

ESTIMATION OF HEMOGLOBIN 3.1

INTRODUCTION Hemoglobin is the major constituent of the red cell cytoplasm, accounting for approximately 90% of the dry weight of the mature cell. It is comprised of heme and globin.

OBJECTIVES After reading this lesson, you will be able to: z describe the structure of hemoglobin z list the function of hemoglobin z explain the various laboratory methods for estimation of hemoglobin z enumerate the advantages and disadvantages of each method z discuss the normal value and interpretation of abnormal results

3.2 STRUCTURE OF HEMOGLOBIN The Hemoglobin molecule is a tetramer consisting of two pairs of similar polypeptide chains called globin chains. To each of the four chains is attached heme which is a complex of iron in ferrous form and protoporphyrin. The major (96%) type of hemoglobin present in adults is called HbA and it has 2 alpha globin chains and 2 beta globin chains ($\alpha_2\beta_2$). The gene that codes for the formation of α globin chains is located on chromosome 16 and that which codes for the formation of β globin chains is on chromosome 11. In adults, a minor amount of HbA₂ ($\alpha_2\beta_2$) is also present and constitutes less than 3.5%. During embryonic and fetal life other different types of hemoglobins predominate. These include Gower-1 (present in early embryonic life), Gower-2 and Hb Portland. After the eighth week of development, embryonic hemoglobins are replaced by Fetal hemoglobin ($\alpha_2\beta_2$). This remains the predominant hemoglobin until after birth and constitutes 50-90% of the total hemoglobin. After birth, its concentration decreases to less than 2% by 30 weeks of age. HbA is then the predominant hemoglobin.

Function of Hemoglobin Heme has the ability to bind oxygen reversibly and carry it to tissues. It also facilitates the exchange of carbon dioxide between the lungs and tissues. Thus, hemoglobin functions as the primary medium of exchange of oxygen and carbon dioxide.

3.3 ESTIMATION OF HEMOGLOBIN IN THE LABORATORY Various methods are available for estimation of hemoglobin in the laboratory. I. Methods based on development of color. These are z Sahli's or acid hematin method z Cyanmethemoglobin method z Oxyhemoglobin method z Alkaline hematin method II. Measurement of oxygen combining capacity III. Measurement of iron content

3.4 METHODS BASED ON COLOR DEVELOPMENT The commonly used methods are Sahli's/ acid hematin method and Cyanmethemoglobin method. The details of these methods are described below.

Sahli's/acid hematin Method Principle:

Blood is mixed with N/10 HCl resulting in the conversion of Hb to acid hematin which is brown in color. The solution is diluted till its color matches with the brown colored glass of the comparator box. The concentration of Hb is read directly.

Equipment required Hemocytometer which consists of z comparator box which has brown colored glass on either side z Hb pipette which is marked upto 20mm³(0.02ml blood) z Tube with markings of Hb on one side z glass rod z dropper

Reagents required N/10 HCl Distilled water Sample: Venous blood collected in EDTA as described earlier Procedure 1. Add N/10 HCl into the tube upto mark 2g%

2. Mix the EDTA sample by gentle inversion and fill the pipette with 0.02ml blood. Wipe the external surface of the pipette to remove any excess blood.

3. Add the blood into the tube containing HCl. Wash out the contents of the pipette by drawing in and blowing out the acid two to three times. Mix the blood with the acid thoroughly.

4. Allow to stand undisturbed for 10min.

5. Place the hemoglobinometer tube in the comparator and add distilled water to the solution drop by drop stirring with the glass rod till it's color matches with that of the comparator glass. While matching the color, the glass rod must be removed from the solution and held vertically in the tube.

6. Remove the stirrer and take the reading directly by noting the height of the diluted acid hematin and express in g%. Fig. 3.1: Hb comparator box with brown glass on either side and tube with acid hematin solution in centre. The color of the solution is matched with the glass and the concentration of Hb is read directly Advantages z Easy to perform z Quick z Inexpensive z Can be used as a bedside procedure z Does not require technical expertise Disadvantages z Less accurate. z All hemoglobins (oxyhemoglobin, sulphemoglobin) are not converted to acid hematin and hence the value of Hb obtained is less than the actual value. z The color of acid hematin develops slowly. z Color of acid hematin fades with time and dilution must be done exactly after 10 min when the color development is maximum z Individual variation in matching of color is seen.

If the matching point is passed, the whole procedure has to be repeated. z Color of glass in the comparator box tends to fade with time . z Lack of a true standard. Cyanmethemoglobin method This is the internationally recommended method for determining hemoglobin Principle: Blood is diluted in a solution containing potassium cyanide and potassium ferricyanide. The latter converts Hb to methemoglobin which is converted to cyanmethemoglobin (HiCN) by potassium cyanide. The absorbance of the solution is then measured in a spectrophotometer at a wavelength of 540nm or in a colorimeter using a yellow green filter.

Equipment required

Hb pipette Spectrophotometer Reagents required Drabkin's solution pH7.0-7.4 which contains Potassium cyanide 50 mg Potassium ferricyanide 200 mg Potassium dihydrogen phosphate 140 mg Nonionic detergent 1 ml Distilled water 1 L The solution should be clear and pale yellow in color. When measured against water as a blank in a spectrometer at a wavelength of 540 nm, the absorbance must be zero. The solution is unstable if exposed to light and can be stored at room temperature in brown borosilicate bottles for several months. However, if the room temperature is higher than 30°C, the solution should be stored in a refrigerator but brought to room temperature before use. The solution must never be frozen. The pH of the solution must be checked every month.

Discard the solution if found to be turbid/if pH is outside range/ it's absorbance is not zero at 540 nm. Do not pipette Drabkin's solution by mouth. Sample: Venous blood collected in EDTA

Procedure

1. Take 5ml of Drabkin's solution in a test tube.
2. Mix the blood sample by gentle inversion and draw 0.02ml of blood into the Hb pipette. Wipe the outer surface of the pipette to remove excess blood.
3. Place the pipette into the tube containing Drabkin's solution and slowly expel the blood into the solution. Mix well and let it stand undisturbed for 5min.
4. Measure the absorbance of this solution at 540nm in a spectrophotometer after adjusting the OD at 0 by using Drabkin's solution as blank.
5. Calculate the hemoglobin concentration using a standard curve. Advantages z All forms of Hb except sulphhemoglobin are converted to HiCN. z Visual error is not there as no color matching is required. z Cyanmethemoglobin solution is stable and it's color does not fade with time so readings may not be taken immediately. z Absorbance may be measured soon after dilution. z A reliable and stable reference standard is available from World Health Organisation for direct comparison. Disadvantages z Diluted blood has to stand for a period of time to ensure complete conversion of Hb. z Potassium cyanide is a poisonous substance and that is why Drabkin's solution must never be pipetted by mouth. z The rate of conversion of blood containing carboxyhemoglobin is slowed considerably. Prolonging the reaction time to 30min can overcome this problem. z Abnormal plasma proteins cause turbidity when blood is diluted with Drabkin's solution. z A high leucocyte count also causes turbidity on dilution of blood. Centrifuging the diluted blood can help overcome the turbidity. Preparation of Standard curve for Hemoglobin estimation by Cyanmethemoglobin method In a laboratory which tests several samples in a day, it is more convenient to prepare a standard curve for Hb.

A WHO International reference HiCN standard is available commercially as 10ml sealed ampoules. This solution is stable for years. The exact concentration of Hb present in the solution is indicated on the label. Preparation of standard curve 1. Make serial dilutions of the reference solution eg 1in2, 1in4, 1in8 and so on with Drabkin's solution. As the Hb present in the solution is known, the Hb concentration of each dilution will also be known. 2. Take the OD of each dilution in the colorimeter against a blank of Drabkin's solution. 3. Plot the OD against the Hb concentration on a linear graph paper. The absorbance is plotted on the vertical axis and the hemoglobin concentration on the horizontal axis. The points should lie in a straight line that passes through the origin. 4. From this graph a table of readings and the corresponding Hb value can be prepared. This is more convenient than the graph specially when a large number of readings have to be taken. After OD of the sample is taken, the corresponding Hb value can be directly read from the table.

3.5 OXYHEMOGLOBIN METHOD Hb is converted to oxyhemoglobin by reaction with ammonia and the color of the solution is measured in a photocolormeter. The advantages of this method are that it is simple, quick and it's reliability is not affected by increased bilirubin level. However, the solution of oxyhemoglobin fades very quickly. It is not possible to prepare a stable standard. The method does

not give satisfactory results in the presence of carboxyhemoglobin, methemoglobin and sulphhemoglobin. 3.6 ALKALINE HEMATIN METHOD Blood is converted to alkaline hematin by addition of alkali such as sodium hydroxide and the color measured in a colorimeter at 540 nm. It gives a true estimate of Hb and is not affected by the presence of plasma proteins and lipids. However, it is not used routinely as it is less accurate than the cyanmethemoglobin method and some forms of Hb such as HbF are resistant to alkali denaturation. 3.7 MEASUREMENT OF OXYGEN CARRYING CAPACITY The oxygen combining capacity of blood is 1.34ml oxygen per gm of Hb. This is a measure of the function of Hb. It is not used routinely as it is not practical and gives results 2% lower than the other methods. 3.8 IRON CONTENT OF HEMOGLOBIN Iron content can be measured and converted to Hb by using the formula 0.347 mg iron 100g Hb. It is however, impractical for routine use.

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Chapter 141Blood Glucose

J. Michael McMillin. <https://www.ncbi.nlm.nih.gov/books/NBK248/>

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Definition

Glucose is the most important carbohydrate fuel in the body. In the fed state, the majority of circulating glucose comes from the diet; in the fasting state, gluconeogenesis and glycogenolysis maintain glucose concentrations. Very little glucose is found in the diet as glucose; most is found in more complex carbohydrates that are broken down to monosaccharides through the digestive process. About half of the total carbohydrates in the diet are in the form of polysaccharides and the remainder as simpler sugars. About two-thirds of the sugar in the diet is sucrose, which is a disaccharide of glucose and fructose. Glucose is classified as a monosaccharide because it cannot be broken down further by hydrolysis. It is further classified as a hexose because of its six-carbon skeleton and as an aldose, because of the presence of an aldehyde group on carbon 1. The aldehyde group condenses with a hydroxyl group so that glucose exists as a hemiacetal ring structure. This ring structure explains many of the reactions of glucose.

Ordinarily the concentration of glucose in the blood is maintained at a relatively stable concentration from 80 to 120 mg/dl. The strong reducing properties of glucose made it relatively easy to measure and thus the clinical estimation of circulating glucose was one of the earliest tests available to the clinician. The recent introduction of microglucose oxidase technology has now made it possible for the patient to measure his or her own blood glucose concentration and undoubtedly makes the estimation of blood glucose the most widely used test of blood chemistry. An understanding of the methods of blood glucose measurement will help the clinician to interpret values and avoid the pitfalls of inaccurate testing.

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Technique

The concentration of glucose is highest in the arterial circulation. Laboratory determinations are usually done on venous samples. If the venous circulation is delayed, such as by leaving a tourniquet on for a prolonged period of time, the concentration falls even further. Thus, samples should be obtained after releasing the tourniquet. Studies have shown that blood glucose concentration may fall as much as 25 mg/dl when a tourniquet has been left in place for 6 minutes. The concentration of glucose in capillary samples is intermediate between venous and arterial. Warming the extremity increases the capillary flow and "arterializes" the sample, while cooling or a tourniquet decreases the flow and lowers the concentration of glucose.

Both red cells and leukocytes contain glycolytic enzymes. Therefore glucose will be consumed and the concentration of glucose in a sample of whole blood will decline with time. The rate of loss is generally said to be approximately 5% per hour, but may be as rapid as 40% in 3 hours. Consumption of glucose in whole blood samples can be prevented by adding sodium fluoride to the specimen to inhibit the glycolytic enzymes. This approach is the generally applied method in the clinical laboratory. It is effective except in situations where the system is overwhelmed, such as in specimens from patients with leukemia, which contain large numbers of leukocytes. Sodium fluoride has a major disadvantage in that its use makes the sample unacceptable for other determinations such as sodium and uric acid.

Rapid separation of the sample or cooling will also prevent glycolysis and will allow the sample to be used for other determinations. Unhemolyzed samples that have been separated within 30 minutes of drawing are generally considered adequate. Rapid cooling of the sample followed by centrifugation is even more effective in preventing glycolysis. These methods require more attention to detail and are therefore not suitable for routine laboratory use. On occasion, circumstances will require that the glucose concentration be determined on an ordinary serum sample. If the specimen has been promptly centrifuged, it is reasonable to ask the laboratory to measure the glucose concentration even though a sodium fluoride (green top) tube was not used.

Glucose concentration may be determined in whole blood, plasma, or serum samples. If whole blood is used, the concentration will be lower than if plasma or serum is used. This is due to the greater water content of the cellular fraction. Under usual circumstances, the concentration of glucose in whole blood is about 15% lower than in plasma or serum, but the difference will be less in patients with low hematocrits.

Blood glucose cannot be determined accurately on postmortem specimens because both glycogenolysis and glycolysis continue after death. A reasonable estimate of the antemortem blood glucose concentration can be obtained by measuring the glucose concentration of the vitreous of the eye, which does not contain glycolytic enzymes.

Home Glucose Monitoring

Glucose oxidase and reagents to measure the generation of hydrogen peroxide can be bonded to filter paper and the system used to measure glucose concentrations in a drop of capillary blood. This has resulted in the most important change in diabetes management since the introduction of insulin.

Patients are instructed to obtain a blood sample by pricking their fingertip with a lancet. Spring-loaded lancets are available. They are easy to use and cause minimal discomfort.

Surprisingly, many patients consider the discomfort of the finger stick preferable to the inconvenience and aesthetic unpleasantness of obtaining a urine sample for testing. A drop of whole capillary blood is then placed on the reagent bonded to the paper strip. Instead of using a known volume of blood, an excess of blood is exposed to a fixed quantity of glucose oxidase for a finite period of time to estimate concentration. After the specified time, usually 1 minute, the excess blood is removed by washing or wiping and the color is allowed to develop. The concentration is then estimated by comparing to a color chart, or by using a portable reflectance meter specific to the reagent strip, to measure the developed color. Reflectance meters for measuring blood glucose are becoming increasingly sophisticated, compact, and reliable. Shirt-pocket-size models are now available, and prototype models that store the time, date, result, and insulin doses for later graphic printing at the patient's home or physician's office have been developed. Undoubtedly, reflectance meters that have access to complex algorithms for recommending changes in insulin dose individualized to a specific patient will be possible in the near future.

Glycosylated Hemoglobin

A test that reflects long-term blood glucose control in diabetics is the concentration of hemoglobin A1c. When hemolysates of red cells are chromatographed, three or more small peaks named hemoglobin A1a, A1b, and A1c are eluted before the main hemoglobin A peak. These "fast" hemoglobins are formed by the irreversible attachment of glucose to the hemoglobin in a two-step reaction. The percentage of hemoglobin glycosylated depends on the average glucose concentration the red cell is exposed to over time. Since the average life of the red cell is 120 days, the percentage of glycosylated hemoglobin gives a good indication of the degree of blood sugar control over the preceding weeks. Hemoglobin A1c is quantifiably the largest peak so that most laboratories measure it selectively, although some laboratories measure all the "fast" hemoglobins. Numerous biochemical methods are used including electrophoresis, mini columns, radioimmunoassay, and high-pressure liquid chromatography. Unfortunately, there are no standards or reference methods for this test. Some methods include a labile hemoglobin Ale in their measurement. This is reversibly glycosylated hemoglobin A that is dependent on the current blood glucose concentration and can falsely elevate results. Therefore it is important to know the local hospital norms and variations.

Glucose Tolerance Testing

In 1979, an international work group sponsored by the National Diabetes Data Group of the National Institutes of Health developed a classification framework and criteria for the diagnosis of diabetes mellitus. The recommendations are endorsed by the American Diabetes Association and other groups. The classification and criteria are summarized in [Table 141.1](#). Frequently, the diagnosis of diabetes mellitus can be made based on elevated random or fasting blood sugars. In special circumstances, a glucose tolerance test is needed to establish the diagnosis.

Table 141.1

Classification of Glucose Intolerance.

The test should be done in the morning, after a 10- to 14-hour fast in which the patient is permitted only water. Patients are not allowed to smoke during the test. A fasting blood sugar

is obtained. Adults are then given a drink containing 75 g of glucose (the 75 g dose is actually a compromise between the European custom of giving 50 g and the United States custom of giving 100 g). Children are given 1.75 g/kg ideal body weight up to 75 g, and pregnant patients are given 100 g of glucose (the older 100 g dose is retained for pregnant patients because all information on pregnancy outcome is based on tests using the 100 g load). Timing is begun when the patient begins to drink the glucose solution. Blood samples are obtained every 30 minutes for 2 hours in adults and children. For pregnant patients, blood samples are obtained every hour for 3 hours. Criteria for interpretation are given in [Table 141.1](#).

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Basic Science

There are three basic approaches to the laboratory measurement of blood glucose concentration: reducing methods, condensation methods, and enzymatic methods. Reducing methods are the oldest and take advantage of the reducing properties of glucose to change the state of a metal ion while glucose is being oxidized. Reducing methods are nonspecific, and any strong reducing agent can cross react to yield spuriously elevated values. While steps can be added to remove most cross-reacting reducing agents, this approach has largely been abandoned in the clinical laboratory.

The aldehyde group of glucose can undergo condensation with aromatic compounds to yield a colored product. In the most commonly used condensation reaction, *o*-toluidine reacts with glucose to form a glucosamine that has an intense green color. The color is then measured spectrophotometrically to estimate the glucose concentration. The reaction is rapid, and the intense color allows a high degree of sensitivity. Other aldoses can cross react, but only mannose and galactose give a highly colored product. These sugars are not found in great concentrations in the blood and their cross reactivity is ordinarily not significant. *o*-Toluidine has the drawback of being highly corrosive and toxic. For this reason, this method is rapidly being phased out of the clinical laboratory.

The enzyme glucose oxidase reacts with glucose, water, and oxygen to form gluconic acid and hydrogen peroxide. The hydrogen peroxide can then be used to oxidize a chromogen or the consumption of oxygen measured to estimate the amount of glucose present. Glucose oxidase is specific for β -D-glucose, so cross reaction with other sugars is not a problem. In aqueous solution, approximately 66% of D-glucose is in the β state and 34% exists as α -D-glucose. The rate of interconversion is pH and temperature dependent. Some methods add a glucomutase to the reagents to speed up the conversion to the beta anomer, but this does not seem to alter the clinical results. The measurement of generated hydrogen peroxide is not as specific as the first glucose oxidase reaction. Numerous reducing substances can potentially inhibit the oxidation of the chromogen. Although uric acid and creatinine, even in uremic patients, seem to have little effect on the results, ascorbic acid will yield spuriously low blood glucose measurements. The high concentration of uric acid found in urine will affect the result and so glucose oxidase methods are not directly applicable to urine samples. The measurement of oxygen consumption using an oxygen-specific electrode avoids the problem of interfering reducing agents. In general, the glucose oxidase method is relatively inexpensive and specific.

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Clinical Significance

Home Glucose Monitoring

In most hands, the glucose oxidase strip method is accurate and reliable. Since whole blood is used, the results tend to be slightly lower than simultaneous venous samples, but this is balanced by the fact that capillary blood has a higher glucose concentration than venous blood. Most patients can visually estimate the correct value, but a few patients consistently misread the visual charts and must use a reflectance meter. This may be due to an unexpectedly high prevalence of disturbances of color perception in diabetics. Most patients feel more comfortable with the digital readout of the reflectance meter, although it is not necessarily more accurate. The major sources of error are in failing to put a large enough drop of blood on the strip and inaccurate timing. For patients who use reflectance meters, another source of error is failure to keep the machine clean and calibrated. Once the color is developed, it is relatively stable, so patients can be instructed to bring developed strips to the physician's office so that the accuracy can be checked.

Glucose oxidase strips cost about 50 cents each and reflectance meters average \$150. It has been estimated that if 20% of the Type I diabetics in the country were to be involved in a 4-time-a-day home glucose measurement program, the approximate annual cost would be \$225 to \$645 million. On the other hand, the estimated expenditure for the care of Type I diabetics in 1982 was in excess of \$6 billion. The cost of reagents is decreasing. In fact, patients who visually read the reagent strips can realize a 50% reduction in cost by cutting the strips in half lengthwise. A patient who has a laboratory determination of blood sugar on a weekly or biweekly basis may save money by learning home glucose measurement. This author believes that all Type I (IDDM) diabetics should be on a frequent home blood glucose monitoring program. Patients with Type II diabetes mellitus should also be taught home glucose monitoring, although the measurements need not be as frequent.

With third-party hospital payments now tied to the diagnosis rather than to services rendered (DRGs), hospitals are looking for ways to reduce the cost of laboratory tests. Increasing numbers of hospitals are training ward staff to use glucose oxidase strips to monitor blood sugars, in the same fashion as urine sugars have traditionally been monitored in the hospital. Before such a plan is instituted, an effective educational program for the staff must be in place as well as an effective means of quality control.

Glycosylated Hemoglobin

Certain conditions, such as uremia, aspirin ingestion, and alcoholism, can cause spurious elevations of glycosylated hemoglobin. Falsely low percentages of glycosylate hemoglobins can be caused by uremia, anemia, variant hemoglobins such as hemoglobin S, and pregnancy. The sensitivity of the measurement of hemoglobin A1c is such that the test cannot be used to diagnose diabetes, but it is a useful means of following the blood glucose control of the diabetic patient. The measurement of other glycosylated proteins are being studied and may eventually supplant glycosylated hemoglobin measurements.

Glucose Tolerance Testing

The oral glucose tolerance test is fraught with potential problems, and strict adherence to protocol must be followed to reach a valid conclusion. Patients must not be experiencing acute medical or surgical stress. They should be tested several months after recovery. Patients who are chronically malnourished or who have been carbohydrate restricted will have exaggerated blood sugar responses. In general, the patient should have at least a 150 g

carbohydrate intake and normal physical activity for 3 days preceding the test. Patients who have been confined to bed for 3 or more days should also have the test delayed until after recovery. If possible, patients should discontinue all medications for 3 days prior to testing. Patients who have undergone a recent gastrectomy should be watched carefully for alimentary hypoglycemia.

An abbreviated screening glucose tolerance test is recommended for all women between their 24th and 28th week of pregnancy. The test consists of 50 g of oral glucose and the measurement of venous plasma glucose 1 hour later. The test may be administered at any time of day and non-fasting. A 1 hour plasma glucose of 140 mg/dl or greater indicates the need for a full-scale glucose tolerance test as described above.

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