Lab 2

Spectrophotometric Measurement of Glucose

Objectives

1. Learn how to use a spectrophotometer.
2. Produce a glucose standard curve.
3. Perform a glucose assay.

Safety Precautions

Glucose Color Reagent and the Glucose Standard are irritants. Hydrochloric acid is a corrosive. Use gloves and goggles.

Materials

Spectrophotometer (340-600 nm)
0.1, 1.0, and 10 mL serological pipettes
15 x 125 mixing tubes
cuvettes
0.1 N Hydrochloric acid
Glucose Kit (Sigma 115-A)
500 mg/dl Glucose standard (Sigma G3761)
Grape Kool-Aid test solution
Blank 1
Blank 2
Grape Kool-Aid Glucose

Introduction

Diabetes mellitus is a serious incurable disease that affects approximately 16 million U.S. citizens, although only 10 million have been diagnosed. It is a major cause of death in the United States, and its complications include kidney failure, blindness, and lower limb amputations. Diabetes mellitus is caused by the inability of body cells to uptake glucose. This inability can be caused either by low levels of insulin, a hormone necessary for the movement of glucose across cell membranes, or by a defect in the insulin-binding receptors on cell membranes. Glucose is a monosaccharide that is used by cells as an energy source, although when it is not available, most cells in the body use fatty acids as their fuel. After a meal, glucose is absorbed into the bloodstream, elevating the concentration of glucose found in the blood. Insulin is released as a result, and all cells switch to burning glucose. Diabetes is diagnosed by measuring the amount of glucose in blood after an 8-hour fast from food. In normal healthy individuals, the fasting glucose ranges between 70 –110 mg/dl. If the fasting blood glucose level is high (greater than 126 mg/dl on two different occasions) and symptoms such as frequent urination or excessive thirst are present, a diagnosis of diabetes is made.

In this laboratory exercise, you will learn how to measure the concentration of glucose using a spectrophotometer. If you have ever made Kool-Aid, you already have observed some of the basic principles of spectrophotometry. If you add a package of Grape Kool-Aid to a pitcher and then begin to add water, you will notice that at first the solution is a very dark purple. The

ratio of Kool-Aid to water (the Kool-Aid's concentration) is very high. As you add more water, the ratio of Kool-Aid to water decreases and the solution becomes lighter in color. Thus, the intensity of color is directly related to concentration of Kool-Aid. A spectrophotometer can measure the amount of light absorbed by a solution, and the quantity of light absorbed is proportional to the intensity of color or pigment in the solution.

A second principle involved in spectrophotometery is that the amount of light absorbed is also dependent on through how much of the solution the light must pass. If you pour the Grape Kool-Aid into a slender test tube and compare its color to Grape Kool-Aid poured into a drinking glass, the Kool-Aid in the drinking glass will appear darker in color. This is because as light travels through the liquid more and more light is absorbed by the Kool-Aid and less light is transmitted.

The spectrophotometer measures absorbance. Absorbance values, by themselves, do not describe the concentration of a substance. However, we can determine the concentration of a substance in a solution using a standard curve. A **standard curve** translates absorbance values into concentration. See Fig 2.1 for an example. We can construct a standard curve by making solutions with a known concentration of the substance we are measuring and then measuring their absorbance. Graphing the concentration on the x-axis and the absorbance on the y-axis, we can see that there is a linear relationship between concentration and absorbance. Thus a standard curve is not really a curve, but a straight line. Beers Law describes this linear relationship:

$$A = \varepsilon CL$$

Where   A = absorbance

ε = molar absorptivity of the substance (how much light it absorbs at the wavelength used)

C = concentration (for clinical measurements, the concentration often used is mg/dl)

L = pathlength (the length the light travels through the cuvette—remember the slim cuvette versus drinking glass)
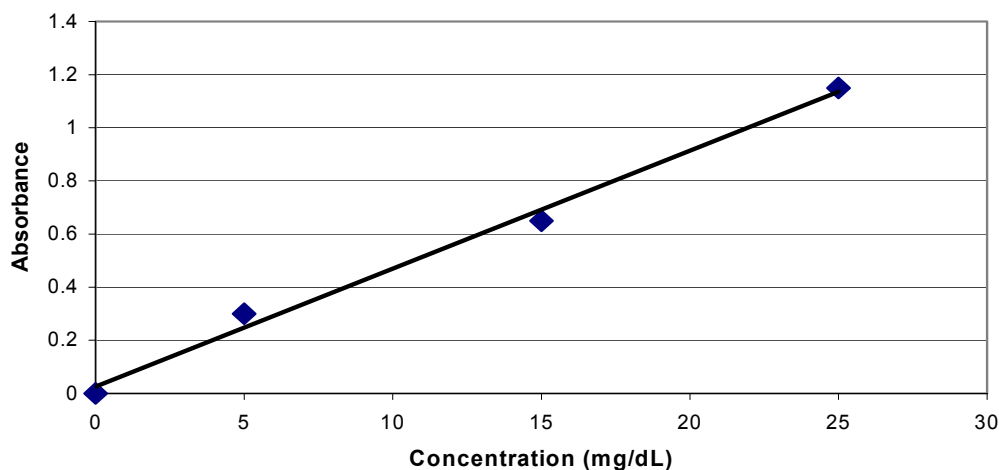

Fig 2-1. Example of standard curve

Using the standard curve, we can determine the concentration of other solutions, by locating the absorbance of that solution on the y-axis and drawing a horizontal line to the standard curve line. Then you can draw a vertical line from that intersection to the x-axis to determine the concentration.

It is also possible to calculate the concentration if we know the slope of the standard curve. In the equation for a straight line, y= mx +b, m is the slope of the line. The equation for Beer's Law also describes a straight line with an intercept of zero. Absorbance is measured on the y-axis, concentration on the x-axis and the value εL comprise the slope (m). An alternative way to determine the concentration of a substance is to rearrange Beer's Law, dividing the absorbance of the solution by the slope of the standard curve:

$$C = \frac{A}{\varepsilon L}$$

We assume that the y-intercept is zero. In other words we are assuming that the absorbance of a solution is zero when none of the substance we are measuring is present. In reality, that is not always the case. If we are measuring the concentration of Grape Kool-Aid, other substances might be present that absorb light. If this occurred, our y-intercept would not be zero. Thus when we are using a spectrophotometer we need to calibrate it so that when none of the substance we are measuring is present, the absorbance that is measured is zero. We do this by using a blank. A **blank** solution contains all the substances present in the solution *except* the substance to be measured. Before making measurements with the Spec 20, the blank is measured, and its absorbance value is set to zero. For example, if we are measuring the concentration of Grape Kool-Aid, the blank should be made of water and sugar, the other constituents in the solution. By using a blank, if water or sugar absorbs light, that absorption will be set to zero, and thus the y-intercept on our linear Beer's Law equation would indeed be zero.

Procedure

Spectrophotometric Technique (See Fig 2-2)
1. Plug in the spectrophotometer and turn it on by turning the left dial clockwise. Allow the spectrophotometer to warm up at least 5 minutes before proceeding.
2. Set the wavelength dial to 600 nm. This is a wavelength of light that gets absorbed readily by the pigment in the Grape Kool-Aid.
3. With no vial in the spectrophotometer, the light path is closed. Therefore no light is transmitted, or in other words there is an infinite absorption. With the on/off dial (left dial) set the needle to read infinity (∞) on the absorbance scale.
4. Now fill a cuvette (a small test tube that is 12 mm in diameter and 100 mm in length) with Blank 1. This solution should contain all the constituents except the substance to be measured. Since we are measuring Grape Kool-Aid, Blank 1 contains the other constituents, sugar and water, in the same proportion as are found in the Grape Kool-Aid.
5. Insert the cuvette containing Blank 1 into the sample chamber. As you do the light path will be opened. This blank solution does not contain any Grape Kool-Aid, and so the absorbance should be set to zero. Use the right knob to set the absorbance to zero.
6. Next fill a cuvette with Grape Kool-Aid test solution. Insert it into the spectrophotometer and record its absorbance in question number one of your assignment.
7. Next, fill a cuvette with Blank 2 solution and repeat steps 5 and 6. Is there a difference in the measurements of the same solution? How can you explain it?
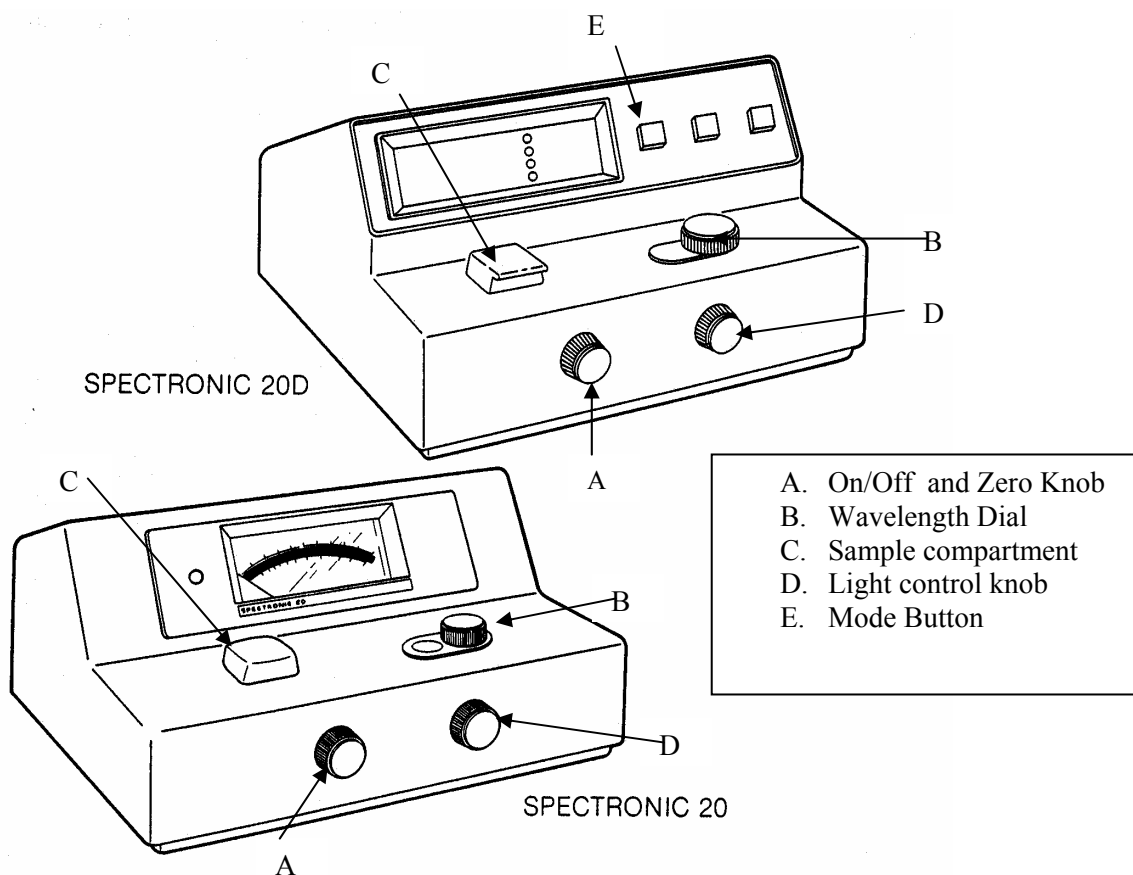
E

C

B

D

SPECTRONIC 20D

A

C

B

D

SPECTRONIC 20

A

A. On/Off and Zero Knob
B. Wavelength Dial
C. Sample compartment
D. Light control knob
E. Mode Button

Fig. 2-2. Diagram of Spec 20 and Spec 20D Spectrophotometers

Glucose Assay

Now we will use the spectrophotometer to measure the concentration of glucose (sugar) in Kool-Aid. When you measured the absorbance of Kool-Aid, you made a direct measurement. The Kool-Aid was a colored product that the spectrophotometer could easily measure. In the glucose assay we will use, you will not measure the concentration of glucose directly. Instead, you will measure the absorbance of colored product created when glucose reacts with the Glucose Assay Reagent. The amount of colored substance is directly proportional to the amount of glucose present. You will create a standard curve that translates the absorbance of the colored substance into concentration of glucose and will use it to determine the amount of glucose in Kool-Aid.

1. First you will make several dilutions of a glucose standard solution that has an initial concentration of 500 mg/dl of glucose. See Fig. 2-3.
   a. Label three large mixing tubes A, B, C.
   b. Into each of the large mixing tubes, pipette 5 ml of deionized water.
   c. To mixing tube A, add 5 ml of glucose standard solution. Cover with Parafilm and mix well.
   d. Into mixing tube B, pipette 5 ml of standard dilution A. Cover with Parafilm and mix well.

e. Into mixing tube C, pipette 5 ml of standard dilution B. Cover with Parafilm and mix well.

Glucose
Standard

Standard
Dilution A   Standard
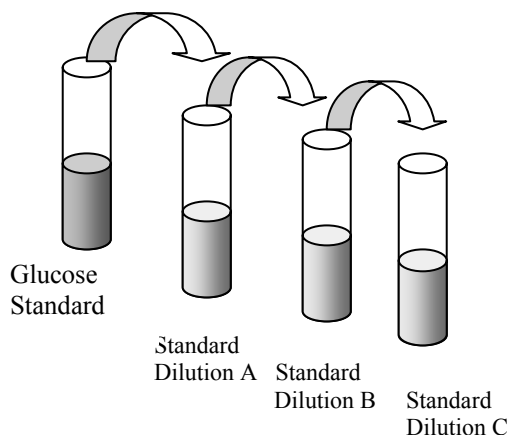Dilution B   Standard
Dilution C

Fig. 2-3. Illustration of the dilutions made.

2. Calculate the concentration of glucose in each tube using the following equation and record it in your assignment:

$$V_1 C_1 = V_2 C_2$$

Where $V_1 =$ volume of the stock solution
C_1 = the concentration of the stock solution
V_2 = the final diluted volume (volume of stock solution and diluent)
C_2 = the final diluted concentration.

For example, we want to find the final diluted concentration (C_2) of Standard Dilution A. V_1 is the volume of the stock solution we are adding to Standard Dilution A. Therefore V_1 is 5 ml or 0.5 dl. The concentration of the stock solution is 500 mg/dl; this is C_1. V_2 is the final diluted volume of Standard Dilution A. Since we are adding 5 ml of stock solution to 5 ml of water, the final diluted volume of Standard Dilution A is 10 ml or 1 dl. Therefore, V_2 is 10 ml or 1 dl. Inserting these values into the formula, we see that:
$$(0.5 \text{ dl})(500 \text{ mg/dl}) = (1 \text{ dl}) C_2$$

$$C_2 = 250 \text{ mg/dl}$$

What is your stock solution for Standard Solution B? What about Standard Solution C?

3. For the glucose assay, we will need seven more mixing tubes. One tube will be labeled "blank." Three additional tubes will be used to test the different concentrations of glucose standard. These tubes can be labeled Standard A, Standard B, and Standard C. Finally, three more tubes will be used to test a sample of Kool-Aid that your instructor or TA will give you. Your instructor or TA will give each lab group a different Kool-Aid sample to assay. Make sure to record in your assignment, which Kool-Aid sample you were assigned. You will test your Kool-Aid sample three times in three different tubes. When the same sample is tested more than one time, the individual tests of that sample are called replicates. By testing the glucose concentration more than one time and averaging the results, we will get a more accurate estimate of its true glucose concentration. For these replicates of your Kool-Aid sample, you can label the remaining mixing tubes Kool-Aid 1, Kool-Aid 2, and Kool-Aid 3.

      a. To each tube, add 1.0 ml of Glucose Assay Reagent
      b. Add 20 microliters (0.02 ml) of water to the Blank tube.
      c. Add 20 microliters (0.02 ml of the appropriate sample (Standard solutions A-C and Kool-Aid solutions 1-3) to each tube.
      d. Mix by gently swirling the tubes.
      e. After 5-10 minutes, add 10 ml of 0.1N hydrochloric acid to each tube, and mix.
      f. Turn the wavelength dial to 520 nm.
      g. With nothing in the sample chamber, adjust the absorbance to ∞ using the left knob.
      h. Pipette or pour the blank solution into a cuvette, until the cuvette is ¾ full. Insert the cuvette into the spectrophotometer. Adjust the absorbance to zero using the right knob.
      i. Pipette or pour each of the sample solutions into cuvettes, until each cuvette is ¾ full. Measure the absorbance of Standards A-C, and the Kool-Aid. Record each absorbance in your assignment. It is important to complete the measurements within 30 minutes after the addition of the hydrochloric acid.

4. Construct a graph of standard curve using Microsoft Excel using the concentration and absorbance of the blank, and standards A-C as data points. Include the equation for a line on your graph if possible, since this will show you the slope of the curve.
5. Examine your standard curve. If your pipetting has been accurate and precise, your data points should be very close to the standard curve line, and the line should go through the origin.   If the line does not go through the origin, you will have to redraw the line ensuring that it passes through the origin. The quality of your standard curve will directly influence how accurately you can determine the concentration of glucose in Kool-Aid.
6. Determine the concentration of glucose in each Kool-Aid sample either by calculating it or using the graph. To calculate it, divide its absorbance by the slope of the standard curve. If you did not have to redraw the standard curve graph, you can find the slope of the standard curve in the equation of a line.
7. Calculate the average glucose concentration in the Kool-Aid you assayed. How much variability is present in your measurement?

References

http://www.cdc.gov/od/oc/media/fact/diabetes.htm [accessed 6/12/01]Center for Disease Control , Office of Communications/Media Relations Diabetes Fact Sheet

http://www.awl.com.au/pharma/drugcalc.html [accessed 6/12/01] "Fundamentals of Pharmacology on-line." Addison, Wesley, Longman.

Sigma Technical Bulletin, Procedure No. 115. 1997. Sigma Diagnostics, St. Louis, MO

Name_____ ***Lab Section Day & Time_____***

Lab 2 Assignment

Spectrophotometric Technique

1. The absorbance of the test solution of Grape Kool-Aid was _____.

2. After blanking with Blank 2, the absorbance of the test solution of Grape Kool-Aid was

    _____.

    Is there a difference between the two measurements of the same solution? If so, why?

Glucose Assay
3. Calculate the concentration of glucose in Standard Dilutions B and C. Show your
    calculations.

4. Transcribe the concentrations you calculated for each standard solution into the table
    below. Then record the absorbance of the standards and of the Kool-Aid in the glucose
    assay.

Solution	Glucose Concentration	Absorbance
Standard A		
Standard B		
Standard C		
Kool-Aid 1		
Kool-Aid 2		
Koolaid 3		

5. Using Microsoft Excel, construct and attach a glucose assay standard curve. Record the identification of the sample of Kool-Aid you tested. (Each group is given a different sample and might obtain different results. For grading purposes, we need to know which sample you received.) What is the concentration of glucose in the three samples of Kool-Aid? What is the average concentration of glucose in the Kool-Aid you tested? Show your calculations.

Kool-Aid Sample #: _____	**Concentration**
Kool-Aid 1	
Kool-Aid 2	
Kool-Aid 3	
Average Kool-Aid Concentration	

Thought questions

6. A physician has ordered that an insulin-dependent diabetic patient be injected with 25 units of insulin. You have a 10-ml vial of insulin that contains 100 units/ml of insulin. With how many milliliters of insulin should the patient be injected? A simple ratio can be set up to solve the problem.
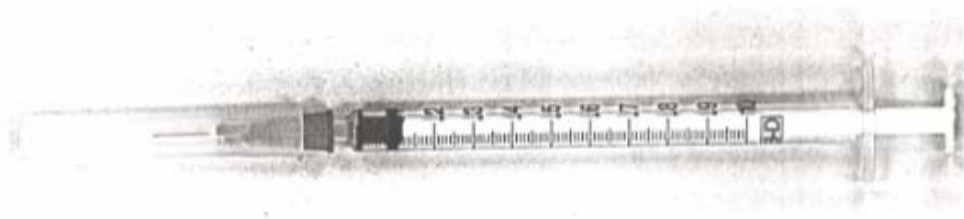
$$\frac{RD}{V} = \frac{SD}{SV}$$

where   RD = Required dose (prescribed by the physician)
V = volume to inject
SD = Stock dose (concentration of insulin in the vial)
SV= Stock volume (volume of insulin in the vial)

This formula can be rearranged:

$$V = \frac{RD \, x \, SV}{SD}$$

Show your calculations and then mark the following picture of a syringe to indicate the amount you should inject.

7. After your experience performing the glucose assay, why do you think the diagnosis of diabetes is made only after measuring fasting blood glucose levels greater than 126 mg/dl on **two different** occasions?