Using Thin Layer Chromatography to Diagnose Disease

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Introduction

There are more than 2,500 genetically transmitted diseases that arise from a mutation in a gene. These mutations can result in loss of activity of an enzyme, a protein that helps to catalyze a chemical reaction. One sub-category of genetic disease is inborn errors of metabolism, where the mutated protein is involved in a metabolic process; metabolism is the sum of all the chemical reactions that happen in a cell. Many of the reactions in metabolism involve converting the energy taken in the form of food to cellular energy and this includes the breakdown of protein. Unlike other energy sources such as carbohydrates and fats, proteins are only broken down when there is an excess amount available. Proteins are first broken down to amino acids, which are often called the building blocks of proteins, and then the amino acids are further broken down.

The standard amino acids are those used to make proteins; there are twenty of them. The general structure is shown at pH 7, figure 1. There is an amino group (shown on left side) and a carboxylic acid group (shown on right side) and these two functional groups were used to coin the name. The α- carbon is attached to both of these functional groups as well as the R group. The R group is what varies from one amino acid to another. Please note that amino acids are usually depicted at pH 7; at this pH, the amino would be in acidic form (basic form is shown) and the carboxylic acid would be in base form (the acid form is shown). Since this amino acid structure was taken from the internet, this is yet another reason to not implicitly trust the internet.

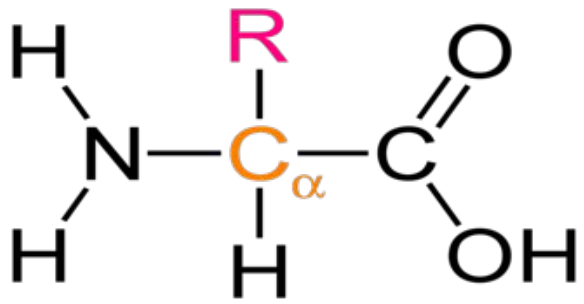


Figure 1. The general structure of an amino acid

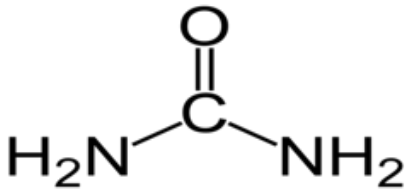
As mentioned, amino acids are broken down when they are present in excess amounts. This can be caused by having large amounts of protein in the diet. Once a protein is degraded to amino acids, they are further catabolized or broken down. Sometimes amino acids share common enzymes when catabolized and

sometimes the enzymes are unique to one particular amino acid. Many of the diseases associated with failure to catabolize an amino acid involve an essential amino acid or one that must come from the diet. While non-essential amino acids can be made in the body, the only source of essential amino acids is the diet. This makes these diseases the result from a failure to catabolize an essential amino acid very treatable. Since amino acids are only catabolized when there is an excessive amount and the essential amino acids only come from the diet, the treatment is to limit the protein intake and particularly that of the affected amino acid.

Since they are so treatable once detected, these diseases related to failure to catabolize an amino acid are included in the 68 diseases that are tested in all newborns in the state of New Jersey. The usual procedure is to do a heel prick on the newborn to collect blood which is then separated into its cellular component and plasma, which contains molecules found in blood. In this project, you will be shown results based on plasma that was collected from seven patients (referred to as patient A through G.) The plasma is typically used in Gas Chromatography-Mass Spectrometry or GC-MS and the increased level of the affected amino acid is detected. A failure to diagnose and treat these diseases means the patient will have increased levels of these amino acids, which can have serious deleterious effects on health.

The Diseases to be Diagnosed

In catabolism, all amino acids must lose the amino group in order to be catabolized and the resulting ammonia (NH_3) enters the urea cycle. In this pathway, the amino group is passed through a series of chemical compounds or intermediates and ultimately is converted to the molecule urea, which is disposed of in the urine. There are a number of diseases associated with the various enzymes of this pathway and collectively, they are called the Urea Cycle Disorders or UCD. A hallmark of all of the UCDs is that there is a decrease in the amount of urea made. If any of the enzymes of the urea cycle are defective, there is a decrease in the amount of the final product, urea, which is made. In addition, there are increased amounts of other intermediates in this pathway. For example, OTC deficiency produces an increase in ornithine and glutamate which are two intermediates before the blocked step. Citrullinemia type I produces an increase in citrulline and glutamate while type II also produces an increase in citrulline as well as the amino acid methionine. Finally, arginemia leads to an increase in arginine. The structure of urea and an overview of the urea cycle is shown, figure 2.



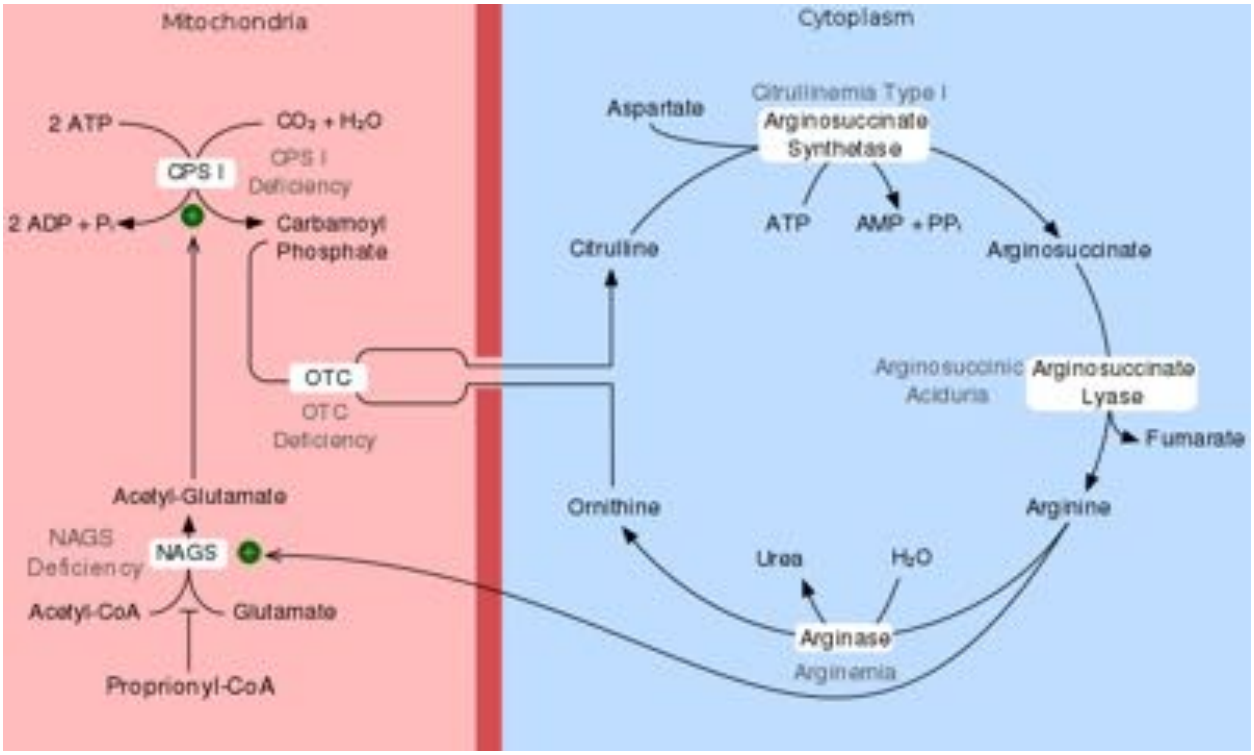


Figure 2. Top) The urea molecule. Bottom) The urea cycle

There are other diseases that are also based on failure to catabolize an amino acid but the defective enzyme is not part of the urea cycle. For example, phenylketonuria (PKU) results from a failure to breakdown the amino acid phenylalanine. This amino acid is one of two that make up aspartame, a common artificial sweetener. For this reason, there is a warning on products that contain aspartame for those with PKU, figure 3. This disease is diagnosed by an increase in the amount of phenylalanine. Tyrosine is another amino acid and failure to breakdown this amino acid produces Tyrosinemia. Since it takes several enzymes to complete this, there are different versions based on defective enzyme. In Tyrosinemia I, it is the first enzyme that is defective and consequently, there is an increase in the amount of tyrosine.

These two amino acids use unique enzymes but there are three amino acids that share a common enzyme. These three amino acids are isoleucine, leucine, and valine and they are collectively referred to as the branch chain amino acids (BCAA). If the enzyme that breaks down these amino acids is defective, it produces Maple Syrup Urine Disease (MSUD) and this disease can be diagnosed by increased levels of the one or more of the BCAAs.

Figure 3. A warning label on a beverage sweetened with aspartame.

Goal of the project

Your goal is this project will be to diagnose one of seven patients using Thin Layer Chromatography (TLC). The diseases that these patients might have are:

- Arginemia
- Citrullinemia Type I
- Citrullinemia type II
- Maple Syrup Urine Disease
- OTC Deficiency
- Phenylketonuria
- Tyrosinemia

You have used TLC in a past experiment. This method involves two phases: the stationary phase, which is the plate itself and the mobile phase, which is the liquid mixture that rises up the plate by capillary action. The theory is that molecules have different tendencies to interact with the mobile or stationary phase and thus separate. The R_f value can be used to identify a molecule by comparing it to standards.

In this project, the stationary phase is the same silica plates that you previously used, the mobile phase is 70/30 (v/v) n-propanol/water. In your previous TLC experiment, the molecules themselves were visible under UV light. That is not the case with most of these amino acids and they must be reacted with a molecule or stain to make them visible. These results used the ninhydrin stain. The reaction is shown in figure 4. While the amino acids are colorless, the product of this reaction is a purple stain that is easily

visible. While ninhydrin reacts with all of the amino acids, it does not necessarily do so equally and even if equal amounts are used, some amino acids produce a more intense color than others.

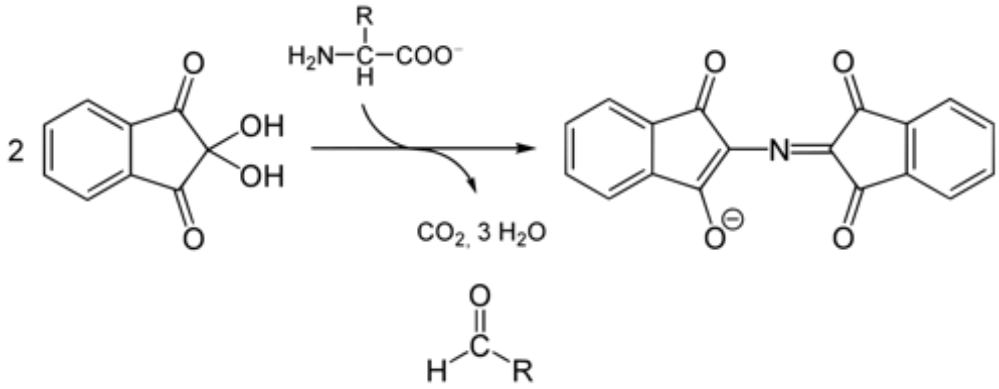


Figure 4. The reaction of ninhydrin with an amino acid to produce a chromophore.

All of the afflicted patients have a disease that results from a failure to break down an amino acid but the affected molecules will not be the same. All of the patients with a urea cycle disorder (UCD) have a decreased amount of urea while those with PKU, tyrosinemia and MSUD have normal levels of urea. The measurement of urea in the blood is called BUN which is an acronym for Blood Urea Nitrogen. The normal levels in blood range from 2.5 to 7.1 mM. Urea reacts weakly with ninhydrin and cannot be detected using TLC. It can be detected using a colorimetric test. In this test, the urea reacts with a reagent to make a colored product that is easily measured. The amount of urea can be determined by comparing to a standard curve, figure 5. All of the samples from the patients were diluted 10-fold prior to analysis.

The plasma from the blood of all patients was tested for BUN and the results are shown in figure 5.

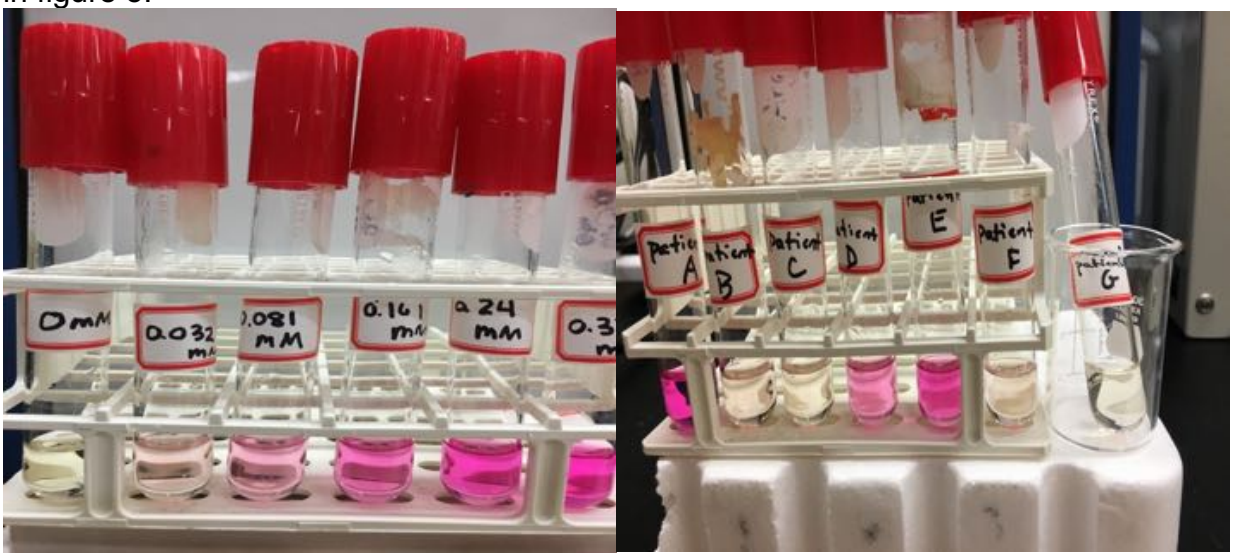


Figure 5. Left) Standard curve for the measurement of BUN. Right) BUN test results for patients A – G.

TLC is needed for the diagnosis of non-urea cycle disorders. One disease results from a failure to breakdown phenylalanine (P) and one from tyrosine (T). The other disease results from a failure to breakdown three amino acids: isoleucine (I), leucine (L) and valine (V) which are collectively known as branched chain amino acids. Figure 6 shows the TLC results for standards of each of these amino acids.

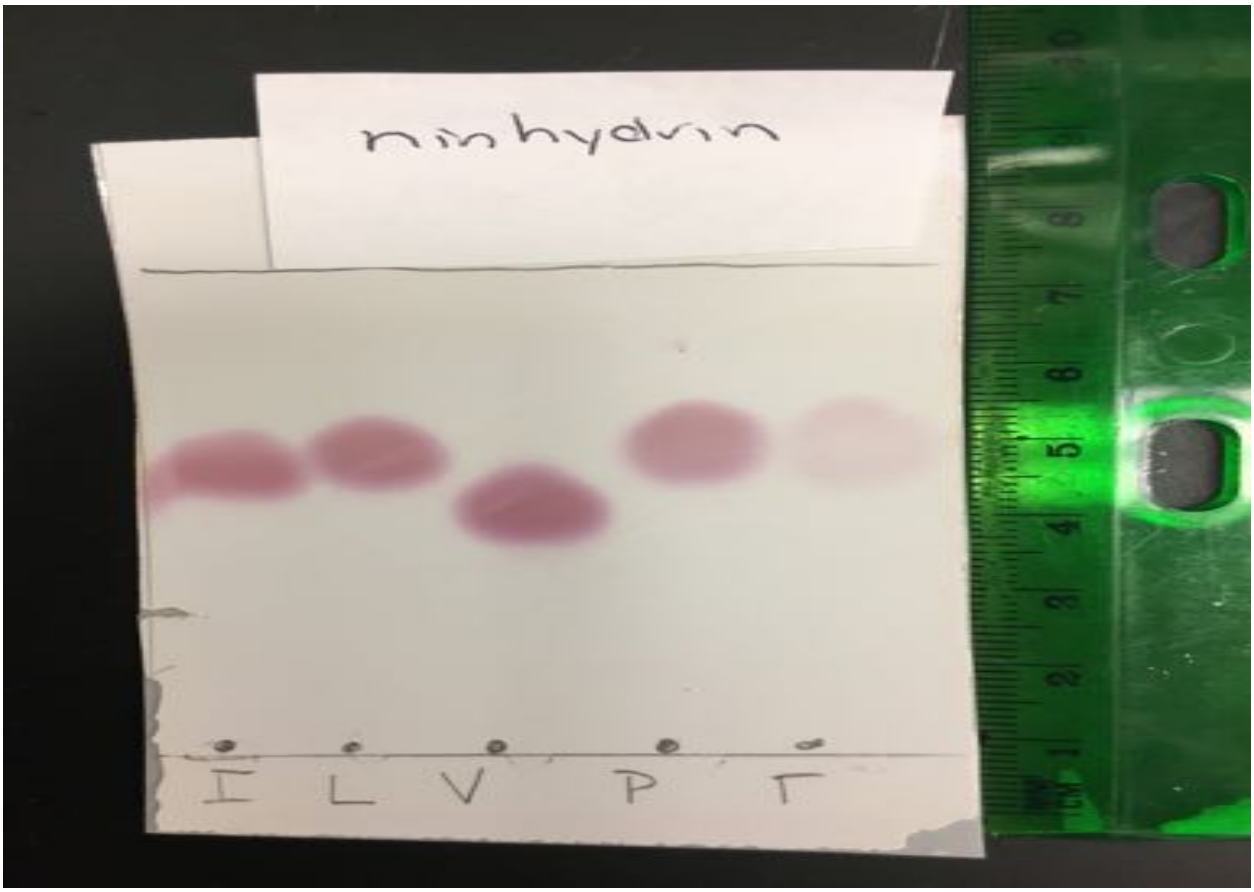


Figure 6. TLC of amino acid standards isoleucine (I), leucine(L), valine (V), phenylalanine (P), tyrosine (T).

The TLC plate for the amino acids affected by UCD are shown in figure 7. The amino acids affected in these diseases include arginine (A), citrulline (C), glutamate (G), ornithine (O) and methionine (M).

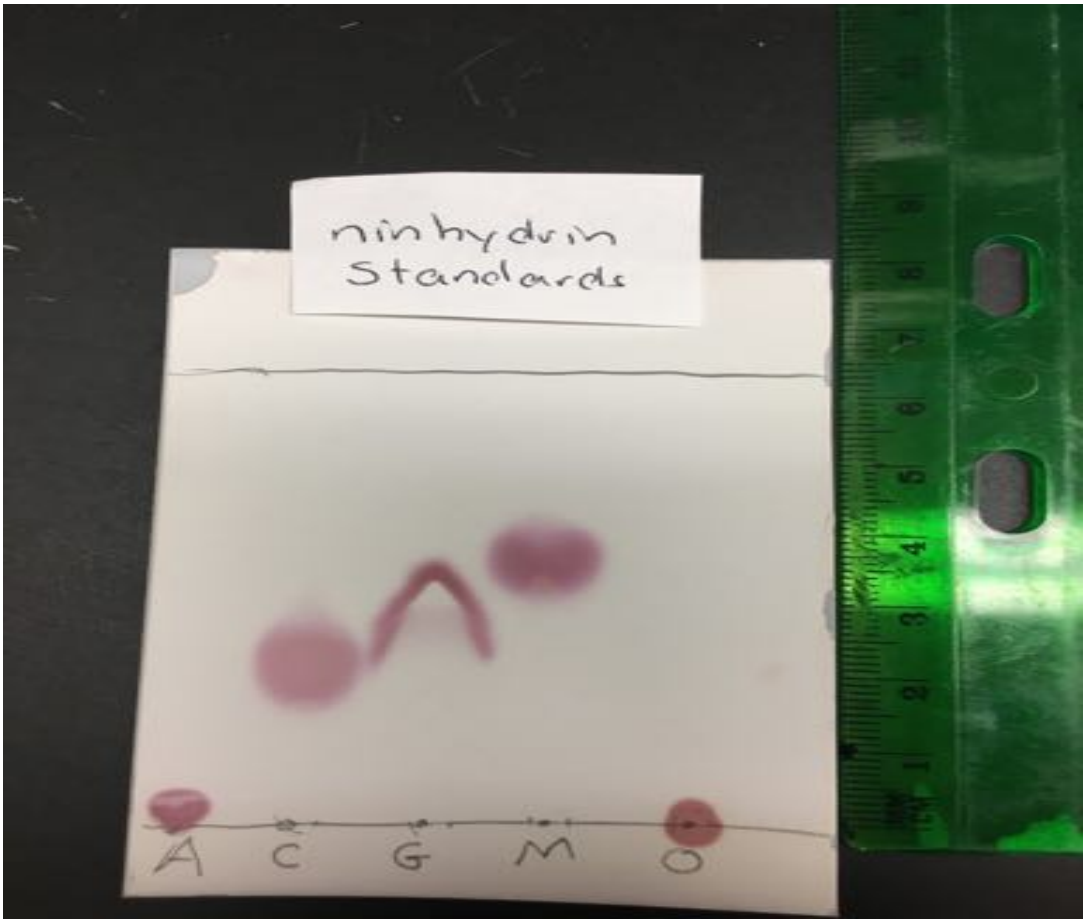
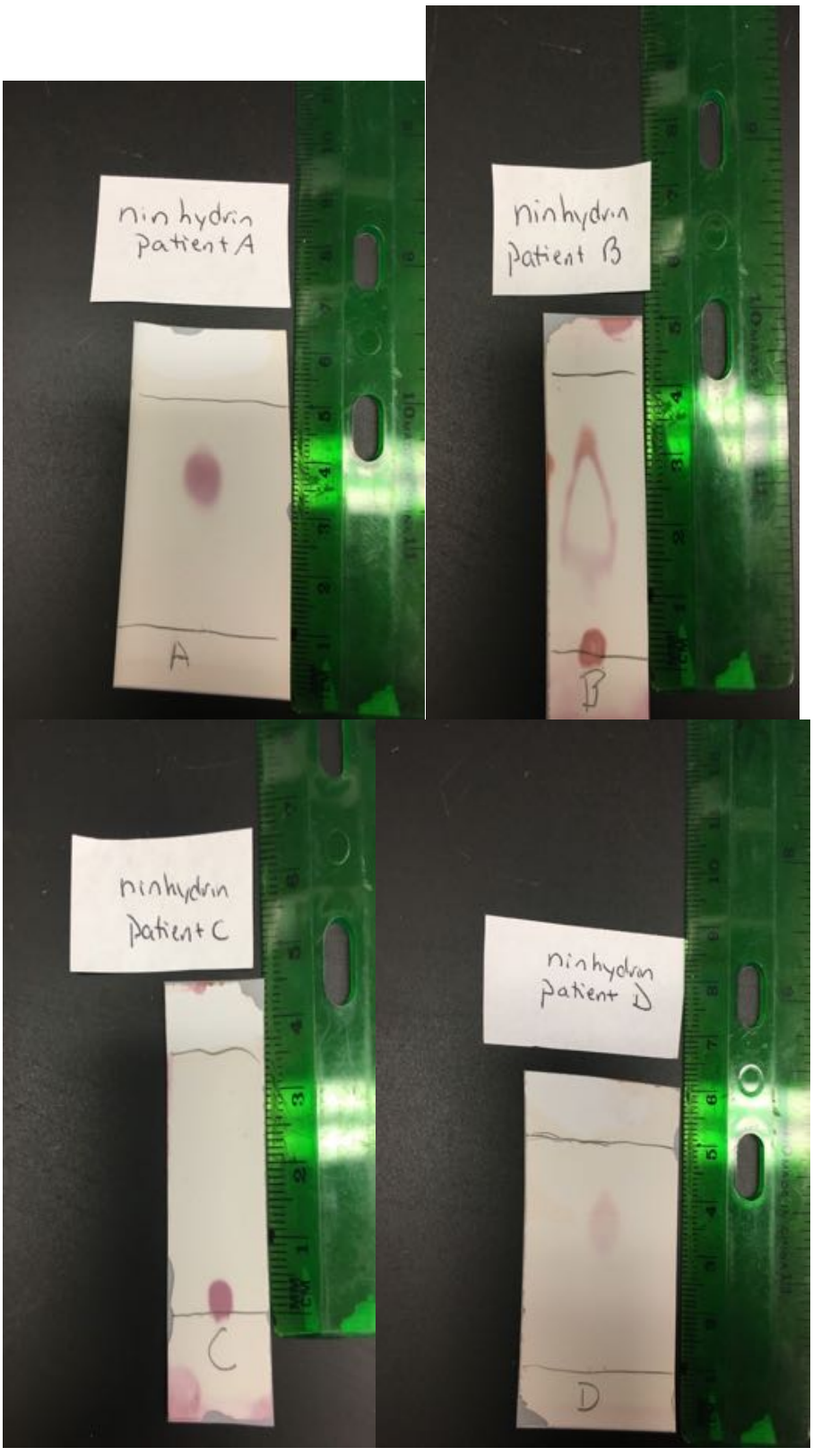
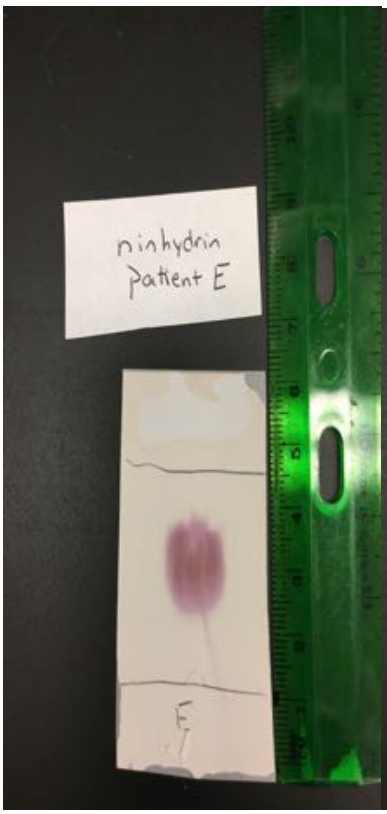
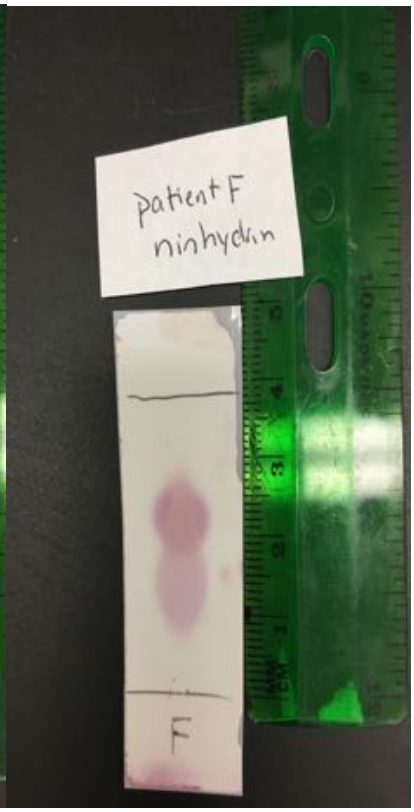


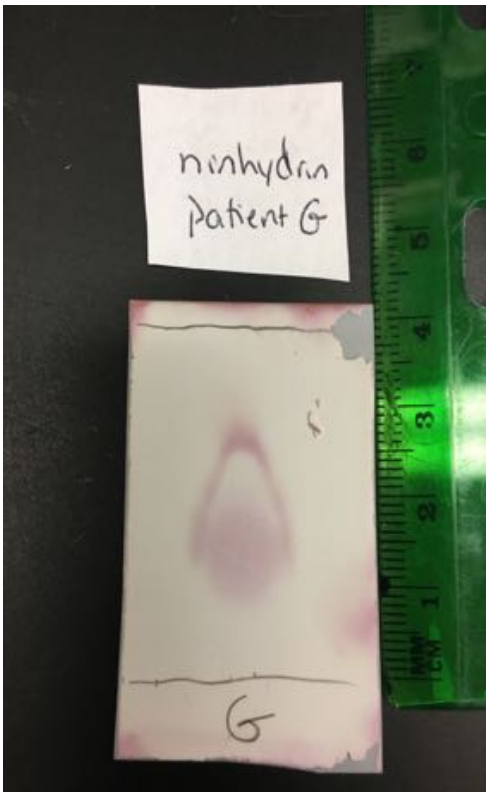
Figure 7. TLC of amino acid standards arginine (A), citrulline (C), glutamate (G), methionine (M) and ornithine (O).

The results for patients A – G are shown in figure 8. Use these and the results from the BUN test to diagnose the patient you were assigned. Justify your diagnosis with explicit reference to the data.




ninhydrin
Patient E
E


Patient F
ninhydrin
F


ninhydrin
Patient G
G