Separation techniques

Practical Lesson on Medical Chemistry and Biochemistry

General Medicine

Task 1: Separation of hemoglobin and potassium ferricyanide using gel filtration

In this experiment, you will separate two colored substances with different molecular weights: red (or brownish) hemoglobin/methemoglobin (Mr 64,500) and yellow potassium ferricyanide (Mr 368). The process of separation will be clearly visible.

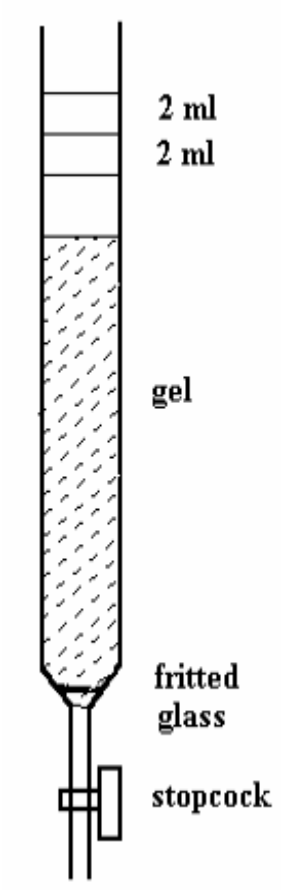
Task:

Determine the concentration of hemoglobin and potassium ferricyanide in a mixed sample.

Reagents and equipment:

Unknown sample (containing hemoglobin and potassium ferricyanide)
Potassium ferricyanide (1 g/L)
Sucrose (20 g/L)
NaCl (0.3 mol/L)
Chromatographic column filled with Sephadex G-50 Medium

Procedure:



- **Absorption spectrum**

Firstly you need to determine appropriate wavelengths which can be used for spectrophotometric determination of your analytes. Measure absorption spectrum of ferricyanide (visible light spectrum is sufficient) and choose appropriate wavelength to be used. Provide a reason for your choice. Use 570 nm for hemoglobin.

- **Calibration curve**

Prepare a set of standards (5-6 points within the range of 1 g/L – approx. 0.05 g/L should be sufficient) by diluting of the provided stock solution of ferricyanide and measure their absorbances at the appropriate wavelength. Plot these data into a chart to generate calibration curve. Use molar absorption coefficient $\varepsilon = 45{,}000$ L . mol^{-1} . cm^{-1} to measure concentration of hemoglobin.

- **Gel filtration + spectrophotometry**

Mix 0.2 mL of unknown sample with 0.2 mL of sucrose solution (to facilitate loading).

Important: Throughout the experiment, ensure the gel bed in the column never dries out! Always keep some solvent above the gel. Air must never enter the gel beads. The flow of the mobile phase is controlled by the stopcock at the lower end of the column. Since no bonds hold the gel beads together, they can be easily disturbed. Therefore, apply the sample or mobile phase (0.3 M NaCl in this experiment) carefully onto the gel surface.

a. Fill the upper end of the column with the eluent (mobile phase) up to the upper mark. Avoid disturbing the gel.

b. Carefully apply 0.2 mL of the sample just above the gel. (The colored mixture has been supplemented with a sucrose solution of high density, so that the sample does not mix with the eluent, but rather sinks onto the gel surface). Use an automatic pipette with an extended tip for application of the sample. Again, take care not to disturb the gel bed.

c. Place a test tube under the column outlet and open the stopcock. When the level of the eluent reaches the nearest lower mark on the column, close the stopcock, take a new test tube and collect another fraction. The volume of the collected fraction in the tube is 2 mL.

d. Once the eluent level reaches the lowest mark, replenish the eluent as in the step a. up to the upper mark.

e. Repeat the previous two steps (c and d) until both colored substances are eluted from the column. Approximately 15 fractions will be obtained.

f. Measure the absorbances of all fractions at wavelengths you have chosen (see above) against deionized water. If an absorbance reading above 2 (or higher than the highest point of calibration) is obtained, dilute the fraction 2× or more and measure the absorbance again. Be sure to account for this dilution in your calculations.

g. Draw the elution curve by plotting fraction No. on the x axis, and absorbance (or concentration) on y axis.

h. Calculate the elution volume of each substance resolved by gel filtration (elution volume is the volume of the mobile phase needed to elute most of the analyte out of the column).

Elution volume Ve = Fraction No. with highest absorbance × volume of one fraction

i. Calculate the concentration of both substances in the original unknown sample. To do it, you need to calculate the amount (not only concentration) of given compound in all relevant fractions, sum it up (to get total weight of given substance in the original sample) and calculate the original concentration (volume of the sample is to be taken into account).

Task 2: Thin-layer chromatography of plant pigments

Reagents and equipment:

Leaf of any plant
Glass powder (or sand)
Mortar and pestle
Sheet of silica-foil
Transparent tape

Acetone

Hexane

Pencil

Procedure:

Extract preparation (one student will prepare the extract for the whole group):

Cut a leaf of any plant into small pieces, put it in a mortar together with a bit of glass powder or fine sand and mash well with a pestle. Add 1-5 mL of acetone and mash again. Transfer the extract into several microtubes (try to transfer only the extract without the solid particles) and spin briefly in a microfuge. The supernatant (should be clear and intensely colored) is the final extract to be used for separation.

Separation:

- Take a sheet of silica-foil and mark the start position using a pencil (the start should be approx. 1.5 cm from the lower end of the foil). Load a small amount of the extract (depending on the color intensity 1-6 μL) on the start using a micropipette. Load it slowly and carefully to get the final spot as small as possible. Ideally, load it in several steps – let the spot dry before re-loading. Avoid scraping the silica layer.
  Prepare two identical sheets.
- Put the foils in the chromatographic chambers, filled with different mobile phases: hexane: acetone 3:2 (v:v) in the first chamber; only hexane in the other one, and cover with a glass lid quickly. The start line must be above the mobile phase level.
- Take the foil out of the chamber when the solvent front migrates approx. 1 cm below the upper end of the foil. Let it dry, contour individual bands, and mark the solvent front with a pencil. Seal the foil with transparent tape to slow down fading of pigments.

Task 3: Dialysis

This experiment demonstrates the phenomenon of limited membrane permeability: cellophane tubing is filled with starch solution (starch is a polysaccharide of very high molecular mass) and immersed in diluted Lugol solution (iodine in potassium iodide). Upon contact of both compounds violet color is developed (you will learn more about this reaction in Saccharides lesson).

Reagents and equipment:

Starch solution (1%)
Diluted Lugol solution (iodine 1 g/L in KI 2 g/L)
Cellophane tubing

Procedure:

Soak the tubing in distilled water to soften it. Close one end of the tubing by making a tight knot. Open the other end of the tubing using a needle, fill it with approx. 2 mL of starch solution, and close with a tight knot. Wash the bag briefly with distilled water (to clean the end of the tubing from any contamination with starch) and place in a beaker with 10 ml of distilled water. Add approx. 0.6 mL of diluted Lugol solution into the beaker.
Observe and explain changes in color.