Physical Biochemistry Lab

Experiment no. 4:

**Analysis and identification of abnormal metabolites in urine using paper and thin layer chromatography**

**Objective:**

1. Get introduced to the technique of paper and thin layer chromatography.

2. Detection of amino acid in urine and the application or use of TLC and paper chromatography as a diagnostic tool.

**Introduction and principle:**

In 1903, the Russian botanist Mikhail Tswett described the separation of plant leaf pigments in solution through the use of solid adsorbents. He named this process chromatography (Greek: chroma, color + graphein, to write). Chromatography is a method of separating a mixture of molecules depending on their distribution between a mobile phase and a stationary phase. The mobile phase (also known as solvent) may be either liquid or gas. The stationary phase (also known as sorbent) can be either as solid or liquid, a liquid stationary phase is held stationary by a solid. The solid holding the liquid stationary phase is the support or matrix. The molecules in the mixture to be separated are the solutes.

Chromatography is used to separate and identify chemical compounds. There are four types of chromatography, Partition chromatography, Adsorption chromatography, Gel filtration, and Ion exchange chromatography. In partition chromatography, the solutes will distribute itself between two immiscible phases according to its solubility in each phase, this is called partitioning. Paper and thin layer chromatography are the most common types of partition chromatography. In both cases (paper and TL chromatography) the stationary phase is a liquid bound to a matrix. In paper chromatography, the stationary phase is the “water molecules bound to a cellulose matrix” the cellulose support contains a large amount of bound water. While in TLC, the stationary phase is the “solvent used to form a layer of matrix spread uniformly over the surface of a glass or plastic plate” so the solvent gets bound to the matrix (support). The mobile phase in both cases is the Solvent. Partitioning occurs between (the bound water in case of paper chromatography) (the solvent used to form the thin layer in case of TLC) and the Solvent which is the mobile phase. Paper chromatography uses paper which can be prepared from cellulose products only as the matrix. In TLC, any substances that can be finely divided and formed into a uniform layer can be used as the matrix. So, both organic and inorganic substances can be used to form a uniform layer for TLC. The organic
substances include cellulose, polyamide, and polyethylene. The inorganic are include silica gel, aluminum oxide, and magnesium silicate.

Partition chromatography is mainly used for separation of molecules of small molecular weight.

The experimental procedure for paper and thin layer chromatography is can be described as shown in following words. A small volume of a solution of a mixture to be separated or identified is placed at a marked spot (origin) on a strip of paper or TLC plate and allowed to dry. The paper or TLC plate is then placed in a closed chamber and one end is immersed in a suitable solvent. The solvent is drawn (moved) through the paper or TLC plate by capillary action. As the solvent passes the origin, it dissolves the sample and moves the components in the direction of flow. After the solvent front has reached a point near the other end of the paper or TLC plate, the paper or TLC plate is removed and dried. The spots are then detected and their positions marked. Detection of spots can be done by four different ways: by their natural color, by their fluorescence, by the chemical reactions that take place after the paper or TLC has been sprayed with various reagents, or may the spots detected by radioactivity. Then the spots are usually identified by comparing with standards of known $R_f$ values. Relative Flow ($R_f$) is the ratio of the distance moved by a solute to the distance moved by the solvent. The $R_f$ values are always less than one, and constant for a particular compound, solvent system, and insoluble matrix.

$$\text{Relative Flow} (R_f) = \frac{\text{distance of migration of solute}}{\text{distance of migration of solvent}}$$

The pattern of separated substances obtained by chromatography is known as Chromatogram.

Paper chromatography can be developed either by ascending or descending solvent flow. Descending chromatography is faster because gravity helps the solvent flow, but its disadvantage is that it is difficult to set the apparatus. Ascending chromatography is simple and in expensive compared with descending and usually gives more uniform migration with less diffusion of the sample "spots".

Advantages of TLC over Paper chromatography:

1. TLC has greater resolving power because spots are smaller.
2. It is faster thus greater speed of separation.
3. A wider choice of materials as sorbents.
4. Easier detection of spots since they are smaller and thus more concentrated.
5. No zonal spreading or diffusion of spots such as that seen in paper chromatography (the problem with paper chromatography is that the fibrous structures and associated capillarity of the fibers tend to increase spot size).
The separation of compounds by chromatography depends on several factors:

1) Partition of solute between a moving solvent phase and a stationary aqueous phase. The solute moves in the direction of a solvent flow at a rate determined by the solubility of the solute in the moving phase. Thus a compound with high mobility is more attracted to the mobile phase than to the stationary phase.

2) Ion exchange effect: any ionized impurities in the support medium will tend to bind or attract oppositely charged ions (solute) and will therefore reduce the mobility of these solutes.

3) Temperature: since temperature can affect the solubility of the solute in a given solvent temperature is also an important factor.

4) The molecular weight of a solute also affects the solubility and hence chromatographic performance.

5) Adsorption of compound (solute) onto support medium: although the support medium (e.g. silica gel) is theoretically inert, this isn't always the case. If a solute tends to bind to the support medium this will slow down its mobility in the solvent system.

6) The composition of the solvent: since some compounds are more soluble in one solvent than in other, the mixture of solvents used will affect the separation of compounds.

In this experiment you will examine the presence of an amino acid (phenylalanine or cystine) in two urine samples, one of them is normal and another is for a patient suffering from a genetic disorder of amino acid (patient with either phenylketonuria or cystinuria) using standard solutions of phenylalanine and cystine. In a number of inherited disorders of amino acids, the concentration of some amino acids may be high in the plasma or urine or both. Phenylketonuria (PKU) is an inborn metabolic disorder caused by a deficiency of the hepatic enzyme phenylalanine hydroxylase (PAH). This enzyme is necessary to metabolize the amino acid phenylalanine to the amino acid tyrosine. When PAH deficient, phenylalanine is accumulates in plasma and excreted in a detectable concentration in the urine.
Cystinuria is a disease of defective membrane transport of cystine and the basic amino acids (lysine, arginine, and ornithine) that results in increased renal excretion of these compounds in urine. Normally these amino acids are filtered by the glumeruli and reabsorbed in the proximal renal tubules. In cystinuria, reabsorption fails because a carrier system that transports all four amino acids is defective. Because the low solubility of cystine, its over excretion in urine often leads to the formation of cystine calculi in kidney.

In this experiment the spots detection will done by using chemical reaction by the ninhydrin reagent. Ninhydrin regent react with amino acid to form a colored product.

![Chemical Reaction Diagram]

Materials and apparatus:

1- Standard solutions (1% solutions of phenylalanine and cystine)
2- Urine samples (diluted 1:10)
3- Chromatography solvent (butanol : acetic acid : water, 60 : 15 : 25 by volume)
4- Ninhydrin reagent (dissolve 0.2 g in 100 ml of acetone just before use) in spray gun
5- Thin layer plate of silica gel
6- Whatman No.1 chromatography paper
7- Chromatography glass chamber
8- Aluminum foil
9- Capillary tubes
10- Oven at 105 °C.

Method:

1- You are provided with two urine samples, and two standard solutions (phenylalanine solution) and (cystine solution).
2- Take a sheet of paper and TL chromatography and (in both) with a pencil draw a line about 2 cm from and parallel to the end, draw the line very gently so as not to break the surface of the gel layer on TLC plate.
3- At equally spaced intervals mark the line at 4 different places using the tip of your pencil, and label it from the right as following: Cys, Phy, U1, U2 (name the points of urine samples as it provided to you).

4- Mix very well each solution and using capillary tube put a one small drop from each to its points, let it to dry and repeat the drops to be concentrated.

5- After you finish, let it to dry in a current of air for a moment. Then insert the TLC plate and the paper chromatography sheet in the solvent inside the tank. The sample spots should be in a position just above the surface of the solvent.

6- Cover the tank with aluminum foil, and let it to stand for 45 minutes.

7- Remove the PC sheet and TLC plate from the solvent tank and directly mark the position of solvent with pencil line, and allow drying in the current air.

8- Apply (spry) ninhydrin reagent to the dried PC sheet and TLC plate completely. (Care should be taken in handling ninhydrin solution as it is carcinogenic).

9- Put the PC sheet and TLC plate in an oven (105 °C), until the amino acid colored spots are develop.

10- Mark the spots with a pencil (and determine the center point of spots) soon after development as the colors gradually fade.

11- Take the distance of migration for each spot from the origin line, and the distance of migration for solvent front from the origin line.

12- Calculate the $R_f$ value for each spot.

Results:

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<th>Sample</th>
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Questions:

1. Thin layer chromatography is widely used compared with paper chromatography. Why?
2. Why is ninhydrin used to develop the spots?