PRACTICAL PHYSIOLOGY

HIGHLIGHTS

- Updated with new clinical knowledge
- Color photograph of clinical examination
- Viva-voce question and OSPE at the end of each chapter
- Original graphs and charts
- University model of practical examination

As per the Competency-based Medical Education Curriculum (NMC)

N Geetha

2nd Edition

JAYPEE

Contents

Section 1: Hematology

Section 2: Clinical Examination

Chapter 6

Estimation of Hemoglobin (Hemoglobinometry)

LEARNING OBJECTIVES

- List the different methods of estimation of hemoglobin
- Estimate hemoglobin content of your blood by Sahli's acid hematin method
- Advantages and disadvantages of Sahli's method
- Principle of Sahli's method
- Mention the normal values of hemoglobin concentration in infants, adult and old age
- Why is hemoglobin content more in males
- Name the conditions where there is variation in the hemoglobin content of blood
- Define anemia and mention the types of anemia depending on hemoglobin content
- Describe the steps in the synthesis of hemoglobin
- List the derivatives of hemoglobin
- Describe different types of anemia

PY2.11: Estimate hemoglobin, red blood cell count, total leukocyte count, RBC indices, differential leukocyte count, blood groups, BT/CT.

COLORIMETRIC ESTIMATION OF HEMOGLOBIN

Different colorimetric methods are:

- Sahli's method
- Tallquist's method
- Haldane's method
- Cyanmethemoglobin method (most accurate method)
- Gasometric method

Principle

The hemoglobin present in a measured sample of blood is converted into a derivative having a definite tinge of color. In Sahli's method acid hematin is prepared. In Tallquist's method the color of oxyhemoglobin is compared. In Haldane's method, carboxyhemoglobin is prepared. Cyanmethemoglobin derivative is also used to give accurate results. The density of this color is compared with a standard solution of the same derivative. Iron estimation or spectrophotometry is used while deciding the strength and the color of the standard.

Such a standard solution of the derivative cannot be prepared every time. They do not maintain their color for long. There is always a difficulty in obtaining a known sample of blood for its preparation.

So, artificially tinted glass rods matching exactly with the color of the standard solution are used as the standard color. These rods maintain their color for long time.

For comparing the color, first a concentrated solution is prepared. This solution is then diluted till its color density matches with that of the standard. The degree of dilution gives a measure of the amount of hemoglobin (as derivative) present in the sample. The tube in which the blood is diluted is calibrated to give a direct reading of hemoglobin in grams.

Sahli's Method

Principle

Sahli's method is the commonly employed method for hemoglobin estimation. The hemoglobin present in a particular volume of blood is converted to acid hematin by treating it with N/10 HCl. Acid hematin is brown in color and the solution is diluted with distilled water till the color matches with the standard color. The standard color is prepared by treating a sample of blood containing 14.5 g

of hemoglobin per 100 mL of blood with N/10 HCl and diluting it 100 times. So 100% reading in the diluting tube corresponds to 14.5 g% of hemoglobin.

Apparatus

Sahli's hemoglobinometer set **(Fig. 6.1)** contains the following:

- A rectangular plastic box with two color standards on either side with a central provision for keeping the diluting tube. The color standard is made of nonfading, standardized, golden brown glass rods **(Fig. 6.2)**.

Fig. 6.1: Sahli's hemoglobinometer showing the box with standard color, brush, diluting tube, pipette, glass rod, N/10 HCl and dropper.

Fig. 6.2: Sahli's hemoglobinometer showing the color standards.

- Specially graduated diluting tube is square or round in shape. It is graduated in percentage (20–140%) on one side and in gm per deciliter (2–24 g%) on the opposite side.
- A glass rod with flat tip to stir the contents.
- Hemoglobin pipette with a 20 mm^3 mark and rubber tubing with a mouthpiece to suck the blood. There is no bulb in the hemoglobin pipette and the 20 mm^3 mark indicates a definite measured volume and not an arbitrary volume as in the case of RBC and WBC pipette.
- Needle or lancet.
- Bottle containing distilled water and a dropper with teat.
- Bottle containing N/10 hydrochloric acid, which is prepared by mixing 3 mL of concentrated HCl and 997 mL of distilled water.
- Brush to clean the tube.

Procedure

- See that the hemoglobin pipette is clean and dry.
- Place the graduated tube between the standards in the plastic box.
- Fill it with N/10 HCl up to the lowest mark.
- Prick the tip of the finger to get a large drop of blood.
- Hold the pipette in the right hand with the graduation in front.
- Place the mouthpiece between the lips.
- Hold the pipette at an angle of 45° above the horizontal and touch the tip of the pipette to one side of the drop.
- Suck the blood till the blood reaches the 20 mm^3 mark (if any air bubble enters the pipette, repeat with a new dry one).
- Remove the pipette and clean the outer surface with a cotton swab by wiping it towards the tip to remove any blood that is present on the outer surface of the pipette. Do not touch the open end while wiping because blood will come out of the pipette due to capillary action.
- If the blood has slightly crossed the mark, take the excess blood out by touching the tip of the pipette on the palm of the hand for a couple of times and the blood will recede back in the pipette. Make sure that you are wearing gloves if blood is taken from another person.
- Do not use a swab or a filter paper to remove the excess blood. It will absorb a very large quantity of blood.
- While transferring the blood to the HCl in the diluting tube, blow down slowly.
- Dip its tip in the acid and blow out gently to transfer all the blood into the tube.
- Suck the superficial acid and rinse the pipette repeatedly till all the blood in the pipette is transferred into the diluting tube.
- Mix the blood and the acid with the glass rod provided.
- Note the time. Wait for 10 minutes to allow the brown color of acid hematin to develop. The color does not develop immediately. Its intensity changes with time. 95% of the color is reached by the end of 10 minutes.

Fig. 6.3: Correct matching of sample with standard color.

Fig. 6.4: Overdilution of blood sample, color not matching with the standard color.

- Add distilled water drop by drop mixing the solution with the glass rod after the addition of each drop.
- After adding two or three drops of water and mixing, compare the color of the solution with the standard color of the glass block. While comparing, lift the stirrer above the level of the solution but it should not be completely taken out of the tube. If comparison is done with the glass rod in the solution, the solution will appear lighter. While comparing the color, avoid viewing through the markings on the tube and hold the apparatus against natural light.
- Add water drop by drop, mix and compare each time after every addition.
- Continue till the colors match **(Fig. 6.3)**.
- Raise the glass rod and take the reading in g/dL.
- Read the lower meniscus. Since the solution is transparent the lower meniscus is clearly seen even though it is colored.

Precautions

- The color of the standard should be examined from time to time to exclude any fading or change in color.
- After pricking, the finger should not be squeezed to obtain blood. This may give a false low value for hemoglobin since the blood gets diluted with tissue fluid. A free flowing drop should be obtained.
- There should not be any air bubble in the blood column in the pipette.
- Blood from the pipette should be immediately transferred into the tube containing N/10 HCl. Otherwise blood may clot.
- Distilled water should be added dropbydrop, mixed well and compared with the standard. Otherwise over dilution may occur **(Fig. 6.4).**
- The diluting tube should be kept in between the standards in such a way that the graduations are not interfering with the comparison.
- Take the reading without delay because on keeping, the color will deepen.
- Matching and reading should be taken after lifting the glass rod from the solution. If it is left in the solution while comparing the color, the solution will appear lighter.
- The glass rod should not be removed from the diluting tube while comparing the color, because it may lead to loss of fluid. Hold it against the side of the diluting tube above the solution.
- Reading should be taken in good daylight.

Result

Read directly from the diluting tube and record the result in grams of hemoglobin per 100 mL of blood.

Disadvantages of Sahli's Method

- There can be visual error.
- This method estimates only oxyhemoglobin and reduced hemoglobin. Carboxy, met and sulfhemoglobin cannot be converted to acid hematin.
- The color of the standard fades over time.
- If the reading is not taken immediately, the color of acid hematin changes.

Cyanmethemoglobin Method

Principle

The basis of this method is dilution of blood in a diluent called Drabkin cyanide–ferricyanide solution. Ferrous ion of hemoglobin is converted to ferric ion by ferricyanide and methemoglobin so produced is combined with potassium ferricyanide to produce cyanmethemoglobin which is red colored. All hemoglobin derivatives of blood except sulfhemoglobin are measured by this technique. But sulfhemoglobin is rarely present in significant amounts in blood. *Cyanmethemoglobin method is one of the most reliable and the most accurate method and is the WHO'S recommended method for determining the hemoglobin concentration of blood.* The color of cyanmethemoglobin in the unknown blood is compared with the standard solution of cyanmethemoglobin.

Procedure

To 5 mL of Drabkin solution add 20 µL of blood. Mix well and after 5 minutes read the absorbance in a spectrometer at 540 nm or in a photoelectric colorimeter with a yellow-green filter. Drabkin solution consists of dihydrogen potassium phosphate (140 mg), potassium ferricyanide (200 mg), potassium cyanide (50 mg) and distilled water to 1,000 mL. The oxy, carboxy and methemoglobin are all converted into cyanmethemoglobin and there is development of pink color. The color intensity is proportional to the concentration of hemoglobin in the blood sample. The color is compared with that of a standard cyanmethemoglobin solution.

Tallquist's Method

A drop of blood is placed on an absorbent paper and is then matched against a series of lithographed color supposed to represent hemoglobin values in grades of 10–100%. Though quick, this method is inaccurate and is only used in mass surveys for anemia.

Haldane's Method or Carboxyhemoglobin Method

Blood is treated with ammonia solution and is then saturated with CO. The carboxyhemoglobin formed is compared with a glass tube containing carboxyhemoglobin as the standard.

Gasometric Method

It is also called van Slyke's oxygen capacity method. It estimates the amount of hemoglobin from the amount of O_2 it absorbs.

OTHER METHODS OF ESTIMATION OF HEMOGLOBIN

Estimation of Iron

The amount of iron present in a known volume of blood can be accurately measured by chemical methods. Normally, 1 g of hemoglobin contains 3.35 mg of iron. By finding out the amount of iron present in the blood sample the amount of hemoglobin can be calculated. This method is time consuming.

Spectrophotometry

This method is based on the ability of hemoglobin to refract definite wavelengths of light giving typical absorption bands. If light is passed through a solution of hemoglobin, definite wave lengths of light are absorbed by hemoglobin. By using suitable wavelengths of light, the con centration of hemoglobin can be measured by a photoelectric colorimeter.

Oxygen Carrying Capacity Determination

The maximum amount of oxygen the sample of blood is capable of carrying is called the oxygen carrying capacity of the blood. The amount of oxygen present in the blood can be measured very accurately with the Van Slyke's apparatus. A sample of blood is exposed to the atmosphere. The partial pressure of oxygen in the atmospheric air is about 150 mm Hg. At this partial pressure the hemoglobin gets completely saturated with oxygen. This oxygenated sample of blood is treated with ferricyanide and exposed to vacuum in the Van Slyke's apparatus when all the oxygen present is evolved out and it is measured. One gram of hemoglobin combines with 1.34 mL of oxygen at normal temperature and pressure. By measuring the amount of O_2 evolved, the amount of hemoglobin in the given blood sample can be calculated.

The above three methods cannot be used for routine estimation of hemoglobin since it is time consuming and requires sophisticated equipment.

DISCUSSION

Hemoglobin is the red coloring pigment and the O_2 binding protein of RBC. It forms 95% of the dry weight of RBC. The function of hemoglobin is to carry O_2 as oxyhemoglobin and CO_2 as carbaminohemoglobin. The iron atoms are in their reduced state, i.e., ferrous state in the hemoglobin molecule and in this state each iron atom can combine with one molecule of O_2. When the iron atoms get oxidized to their ferric state then it is called methemoglobin. Methemoglobin has no ability to carry O_2.

Viva Questions

1. Give the normal hemoglobin content.
- At birth: 23–25 g%
- Adult male: 14–16 g%
- Adult female: 12–15 g%

2. Mention the variations in hemoglobin content.
- Increase in hemoglobin content is seen in newborn, at high altitude, after exercise, hemoconcentration, polycythemia vera, etc.
- Technical error like increase in the amount of blood taken in the pipette (>20 µL) gives a higher value.
- Decrease in hemoglobin content is anemia. All conditions producing anemia will result in decrease in hemoglobin content.
- In hemodilution as in pregnancy, Hb concentration will be less than normal.
- Technical errors like less amount of blood taken in the pipette and blood sample obtained by squeezing the fingertip will give a lower value. When the fingertip is squeezed, the fluid coming will contain more of tissue fluid and less number of RBCs. Failure to wait for 10 minutes after mixing blood with N/10 HCl will give a low hemoglobin value. If the amount of N/10 HCl taken is not adequate, all the hemoglobin present in the sample will not be converted to acid hematin and a low value will be obtained.

3. What happens if hemoglobin is found in plasma as the free hemoglobin?
If Hb is present dissolved in plasma and not confined within the RBC, it leads to the following problems:
- It increases the viscosity of blood leading to an increase in the blood pressure.
- Increase in the osmotic pressure of blood.
- Free hemoglobin will be filtered and excreted by the kidneys leading to hemoglobinuria and precipitation of hemoglobin in the renal tubules leading to renal failure and anemia.
- Free hemoglobin will be rapidly destroyed by the reticuloendothelial cells leading to increase in the bilirubin content of blood.

4. What is the reason for increase in hemoglobin content in the newborn?
During fetal life the type of hemoglobin in blood is HbF (fetal hemoglobin). It contains two α and two γ chains. The γ chain does not combine with 2, 3bisphosphoglycerate (2, 3 BPG) and so HbF has greater affinity for oxygen. It releases less O_2 at the tissue level and tissues suffer from hypoxia, which is the most potent stimulant for erythropoietin secretion. Since erythropoiesis is increased there is increase in hemoglobin content in newborn.

5. What is physiological jaundice?
Yellowish discoloration of skin and mucous membrane seen in newborn is called physiological jaundice. It disappears in 2 weeks. After birth fetal hemoglobin is replaced by adult hemoglobin. There is increased destruction of red blood cells after birth in order to bring the RBC count to normal level. This leads to increase in the level of bilirubin in blood leading to jaundice.

6. What is the principle behind Sahli's method of hemoglobin estimation?
Refer page 28.

7. How is the standard color in Sahli's method obtained?
The standard color is prepared by treating 20 mm^3 of blood containing 14.5 g of hemoglobin per 100 mL with N/10 HCl and diluting it 100 times.

8. What is the reason for waiting for 10 min after adding blood into the diluting tube?
This much time is needed for the complete hemolysis of RBCs and for the conversion of all the hemoglobin present to form acid hematin.

9. What are the functions of hemoglobin?
- It helps in the transport of O_2 from lungs to tissues by forming oxyhemoglobin.
- Helps in the transport of CO_2 from tissues to lungs by forming carbaminohemoglobin.
- It acts as a buffer to maintain the normal pH of blood. Hemoglobin being a protein is responsible for 70% of the buffering capacity of whole blood.
- The β-chain F hemoglobin has an additional nitric oxide (NO) binding site. The affinity of hemoglobin for NO is increased by O_2. So hemoglobin binds with NO in the lungs and releases it in the tissues where it promotes vasodilatation.

10. What are the various forms of hemoglobin present in blood?
- Oxyhemoglobin (HbO_2)—Hb in combination with O_2.
- Carbaminohemoglobin (HbNHCOOH)—Hb combined with CO_2.
- Carboxy hemoglobin (HbCO)—Hb in combination with CO.
- Sulfhemoglobin (SHb)—in combination with sulfur containing compounds.
- Methemoglobin—when the ferrous form of iron is oxidized to the ferric form.
- Adult hemoglobin are HbA ($\alpha_2\beta_2$) and HbA_2 ($\alpha_2\delta_2$).
- Fetal hemoglobin or HbF ($\alpha_2\gamma_2$).

11. What is anemia? How is it graded?
Reduction in the hemoglobin content of blood or RBC count or both below the normal range for that age and sex is called anemia. When the RBC count is less than 4 million/mm^3 of blood or hemoglobin content less than 12 g/dL in an adult the condition is anemia. Severity of anemia can be graded depending on the hemoglobin content as follows:
Mild anemia where the hemoglobin content is 10–11 g%. **Moderate anemia** where the hemoglobin content is 7–9 g%. **Severe anemia** when the hemoglobin content falls below 6 g%.

12. What is the etiological classification of anemia?
- » Anemia due to increased destruction of RBC (hemolytic anemia).
- » Anemia due to defective formation of RBC (deficiency anemia and anemia due to bone marrow depression).
- » Posthemorrhagic anemia (acute and chronic blood loss).

13. What are the disadvantages of Sahli's method?
- » This method estimates only oxyhemoglobin and reduced hemoglobin. Other forms such as carboxyhemoglobin and met hemoglobin are not estimated.
- » The color of the standard may fade and become lighter after a few years and this will give a wrong result.

14. Which is the most accurate method for estimating hemoglobin content? Why?
Cyanmethemoglobin method. All hemoglobin derivatives of blood except sulfhemoglobin are measured by this technique. But sulfhemoglobin is rarely present in significant amounts in blood.

15. What is oxygen carrying capacity of blood?
The amount of oxygen present in 100 mL of blood, when all the hemoglobin present in it is completely saturated with O_2 is the oxygen carrying capacity. Normally it is about 21 mL/100 mL of blood. One gram of hemoglobin can combine with 1.35 mL of O_2.

16. At what stage of erythropoiesis does hemoglobin appear in red cells?
Hemoglobin appears in the early normoblast stage and maximum level is reached in the late normoblast stage.

17. Which is the most common cause of anemia in India?
Worm infestation especially hook worm infestation is the most common cause. It produces iron deficiency anemia.

Objective Structured Practical Examination

I. Suck blood in the hemoglobin pipette for estimating your hemoglobin concentration
1. Take a clean and dry hemoglobin pipette
2. Sterilize the finger tip
3. Give a deep prick using a sterile lancet (do not squeeze the finger tip for obtaining blood)
4. Wipe off the first drop using sterile cotton
5. Apply gentle pressure so that a drop of blood appears on the finger tip
6. Suck blood into the pipette exactly up to the 20 mm^3 mark on the pipette
7. Wipe the tip of the pipette
8. Press the finger tip with clean cotton

II. Do the hemoglobin estimation with the blood taken in the hemoglobin pipette
1. Take a clean diluting tube
2. Take N/10 HCl up to the lower mark in the tube
3. Blow the blood in the pipette into the acid solution in the diluting tube.
4. Rinse the pipette in the acid solution 2 more times and blow out into the diluting tube so that whole of blood remaining in the pipette is transferred to the diluting tube
5. Place the diluting tube between the standard colors
6. Note the time and wait for 10 minutes for the formation of acid hematin
7. Add distilled water drop by drop mixing the solution with the glass rod after the addition of each drop.
8. After adding two or three drops of water and mixing, compare the color of the solution with the standard color of the glass block. While comparing, lift the stirrer above the level of the solution but it should not be completely taken out of the tube.
9. Add water drop by drop, mix and compare each time after every addition.
10. Continue till the colors match.
11. Raise the glass rod and take the reading in g/dL.
12. Read the lower meniscus.

PRACTICAL PHYSIOLOGY

Salient Features

- The book is clinically oriented, laying emphasis on the physiological basis of the observation.
- The full colored photographs and illustrations help to precisely understand the clinical condition.
- Clinical examination follows the basic pattern, which includes a thorough and systematic approach in examining the patients.
- The book also covers practical aspects on animal experiments and provides graphs and records of the same.
- Viva-voce questions with answers are included at the end of each experiment.
- Scheme of practical examination with sample questions is provided in the last chapter.
- Detailed index is provided at the end of the book.
- This book is useful not only for the medical students, but also for dental, paramedical, and allied health sciences students.

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