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 hemoglobin (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.
Draw an elution curve and determine elution volume (Ve) of both compounds.

Reagents and equipment:

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

Procedure:

- **Absorption spectrum**

First you need to determine appropriate wavelengths that can be used for spectrophotometric determination of your analytes. Measure absorption spectrum of potassium ferricyanide (use the visible light range) and choose a suitable wavelength. Hemoglobin will be measured at 570 nm.

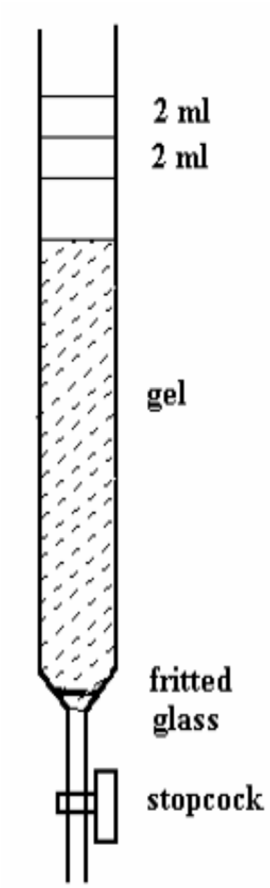
- **Calibration curve**

Prepare a set of potassium ferricyanide standards (5-6 points within the range of 1 g/l – approx. 0.05 g/l should be sufficient) by diluting provided standard solution and measure their absorbances at the appropriate wavelength. Plot these data into a chart to generate calibration curve. No calibration curve for hemoglobin is needed; determine its concentration using molar absorption coefficient ε=45,000 $L*mol^{-1}*cm^{-1}$.

- **Gel filtration + spectrophotometry**

Mix 0.2 ml of unknown sample with 0.2 ml of sucrose solution (facilitates loading).

Throughout the experiment, take care and never allow the gel bed in the column to get dry! There should always remain some solvent on top of the gel. Air must never enter the gel bed! The flow of mobile phase is controlled by the stopcock at the lower end of the column. As no bonds hold the gel beads together, they are easily disturbed! Therefore, the sample or the mobile phase (0.3 M NaCl in our experiment) must be applied onto the gel surface very carefully.



a. Fill the upper end of the column with the eluent (0.3 M NaCl) up to the upper mark. Avoid disturbing the gel.
b. Apply very carefully 0.2 ml of the sample 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 one test tube under the column outlet and open the outlet. When the level of the eluent reaches the nearest lower mark on the column, close the outlet, take a new test tube and start to 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.-d.) until all the colored substances, including the yellow ferricyanide, are eluted from the column. About 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 2x or more, and measure the absorbance again. Do not forget to include 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 mobile fraction needed to elute most of the analyte out of the column).

Elution volume Ve = Fraction No. with highest absorbance x volume of each fraction

i. Calculate the concentration of both dyes 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 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:

Acetone
Leaf of any plant
Sheet of silica-foil
Glass powder (or sand)

Hexane
Mortar and pestle
Transparent tape
Pencil

Procedure:

Extract preparation (one student will prepare extract for the whole group):
Cut a plant leaf 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 1-2 ml of acetone and mash again. Transfer the extract into several microtubes (try to transfer just the extract without the solid particles) and spin briefly in a microfuge. The supernatant (should be clear and intensively 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 (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. Narrow horizontal band is much better than a huge circular spot. 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; hexane in the other) and cover with a glass lid quickly. The start line must be above the mobile phase surface!
- Take the foil out of the chamber when the solvent front reaches approximately half of the foil. Let it dry, contour individual bands, and mark the solvent front with a pencil. Seal the foil with a transparent tape to slow down fading of pigments.

Task 3: Dialysis

This experiment demonstrates the phenomenon of limited membrane permeability: a 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 with 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 eventual 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.