National University-Sudan

Faculty of Medical Laboratory Sciences

Student Practical Manual-
Haematology and Immunohaematology Department

Fourth Year, Semester (7)
Haemostasis and Bleeding Disorders Investigations

MLS-HAEM-413

Student's Name:……

ID: ……**Batch**……...…...

Instructions

- Wear lab coat
- Wear Gloves
- Avoid swallow any chemical
- Follow the procedures provided
- Write your results in this manual

Content

Bleeding Time (BT)

Aim

The bleeding time test is a useful tool to test for platelet plug formation and capillary integrity. Occasionally, the bleeding time test will be ordered on a patient scheduled for surgery.

Four procedures are currently in use for determining the bleeding time:

1. The Duke method.
2. The Ivy Method.
3. The Mielke Method.
4. The Template or Surgicutt Method.

Duke Method

Requirements:

1. Sterile lancet,
2. Cotton
3. rectified spirit
4. filter paper
5. Stop watch.

Procedure:

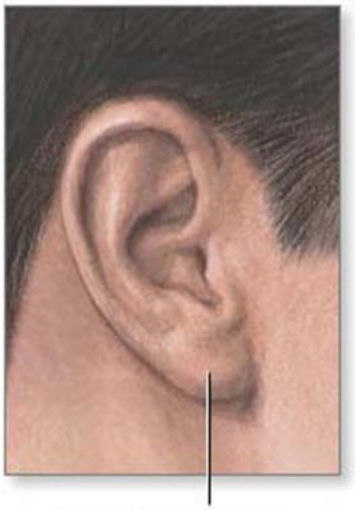
1. In Duke Method, the patient is pricked with a special needle or lancet, on the earlobe, after having been swabbed with alcohol.
2. The prick is about 3–4 mm deep.
3. Then wipes the blood every 30 seconds with a filter paper.
4. The test ceases when bleeding ceases.
5. The test causes nervousness in the patient.

Normal range: The usual time is about 1–3 minutes.

Limitations

a. No repeat testing is allowed due to space.

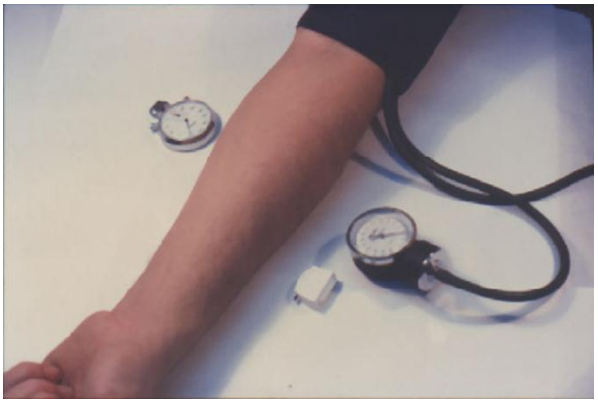
b. This test method is the easiest to perform, but is the least standardized and has the less precision and accuracy.

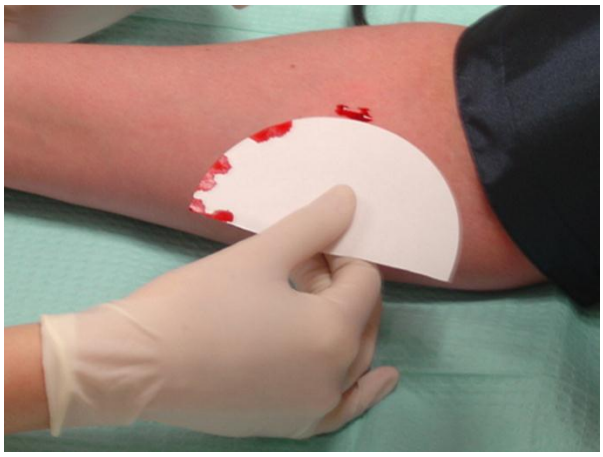


Ivy Method

1. In the Ivy method, a blood pressure cuff is placed on the upper arm and inflated to 40 mmHg to control capillary tone and to improve the sensitivity and reproducibility– this will maintain constant pressure within the capillaries and help standardize the procedure- .
2. A sterile, disposable blood lancet is used to make a shallow incision that is 1 millimeter deep on the underside of the forearm.
3. Every 30 seconds, filter paper is used to draw off the blood.
4. The time from when the incision is made until all bleeding has stopped is measured.
5. The test is finished when bleeding has stopped completely.

Normal value: 2 – 7 minutes.





Interpretation of bleeding time

- A prolonged bleeding time may be a result from decreased number of thrombocytes, abnormal platelet function or impaired blood vessels.
- The greatest source of variation in this test is largely due to difficulty in performing a standardized puncture. This usually leads to erroneously low results.

Bleeding Time Abnormalities:	
Collagen disorders	e.g. Ehlers Danlos syndrome
Thrombocytopaenia	A platelet count of <50 x 10/L is generally considered to prolong the BT.
Qualitative platelet disorders	• Inherited and acquired platelet disorder including the use of anti-platelet drugs such as aspirin and clopidogrel will prolong the BT. • Paraproteinaemias can also lead to defective platelet function and may, therefore, prolong the BT. • Other acquired disorders of platelet function such myelodysplastic syndromes (MDS) and myeloproliferative disorders (MPD) will also prolonged the BT.
Von Willebrand Disease (VWD)	A deficiency of Von Willebrand Factor (VWF) may prolong the BT but not in all cases.
Hypofibrinogenaemia	Fibrinogen is required for platelet-platelet interaction and the BT will, therefore, be prolonged in cases of hypofibrinogenaemia.

Clotting Time CT:

Aim :

Clotting time was used as a screening test to measure all stages in the intrinsic coagulation system and to monitor heparin therapy.

Principle:

Clotting Time is the time required for blood to form a clot in vitro.

It based on that whole blood will form a solid clot when exposed to a foreign surface such as a glass tube.

Methods:

1. Capillary Method.
2. Slide Method.
3. Tube Method

Tube Method (Lee-White method)

Reagent & equipment

1. Water bath, 37^{o} C.
2. Glass test tube (10 x 75 mm)
3. Stopwatch.
4. Plastic syringe.

Specimen: 4 ml of fresh whole blood.

1. Label 3 glass test tubes with patient name and number them, 1, 2, and 3.

2. Perform a clean, Untraumatic venipucture using a 20-gauge needle and drawn 4 mL of blood.
3. Start the stopwatch as soon as the blood enters the syringe.
4. Remove the needle from the syringe, and fill each of the three tubes with 1 ml blood.
5. The last 1 ml of blood may be discarded.
6. Place the three test tubes in a 37°C water bath.
7. At exactly 3 min., Remove the first tube form water bath and tilt gently to a 45° angle to see whether the blood has clotted.
8. If Blood not clotted return it to the water bath and examine it at 30 second intervals.
9. After the blood in the first tube has clotted, examine the second tube immediately.
10. Then examine the 3rd one.
11. Record the time it took the blood in the 3rd test tube to clot.
12. Then one tube should remain in the 37°C water bath to be checked for **clot retraction**. Also, this same tube may be allowed to remain in the water bath overnight and checked the next day for **clot lysis**.

Normal Range:
5 – 10 Minutes

Interpretation:

Conditions accompanied by increased Clotting Time:

1. Factors V, VII, VIII, IX, XI, XII Deficiencies.
2. Hemorrhagic disease of Newborn
3. Vitamin K deficiency.
4. Heparin Therapy.
5. Presence of Circulating antibodies (inhibitors)
6. Afibinogenemia

Limitations:

1. Variations are wide and the test sensitivity is limited.
2. The test is the least effective test in the diagnosis of actual haemostasis failure; so it has been replaced by APTT.
3. Poor venipuncture technique, causing hemolysis or tissue thromboplastin to mix with the blood, shortens the clotting time.

Bubbles entering the syringe when the blood sample is being obtained increase the rate of coagulation.

Student's findings (measurements or observations):

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comments and interpretation:

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Evaluation (carried out by the instructor):

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Name and signature of the instructor:

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Date: ……..\.....\.....

Prothrombin time (PT):

Principle:

The PT test measures the clotting time of plasma in the presence of optimal concentration of tissue extract thromboplastin, and indicate the overall efficiency of the extrinsic and common clotting factors.

Regents:

1. Patient and control plasma (platelet poor plasma)
2. Thromboplastin reagent with calcium (CaCl2 0.025 mol\L)

Preparation of platelet poor plasma (PPP):

1. Platelet rich plasma (PRP) is obtained by centrifuging blood at room temperature (20 C) for 10 – 15 min at 2000 rpm. The PRP is carefully removed without contamination with red cells or buffy coat;
2. Remover plasma is centrifuge at 2ooo rpm to obtain PPP

Method:

1. 0.1 ml of patient and control into a glass test tube placed in a water path
2. 0.2 ml of thromboplastin reagent with CaCl2 and start the stopwatch
3. Mix the content of the tube and record the end point

Expression of the result:

1. As the mean of the duplicate reading in second
2. As the ratio of the mean patients plasma time to the mean of normal control plasma time
3. As an international normalized ratio (INR)

Normal range:

11 – 16 secs.

Interprétation:

1. Oral anticoagulant
2. Liver diseases especially obstructive
3. Vitamine K deficiency

4. Disseminated intravascular coagulation (DIC)

Student's findings (measurements or observations):

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comments and interpretation:

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Evaluation (carried out by the instructor):

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Activated partial thromboplastin time (APTT):

Also known as the partial thromboplastin time with kaolin (PTTK). And the kaolin cephalin clotting time (KCCT).

Principle:

APTT test measures the clotting time of plasma after the addition of the activation of contact factors but without added tissue thromboplastin. And indicate the overall efficiency of intrinsic pathway.

Reagents:

1. Platelet poor plasma (PPP)
2. Kaolin: 5g in barbitone buffer saline PH 7.4
3. Phospholipid
4. $CaCL_2$ (0.025 mol\L)

Method:

1. Mix equal volume of the phospholipid reagent with kaolin suspension and leave it in the water path
2. 0.1 ml of patient and control PPP in glass test tube
3. Add 0.1 ml of phospholipid reagent with kaolin
4. Incubate 2 – 3 min at water path 37 C
5. Add 0.1 ml of $CaCL_2$ reagent and start the stopwatch
6. Mix the content of the tube and record the end point

Normal range:

26 – 40 sec.

Interpretation:

1. DIC
2. Liver diseases
3. Hemophilia
4. Administration and contamination of heparin and other anticoagulant
5. Circulating antibodies and inhibitors

Student's findings (measurements or observations):

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comments and interpretation:

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Evaluation (carried out by the instructor):

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Thrombin time (TT):

Also known as thrombin clotting time (TCT)

Principle:

Thrombin is added to plasma and the clotting time is measured.

TT is affected by the concentration of fibrinogen, and the presence of fibrinogen or fibrin degradation products (fdp).

Reagents:

1. PPP
2. Thrombin solution

Method:

1. 0.1 ml of thrombin is added to 0.2 ml of patient and control PPP at 37 C water path
2. Start the stopwatch
3. Record the end point

Expression of the result:

As means of duplicate clotting time in second for patient and control

Normal range:

11 – 18 sec.

Student's findings (measurements or observations):

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comments and interpretation:

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Evaluation (carried out by the instructor):

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Mixing study (correction test) using the PT and APTT

Principle:

By mixing the patient plasma with normal plasma correction indicates a possible factor deficiencies whereas failure to correct suggest the presence of inhibitors.

REAGENTS:

1. Normal plasma contains all the coagulation factors (Control) For Correction.
2. Plasma Poor Platelet (PPP) from the patient
3. Reagent of PT or APTT

METHOD:

1. Perform 50:50 Mixture of the control plasma and patient plasma
2. Perform PT and\or APTT

INTERPRETATION:

If the prolongation is Correct indicate factors deficiency. If the normal plasma fails to correct the prolongation indicate factors inhibitors.

Correction tests with Aged and Adsorbed plasma:

- **Aged plasma:**

  Contain factors II, VII, IX, X

  Poor for factors VIII, V

  Using for both PT \APTT

- **Adsorbed plasma:**

  Contain factors VIII, V

  Poor for factors II, VII, IX, X

  Using for both PT\APTT

Plasma adsorbed by Aluminum hydroxide gel that adsorbs Vitamin K dependent factors

REAGENTS:

1. Aged plasma
2. Adsorbed plasma
3. PPP from patient
4. Reagents of PT\APTT

METHOD:

1. Perform 50:50 mixture of the patient sample with aged and adsorbed plasma respectively.

NO	Aged Plasma (for APTT)	Adsorbed Plasma	Interpretation
1	Correction	Abnormal	F IX Deficiency
2	Abnormal	Correction	F VIII Deficiency
3	Correction	Correction	F IX, VII Def.Or XI

2. Perform PT\APTT

INTERPRETATION:

Ability of used plasma to correct patient plasma indicate that the factors deficient in used plasma are not that in patient plasma.

No	Aged Plasma (For PT)	Adsorbed Plasma	Interpretation
1	Correction	Abnormal	F X Deficiency
2	Abnormal	Correction	F V Def.

3	Correction	Partially correction	F II Def

Student's findings (measurements or observations):

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comments and interpretation:

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Evaluation (carried out by the instructor):

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Name and signature of the instructor:

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Coagulation factor assay:

Factor assay can be performed by three ways:

1. Immunological method: ELISA
2. Assay using chromogenic substrate
3. Coagulation assay

The coagulation assay:

Principle:

If two material containing the same coagulation factor are assayed in specific assay system in arrange of dilution and the clotting time are plotted against the plasma concentration on double Log paper a sigmoid curve with a straight middle section is obtained.

In some cases like factor VIII assay the semi – log paper is required.

If the dilution of the test and control plasma are chosen carefully it should be possible to draw two straight parallel lines. The horizontal distance between two lines represent the difference potency strength or concentration of the factor assay.

If the test line is to the right of the standard, it contain less of the factors than the standard

If is to the left of the standard, it contain less of the factors than the standard

At least three dilution of the standard and test plasma should be assayed

Assay based on the PT:

Used to assay factors VII, X and V

Assay based on the APTT:

Used to assay factors VIII, IX, XI and XII

Student's findings (measurements or observations):

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comments and interpretation:

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Evaluation (carried out by the instructor):

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