UNIT: Spectrophotometry

Instrumentation II

Task

Operation of the spectrophotometer, Beer's Law, and spectral transmittance curves.

Objectives

Upon completion of this exercise, the student will be able to:

1. Use proper technique in operating a spectrophotometer and make accurate readings on absorbance and transmittance scales.
2. Calibrate the spectrophotometer with a didyium filter.
4. Explain the method for development of a spectral-transmittance (S-T) curve and plot an S-T curve.
5. Be able to define and use range of linearity, blank solutions, and standards and controls.

Supplies and Equipment

1. Spectrophotometer & cuvettes
2. Hemoglobin solution
3. Graph paper (linear and semi-log)
4. Filter paper
5. Didymium filter
6. Blood drawing supplies (lavender tops)

Principle

The spectrophotometer has proven to be the most versatile, reliable and widely used of all laboratory instruments in clinical chemistry. The majority of clinical chemistry procedures have been developed to produce a colored end-product which can be detected and measured by some sort of photometer. Even highly automated analyzer systems utilize a spectrophotometer as a read-out device.

Because of the heavy usage of spectrophotometers, it is imperative that laboratory workers have a firm understanding of the principles of spectrophotometry as well as the technical abilities to operate these instruments properly and perform basic calibration and maintenance procedures.

Photometry (direct colorimetry) measures absorbance (A) also called optical density (OD), or percent transmittance (%T).

The depth of the solution is held constant using a standard cuvette. (Properties of cuvettes are critical.) The substance molecules can be thought of as occurring in innumerable monomolecular layers of absorbing material. As the absorbance of a solution increases, %T decreases logarithmically and will plot as a straight line on semi-log paper. The radiation not transmitted is
absorbed characteristic of a given molecule and is called molar absorptivity. Molar absorptivity is a characteristic of a substance.

In a double beam system, the light is divided/split into two paths. One path passes through the reference cuvet (incident light) while the other is directed through the sample (transmitted). A major advantage of a double beam system is that the reference (blank) is periodically checked and any changes are immediately compensated. Refer to your classroom notes for further information on double beam systems.

The basic concept of light transmittance through a solution is important, because only transmitted light can be measured in a spectrophotometer. The transmittance (T) is defined as the proportion of the incident (original or incoming) light that is transmitted.

\[
T = \frac{\text{transmitted light}}{\text{incident light}} = \frac{T}{T_i} \times 100 = \%T
\]

\[
\frac{T}{T_i} = \frac{2}{4} = 0.5 \times 100 = 50\%T
\]
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The absorption and/or transmission of light through a specimen is used to determine molar concentration of a substance.

The relationships between absorption, transmittance and concentration is stated using the Beer-Lambert Law:

The concentration of a substance (or strength of “color”) is directly proportional to the amount of light absorbed by the chromogen and inversely proportional to the logarithm of the transmitted light. The mathematical formula showing this relationship is:

$$A = 2 - \log \%T.$$  

Scale showing relationship between absorbance and percent transmittance

Not all chromagens produced in clinical chemistry reactions follow the Beer-Lambert Law (or do so for limited concentration levels).

In order to follow Beer's Law:

1. keep light path constant by using matching sample cuvettes standardized for diameter and thickness
2. solution demonstrates a straight line relationship between two quantities in which the change in one (absorption) produces a proportional change in the other (concentration) is called linearity. Not all solutions demonstrate a straight line graph at all concentrations.

Errors or variation in reading can occur in three places: setting zero %T, setting 100%T, and reading the sample %T.

The relative error in determining concentrations increases as readings are taken at the left end of the %T scale, even when light absorption follows Beer's law exactly. Look at the scale on the Coleman Spectrophotometer and notice how very close together absorbance values are at the low transmittance end of the scale and remember that it is absorbance that is directly related to concentration.

The increased relative error from reading at the low %T (left) end of the scale is due to large error in calculated absorbance with any small error in %T readings. Increased relative error at the high %T (right) end of the scale, however, is due to the very low concentrations of the solutions being measured.

Three Approaches to Determining Concentration

When performing quantitative analysis (i.e., finding the concentration of an unknown) using spectrophotometry, three different procedures may be used:
1. Calculate directly using absorbance or %T readings, and known molar absorption constants of the substance.

2. Analyze a standard of known concentration and calculate the unknown concentration:

\[
\text{concentration of unknown} = \frac{\text{Abs (unknown)}}{\text{Abs (standard)}} \times \text{concentration of the standard}
\]

3. Plot Abs (on linear) or %T (on semi-log) of known standard values vs. concentration, a standard calibration curve.

**Preparation of a Standard Curve**

A standard curve is prepared using several known concentrations of a substance in solution to plot points on a graph. The concentration is plotted vs. either absorbance or % transmittance. The curve is then used to determine the concentrations of unknown samples after obtaining the absorbance or transmittance values of the unknowns.

A graph of absorbance versus concentration will result in a straight line relationship when plotted on coordinate graph paper and a graph of %T vs concentration results in a straight line relationship when plotted on semi-log graph paper. Absorbance plotted on a coordinate scale is usually preferred to %T plotted on semi-log graph paper. However, both result in a straight line and either can be used to determine concentration.

Even though a procedure follows Beer's Law, deviations from linearity will occur at very low or very high concentrations. **The linear part of the curve will still be useful.**

1. If the concentration is too low, the result usually is reported as less than the lowest concentration on the linear portion.

2. If the concentration is too high, the specimen can be diluted, assayed and the result multiplied by the dilution factor.

**Blank Solutions**

1. Water blank - deionized water.

2. Reagent blank
   a. Must contain all constituents of a test except the unknown sample
   b. Adjusts the instrument to account for absorbance of light by the solution that the solute (substance being measured) is in.
   c. Setting the photometer at a value of 0.00 O.D. or 100%T, effectively cancels the absorbance of reagents being used.

3. Patient blank
   a. Used when patient specimen is lipemic, icteric, or hemolyzed.
   b. Contains all test reagents and patient sample (usually added last). Follow manufacturer's directions.
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c. The O.D./Abs reading must be subtracted from the patient's test reading before determination of concentration can be made.

**Bandpass** – the distance between two points where the wavelength is \( \frac{1}{2} \) the intensity as the peak: (Range of wavelengths)

![Diagram of bandpass](image)

**Advantages of Narrow Bandpass Spectrophotometers**

1. Greater potential accuracy.
2. Greater potential sensitivity.
3. Can use high intensity light source.

**Disadvantages of Narrow Bandpass Unit**

1. Must be accurately calibrated.
2. More expensive equipment.
3. Complex optical system.

**Concerns/Problems with Spectrophotometry**

1. **Certainty of Wavelength**

   Verification of wavelength calibration should be done periodically, especially if a parameter in the instrument has been changed, such as the lamp being changed or the instrument has been bumped or traumatized. Wavelength calibration verifies that the wavelength indicated on the dial is what is being passed through the monochromator.

   Solution: Calibrate with appropriate filter (ex., didymium, holmium oxide)

2. **Too Broad/Narrow Bandpass**

   The problem is inherent in spectrophotometer as determined by monochromater. The solution is to change the monochromater **OR** use a different spectrophotometer.

3. **Linearity** -
Solution: Determine range of linearity using standard curve.

4. **Stray Light** - (light energy measured outside the spectral region defined by monochromater) causing non-linearity and insensitivity.
   Solution: Use sharp cut off filters. If dirty, clean optical system. Replace monochromator grating if defective.

5. **Old/New Bulbs**
   Solution: Wavelength calibration. Replace old bulb.

6. **Low Concentration of Analyte Increases Instrument Error**
   Solution: Alter/adapt new procedure

**Procedure I: Use and Calibration of the Sequoia-Turner Spectophotometer Model 340**

*Initial Operation Instructions*

1. Turn mode switch to **TRANS**. Allow a 15 minute warm-up.
2. Adjust instrument to desired wavelength.
3. Insert appropriate **stray light filter** into position.
4. Place cuvette with water or other blank solution into cuvette holder.
5. Press and hold **ZERO SET** button while adjusting **ZERO** knob until display indicates **0.0**.
6. Carefully release **ZERO SET** button.

*Absorbance Mode Instructions*

1. Set **MODE** switch to **ABS**.
2. Adjust 100% T/OA **COARSE** knob to approximately 0.000.
3. Adjust 100% T/OA **FINE** knob to exactly 0.000.
4. Replace blank cuvette with sample cuvette and read absorbance from digital display.
5. When finished, turn **MODE** switch to off, remove and properly store **stray light filter**, and cuvette.
6. Protect the spectrophotometer from dust and spills when not in use with its plastic dust cover.
Directions for Use of Selected Cuvets

Each Coleman selected cell bears a trademark with the word COLEMAN immediately above that surface of the cell that has been found optically best and selected for use. The cell has been accepted and classified on the basis that this indicated surface shall face the light source. The excellent precision of which these cells are capable will only be attained if this condition is met: **Always use Coleman cells with the trademark facing the light source.**

Coleman selected cells (round) are subdivided into classes on the basis of their outside diameters, and are identified by the letters A, B or C appearing on the cell immediately below the trademark. These cells are delivered only in sealed packages containing twelve cells on the same classification. All twelve cells in one package are matched to ±0.3 %T at a nominal 41%T and a wavelength in the region 500-600 nm. Excepting cells for nephelometry (Cat. Nos. C 007-0302 and C 007-0304), any package of cells within a single classification will match other packages of the same classification within ±0.6%T and the mean transmission of adjacent classes will differ by approximately 0.6%T at a nominal 41%T and a wavelength in the region 500-600 nm. Nephelometry cells are matched on the basis of light-scattering characteristics – their classification letters refer only to outside diameter. Square cells are manufactured to a single specification and do not carry classification letters. Matched pairs of round or square cells are also available.

Particular care should be taken to avoid the scratches certain to occur if the cells are allowed to rub against one another or against other hard surfaces. Avoid abrasive cleaning agents and make sure that the exposed surfaces of the cell are optically clean by wiping with a soft cloth or with cleansing tissue just before the cell is put in the well, and therefore, handling only by the top edge.

Avoid diluting the sample solution with wash water by always rinsing the cell with at least one portion of sample, unless the cell is known to be perfectly dry. One drop of wash water in the 10 mm cell means an error of over five per cent.

1. Always clean the cell thoroughly and then rinse at least once and preferably twice with a portion of sample before making a reading.
2. Always wipe the lower third of the cell dry and free from lint and finger marks before placing in the cell adapter or well.
3. Do not attempt measurements at temperatures below the dew point.
4. Make sure that no bubbles cling to the inner surface of the cell.
5. For maximum precision standardize and test with cells of the same class.
6. Handle and clean these cells carefully to avoid injuring the optical surfaces.
7. Always place the cell in the instrument with the trademark word COLEMAN squarely facing the light source.

![Diagram of cuvette classification](image)
Procedure II

The Spectral Curve (Hemoglobin)

**Principle:** The spectral transmittance curve (S-T) allows the technologist to select the optimum wavelength for photometric measurements so that analytical methods will follow the Beer-Lambert law.

**Example – Hypothetical S-T Curve**

1. $\lambda 1 = 0$
2. $\lambda 2$ – rising too rapidly, therefore, increasing error
3. $\lambda 3$ – BEST (absorbance maxima, broad peak)
4. $\lambda 4$ – may also be used; absorbance minima
5. $\lambda 5$ – peak too narrow (too specific) although maximum absorption occurs here

In this example, the absorbance maxima (wavelength maximally absorbed) of each type of hemoglobin (Hb, oxyhb, carboxyhb, and methb) have at least two absorption peaks in the visible spectra.

Based on this principle, serum or blood can be screened for hemolysis, CO poisoning, and methemoglobinemia.

**Procedure:**

1. Prepare a 1:200 dilution of freshly drawn EDTA whole blood using deionized water. Mix sample and set aside.


4. Pour hemoglobin solution into a matching cuvet. Read and record the absorbance value at 450 nm.

5. Place the blank (DI water) back into the instrument, change the wavelength to the next appropriate one (460nm), zero, place the cuvet with the hemoglobin solution back into the instrument, record etc.

6. According to the instructions provided, plot the spectral curve on the graph provided.

7. **NOTES:**
   1) Non specific absorption will occur in the UV wavelengths range, do not select this peak as the absorbance maxima for the hemoglobin.
   2) Draw this curve from point to point.
Results Sheet

Name_________________________________ Date__________________________________

Spectrophotometer used________________________________

<table>
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<tr>
<th>Wavelength (nm)</th>
<th>Measured Absorbance</th>
<th>Wavelength (nm)</th>
<th>Measured Absorbance</th>
<th>Wavelength (nm)</th>
<th>Measured Absorbance</th>
<th>Wavelength (nm)</th>
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</tr>
</tbody>
</table>

1. What wavelength was maximally absorbed by the hemoglobin?

2. Were there other absorption peaks? If so, at what wavelength?
Wavelength
Study Questions

Instructions: Legibly write your answers in the space provided. Unless otherwise indicated, each question is worth one point.

1. What is a monochromator?

2. Explain / define the absorbance maxima and for what it is used. (2 points)

3. What is meant by the term "stray light"?

4. If an instrument provided only %T readings, state two ways you could obtain the concentration of the substance being tested.

5. What is the advantage of the double beam system?

6. In your own words, briefly summarize Beer's Law.

7. Why must standardized cuvettes be used with a spectrophotometer?

8. What is the purpose of a readout device such as a galvanometer?

9. For what purpose is a didymium filter used?

10. State the mathematical formula that shows the relationship between concentration, absorption and transmittance.

11. Define “range of linearity.”

12. A technician performs a glucose analysis and reads results from a previously prepared glucose standard curve. The range of linearity for the procedure is 30 mg/dl to 350 mg/dl. The glucose results for one patient calculates as 20 mg/dl and for another 610 mg/dl.
   a. How should the 20 mg/dl value be reported?
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b. How should the 610 mg/dl value be reported or handled?