN)]^{2+}$. 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 enough distilled water so that the flask is about two-thirds full.
3. Cover the flask with a stopper or Parafilm and invert the flask 10-15 times to promote mixing.
4. Add distilled water CAREFULLY until the water level approaches the dilution mark on the flask (see figure below). DO NOT go past the mark!
5. 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.
6. Cover the flask with a stopper or Parafilm and invert the flask 10-15 times to promote mixing. It is important that the solution is homogenous before it is used for a measurement.

General Properties of Spectrophotometers

A spectrophotometer directs light through your sample, and measures how much of it passes through the sample (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 laboratories. The Spectronic 20 Genesys™ Spectrophotometer that we will use in is a robust, user-friendly instrument that is well suited for the teaching lab.

Using Spec 20 Cuvettes

The Spectronic 20 Genesys™ Spectrophotometer uses rectangular sample tubes called cuvettes. You will be provided with (2) cuvettes.

1. In one of the cuvettes, fill the tube with distilled water until it is three-quarter full. This will be your "blank" and it is to contain ONLY distilled water.

2. The second cuvette 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.

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.

Your instructor may complete some of the steps below prior to the start of the lab period. Pay attention to your instructor's directions and do not press any keys on the spectrophotometer unless instructed to do so. 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

1. Press 'A/T/C' to select the absorbance or % transmittance mode. The selected mode will appear on the display. We will work in Absorbance mode (A).
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. 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.

SAFETY AND DISPOSAL

- The Hemoglobin Stock Solution ("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 solutions one at a time in a 100 mL volumetric flask using the stock solutions on the side benches.

1. Bring two clean 100 or 150 mL beakers to the side bench and transfer about 70 mL of the Hemoglobin Stock solution to one beaker and about 50 mL of the NH_4SCN solution to the second beaker and return to your bench.
2. After returning to your bench, use your 10 mL graduated cylinder to transfer the volumes of Hb stock solution and NH_4SCN solution to your 100 mL volumetric flask, as shown in the table below.
    a. For each transfer, pour the stock solution from the beaker into the graduated cylinder, stopping prior to reaching the target volume and using a dropper to add the remaining solution dropwise to reach the target volume. Rinse the graduated cylinder with a few mL of distilled water after each transfer and pour the rinse into the volumetric flask as well.
    b. For volumes greater than 10 mL, you will need to make multiple transfers of the solution. For instance, to transfer 16.0 mL, first transfer 10.0 mL with the graduated cylinder, then transfer the remaining 6.0 mL for a total volume of 16.0 mL

Solution	Concentration	Volume Hb Stock	Volume NH_4SCN	Volume HNO_3
1	4 g Hb/100 mL	4.0 mL	3.0 mL	2 drops
2	8 g Hb/100 mL	8.0 mL	6.0 mL	4 drops
3	12 g Hb/100 mL	12.0 mL	9.0 mL	6 drops
4	16 g Hb/100 mL	16.0 mL	12.0 mL	8 drops
5	20 g Hb/100 mL	20.0 mL	15.0 mL	10 drops

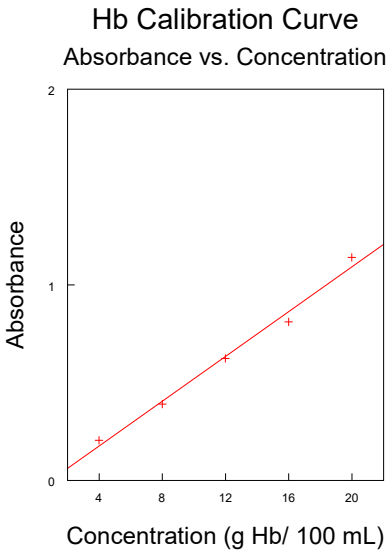
3. Once the appropriate quantity of Hb Stock, NH_4SCN, and 10% HNO_3 have been added to the 100 mL volumetric flask, dilute to 100 mL as described in the TECHNIQUES section.
4. Put the stopper into the volumetric flask and invert several times to mix the solution.
5. Measure and record the absorbance of the solution on the Spectronic 20 immediately after preparation.
6. Save a portion of each solution in a clean test tube, rinse the volumetric flask thoroughly with distilled water, and proceed to the next solution.

a. **Do not discard your solutions until your calibration curve has been completed and approved.**

7. Repeat steps 2-5 for solution 1 two more times so that you have a total of three measurements for the 4 g Hb/100 mL solution.

B. Prepare graph using computer

1. An Excel worksheet will be set up on a computer in the lab.
2. Input your 5 absorbances on the worksheet. A graph will be automatically created, plotting concentration in g Hb/100 mL on the horizontal (x) axis and absorbance on the vertical (y-axis). Confirm with your instructor that the plot looks reasonable before continuing.
3. An equation will print on your graph. The equation is derived from the data you entered for the graph. **Record the equation on your data sheet.**
4. Double click inside the graph to highlight the graph. Before you print, check print preview to ensure that the graph is set to print.
5. Once you have a satisfactory plot, discard your solutions and 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 5.0 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)

Male	13-18 g Hb/100 mL
Female	12-16 g Hb/100 mL