TECHNICAL SERIES

Quality Assurance for routine Hemostasis Laboratory

...Setting trends

Introduction

Coagulation tests for the routine assay parameters in laboratories are fairly simple to perform and master.

The performance of basic tests requires simple apparatus such as water bath, test tubes, pipettes and stop watch and / or automatic clot timer. And it's precisely for this reason that these techniques appear deceptively easy.

There are a number of pre test variables that effect the accuracy and precision of coagulation results. These may relate to collection techniques, processing of samples, selection and preparation of reagents. In order to achieve optimum and reproducible results the impact of variables needs to be understood and controlled so as to reduce variability and errors and improve accuracy and reproducibility. Various variables that impact the results are discussed, along with the basis that leads to such recommendation.

Preparation of patients

Although no special preparation of patients is required prior to approved techniques it is preferable that patients are not heavily exercised before blood collection. Fasting patients or patients on a light non-fatty meal are preferable.

- Patients who are fasting or on a light non-fatty meal prior to blood collection provide samples with desirable lower opacity this improves the sensitivity of clot detection especially when photo optical instruments are being used.

  Turbid, icteric, lipemic or grossly haemolysed samples generate erroneous results due to varying opacity.

Sample Collection techniques

(Phlebotomy)

- Blood should be withdrawn without undue venous stasis and without frothing into a plastic syringe with a short needle of 19 to 20 SWG.
- The venepuncture must be a 'clean' one and incase of difficulty with a new syringe and needle another vein should be tried. The tourniquet should not be placed too tightly or for extended lengths of time. Patting the venepuncture site should also be avoided.
- Distribute blood into test tubes (preferably plastic) after detaching the needle from the syringe. Do not delay mixing blood with anticoagulant by gentle inversion of the tube.
  - 'Clean' venepuncture is essential to avoid formation of microclots at the site of venepuncture and consumption of factors, which will lead to artificially prolonged results.
  - Usage of short bigger bore needle allows free flow of blood within the syringe and reducing blood contact with metal surface. With smaller bore longer needles blood will remain in contact with metal surface for longer time. This will lead to initiation of clotting or partial consumption of factors being assayed leading to erroneous results during test procedures.
  - Frothing when distributing the blood into anticoagulant tube should be avoided because frothing induces microclot formation.

Sample Preparation

- The anticoagulant of choice for most coagulation procedures is sodium citrate or preferably buffered sodium citrate.
  - Sodium citrate is an ideal anticoagulant since FactorV and FactorVII are more stable in citrate. These factors are more labile in sodium oxalate. Heparin neutralizes thrombins action on fibrinogen.
- The recommended molarity of sodium citrate for coagulation studies is 0.109M, which equates to 3.2% of tri-sodium citrate.
- Use of buffered sodium citrate is preferred over plain sodium citrate solution.
  - After collection of blood in citrate during centrifugation for preparation of PPP or PFP the pH of the solution shifts releasing carbondioxide (CO_2). This shift in pH affects the labile Factor V leading to erroneous results during test. Use of appropriately formulated buffered citrate overcomes this phenomenon.
  - When samples are collected in 3.8% citrate (129Mm) the prothrombin time of samples, especially with patients receiving oral anticoagulants give prolonged results. Also the ISI of thromboplastins is lowered. It is for this reason 3.2% citrate is recommended universally instead of 3.8% for increasing accuracy of test results.

- The optimum ratio of citrate to blood is 1 part of anticoagulant to 9 parts of blood.
  - When the molarity of citrate is accurate the anticoagulant supplied in this amount and ratio is sufficient to bind all the available calcium in the collected sample so as to prevent clotting. A shift in this ratio leads to erroneous results as follows:
  - More blood less citrate: The chelating activity of citrate will not be sufficient to bind the calcium present in the sample. This will lead to formation of clots, consumption of factors and subsequent prolongation of results during test.
  - More citrate less blood: Excess citrate remaining in the blood sample would consume the calcium from the reagents thereby giving prolonged results.

- The optimum concentration of Calcium chloride to be used for APTT test should be 0.02M.
  - The concentration of 0.02M $CaCl_2$ replaces the calcium necessary to activate the intrinsic coagulation cascade. This ultimately generates thrombin from prothrombin via the coagulation cascade.
  - Appropriate volumes of $CaCl_2$ should be aspirated for the days work. Prewarmed $CaCl_2$ should always be discarded at the end of the working day.

- The standard ratio of blood to anticoagulant of 9:1 is for normal haematocrit or PCV
  - For occasional patients with PCV less than 20% (e.g.: Microcytic hypochromic anemia) and greater than 55% (e.g.:Polycythemia vera) the anticoagulant to blood ratio must be readjusted using the following formula,

$$C = 1.85 \text{ X } 10\text{-}3 \text{ (100-H) V}$$

C = Volume of sodium citrate in ml.
V = Volume of whole blood - Sodium citrate in ml
H = Hematocrit in percentage

  - When the PCV is higher than 55% the patient blood contains so little plasma that excess unutilized anticoagulant remains and is available to bind reagent calcium leading to prolongation of test results.
  - On the other hand if the PCV is less than 20% the patient blood contains excess of plasma but less of anticoagulant and the chelating activity of citrate will not be sufficient to bind the calcium present in sample. This will lead to formation of clots invitro, consumption of factors and prolongation of results.

Sample Processing and Storage

- Containers for collection and processing of plasma should be ideally made out of plastic or siliconized glass tubes. They should be scrupulously clean and dry.
  - The containers should be ideally made out of plastic and not from glass as scratched glass surfaces can activate invitro the coagulation mechanism within the sample due to contact with silica. While plastic tubes overcome this problem they should be free from leavening chemicals used by the plastic industry during moulding. These chemicals usually have an inhibitory effect. Scrupulous washing and drying overcomes this problem.
  - All the containers used for collection, storage and test should be free from detergents, acids and alkalies. These chemicals have a varying effect on pH. Change in pH affects factor stability. Detergents inhibit reactive characteristics of the Sample/ Reagent mixture.
  - Ideally the cleaning of glassware used in coagulation tests should be the responsibility of one individual and should be handled separately from routine laboratory glassware. Alternatively disposable labware should be used.

- The specimen to be tested for coagulation studies must be used preferably immediately.
  - As most of coagulation factors are time as well as temperature labile it is of utmost importance that they should not be subjected to high temperatures and tests be performed as early as possible, preferably immediately.
  - If specimen are held at 22°C-24°C then they must be tested within 2 hours and if the specimen are held at 2°C-4°C then they must be tested within 3 hours.
  - Plasma samples held at 4°C-8°C for prolonged periods may undergo cold activation leading to erroneous results.
  - Samples obtained for factor assays and tests for fibrinolysis should be stored in crushed ice if a delay in testing is anticipated.
  - Citrated blood for platelet aggregation studies should remain in capped tubes at R.T. (20°C-25°C) before testing

- The samples collected must be stored tightly capped.
  - The samples collected must be stored tightly capped. If the tubes are not capped the samples will absorb atmospheric CO2 leading to shift in pH to an unacceptable range. This hampers factor stability and accuracy of results.

- Centrifugation speed and time are of absolute importance in coagulation studies. The PT test uses PPP while the APTT test uses PFP.
  - Excessive centrifugation may destroy clotting factors due to the heat generated during centrifugation.
  - Under centrifugation would lead to the presence of platelets in the plasma sample which could lead to activation of clotting mechanism invitro which leads to erroneous results.
  - Normally centrifugation for 15 minutes at approximately 1500g yields PPP (platelet poor plasma) and centrifugation at approximately 2000g for 15 minutes yields PFP (platelet free plasma). The 'g' is a function of length of rotor head and RPM. It is for this reason each laboratory must calibrate its own equipment to achieve satisfactory samples depending on test performed and kind of plasma sample required.

Calibration of instruments / equipments

- Waterbaths or heating blocks calibrated and preset at 37°C + 0.5°C are an important requirement to achieve accuracy and reproducibility.
  - The whole process of the coagulation tests is based on a series of enzymatic reactions which are dependent on pH, ionic strength, and the temperature of the reaction process. A correct temperature at 37°C + 0.5°C is critical as most of the reagent systems are standardized at this temperature. Day to day shift in reaction temperature of equipment will introduce uncontrolled variation into test conditions.Therefore temperature of all equipments must be calibrated daily and diligently to avoid erroneous results and ensure accuracy and reproducibility.

- Sample/reagent dispensing mechanisms must be accurate and precise
  - Well-calibrated dispensing mechanisms are required for all coagulation-based tests to accurately dispense samples as well as reagents. Any shift in ratio or individual volumes of the sample and or reagent can lead to shortening or prolongation of results.
  - Straight 0.1 and 0.2ml glass pipettes are usually satisfactory, provided they are scrupulously clean and dry.
  - Automatic micro pipettes which are able to deliver the required volumes are replacing the glass pipettes, provided these pipettes are calibrated frequently. The use of clean disposable tips places this system at an advantage over the older mechanisms.

Storage of Reagents

- Usually reagent manufacturers recommend aspiration of adequate reagent for the days use in a thoroughly clean and dry tube instead of intermittent aspiration from reagent vials at the time of test.
  - Most coagulation reagents are extremely delicate reagents.
    For them to maintain their sensitivity and performance the reagent formulations must maintain reagent integrity over the usage period. Repeated intrusions into the reagent vial exponentially increases the chances of reagent contamination and destruction of reagent formulations and integrity. Undried and or contaminated pipettes, tips, glassware are usually the main culprits. Such contaminated reagents perform suboptimally.
  - The reagent vials must be immediately stored back to the recommended storage temperatures after the aspiration of the days requirement separately so that the remaining reagent remains at optimal temperature for future use. Keeping unused reagents at higher ambient temperatures during the day causes steady deterioration of the reagent due to thermal stress.

- The recommended storage temperature for reagents should be strictly complied to.
  - Most of the liquid stable or reconstituted reagents such as PT and APTT are colloidal suspensions of lipoproteins and or phospholipids. Subjecting them to elevated temperatures through repeated freeze-thaw cycles stresses the colloidal system. Especially detrimental are the effects of freezing (below 2°C). After freezing the reagent colloidal suspension undergoes an irreversible change and precipitates out or presents itself as a particulate mass. Such reagents give erroneous results.

- Bringing reagents/samples to room temperature should be a two step process.
  - When enough reagent is aspirated out for the days testing as recommended the reagent and samples stored at 2-8°C should be first allowed to attain room temperature (25°C-30°C) and then they should be subsequently brought to the optimal test temperature of 37°C + 0.5°C.
  - When reagents/samples from 2-8°C are directly brought to 37°C the required time of 3-5 minutes may not be sufficient for the reagent/samples to attain a homogeneous temperature of 37°C within the recommended time. This affects the reaction kinetics leading to erroneous results.

End Point Reading

- Reading of endpoint of clot based tests varies from user to user.
  - Usually when manual techniques are followed the definition of “end point” is important. Ideally the end point tests should be read “as soon as the first fibrin strand is visible and the gel clot formation begins”.
  - When some users use a fully formed gel clot as an end point, there is a variation of 1-3 seconds between the end points as recorded by the ideal method and user based variation.
  - It is advisable to have well illuminated background for reading the clot based end points. Since user variations based on proficiency continue to influence results, it is advisable not to change personnel involved in coagulation tests off and on.

- Manufacturers instructions must be followed meticulously when instrument based or automatic clot detection systems are used.
  - Each clot detection system works on a different principle such as electro mechanical, turbidimetric or photo-optical. Each system of clot detection has its requirements for optimum functioning. Special care must be taken while using optical instruments for clot detection since reagent induced turbidity can influence the results dramatically. Usually low turbidity reagents are preferred for manual as well as instrument based clot detection.

Drugs/Clinical Conditions influencing patient results

- Drugs/clinical conditions influence results of patients coagulation studies
  - PT tests are influenced on administration of following drugs

PT may be shortened	PT may be Prolonged
DRUGS ● Antihistamines ● Butabarbital ● Phenobarbital ● Caffeine ● Oral contraceptives ● Vitamin K	**DRUGS** ● Corticosteroids ● EDTA ● Asparaginase ● Clofibrate ● Erythromycin ● Ethanol ● Tetracycline ● Aspirin ● Anticoagulants such as warfarin, heparin

- APTT tests are influenced on administration of following drugs

APTT may be shortened	APTT may be Prolonged
DRUGS ● Oral contraceptives ● Conjugated estrogen therapy	**DRUGS** ● Diphenylhydantoin ● Heparin ● Warfarin ● Naloxone ● Radiographic agents

- Thrombin Time Test is prolonged in the following clinical conditions

CLINICAL CONDITIONS	
● Normal newborn infant ● Systemic lupus erythematosus ● Macroglobulinemia ● Presence of exogenous/ endogenous circulating anticoagulants	● Hepatic diseases ● Toxemia of pregnancy ● Multiple myeloma

MNPT and INR

- MNPT is a critical requirement in the derivation of INR
  - MNPT is a critical requirement in the derivation of INR. Ideally each laboratory must derive its own MNPT from 20 or more normal patients for a given PT reagent and Lot under use. This corrects within laboratory test variables that influence PT results.

By definition INR represents the PT ratio which would have been obtained for a particular patient sample as if the WHO reference thromboplastin itself (ISI=1.0) had been used in the PT determination.

$$INR = [R]^{ISI}$$

$$INR = \left[\frac{\text{Patient PT in seconds}}{\text{Mean of the normal range}}\right]^{ISI}$$

A PT ratio is obtained by dividing the patient PT in seconds by the **"Mean of the normal range"** (MNPT). This ratio is then "normalized" by raising the results to the power of the ISI of the PT reagent used.

If "normal control plasmas" are used in place of patient plasma for arriving at the MNPT it can effect the evaluation of the patients level of anti coagulation.

For eg:

Reagent ISI=2.5	Test Day 1	Test Day 2	Test Day 3
Patient PT (sec)	16.0	16.0	16.0
Normal Control (10.4 - 12.3 sec)	11.5	10.4	12.3
INR Formula $[R]^{ISI}$	$\left[\frac{16}{11.5}\right]^{2.5}$	$\left[\frac{16}{10.4}\right]^{2.5}$	$\left[\frac{16}{12.3}\right]^{2.5}$
Resulting INR	2.27	2.89	1.92

If the control time is greater than the mean normal range (MNPT), the PT ratio for any patient PT will be smaller, potentially leading to over coagulation. If the control time is lesser than MNPT the ratio for any patient PT will be greater, leading to under coagulation.

On the other hand MNPT for a particular laboratory using the same combination of methodology, reagent and instrument would remain constant.

Quality Control Aspects

- Quality of water used for reconstituting lyophilized coagulation reagents must be good.
  - The water used for reconstitution of lyophilized coagulation reagents should be atleast distilled twice and kept separately labelled for "coagulation studies". The reagents employed for coagulation studies are extremely delicate and inability to use good quality distilled water could lead to incorporation of metallic impurities in the reagent formulation as well as change in pH.
    Such changes can alter reaction kinetics and overall stability and performance of reagents.

- Quality assurance for coagulation based reagents must be performed preferably on a daily basis.
  - Each laboratory should test coagulation reagents with normal and abnormal control plasma specimens at the beginning of each days work to verify instruments, temperature calibration and also reagent performance.
    If the control results fall within the stated limits, the test results are considered valid.
    But if the results fall outside the stated control limits then the reagents, control and equipments are checked and the problem should be corrected.
    Control results should be recorded and analyzed after regular intervals to ascertain the long term validity of results.

References and Suggested Readings

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