METHODS OF BLOOD GLUCOSE ESTIMATION

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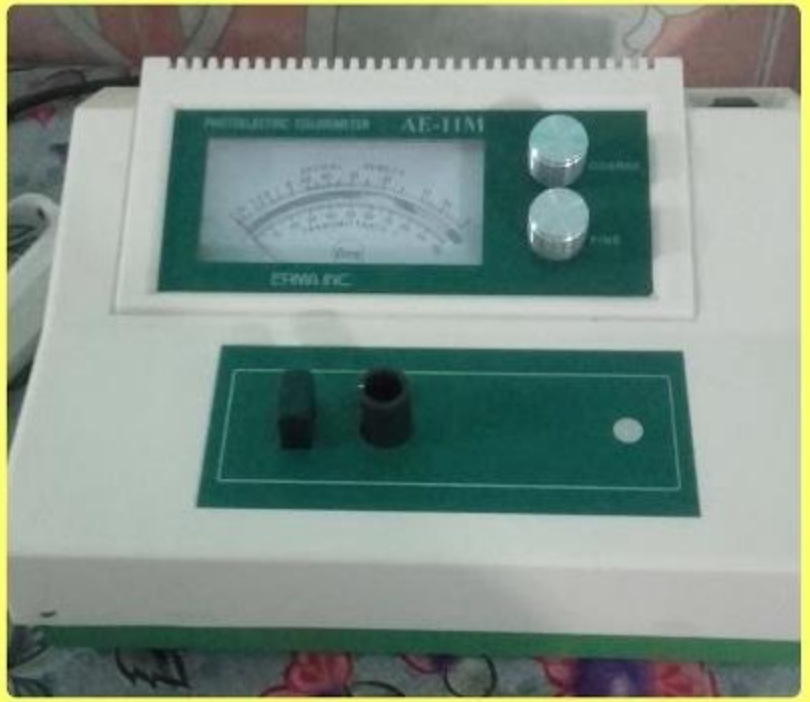


Estimation of Blood Glucose

Methods

Non Enzymatic

1-Alkaline copper reduction method

2-O-toluidine test (method of Dubowski)

3-Method of Asatoor and King

Enzymatic

Glucose oxidase method commonly known as Glucose Oxidase/ Peroxidase (GOD/POD method).

Electrochemical methods:

Glucometer

Non Enzymatic Methods

- Most of these methods utilize the reduction of metals like copper (II) or iron (III) by glucose and measure glucose concentration indirectly.

1. Alkaline Copper Reduction (Folin-Wu) Method :-

Folin – Wu devised a method to estimate sugar in blood by alkaline copper reduction exploiting the reducing property of glucose.

Principle:

Glucose in hot alkaline medium forms a strong reducing agent **enediol** which reduces cupric ion to cuprous ion. Initially it forms a yellow coloured cuprous hydroxide which upon heating produces red precipitate of cuprous oxide. Addition of phosphomolybdic acid result in oxidation of cuprous ion to cupric accompanied by reduction of phosphomolybdic acid to water soluble molybdenum blue which is measured colorimetrically .

Folin - Wu

$Glucose + Cu^{2+} >>> Gloconate + Cu^{+}$

pH > 7 , heat

Cu^{+} + phosphomolybdate

Blue molybdenium complex

λ = 660 nm

Thus glucose is estimated indirectly by the intensity of the color of molybdenum blue. The intensity of blue color is proportional to glucose concentration.

Note- Whole blood is used in this technique and the estimation is done following hemolysis.

Disadvantage :

Since this is an oxidation reduction method, apart from glucose, other reducing substances present in blood namely glutathione (most imp.) , glucuronic acid, uric acid, threonine, ascorbic acid etc. are also estimated. This give a higher value (about 30mg/dl). Therefore this method measures total blood sugar not blood glucose.

O-toluidine test (method of Dubowski) & Method of Asatoor and King

- These chemicals methods have been modified to estimate glucose (true sugar) level by using isotonic solutions so that cells may not be hemolyzed and release non glucose reducing substances into the solution and using such protein precipitants which are capable of precipitating all such substances .
- The result obtained by theses methods are only slightly higher (5-7 mg/100ml) than the enzymatic methods.
- These methods are simple accurate and convenient for most of the laboratories .

These involve three steps:

- Precipitation of blood proteins and other reducing sugar substances.
- Heating the supernatant in alkaline buffer medium to reduce the metal to its lower valency state by glucose.
- Development of color by treating it with phosphomolybdic acid or arsenomolybdic acid solution . The use of arsenomolybdic acid is preferred because the color formed is quite stable where as the color formed with phosphomolybdic acid fades with time and so should be measured soon.

In some methods Orthotoluidine is used as color developing reagent this is corrosive and requires carefully pipetting.

O- toluidine method (method of Dubowski):-

Principle:

The glucose in a protein free supernatant from blood, plasma or serum is heated with a solution of a primary aromatic amine(O-toluidine) in glacial acetic acid. Green color is produced (probably a glycosylamine) which is measured colorimetrically. The reaction is not seriously affected by other reducing sugars except galactose the reagent is stabilized with thiourea.

Reagents-

1. Trichloroacetic acid (TCA)
2. O-Toluidine reagent
3. Stock glucose standard
4. Working glucose standard

Procedure:

- Take 1.9 ml TCA in a centrifuge tube add 0.1ml blood.
- Mix well and centrifuge after about 10 minutes.
- Measure 1.0 ml clear supernatant (serum) in a tube.

	Blank	Standard	Test
Serum	-	-	1 ml
Distilled water	1ml	-	-
Working Glucose Standard	-	1ml	-
O-toluidine	5ml	5ml	5ml

- Mix well cap the tube with cotton wool and heat in boiling water for 10 minutes.
- Cool in cold tap water.
- Measure the absorbance using red filter (630 nm) against reagent blank.

Calculation:

Glucose(mg/100ml) =T/S x 200

Note:

- The use of cheaper grades of O-toluidine will give unacceptably high blanks.
- Due to corrosive nature of toluidine reagent it should be handled with care.
- Minor degrees of opalescence in the supernatant usually clear after boiling with the toluidine reagents.

Method of Asatoor and King

Reagent

1) Isotonic solution
2) Sodium tungstate
3) Alkaline tartrate solution
4) Arsenomolybdate reagent
5) Stock glucose standard
6) Working glucose standard

Procedure :

Collect about 0.5 ml blood in a fluoride vial, mix by gentle rotation.

Test:

Measure 3.8 ml isolating solution in a centrifuge tube add 0.1 ml blood, mix and add 0.1 ml sodium tungstate solution .

Mix well by inverting the tube and centrifuge after sometime, pipette 1.0 ml supernatant in a 15 ml tube.

Standard:

1.0 ml working glucose standard in a 15 ml tube.

Blank:

1.0 ml isotonic solution in a similar tube.

- All these test tubes add 1.0 ml Alkaline tartarate solution, mix and plug the tube with cotton. Place them in boiling water in a beaker for 10 minutes.
- Remove cotton and cool the tube then add 1.0 ml arsenomolybdate reagent to each tube mix by shaking till effervescence ceases.
- Allow to stand for 10 minutes then add 5.0 ml water to each tube and mix well.
- Read absorbance after 10 minutes on 680 nm (red filter) against the reagent blank.

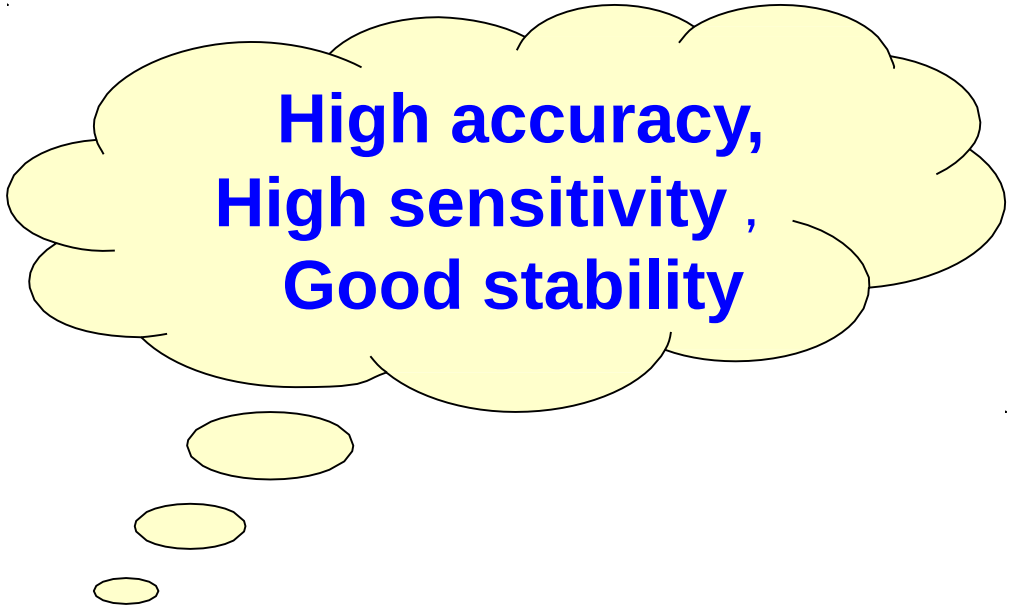
Calculation:

Blood glucose(mg/100ml)= T/S X100

Chemical reactions-

- Isotonic solution is used to prevent hemolysis so that non glucose reducing substances may not released from the cells.
- Copper reacts with tungstate forming copper tungstate which precipitates proteins as well as other substances behaving like glucose in the reaction.
- Alkaline tartrate favors the reduction of copper(II) to copper(I) by glucose and glucose is oxidized to gluconic acid.
- It also prevents reoxidation of copper(I) by binding it to tartrate which is present in alkaline tartrate reagent.
- Arsenomolybdic acid oxidizes copper(I) to cupric and is being reduced to molybdenum blue which is measured.

Enzymatic Method- GOD- POD



Why do we chose Glucoseoxidase-peroxidase method(GOD-POD) to measure blood glucose?

Enzymatic method

- This method is adopted by most of the laboratories because it offers several advantages.
- It is specific for glucose and single step method requiring less sample.
- No deproteinization and boiling is needed.

Principle:

Glucose oxidase method:- commonly knowns as Glucose Oxidase / Peroxidase (GOD/POD) method.

- This utilize a specific bacterial enzyme glucose oxidase which promotes oxidation of glucose into gluconate and H_2O_2 . In presence of another enzyme peroxidase , H_2O_2 reacts with a chromogenic O_2 acceptor e.g.-O-dianisidine or oxidizes phenol which then reacts with 4-aminophenazone to give a colored compound which is measured colorimetrically.

Glucose + H_2O + O_2 **GOD** → Gluconic acid + H_2O_2

4 Amino Phenazone + Phenol + $2H_2O_2$ **POD**→ **4-(P-**benzoquinone- monoimino)-phenazone + $4H_2O$.

4-(P-benzoquinone- monoimino)-phenazone is a colored compound which is measured colorimetrically.

Protocol:

Since different companies are commercially producing kits employing this principle, the literature of kits and adopt a protocol depending on the infra structure of laboratory including photometer.

Specimen:

- Serum, or plasma free of haemolysis.
- Sodium fluoride is preferred as an anticoagulant due to its antiglycolytic activity.

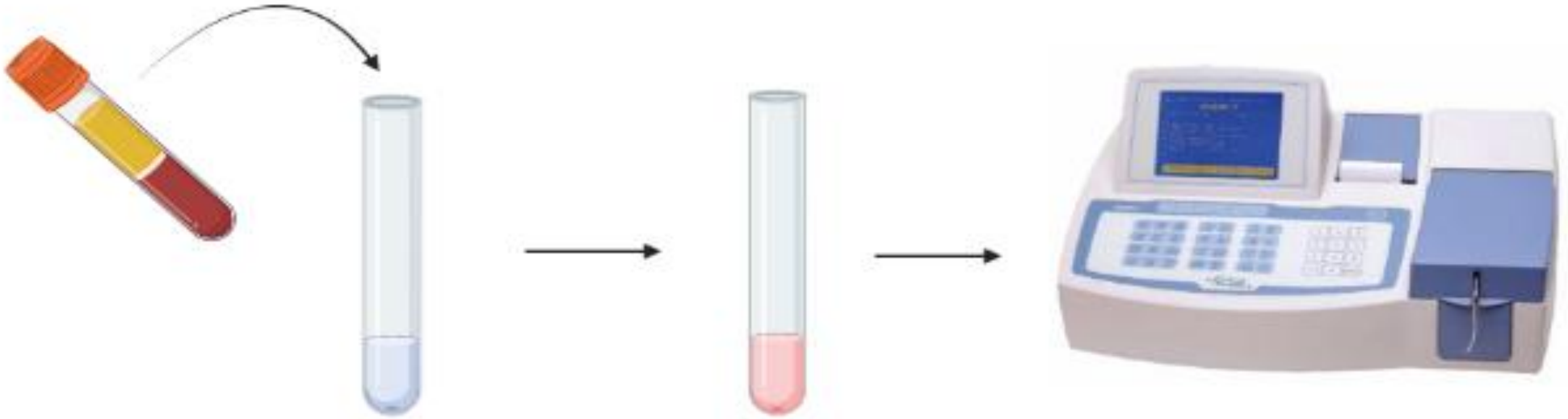
Reagents:

1. Glucose standard (100 mg/dl)
2. **GOD-POD reagent**: Enzyme reagent mixture containing glucose oxidase (GOD), peroxidase (POD), 4-aminoantipyrine, phenol, and phosphate buffer (pH≈7.0), some stabilizers and activators.

Instruments:

1. Test tubes
2. Pipettes, disposable tips, rack
3. Water bath
4. Colorimeter

Estimation of Blood Glucose By GOD-POD Method



Procedure

1. Label three clean, dry test tubes as **Blank (B)**, **Standard (S)**, and **Test (T)**.
2. Pipette as follows

	Blank	Standard	Test
GOD-POD Reagent	1 ml	1 ml	1 ml
Distilled water	10 µl	–	–
Glucose standard	–	10 µl	–
Sample	–	–	10 µl

Mix well and incubate at 37°C for 10 minutes. Or, at room temperature (25°C) for 30 minutes then use cuvette to measure their absorption value.

Measure the absorbance of the standard and test sample at 540 nm (green filter) against reagent blank within 60 minutes.

Calculation

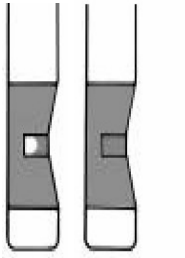
$$\text{Conc. of Glucose in the specimen (mg/dl)} = \frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} \times 100$$

Blood Glucose Meter- Glucometer

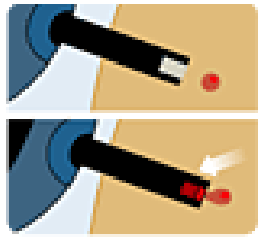
People who have diabetes should be testing their blood glucose regularly at home.

- Lancing the finger
- Apply blood to strip

Drop,
not smear



Cover ALL of
test strip
window



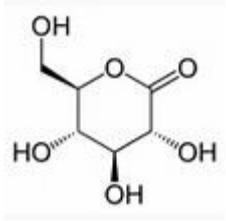
Some strips wick
blood onto the
strip

Mechanism for catalytic oxidation of glucose *With Glucose oxidase (GO) and Fc mediator*

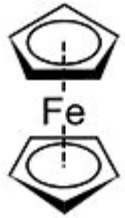
Glucose + GO(FAD) + 2 H^+ - - > gluconolactone + GO($FADH_2$)

GO($FADH_2$) + 2 Fc^+ --> GO(FAD) + 2 Fc + 2 H^+

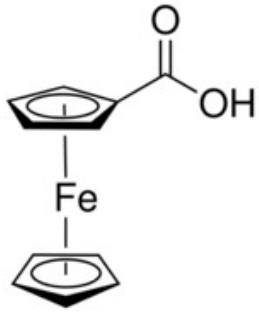
Fc --> Fc^+ + 2 e^- (at electrode)



Gluconic acid/gluconolactone



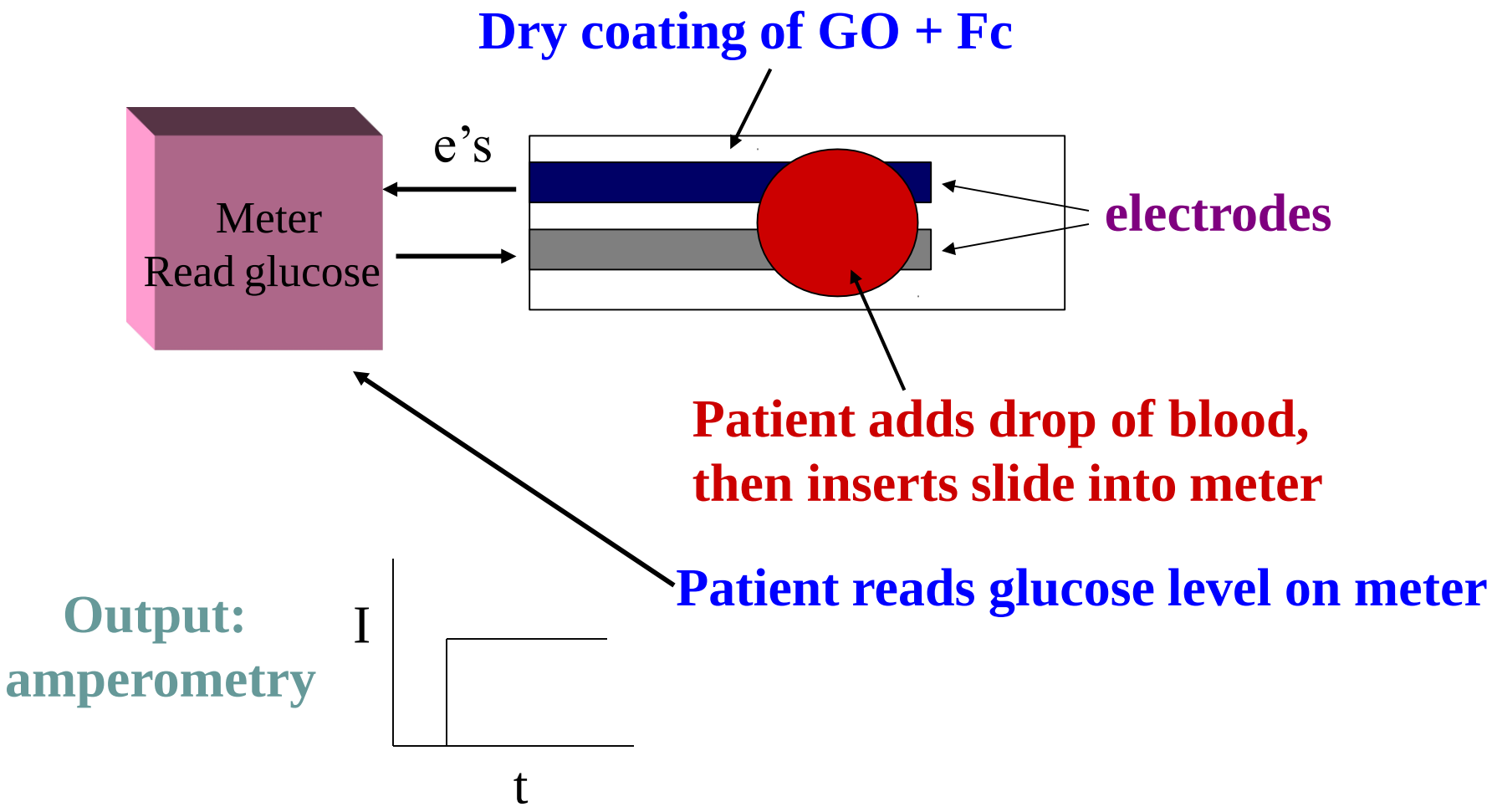
ferrocene



Fc = ferrocene carboxylate

Signal can also be measured by amperometry: Hold const. E where oxidation occurs, measure I vs time

Glucose biosensor test strips




THANK
YOU!