CHAPTER

4

Estimation of Haemoglobin

Learning Objectives

After completion of practical the student should be able to:

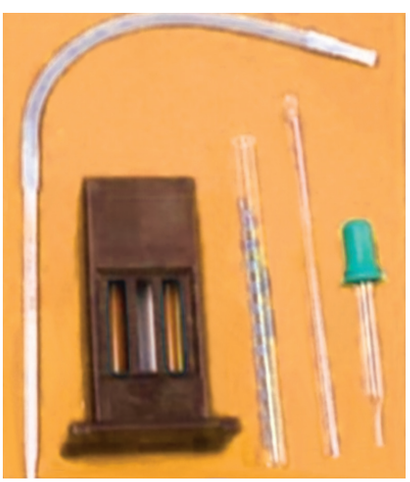
1. Estimate the haemoglobin using Sahli's method.
2. Discuss the advantages and disadvantages of Sahli's method.
3. Enlist the precaution while estimating haemoglobin by Sahli's method.
4. Discuss the other methods for estimation of haemoglobin.
5. State the normal haemoglobin levels in males and females
6. Enlist various clinical conditions in which the haemoglobin levels are altered?
7. Able to classify anaemia on the basis of haemoglobin levels.
8. Describe the method of estimation of haemoglobin by internationally accepted cyanmethaemoglobin method.
9. Estimate oxygen carrying capacity of blood and iron content of haemoglobin.

Aim: Estimation of the haemoglobin concentration.

SAHLI'S ACID HAEMATIN METHOD

Material, apparatus and chemicals: Sahli's haemoglobinometer: It is a box containing comparator, haemoglobin tube, haemoglobin pipette, and stirrer.

Haemoglobin tube: It is graduated and calibrated on one side as gram per cent (gm%), from 2–22, and on in percentage (%), from 10 to 140 on other side. This tube is also known as Sahli-Adams tube.



R-L dropper, stirring rod, haemoglobinometer tube, comparator box and haemoglobin pipette

Fig. 4.1: Haemocytometer

Comparator: It is a rectangular box of processed plastic polymer material having a centre slot for haemoglobin tube and two-colour standards for matching on either side. These standard brown tinted glass frame slots are provided on either side of the centre slot for the colour matching.

Haemoglobin pipette: The pipette bears a mark indicating 20 µL. It consists of tubing of rubber with mouth piece attach to it. The pipette is devoid of any bulb.

Stirrer: It is a thin glass rod which is used for stirring the solution.

Chemical solution: N/10 HCl bottle and distilled water.

Other Materials

Dropper: Ordinary glass dropper or Pasteur pipette which is an 8–10 inches glass tube with a long thin nozzle having a rubber teat is used as dropper.

Materials for a sterile finger prick: Sterile lancet, sterile gauze, cotton swabs and methylated spirit.

Principle: The Hb present in blood is converted to acid haematin on addition of N/10 HCl (hydrochloric acid). The observed golden brown colour depends on the concentration of Hb. The colour of the solution is matched against golden brown tinted glass colour of the comparator. The formed acid haematin appears golden brown in colour and this solution is diluted with distilled water till the colour matches with the standard colour. The Hb concentration is noted in gm% at the level where the colour of solution matches with that of comparator.

Procedure

1. Ensure that haemoglobinometer tube and pipette are clean and dry.
2. Add N/10 HCl in the haemoglobinometer tube up to its lowest mark (2 gm/dl or 10%) with the help of a dropper.
3. Clean the finger with methylated spirit using the cotton gauze and prick the finger under all aseptic precautions. Discard the first drop of blood.
4. Allow a large drop of blood to form on the fingertip, and then suck the blood up to 20 µL of the pipette.
5. Wipe the tip of the pipette to remove any blood which may remain on outside of the tube.
6. Transfer the blood from the haemoglobin pipette into the haemoglobinometer tube containing N/10 HCl by immersing tip of the pipette in N/10 HCl solution by blowing out blood from the pipette. Rinse the pipette by drawing N/10 HCl and blowing it for couple of time for uniform mixing. Ensure uniform mixing of content by using a stirrer.
7. Leave the solution in the haemoglobinometer tube for about ten minutes (for conversion of haemoglobin to acid haematin which occurs in the first ten minutes).
8. Dilute the acid haematin by adding distilled water drop by drop. Mix it with the stirrer. Match the colour of the solution in the haemoglobinometer tube with the standards of the comparator. After addition of every drop of distilled water, the solution should be mixed and the colour of the solution and should be compared with the standard of the comparator till it matches with that of the standard. Take care to hold the stirrer above the level of the solution while reading is being matched, otherwise at no stage should the stirrer should be taken out of the tube.
9. Note the reading when the colour of the solution exactly matches with the standard and express the haemoglobin content as gm per 100 ml of blood.

Precautions

1. All aseptic precautions should be used during pricking.
2. The first drop of blood should be discarded and do not squeeze the finger because tissue fluids which comes out gives lower values of haemoglobin.
3. Wait for at least ten minutes for the formation of acid haematin by the action of hydrochloric acid on Hb.
4. Avoid over dilution as later the solution cannot be concentrated to match with comparator colour.
5. Golden brown colour of the solution should be compared with the standard of the comparator till it matches with that of the standard of the comparator.
6. Match the colour in the comparator box in natural source of daylight.
7. Take care to hold the stirrer above the level of the solution. At no stage should the stirrer be taken out of the tube.

Advantages of Sahli's Acid Haematin Method

1. Shali's haemoglobin meter is portable, easy to carry anywhere (bed side in case of critical patient, outpatient department in hospital, at field visit during clinical studies, etc.) and hence test can be conducted as per convenience.
2. It is easy to perform and handle.
3. It is quick procedure and result can be noted immediately.
4. It is inexpensive test.
5. No technical expertise is required to conduct this test.

Disadvantages of Shali's Acid Haematin Method

1. The standard colouration on the haemoglobinometer fades away over the year and this will give wrong results.
2. It gives results in approximation only and not accurately and cannot be fully relied upon.
3. All types of haemoglobin's (oxyhaemoglobin, sulphaemoglobin) do not get converted to acid haematin and hence the value of Hb may be determined lesser than the actual value.
4. Individual variation in matching of colour is seen

Oxygen Carrying Capacity

1 gm of haemoglobin carries 1.34 ml of oxygen. Hence the oxygen carrying capacity is haemoglobin level × 1.34 ml

RESULTS

Hb concentration in the volunteer subject is ..... by Sahli's method.

1 gm of haemoglobin carries 1.34 ml of oxygen, hence the oxygen carrying capacity in the subject is ..... .

NORMAL VALUES

Adult males: 14–18 gm/dl of blood (average is 15.5 gm/dl)
Adult females: 12–16 gm/dl of blood (average is 14 gm/dl)

OSPE I—SKILLED

Question: Draw the blood into haemoglobin tube for estimation of haemoglobin.

Steps to be followed by student

1. Sucks the blood into haemoglobin pipette and empty it into haemoglobinometer tube containing N/10 HCl. (Yes/No)
2. Ensures haemoglobinometer tube and pipette is clean and dry. (Yes/No)
3. Adds N/10 HCl in the haemoglobinometer tube up to its lowest mark (10 per cent or 2 gm %) with the help of a dropper. (Yes/No)
4. Draws blood up to 20 µL mark of the pipette from the given sample. (Yes/No)
5. Wipes the tip of the pipette and transfer the 20 µL of blood from the pipette into the haemoglobinometer tube containing N/10 HCl by immersing tip of the pipette in the acid solution and blowing out blood from the pipette. (Yes/No)
6. Leaves the solution in the haemoglobinometer tube for about ten minutes (for conversion of haemoglobin to acid haematin which occurs in the first ten minutes). (Yes/No)

The student should leave the tube in the comparator for acid haematin formation. The technician will take care of further step.

OSPE II—ANOTHER STUDENT

Note: A technician after waiting for 10 minutes has kept the haemoglobinometer tube ready. Golden brown colour of acid haematin is visible.

Question: Dilute the blood from given sample for estimation of haemoglobin.

Steps to be followed by student:

1. Dilutes the acid heamatin formed blood by adding distilled water drop by drop. Mixes it with the stirrer. Matches the colour of the solution in the haemoglobinometer tube with the standards of the comparator. Takes care to hold the stirrer above the level of the solution. (Yes/No)
2. After addition of every drop of distilled water, the student mixes the solution and compares the colour of the solution with the standard of the comparator till it matches with that of the standard. Takes care to hold the stirrer above the level of the solution. (Yes/No)
3. Throughout matching procedure takes care to hold the stirrer above the level of the solution. At no stage should the stirrer is taken out of the tube. (Yes/No)
4. Notes the reading when the colour of the solution exactly matches with the standard and expresses the haemoglobin content in gm%. (Yes/No)

Snap box 1

Key Information

WHO Haemoglobin Colour Scale: The colour of a drop of blood is absorbed on a chromatography paper and the colour of the drop of blood is matched against a printed scale of colour corresponding to varied levels of haemoglobin ranging from 4 to 14 gram/dl.

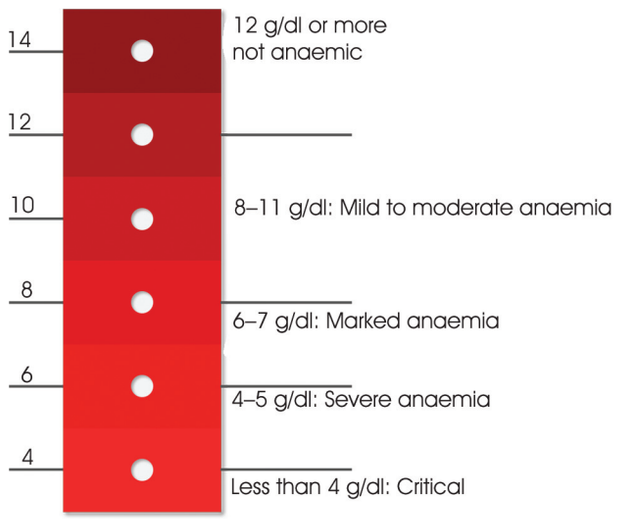


Fig. 4.2: WHO haemoglobin colour scale

Bibliography

Cherian M, Emmanuel JC, Lewis SM et al. Evaluation of the haemoglobin colour scale.*Bull World Health Organ.* 2002; 80: 839

VIVA VOCE QUESTIONS

Q1. What is the principle of Hb estimation?

Ans. In this method Hb is converted to acid haematin by N/10 HCl. The golden brown colour of the solution is matched with colour of a standard comparator to give rough estimate of Hb in gm%.

Q2. What is the function of N/10 HCl?

Ans. Hydrochloric acid acts with haemoglobin to form acid haematin.

Q3. What are the advantages and disadvantages of Sahli's method?

Ans. *The advantages of Sahli's method are*:

Sahli's haemoglobin meter is portable, easy to carry anywhere (bed side in case of critical patient, outpatient department in hospital, field visit during clinical studies, etc.) and it is easy to perform and handle.

Disadvantages of Sahli's method are:

a. The standard colouration on the haemoglobinometer fades away over the year and this will give wrong result.
b. It gives results in approximation only and not accurately and cannot be fully relied upon.

Q4. Which of the methods is most accurate method for haemoglobin estimation?

Ans. Cyanmethaemoglobin method is most accurate because estimation is done with photoelectric colorimeter.

Q5. Enlist three main causes for error reporting while estimating haemoglobin level.

Ans. *The error in reporting haemoglobin levels while estimating haemoglobin concentration in blood is*:

1. Technical errors—improper mixing of blood.
2. Errors in pipetting—tissue fluid contaminating capillary blood.
3. Visual errors—taking the reading is very subjective, as it is a comparison of colours. It can vary from person to person. Hence the results may not be accurate.
4. Quality of the colour comparators can affect the reading—if the glass blocks are old or faded it can cause wrong results.

Q6. What is the adequate time required for conversion of Hb to acid haematin?

Ans. Minimum time period of ten minutes is required for complete conversion of Hb to acid haematin; otherwise it will lead to false negative result. The 95% of Hb is converted acid haematin at the end of 10 minutes, 98% at the end of 20 minutes, and the maximum colour is reached in about 1 hour.

Q7. What are the types of haemoglobin which do not get converted to acid haematin?

Ans. The types of haemoglobin which do not get converted to acid haematin are carboxyhaemoglobin, methaemoglobin and sulphaemoglobin.

Q8. What will time delay in noting the haemoglobin levels by Sahli's method lead to?

Ans. The golden brown colour of acid haematin is unstable, hence undue delay in reading the test result will lead to false result.

Q9. What are the precautions to be taken while making the reading for haemoglobin value with that of comparator?

Ans.

1. The most important precaution to be taken is that the glass rod should not be left inside the haemoglobin tube.
2. Note the reading only when the colour of the solution in the haemoglobin tube is same as that of the comparator.
3. The matching should be done against natural light.

Q10. Hb concentration of a given subject was found to be 14 gm%. Calculate its oxygen carrying capacity %.

Ans. The normal oxygen carrying capacity of blood per gram of haemoglobin is 1.34 ml; hence the oxygen carrying capacity of the subject is $14 \times 1.34 = 18.76$ ml/dl.

Q11. What are the functions of haemoglobin?

Ans. *The functions of haemoglobin are*: Haemoglobin transports oxygen from the lungs to the tissues and carbon dioxide from the tissues to the lungs. It also acts as a buffer in and helps maintaining the blood pH. Haemoglobin in tissue regulates iron metabolism and mediates antioxidant effects.

Q12. What is the name of the molecule that transports oxygen in red blood cells?

Ans. The respiratory pigment of the red blood cells is haemoglobin.

Q13. What is the molecular composition of haemoglobin? Does the functionality of haemoglobin as a protein depend upon its tertiary or upon its quaternary structure?

Ans. Haemoglobin is a molecule made of four polypeptide chains, each bound to an iron-containing molecular group called a haem group. Thus, the molecule contains four polypeptide chains and four haem groups. As a protein it is composed of association of polypeptide chains, the functionality of haemoglobin depends upon the integrity of its quaternary structure.

Q14. What is the molecular weight of haemoglobin?

Ans. Haemoglobin is a globular molecule having a molecular weight of 68,000 daltons.

Q15. What is the normal blood haemoglobin level in adult male and adult female?

Ans. The normal haemoglobin concentration in adult male is 15.5 g/dl (range 14–18 g/dl) and females it is 14 g/dl (range 12–15.5 g/dl).

Q16. What is the normal haemoglobin concentration at birth and 1 year of age?

Ans. At birth the concentration of haemoglobin increases and may reach up to 23 g/dl. After two days of birth the Hb level starts decreasing and stabilizes at the end of three months to 10.5 g/dl. At 1 year of age the Hb concentration rises to 12 g/dl.

Q17. Enlist the normal variant of haemoglobin.

Ans. The normal variants of haemoglobin are adult haemoglobin (Hb A) and haemoglobin A2 (Hb A2) and foetal haemoglobin (Hb F2).

Q18. Enlist the various methods of estimation of haemoglobin concentration.

Ans. *Haemoglobin can be estimated by various methods and categorically can be classified as*:

1. Visual methods include Sahli's method, Wintrobes method, Haldane's method and tallquists method.
2. Gasometric method includes cyanmethaemoglobin method, Van Slyke method, spectrophotometric method and oxyhaemoglobin method.
3. Automated haemoglobinometry
4. Other methods include alkaline-haematin method, specific gravity method and comparator method.

Q19. Describe the various methods of estimation of haemoglobin.

Ans. *The various methods of estimation of haemoglobin are*:

Other Methods

A. Spectrophotometry (photoelectric colorimeter method): It measures the amount of light which gets absorbed by a solution. The wave band of light corresponds with portion of the spectrum which is absorbed to maximum in the test solution. These methods are rapid and give accurate results.

1. **Oxyhaemoglobin method:** Principle—ammonium hydroxide (0.04 ml/dl) haemolyse the red cells and converts the haemoglobin to oxyhaemoglobin and the absorbance of the solution, then it measures in the spectrophotometer (photoelectric colorimeter). This conversion is immediate, and the resulting colour is stable.
2. **Cyanmethaemoglobin method:** In this method blood is diluted in a solution containing Drabkin's reagent (1 gm of sodium bicarbonate, 50 mg potassium cyanide, 200 mg potassium ferricyanide in one liter of distilled water). Hb is oxidized to methaemoglobin by potassium ferricyanide and combined with potassium cyanide to form cyanmethaemoglobin. The absorbance of the solution measures in a spectrophotometer at wavelength 540 nm against Drabkin's solution as a blank. The result is expressed in gm/litre or mg/dl.

Fig. 4.3

Note: Measure the absorbance of this solution (Reaction of blood with Drabkin's solution) at 540 nm for estimating haemoglobin concentration.

B. Gasometric method: Gasometric method of estimation of haemoglobin is by using Van Slyke apparatus. This method is not used routinely in clinical laboratories as it is time-consuming, and the process of estimation is complex.

C. Automated haemoglobinometry: Various automated techniques have been employed to measure haemoglobin. Nowadays with developing health technology automated haemoglobinometer are also being used for haemoglobin estimation due to its simple and quick method of assessment.

D. Tallquists method: The method involves direct matching of the red colour of a drop of whole fresh blood on a filter paper with colour standards on the paper. Depending on the colour haemoglobin concentration value as depicted in the standard is noted as result.

E. Comparator method: This is a visual method and diluents used are an alkali solution (ammonia solution 0.04 percent). After mixing with dilute ammonia solution, the intensity of the colour of the haemolysed solution of red blood cells is compared against a standard colour disc in the comparator.

F. Haldane method using *Haldane's haemoglobinometer* (Haldane's modification of Gower's method): The instrument consists of two tubes, one of which contains 20 cu mm of blood haemolysed with distilled water and saturated with CO gas. The colour of this tube is used as standard. In the other tube a little distilled water is taken and 20 cu mm of patient's blood, collected from the fingertip by a special pipette, is added. When blood becomes fully haemolysed, it is saturated with CO by passing coal gas through it. The colour developed is compared against that of the standard. If the colour of the unknown is stronger, it is diluted with distilled water until the tinge is same in both. The graduation up to which the blood has been diluted gives the percentage haemoglobin.

G. Alkaline haematin method: In this method 50 ml of blood is added to an alkaline solution of 4.95 ml N/10 NAOH. The mixed solution is kept warmed at 37°C and kept in cold bath. The haemoglobin is converted to alkaline haematin and a stable colour compound is formed. The absorbance of alkaline haematin is measured using photoelectric colorimeter.

Note: The student should refer Appendix 1 for colorimetry.

Q20. What are the types of haemoglobin and its variant?

Ans. *The types of haemoglobin and its variants are*:

In the embryo haemoglobin types are: Gower 1 ($\zeta_2\varepsilon_2$) and Gower 2 ($\alpha_2\varepsilon_2$) haemoglobin

Portland I ($\zeta_2\gamma_2$) and Haemoglobin Portland II ($\zeta_2\beta_2$).

In the fetus haemoglobin type is: Haemoglobin F ($\alpha_2\gamma_2$)

In the adult haemoglobin types are: Haemoglobin A ($\alpha_2\beta_2$), Haemoglobin A2 ($\alpha_2\delta_2$) and Haemoglobin F ($\alpha_2\gamma_2$).

Haemoglobin variant forms that cause disease:

- Haemoglobin H (β_4)—which may be present in variants of α thalassemia.
- Haemoglobin Barts (γ_4)—may be present in variants of α thalassemia.
- Haemoglobin S ($\alpha_2\beta_2^S$)—a variant form of haemoglobin found in patients of sickle cell anaemia.
- Haemoglobin C ($\alpha_2\beta_2^C$)—this variant causes a mild chronic haemolytic anaemia.
- Haemoglobin E ($\alpha_2\beta_2^E$)—this variant also causes a mild chronic haemolytic anaemia.
- Haemoglobin AS—a heterozygous form in sickle cell traits and has one adult gene and one sickle cell disease gene.

Q21. Define anaemia.

Ans. The decrease oxygen carrying capacity due to decreased haemoglobin and red blood cell count is called anaemia.

Q22. How foetal haemoglobin (HbF) does differ chemically and spectroscopically than adult haemoglobin (HbA) and what is the advantage of foetal haemoglobin in terms of oxygen carrying capacity?

Ans. Foetal haemoglobin differs chemically and spectroscopically from the adult haemoglobin. It has a greater affinity for oxygen and releases CO_2 more readily. This is due to some difference in the globin fraction. This property helps to compensate the relative anoxia of foetal blood. At low O_2 pressure foetal haemoglobin can take up larger volumes of O_2 than adult haemoglobin.

Advantage of foetal haemoglobin: HbF (foetal haemoglobin) is 70% saturated at 20 mm Hg of PO_2 pressure, whereas adult haemoglobin is only 20% saturated at this pressure.

Q23. What is haemoglobinuria and what are causes for the condition?

Ans. *Haemoglobinuria* is the condition when free haemoglobins excreted through the urine. If Hb is free in the plasma, then it is excreted through the urine. In plasma, haemoglobinuria may be caused under the following conditions:

1. In strenuous exercise.
2. Due to mismatched blood transfusion.
3. Black water fever due to virulent type of malaria and red water fever due to another type of parasite which invades the erythrocyte causing release of Hb in the plasma.
4. Paroxysmal nocturnal haemoglobinuria
5. Hypotonicity of plasma
6. Thermal or chemical injuries
7. Paroxysmal cold haemoglobinuria.

Q24. Who shared the Nobel Prize for the studies of the structures of haemoglobin and myoglobin?

Ans. Max Ferdin and Perutz Wasan Austrian-born British molecular biologist who shared the Nobel Prize for Chemistry with John Kendrew for their studies of the structures of haemoglobin and myoglobin in 1962.

Q25. Discuss the causes for variation in haemoglobin concentration.

Ans. *The causes for variation in haemoglobin concentration are*:

1. *Age:* In the foetus, the concentration is highest. At birth, the average concentration is about 23 g per 100 ml. By the end of the third month it falls below normal, probably, because of deficiency of iron in milk. After this gradual recovery takes place and at the end of the first year, the average amount is 12.5 g. Then it rises gradually up to normal adult (physiological) range.
2. *Sex:* In females, the amount of haemoglobin is slightly lower than in males. In adult females, the average is 14 gm%, in adult males the average is 15.5 gm%.
3. *Diurnal variation:* Variation of at least 10% occurs throughout the day. In the morning it is the lowest; in the evening it is the highest.
4. *Altitude:* At higher altitude haemoglobin percentage rises.
5. *Exercise, excitement, adrenaline injection,* etc. increase the amount of haemoglobin.

> **Note:** It should be noted from the above that normal variation of haemoglobin is mostly due to alteration of number of red cells and not due to any change in the absolute quantity of haemoglobin in each cell. Anything that alters the red cell count will alter the percentage of Hb proportionally.

26. Describe the various derivatives of haemoglobin compound.

Ans. *The various derivatives of haemoglobin are*:

a. **Oxyhaemoglobin:** It is a compound of haemoglobin with oxygen. Iron remains in the ferrous (Fe^{++}) state in haemoglobin. It is not a stable compound.

b. **Methaemoglobin:** It is metalloprotein haemoglobin. It has iron in the heme group which is in the Fe^{3+} (ferric) state and not the Fe^{2+} (ferrous) state as in normal haemoglobin. It can be produced after treating the blood with potassium ferricyanide. It is chocolate brown in colour. It is a stable compound.

c. **Carbohaemoglobin:** It is a compound of haemoglobin with CO_2. The compound is formed by union of CO_2 with the globin portion.

d. **Carboxyhaemoglobin or carbon monoxyhaemoglobin:** Haemoglobin combined with CO instead of oxygen. The affinity of human haemoglobin at 38°C, for CO is 210 times greater than oxygen, and is extremely poisonous.

e. **Sulphaemoglobin:** It is formed by the reaction of haemoglobin with a sulfide in the presence of oxygen or hydrogen peroxide and found in putrefied organs and cadavers.

f. **Nitric oxide haemoglobin:** Haemoglobin combined with NO instead of oxygen.

Bibliography

1. Gamperling N, Mast B, Hagbloom R, Houwen B: Performance Evaluation of the Sysmex KX-21 [TM] Automated Hematology Analyzer. Sysmex J Int. 1998, 8: 96–101.
2. Morris SS, Ruel MT, Cohen RJ, Dewey KG, de la Briere B, Hassan MN: Precision, accuracy, and reliability of haemoglobin assessment with use of capillary blood. Am J Clin Nutr. 1999, 69 (6): 1243–1248.
3. Sari M, dePee S, Martini E, Herman S, Bloem MW, Yip R: Estimating the prevalence of anaemia: A comparison of three methods. Bull World Health Org. 2001, 79: 506–511.
4. Zhou X, Yan H, Xing Y, Dang S, Zhuoma B, Wang D: Evaluation of a portable haemoglobin photometer in pregnant women in a high altitude area: A pilot study. DMC Public Health. 2009, 9 (1): 228–10.1186/1471–2458–9–228.

EXERCISE FOR STUDENTS: OBSERVATIONS: NOTE YOUR RESULTS

Results

1. The Hb concentration in the volunteer subject is _____ by Sahli's method.
2. Oxygen carrying capacity —1 gm of haemoglobin carries 1.34 ml of oxygen, hence the oxygen carrying capacity in the subject is