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BCH 471
Experiment (8)

Quantitative determination of G6PDH deficiency in hemolysed RBC sample

Qualitative determination of HbS in blood.

Quantitative determination of iron in serum

Experiment (1): Quantitative determination of G6PDH deficiency in hemolysed RBC sample

Objective:

Quantitative determination of glucose 6-phosphate dehydrogenase (G6PDH) activity in erythrocytes (hemolysate).

Principle:

Glucose + NADP+ $\xrightarrow{G6PDH}$ 6-Phosphogluconate + NADPH + H+

- The enzyme G6PDH is catalyses the dehydrogenation of glucose 6-phosphate as the first step in pentose phosphate pathway (PPP).
- NADP+, the electron acceptor, is reduced to NADPH in the reaction.
- The optimum pH for G6PDH reaction is 8.3.
- The rate of formation of NADPH is a measure of the G6PDH activity and it can be followed by means of the increase in the Absorbance at 340 nm .

Note: A red cell hemolysate is used to assay for deficiency of the enzyme, while serum is used for evaluation of enzyme elevations.

Procedure

- Pipette into clean and dry test tubes

Reagent	Volume
G6PDH Buffer	3 ml
NADP reagent	100 µl
Hemolysate	50 µl
Mix and incubate for 5 min at 25°C, the add	
G6PDH Substrate	50 µl
Mix and after 30 seconds read initial absorbance at 340 nm against distilled water. Repeat absorbance reading every min for 3 min and calculate ΔA/min	

Calculations

G6PDH Activity in mU/erythrocytes/ml of blood(P)= ΔA/min x 30868

- Note: If the erythrocytes count per ml of blood is 4.5 X 10^9

→ Then the G6PDH activity in mU/ 10^9 cells = P/4.5

Abnormal value= 0- 11 mU/ 10^9 cells.

Expected value= 80- 180 mU/ 10^9 cells

Experiment (2): Qualitative determination of hemoglobin S (HbS) in blood.

Objective:

Qualitative determination of hemoglobin S (HbS) in blood using a phosphate solubility method.

Introduction

There are hundreds of Hb variants, and the most common and important are:

- **Hemoglobin A**
  - It is normal hemoglobin that exists after birth and consist of (α2β2).
  - In normal adult 95% of Hb is present as HbA
- **Hemoglobin A2**
  - It is a minor component of the hemoglobin found in red cells after birth and consists of (α2δ2)
  - less than 3% of the total red cell hemoglobin.
- **Hemoglobin F**
  - Hemoglobin F is the predominant hemoglobin during fetal development and consists of (α2γ2).

Example of an abnormal Hb

- **Hemoglobin S (HbS).**
  - The alpha chain is normal, while the beta chain is mutated, giving the molecule the structure, α2βS2.
  - A point mutation in the **Hb β gene** is responsible for the sickling of RBCs seen in sickle cell anemia .The abnormality is due **to Substitution of non polar valine for a charged Glutamic acid in position 6 in the β chain .**
  - HbS can be inherited in the **homozygous state (S/S)** produce sickle cell anemia , **or in heterozygous (A/S)** ,also called sickle cell trait, usually don't exhibit symptoms of the sickle cell anemia disease (unless under extreme hypoxia).

- Individuals with HbS will be at high risk when exposed to conditions of **low oxygen tension** such as surgery, high altitude or athletics which may results in serious and fatal clinical complications.

Principle

- Erythrocytes are lysed (by saponin) and the released hemoglobin is reduced (by dithionite) in phosphate buffer.
- Reduced HbS is characterized by its very low solubility → So that in the presence of HbS, the solution become **turbid** and the lines behind the test tube will not be visible while, if no sickling hemoglobin was present the clear solution will permit the lines to be seen through the test tubes.

Procedure

Reagent	Volume
Sickling solution	2 ml
Patient sample (whole blopd)	0.02 ml (20 µl)
Mix by inversion and allow stand at room temperature for 5 to 10 min	
Read the test by holding the test tube approximately 3 cm in front of a lined scale on the card.	

Experiment (3): Quantitative determination of iron unsaturated iron binding capacity (UIBC) and total iron binding capacity (TIBC) in serum using a colorimetric method.

Objective:

- To determine the normal level of serum iron.
- To determine the use of this test in diagnosis of anemia (iron deficiency).

Introduction

- Iron is the metal component of haemoglobin, myoglobin, cytochromes and some proteins of the electron transport chain.
- The total iron of an **adult male is 4-5g** and of a **female 3-4g**. of this:
  - 65% is as haemoglobin,
  - 25% as stored iron (ferritin and haemosiderin),
  - 10% as other forms (myoglobin, cytochromes etc.)
  - and only 0.1 %as serum iron.
- Iron is carried in **Fe^{3+} state** bound to a specific iron transport protein known as transferrin.

Transferrins

- They are iron-binding blood plasma glycoproteins that control the level of free iron in biological fluids (When iron stores become low, transferrin levels will increase. When there is too much iron, transferrin levels are low).
- It contains **two** specific high-affinity Fe(III) binding sites.
- Transferrin distributes iron to those tissues which have a demand for its utilization.
- Individuals who lack transferrin show severe **hypochromic anemia** and are also susceptible to bacterial and viral infections.

Total iron-binding capacity (TIBC)

- It is a medical laboratory test that measures the blood's capacity to bind iron with transferrin.
- it is measuring the maximum amount of iron that it can carry, which indirectly measures transferrin
- It is calculated by adding serum iron and unsaturated iron binding capacity (UIBC)
- It is most frequently used along with a serum iron test to evaluate people suspected of having either iron deficiency anemia or iron overload **(hemochromatosis)**

- **Defect in Serum iron**
  - **Serum iron is low** in iron deficiency anaemia whether due to insufficient intake, malabsorbtion or blood loss.
  - **Serum iron concentration is high** when marrow cannot utilize iron as in **pernicious anaemia**, in **thalassemia and hemolysis**. High values are also found in **severe hepatitis** due to release from liver cells.

- **Defect in Iron binding capacity (TIBC)**
  - Increase in iron deficiency anemia
  - Decrease in hemochromatosis, malignant or rheumatic fever .

Normal range of serum iron
50 -160 μg/dl
Normal range of TIBC
250 - 450 μg/dl

CONDITION	SERUM IRON (50-160μg/dL)	TRANSFERRIN (200-400 mg/dL)	% SATURATION (20-55)	FERRITIN (20-250 μg/L)
Iron deficiency	Decreased	Increased	Decreased	Decreased
Iron poisoning/overdose	Increased	Decreased	Increased	Increased
Hematochromatosis	Increased	Decreased	Increased	Increased
Malnutrition	Decreased	Decreased	Variable	Decreased
Malignancy	Decreased	Decreased	Decreased	Increased
Chronic infection	Decreased	Decreased	Decreased	Increased
Viral hepatitis	Increased	Increased	Normal/increased	Increased
Anemia of chronic disease	Decreased	Normal/decreased	Decreased	Normal/increased
Sideroblastic anemia	Increased	Normal/decreased	Increased	Increased

Principle:

- ***Serum iron :*** The iron dissociated from its Fe-III-transferrin complex by addition of acidic buffer containing hydroxylamine which reduces the Fe(III) to Fe(II) . Then the chromogenic agent (PDTS) form a highly colored Fe(II) complex that is measured spectrophotometrically at 565nm .
- ***UIBC:*** Determined by adding Fe(II) to serum so that it binds to unsaturated iron binding site on transferrin . The excess Fe(II) react with PDTS to form color complex which is measured spectrophotometrically at 565nm. The difference between the amount of Fe(II) **added** and the amount of Fe(II) measured represent the UIBC
- ***TIBC*** is determined by adding serum iron to UIBC value.

Procedure:

	IRON			UIBC		
	BLANK	STD	TEST	BLANK	STD	TEST
IRON BUFFER	2.5 ml	2.5 ml	2.5 ml	-	-	-
UIBC BUFFER	-	-	-	2 ml	2 ml	2 ml
IRON STD	-	0.2 ml	-	-	0.2 ml	0.2 ml
SAMPLE	-	-	0.2 ml	-	-	0.2 ml
WATER	0.2 ml	-	-	0.4 ml	0.2 ml	-
Iron reagent color	0.05 ml	0.05 ml	0.05 ml	0.05ml	0.05 ml	0.05 ml
	Mix and incubate at 37c for 10 min Mix and read the absorbance of std and test (iron) at 565 nm against blank. Also read the absorbance of std and test (UIBC) against blank					

Calculations

- Iron conc. (µg/dl) =(Abs. of test/Abs. of std) X Std conc.
  - The std conc. = 500 µg/dl

- SERUM IRON + SERUM UIBC = SERUM TIBC
  (µg/dl) (µg/dl) (µg/dl)

- Transferrin saturation % = $\frac{\text{serum iron concentration}}{\text{TIBC}} \times 100$