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Determination of Prothrombin time and International Normalized Ratio by Using Manual and Semi automated Methods in Khartoum Teaching Hospital Laboratory

تحديد زمن البروثرومبين ونسبة الضبط العالمية بإستخدام الطريقة اليدوية وشبه الأتوماتيكية بمعمل مستشفى الخرطوم التعليمي

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الآية

قَالَ تَعَالَىٰ:

﴿إِنَّمَآ أَمۡرُهُۥٓ إِذَآ أَرَادَ شَيۡـًٔا أَن يَقُولَ لَهُۥ كُن فَيَكُونُ ﴿٨٢﴾ فَسُبۡحَٰنَ ٱلَّذِي بِيَدِهِۦ مَلَكُوتُ كُلِّ شَيۡءٖ وَإِلَيۡهِ تُرۡجَعُونَ ﴿٨٣﴾﴾

صدق الله العظيم

سورة يس الآية ﴿82-83﴾

DEDICATION

To my beloved mother and father

To my dear husband

To my brothers and sisters

To my all greatly and lovely friends

To my lovely little children's

I dedicate this research

Acknowledgment

Praise be to Allah, the Almighty who support me and gave me strength to complete this work.

I would like to express my deep thanks and gratitude to my supervisor:

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List of Contents

List of Tables

List of Figures

List of Abbreviations

Term	Abbreviation
PT	Prothrombin time
APTT	Activated Partial Thromboplastin Time
INR	International normalized ratio
ISI	International sensitivity index
OAT	Oral anticoagulant therapy
MNPT	Mean normal prothrombintime
IRP	International reference preparation
WHO	World health organization
MTS	Medical test site
CLSI	Clinical laboratory standard institute
VKA	Vitamin k antagonist
HIPAA	Health insurance portability and accountability act

Abstract

This is descriptivecross sectionalstudy carried out in the Khartoum teaching hospital research and laboratory unit to determine the Prothrombin time and International normalized ratio.

The Prothrombin time and International normalized ratio was measured in100 blood samples.4.5ml of venous blood was drown from each patients placed in tri sodium citrate containers then centrifugated to get platelet poor plasma.

PT measured by the manual method and semi automated method (DiagnosticaStago).

The aim of this study to compare the reliability of prothrombin test using manual and semi-automated method coagulation analyzer.

Results were analyzed by Statistical package for social science average ±SD and P.value was obtained.

It has been found that there was no significant variation between PT and INR values when using the manual and semi automated with P.value (≥0.05).

The result for the prothrombin time test obtained by semi automated is reliable and is statistically equivilant to those obtained by the manual method.

The study concluded that there weretwo methods gave the same results indicating good calibrated machine.

الخلاصة

أجريت هذه الدراسة التحليلية الوصفية في مستشفى الخرطوم التعليمي وحدة المعامل والأبحاث ،لتحديد زمن البروثرومبين ونسبة الضبط العالمية.

تم قياس زمن البروثرمبين ونسبة الضبط العالمية في 100 عينة دم. تم أخذ 4.5 من الدم الوريدي من كل مريض تم وضعها في أنابيب إختبار تحتوي على مانع التجلط ثلاثي سترات الصوديوم ،وإستخلص المصل الدموي تم القياس بالطريقتين اليدوية ،والشبه أوتوماتيكية بإستخدام جهاز (ستاقو التشخيصي).

الهدف من هذه الدراسة مقارنة موثوقية قياس زمن البروثرومبين باستخدام الطريقة اليدوية وشبه الآلية،وقد تم تحليل النتائج بواسطة برنامج الحزم الإحصائية للعلوم الاجتماعية ،تم قياس متوسط زمن البروثرومبين ونسبة الضبط العالمية والقيمة الإحتمالية.

وجد أنه لايوجد تفاوت كبير بين قيم زمن البروثرومبين ونسبةالضبط العالميةعند استخدام الطريقة اليدوية وشبه الآلية ،حيث وجد أن القيمة الإحتمالية أكثرمن (0.05).

نتائج زمن البروثرومبين التي حصل عليها بإستخدام الطريقة الشبه أوتوماتيكية يمكن الإعتماد عليها وإحصائيا يعادل النتائج التي حصل عليها بإستخدام الطريقة اليدوية.

أبرمت هذه الدراسة بأن الطريقتين أعطت نفس النتائج وهذا يدل على أن الجهاز مضبوط جيداً.

Chapter one

1. Introduction and Literature review

1.1Introduction

Haemostasis is one of a number of protective processes thathave evolved in order to maintain a stable physiology (victor *etal,* 2011)

The haemostatic mechanisms have several important functions: (a) to maintain blood in a fluid state while it remains circulating within the vascular system; (b) to arrest bleeding at the site of injury or blood loss by formation of a haemostaticplug; and (c) to ensure the eventual removal of the plug when healing is complete. Normal physiology thus constitutes a delicate balance between these conflicting tendencies, and a deficiency or exaggeration of any one may lead to either thrombosis or haemorrhage. There are at least five different components involved: blood vessels, platelets, plasma coagulation factors, their inhibitors, and the fibrin lytic system. (Dacie and Lewis, 2006)

Tests of the hemostatic system are used in a variety of clinicalsituations. Hemostasis assays are vital in the evaluation ofpatients who present with either a bleeding diathesis or aclotting disorder. These assays are also used in the evaluationof patients who are at risk for bleeding or clotting, such asin the preoperative evaluation of, or anticipation of, aninvasive procedure. Coagulation testing is imperative whenmonitoring antithrombotic and factor replacement therapies. Monitoring of anticoagulant therapy is, in fact, one ofthe most common and important indications for hemostasistesting. Inaccuracy in results can lead to excessive over orunder -anticoagulation with a resultant increased risk forbleeding or clotting. It is critical therefore to provide accurate and reliable laboratory results for optimum patient care.Inaccurate or misleading results may lead to inappropriatediagnosis and therapy. (Kandice*et al*, 2012)

Coagulation test methodologies available on current laboratory analyzers vary according to the analyte or process being measured. Routine clot-based assays include the prothrombin test (PT)/International Normalized Ratio (INR), the partial thromboplastintest(PTT), the thrombin time (TT), the activated clotting time (ACT),coagulation factor assays (including

fibrinogen), and lupus anticoagulant tests. Chromogenic assays, tests based on the production of achromophore secondary to the intrinsic enzymatic activity of the analyte (e.g., antithrombin, proteins S and C, factor VIII), or the inhibitionof that activity (e.g., heparin anti-factor Xa activity) are available onmany instruments. Finally, immunologic-based testing is also availableon some platforms (e.g., D-dimer, von Willebrand factor, protein S).Most laboratory analyzers have an open reagent system that allows useof reagents not produced by the instrument manufacturer. However, thevendor may indicate that the system is optimized for their reagents,and therefore will not guarantee results using other vendors' reagents.

Regardless of the source of reagents, each individual reagent-instrument test combination must be validated due to the known variability of test results between reagents – even for different lots of the same reagent,and from analyzer to analyzer – even of the same make and model.(Sterling *etal* ,2007)

Automated and semi-automated coagulation analyzers havereplaced the manual methods to meet the increasing testload. The modern analyzers adopt different technologies tomeasure the prothrombin time. The advantages conferredinclude minimal manual interference, increased precision,reduced manpower and a large number of samples canbe processed to meet the ever-increasing sample load.

ofthe different methods employed in this regard, two distinctmethods exist which are utilized in most laboratories. Theyare based on the principles of photo-optical and mechanical clot detection.(Bai*et al*, 2008)

In the present study we more closely investigated semi automation ofprothrombin time testing based onsemiautomated method(DiagnosticaStago)for detection of clot formation.

1.2 Literature review

1.2.1 Hemostasis

Hemostasis is a dynamic process in which the platelet and the blood vessel wall play key roles. Platelets become activated upon adhesion to von Will brand's factor (VWF)and collagen in the exposed sub endothelium after injury. Platelet activation is also mediated through shear forces imposed by blood flow itself, particularly in areas wherethe vessel wall is diseased, and is also affected by theinflammatory state of the endothelium. The activatedplatelet surface provides the major physiologic site forcoagulation factor activation, which results in furtherplatelet activation and fibrin formation. Genetic andacquired influences on the platelet and vessel wall, as well as on the coagulation and fibrinlyticsystems, determinewhether normal hemostasis, or bleeding or clotting symptoms, will result.(Danl*et al*, 2010)

- **Hemostasis process**

Several processes are involved in hemostasis following injury to a small blood vessel:

1. Blood vessel spasm

2. Formation of a platelet plug

3. Contact among damaged blood vessel, blood platelet, and coagulation proteins

4. Development of a blood clot around the injury

5-Fibrinolytic removal of excess hemostatic material to reestablish vascular integrity (Mary,2012)

- **Primary versus secondary hemostasis**

Coagulation (clotting) is traditionally divided into twosystems: primary hemostasisand secondary hemostasis. This division is artificial, but it helps organize our thinking about hemostasis and corresponds to relatively distinct clinical syndromes. Recognition of bleeding as involving primary or

secondary hemostasis is critical in organizing the diagnostic and therapeutic approach to bleeding disorders. (William, 2002).

- **Primary Hemostasis**

Primary hemostasis primarily involves platelets andvWFand results in the formation of a platelet plug. If the endothelial injury is small, this may be adequate to stop bleeding. However, if the injury is greater, participation by

The coagulation cascade is required.(William, 2002)

- **Secondary Hemostasis**

Secondary hemostasis primarily involves the coagulation cascade proteins, which ultimately results in the conversion of fibrinogen to fibrin; fibrin polymerizes to form a clot. The fibrin clot is cross-linked and stabilized by factorXIIIa. (William , 2002)

1.2.1.1Current Concept of the Coagulation Cascade

In the current concept of the coagulation, thekey initiating step is the exposure of TF to the circulation and reaction of TF with factor VIIa. The TF-factor VIIa complex can enzymatically activate factor X to Xa, factor IX to IXa, and factor XI to XIa. The initial activation of factor X to Xa may be important in getting the coagulation cascade started; however, a specific inhibitor produced by endothelium called tissue factor pathway inhibitor (TFPI) rapidly inactivates the TF-VIIa-Xa complex. Therefore, the major action of the TF-VIIa complex in vivo is the activation of factor IX to IXa, which then activates factor X to Xa. Activation of factor XI to XIa by the TF-VIIa complex appears to play a relatively minor role in the coagulation cascade Activation of factor X to Xa and prothrombin (II) to thrombin (IIa) are key steps in the coagulation cascade since both Xa and thrombin have positive feedback activity on earlier steps of the cascade. Factor Xa activates VIItoVIIa, increasing the amount of VIIa available to complex with TF.Thrombin converts factor V to Va and factor VIII to VIIIa. It also activates factor XI to XIa and XIII to XIIIa. Thrombin is also a potent platelet ago-nist. Factor X is activated by a complex of factor IXa, VIIIa,

phospholipid, and calcium. Prothrombin is activated by a complex of factor Xa, Va, phospholipid, and calcium.

Thrombin cleaves off two small peptides from fibrinogen (fibrinopeptides A and B), converting fibrinogen to fibrin monomer,Fibrin monomer spontaneously polymerizes to form soluble fibrin polymer, this is then covalently cross-linked by factor XIIIa, converting it to a Stable fibrin clot.(William, 2002)

1.2.1.1.1The intrinsic pathway

Coagulation can be activated via the intrinsic pathway, so called because its components, factors XII, XI, IX, VIII, prekallikrein, and high molecular weight kininogen, are all plasma proteins locatedintrinsicallyinside the lumen of the blood vessel. The intrinsic pathway is activated through processcalled contactactivation when factor XII undergoes auto activation to factor XIIa on a negatively charged surface.

Negatively charged surfaces include the artificial reagents in the APTT assay, such as kaolin, celite and silica, which explains the dependence of the APTT on the contact activation factors.(Kandice*et al*, 2012)

1.2.1.1.2 The extrinsic Pathway

The extrinsic pathway (also called the tissue factor pathway) starts with exposureof tissuefactor (TF) to blood. Tissue factor is a transmembraneprotein that is highly expressed in the adventitia of blood vessels, the brain, glomeruli, and other tissues. It is not normally present on endothelial surfaces or blood cells. Exposed tissue factor reacts with trace amounts of factor VIIa, which are normally present in the circulation. The TF-factor VIIacomplex then activates factor X to Xa, starting the common path way .Deficiencies of factor VII result in prolongation of the PT. Deficiencies of factors in the common pathway also result in prolongation of the PT, but thePTT is also prolonged(William, 2002)

1.2.1.2Inhibitors of the plasmatic coagulation system

Human plasma contains a number of anti-proteases that inhibit the activity ofmost of the activated coagulation factors and fibrin lytic enzymes. These inhibitors include anti thrombin (AT), protein C and S, TFPI, and PAI, among others. All belong to the serine protease inhibitors (SERPINs). Their task is to limit thrombosis on the one side and fibrinolysis on the other side.

A defect or decrease of activity of these inhibitors can thus lead to thrombosis or hyper fibrinolysis. (Reinhold *et al*, 2007)

1.2.1.2.1 Antithrombin

This glycoprotein with an MW of 65 kDa is synthesized in the liver, is composed of a single polypeptide chain, and migrates with the α2-globulins. The normal concentration of AT in plasma is in the range of 18 to 30 mg/dL. It is the major inhibitor of thrombin, but it also inhibits the factors XIIa, XIa, Xa, IXa,VIIa, plasmin, and plasma kallikrein.(Reinhold *et al*, 2007)

1.2.1.2.2 Protein C and Protein S

Protein C is the zymogen of a serine protease with an MW of 56kDa and a plasma concentration of about 0.4 mg/dL. Protein S (MW 69 kDa) serves as a co-factor for activated Protein C and like protein C is a vitamin K-dependent protein. Its plasma concentration is about 2.5 mg/dL. Protein S exists in plasma either in afree form or bound to the C4b-binding protein,

Acomponent of the complement system. Only the free form of protein S can serve as a co-factor for activated protein C. When thrombin escapes the localized area of vascular injury, it must be kept from freely circulating in blood. This is accomplished by the up regulation ofthrombomodulin on the cell surface of the vascular endothelium, primarily in the microcirculation. Thrombomodulin binds thrombin, thus switching off theprocoagulant activity of thrombin. Thrombin in this bound form changes its substrate specificity from fibrinogen to protein C.

The thrombomodulin/thrombin-complex activates protein C. Following activation, activated protein C formsa complex with protein S. This complex

degrades factor Va and factor VIIIa bylimited proteolysis, dramatically reducing the local generation of thrombin. Activated protein C also increases fibrinolysis by inactivating PAI-1. (Reinhold *et al*, 2007)

1.2.1.3Tissue Factor

TF is the cofactor for the extrinsic pathway and the physiological initiator of coagulation. It is a lipoprotein that is membrane bound and constitutively present in many tissues outside the vasculature and on the surface of stimulated inflammatory cells such as monocytes and, under some conditions, endothelial cells. Factor VIIa binds to TF in the presence of calcium ions and then becomes enzymatically active. Small amounts of factor VIIa are present in the circulation but have virtually no enzymic activity unless bound to TF. The factor VIIa–TF complex can activate both factor X and factor IX, and therefore two routes to thrombin production are stimulated. Factor Xa subsequently binds to TFPI and then to factor VIIa to form a quaternary (Xa–TF–VIIa–TFPI) complex. This mechanism therefore functions to shut off the extrinsic pathway after an initial stimulus to coagulation has been provided. (Dacie and Lewis,2006)

1.2.1.4The Vitamin K–Dependent Factors

The vitamin K–dependent factors group includes coagulation factors II, VII, IX, and X. However, it is important to remember that the anticoagulant proteins S, C, and Z are also vitamin K-dependent. Each of these proteins contains a number of glutamic acid residues at its amino terminus that are γ-carboxylated by a vitamin K–dependent mechanism. This results in a novel amino acid, γ-carboxyglutamic acid, which is important in promoting a conformational change in the protein that promotes binding of the factor to phospholipid. Because this binding is crucial for coordinating the interaction of the various factors, the proteins produced in the absence of vitamin K (PIVKAs) that are not γ-carboxylated are essentially functionless. The vitamin K–dependent factors are proenzymes or zymogens, which require cleavage sometimes with release of a small peptide (activation peptide) to become functional. Measurement of these activation peptides has been post-production modification of six hemostatic factors. These are II, VII, IX, and

X used as a means of assessing coagulation activation. (Dacie and Lewis, 2006)

1.2.1.4.1The Role of Vitamin K

Vitamin K is essential for procoagulant; and proteins C and S: natural anticoagulant. Without vitamin K, the liver produces inactive molecules called PIVKAs (Proteins Induced by Vitamin K Absence). Developing deficiency or antagonism of vitamin K will affect all these factors, starting with those with the shortest half-life, i.e., protein C, then factor VII, then progressing steadily. Clearly the full effect of deficient vitamin K will only be seen when the production of the factors with the longest half-life is affected. At the same time, levels of PIVKAwill rise progressively. (Normanbeck,2009).

1.2.2History of the PTand the INR scale

The PT test, defined as the coagulation time (seconds)of a mixture of platelet-poor plasma, tissue factor(thromboplastin) and calcium chloride was developed by A.J. Quick in 1935 as amean to investigate the coagulopathy associated with obstructive jaundice.(Quick AJ 1935)Being responsive to the deficiencies of many coagulation factors (VII, X, V, II and fibrinogen), PT became over the years the test of choice to investigate congenital or acquired coagulopathies as well as to monitor the treatment with vitamin K antagonists (VKA). The key component for the PT test is thromboplastinwhich may be extractive or recombinant. The variable reagent composition makes coagulation times dependent on the reagent used for testing, thus making inter-laboratory comparability of results difficult. However, this drawback was not considered detrimental to theuse of the PT at least until the treatment with VKAbecame widely used. (Ansell *et al,* 2004).

Restriction of patients to movefrom one laboratory to another and the difficult adoption of the therapeutic intervals (i.e., optimal level of anticoagulation to prevent thrombosis, while minimizing the haemorrhagic risk) became important limitations. Early attempts at expressing PT results as either percentage activity, or the PT-ratio (patient-to-normal coagulation

time) failed to harmonize results which were still dependent on the thromboplastin/coagulom-eter used for testing. At the beginning of the 1980's a system of calibration was devised (KirkWood,1983)

1.2.2.1 Prothrombin Time

The PT is a clot -based test of the extrinsic and common coagulation pathways, first described by Armand Quick in 1935 (Quick AJ*et al*, 1935).

The PT is sensitive to decreased levels of factorsVII, X, V, II, and fibrinogen. It is commonly used to screen for inherited and acquired coagulation disorders and to monitor vitamin Kantagonist therapy. The PT test is initiated by the addition of thromboplastin, which consists of tissue factor plus phospholipid, with the addition of calcium chloride (CLSI,2008). The time to formation of a fibrin clot is then measured by either an optical or mechanical method. ThePT results are expressed in seconds, with typical values of approximately 10–13 seconds. All PTreagents containthromboplastin and calcium chloride, but vary in their phospholipid source and the concentration and type of tissuefactor. Many thromboplastins are extracted from biologic sources, such as rabbit brain or human placenta, while others are derived from a recombinant human tissue factor source. The sensitivity of the reagents to factor deficiencies varies as reflected by the international sensitivity index (ISI).(Poller L,2004). The ISI is a measure of the sensitivity of a reagent and isameasure of a reagent's responsiveness to depressed functional levels of vitamin K -dependent coagulation factors compared to the primary World Health Organization (WHO)international reference preparation (IRP). Reagents with anISI near 1.0 are similar to the WHO IRP and are considered sensitive reagents. Reagents with higher ISIs are considered less sensitive (Krishnan J, 2008). The PT is most sensitive to factor VII deficiency and least sensitive to factor II (prothrombin) and fibrinogen deficiency. Different thromboplastin reagents may vary in their sensitivity to warfarin, in the assessment of liver disease severity, and in the detection of mild factor deficiencies.(Smith SA,2004)Some commercial PT reagents include a heparin neutralizing substance, such as polybrene, in the reagent to neutralize any effect of heparin on the PT. In reagents that do not contain a heparin neutralizing agent, the PT will be progressively prolonged

at higher heparin concentrations.PT reagents are typically insensitive to lupus anticoagulants due to the high phospholipid concentration, but this varies between reagent preparations(Tripodi*et al*, 2001).

The PT result is reported in seconds and/or as the international normalized ratio (INR).

A typical reference range for the PTis 11 to 13 seconds. Each laboratory should determine its own reference range for its specific instruments and reagents.

Some people believe that because we use the PT to follow warfarin therapy and the PTT to follow heparin therapy, the PT is not affected byheparin and the PTT is not affected by warfarin. That is incorrect. ThePTT is more sensitive to heparin than the PT, but the PT is also affected by heparin. The PTT is also affected by warfarin, but the PT changes first. (William, 2002).

The prothrombin time (PT) is commonly measured by either the "Quick method", which is based on the technique described by Quick and co workers in 1935(Quick *et al*, 1935),or by the Owren method (Owren, 1959)(combined thromboplastinreagent). The latter is the predominant approach used in the Nordic countries, Benelux, and Japan.(Hirsh*et al*,1998), more attention is paid to the thromboplastin in the reagent and to its origin (Hermans*et al,* 1994).

The Owrenmethod is sensitive to coagulation factors II,VII, and X. Fibrinogen (factor I) and factor V are in thereagent. The Quick method is affected by deficiencies offibrinogen and factors II, V, VII, and X. Both methods aresuitable for the control of anticoagulant treatment. Themost important technical difference between the two isthe sample volume in the reaction mixture: 5% in theOwren method and 33% in the Quick method. This hasthe effect of making the coagulation reaction in the Quickmethod more sensitive to many preanalytical variables,e.g., citrate concentration in the sample(Duncan EM *et al*, 1999). The small sample volume in the reaction mixture with the Owrenmethod makes it possible to use even EDTA plasma(Horsti,2000).

- **Clinical use of the prothrombin time**

The PT is a useful screening test to detect a deficiency or inhibitor that involves one or more of the clotting factors of the extrinsic or common pathways of the coagulation cascade, namely factors VII, X, V, II, or fibrinogen (Krishnan J, 2008).Apart from hereditary deficiencies, an abnormal PT may also be due to acquired causes such as liver disease, vitamin K deficiency, or specific factor inhibitor. An isolated elevated PT/INR is most indicative of deficiency of factor VII. Deficiencies that occur inthe common pathway factors, namely fibrinogen, factorsII, V, and X.However, mild deficiencies of the common pathway factors may manifest with an isolated elevated PT/INR. This is often observed in patients with mild liver disease or non -overt disseminated intravascular coagulation (DIC)The PT/INR is useful in monitoring oral anticoagulant therapy and thereby maintaining a patient within a safe therapeutic range because of its sensitivity to the decreases in factors II,VII, and X that are typically observed with warfarin. The therapeutic range for most indications is a target INR of 2.5(range 2.0 –3.0), but higher ranges may be required for patients with a higher risk of thrombosis, such as those with mechanical heart valves (Ansell*et al*, 2008).

1.2.2.2 PT method and modification

1.2.2.2.1The Quick prothrombintime

Armand Quick (1894-1978) was a pioneer in developing a modernprothrombin time method (Quick 1935, Quick 1937).The Quickmethodis clearly the most widely used PT principle in world today and most studies reported in the literature have used this method .since the Owren method coverperhaps only about 10%of the market .The Quick method measure Fibrinogen and factorsII, V, VII and X

In Quickmethod 50µL plasma is added to100µLreagent and the coagulation timeis measured .The test can be done by manual method or automated instrument .sample seconds are converted to INR unit. (Juha,2002)

1.2.2.2.2The Owrenprothrombin time

Paul Owren (1905-1990) studied factor V (Owren1944-Owren1947) and began to develop athrombotest (combined thromboplastin reagent) making long series of experiment with OAT patients.He attempted to overcomethe drawbacks of the Quick method (Owren and Aas 1951,Owren 1959).The Owren method is predominantin the Nordic countries, Benelux andJapan ,but is also used on lesser scale in other countries .This method measures coagulation FactorII, VII and Xbovine plasma (adsorbed with BaSO4) is used as source to add fibrinogen (Factor I)and Factor V to the thromboplastin reagent .in the Owren method 10of plasma and 50 of diluents are added to 140 of reagent ,where after the coagulation time is measured.Sample seconds are converted to INR unit. The normal plasma coagulation time is about 20second, which is twice as long as Quick normal coagulation time.(Juha,2002)

1.2.2.2.3Point-of-care prothrombin time testing

Measurementbased on point-of-care testing (POCT) of unmeasured finger-prick whole blood samples have recently become available. The use of these POCT whole-blood PT monitors in the community or in-home PT testing would allow the expansion of anticoagulant services to meet, within the existing resources, increased demand, estimated to be 10% per year (Tripodi*et al*, 2001). Control of treatment in the community distant from hospital would also provide greater convenience to patients (Ansell *et al*, 2001)Average success in the hospital clinic, expressed as the percentage of time in target INR range (Rosendaal*et al,* 1993).

1.2.2.3Various laboratory methods for performing the PT

1.2.2.3.1Clot-based coagulation testing principles

During the coagulation process the clear, liquid plasma becomesa turbid gel due to the formation of a fibrin polymer. The end point for clot -based assays, such as the PT, is reached at the formation of the fibrin clot. This can be detected optically, by change in turbidity, or mechanically, because of the

viscosity changes that occur as the liquid plasma becomesagel.Clot detection can be determined manually or by semi -automated or automated methods. (Chandler ,2009).

1.2.2.3.2Manual clotting techniques

The basic manual tube tilt technique for clot detection that relies on observing for decrease in movement of the test mixture (mechanical) or increasing turbidity (KitchenS, 2000)can be accurate and reliable in well-trained hands. To ensure quality, all tests should be performed induplicate and with both normal and abnormal plasmaas controls. Essentially, these tests only require very inexpensiveequipment's such as a circulating water bath, pipettes, stopwatch, thermometer, centrifuge, weighing balance, refrigerator and pH meter which are obtained quite easily anywhere in the world. But, unstable power supply and improper maintenance an lead to instrument malfunction and poor quality.(Mammen*et al,*2007).

The original method for clot -based coagulation testing is atilt tube method, where the operator pipettes the plasma and reagents into a clear test tube, starts a timer, and tilts the tube back and forthuntil a clot is formed, at which point the timer is stopped.While infrequently performed today, it is still considered thegoldstandardfor instrument comparison.(Kandice*et al*, 2012).

1.2.2.3.3Automated Coagulation Analyzers

A wide variety of automated and semi-automated coagulation analyses are available. The choice of analyzer depends on predicted workload, repertoire, and cost implications. A thorough evaluation of the current range of analyzers is recommended. This is aided by reports of instrument evaluation, e.g., from the National Health Services Medical Devices Agency. If coagulation analyzers are used, it is important to ensure that their temperature control and the mechanism for detecting the end-point are functioning properly. Although such instruments reduce observer error when a large number of samples are tested, it is important to apply stringent quality control at all times to ensure accuracy and precision (DacieandLewis, 2006).

Laboratories can choose from manual, semi-automated or fully automated, moderate or high-throughput analyzers with narrow or broad assay menus depending upon clinical and test volume requirements. These laboratory-based instruments require plasma prepared from spun, anti-coagulated whole blood for analysis. In addition, a number of point of care (POC) devices designed to analyze fresh whole blood are available for use on hospital patient care units, in clinics and doctors' offices, and even in patients' homes. (Sterling *et al*,2007).

1.2.2.4 Detection Methods

A key factor in assessing the clinical utility of a coagulation analyzer is a consideration of the detection modes available. Detection methods in laboratory instruments can be classified into two general categories: photo-optical and electro-mechanical. Clot-based, chromogenic and immunologic assays may all utilize a photo-optical detection method, while electro-mechanical detection of clot formation is obviously limited to clot-based assays. Photo-optical detection of clot formation involves measurement of light that has emanated from a source (generally monochromatic) and has passed through, been absorbed by or scattered from a reaction vessel containing a mixture of reagent, patient sample and fibrin clot. In the case of the PT, PTT, TT and ACT tests, the time required to reach a pre-defined, optical endpoint determines the clotting time. Any factor, other than fibrin clot, that diminishes the relative amount of light passing through the test mixture can adversely affect the result. This includes interfering colored substances (e.g., hemoglobin, bilirubin) that may absorb light at defined frequencies, or suspended particles such as lipoproteins that scatter light. The same substances may also interfere with chromogenic assays that use spectrophotometric detection, as well as immunologic methods that employ turbidimetry.(Sterling *et al*, 2007).

1.2.2.5Assay Monitoring and End-Point Detection

Detecting clot formation as the end-point depends to some extent on the rate of its formation: the shorter the clotting time the more opaque is the clot and

the easier it is to detect. A slowly forming clot may appear as mere fibrin wisps, which are difficult to detect by eye or machine.

In manual work, the observer must try to adopt a uniform convention in selecting the moment in clot formation that will be accepted as the end-point. It is also important to ensure that the tube can be watched with its lower part under the water or while being quickly dipped in and out so as to avoid cooling and a slowing down of the clot formation. Bubbles also make the determination of the end-point difficult Manual clotting techniques are still used in WHO calibration schemes and therefore should be viewed as an essential skill despite the ever-increasing reliance on automation. It is worth remembering that not all results produced by an automated analyser are correct and sometimes they may be spurious; dubious or inconsistent results should be checked manually(Dacie and Lewis, 2006) .

In instrumental work the coagulometer must be shown to detect long clotting times reliably and reproducibly. The various coagulometers available have different means of detecting the end-point, which may make comparison of results difficult. Some commonly used techniques are as follows.

1.2.2.5.1Electromechanical

- Impedance, Steel Ball (e.g., Amelung KC10)

The sample cuvette rotates and a steel ball remains stationary in a magnetic field until the formation of fibrin strands around the ball produces movement. This is detected by a change in the magnetic field, and the coagulation time is recorded.

- Impedance, Steel Ball (NycomedThrombotrack)

A steel ball rotates under the influence of a magnet until the formation of fibrin strands around the ball stops it rotating. This is detected by a sensor, and the coagulation time is recorded.

1.2.2.5.2Photo Optical

- Scattered Light Detection for Clotting Assays (660 nm)

The turbidity during the formation of a fibrin clot is measured as an increase in scattered light intensity when exposed to light at a wavelength of 660 nm.

Transmitted Light Detection for Chromogenic Assays (405 nm, 575 nm, 800 nm)

Colour production leads to a change in light absorbance, which is detected as a change in transmitted light. Over time the change in absorbance per minute is calculated (Δ OD/min). Various wavelengths can be used such as 405 nm, 575 nm, and 800 nm.

- Transmitted Light Detection for Immunoassays (405 nm, 575 nm, 800 nm)

The change in light absorbance caused by the antigen antibody reaction is detected as the change in transmitted light. Over time the change in absorbance per minute is calculated (Δ OD/min).

- Waveform Analysis (Biomerieux)

Detection of light transmittance with Tungsten-Halogen light is performed at 35 wavelengths between 395 and 710 nm. Waveform analysis provides additional information from routine clotting assays. For example, the biphasic waveform A2 Flag is associated with sepsis and disseminated intravascular coagulation.

- Nephelometry (IL ACL Analysers)

Nephelometry is the determination of the intensity of light scatter using a detector placed at right angles to the incident light path but of the same wavelength as the incident light. The procedure is particularly useful in measuring complexes of antigen and antibody produced by immune precipitation.

1.2.2.5.3Electrochemical

- INRatio Meter (Hemosense) Near Patient Testing Devices

The INRatio single-use test strip is made of laminated layers of transparent plastic. Each test strip has a sample well where blood is applied, three channels through which the blood sample flows to reach the testing areas, reagents to start the coagulation process, and electrodes that interface with the INRatio meter. The device detects a change in electrical resistance when blood clots. (Dacieand Lewis, 2006).

Collection of Coagulation Specimens, lipemic, hemolyzed or icteric specimens may interfere with assays employing photo-optical detection methods. The simplest approach to dealing with samples containing interfering substances is to re-collect the specimen to avoid the interference (e.g., avoid hemolysis, avoid post-prandial lipemia). However, this may be either impractical (e.g., patient availability) or impossible (e.g., hyperlipidemic or hemolytic disease). Ultracentrifugation of samples to remove lipoprotein particles is a common approach to dealing with lipidemia. Use of a blank measurement to zero out the interference, measurement at a less susceptible wavelength, or measurement at multiple wavelengths are other potential approaches to modify photo-optical methods to compensate for interferences. An alternative approach is to use a mechanical clot detection system. Mechanical detection methods employed in automated laboratory analyzers detect clot formation through changes in viscosity (movement of a metal ball), and are generally not considered to be susceptible to interference from colored solutes or suspended particles. However, it is worth noting that large concentrations (>3g/dL) of dissolved hemoglobin, in the form of hemoglobin-based oxygen carriers, can cause artificial prolongation of clotting times when employing amechanical-based detection method. In addition, high concentrations offibrin degradation products act to impede fibrin strand polymerization,and may result in falsely low fibrinogen measurements.(Sterling *et al*, 2007).

1.2.2.6Sampling

1.2.2.6.1Anticoagulant type and concentration

Sodium citrate is the anticoagulant of choice for most hemostasis testing it is the type of anticoagulant found in a light -blue stopper tube. The World

Health Organization (WHO) and Clinical Laboratory Standards Institute (CLSI) recommend 105 –109 mmol/L, 3.13–3.2% (commonly described as 3.2%), of the dihydrateform of trisodium citrate (Na 3C6H5O7 • 2H 2O), buffered ornonbuffered, rather than 129 mmol/L, 3.8% (commonly described as 3.8%), although either is acceptable for hemostasis testing (WHO1983).Other anticoagulant containing evacuated tubes such as ethylenediaminetetra acetic acid (EDTA) or heparin tubesare not acceptable for hemostasis testing. Samples collected in purple (EDTA) or green (heparin) stopper tubes will lead to aberrant results in many hemostasis assays Serum samples are not acceptable for many hemostasis assays, especially those that are clot based. Serum samples subjected to APTT and PT testing yield markedly prolonged results.(Kandice*et al*, 2012).

Other anticoagulants, including oxalate isunacceptable the labile factors (factors V and VIII) are unstable in oxalate, whereas heparin and EDTA directly inhibit the coagulation process and interfere with end-point determinations. Additional benefits of trisodium citrate are that the calcium ion is neutralized more rapidly in citrate, and APTT tests are more sensitive to the presence of heparin.

For routine blood coagulation testing, 9 volumes of blood are added to 1 volume of anticoagulant (i.e., 0.55 ml of anticoagulant for a 5 ml specimen). When the haematocrit is abnormal with either severe anemia or polycythaemia, the blood: citrate ratio should be adjusted. For a 5 ml specimen, the amount of citrate should be as follows in the table below on the next page.(Dacie and Lewis, 2006).

The amount of citrate according to hematocrit

Haematocrit	*Citrate (ml)*
0.20	0.70
0.25	0.65
0.30	0.61
0.55	0.39
0.60	0.36
0.65	0.31
0.70	0.27

The Clinical and Laboratory Standards Institute (CLSI) has defined high hematocrit as being greater than 0.55L/L (55%) In this case, an alteration in the blood-to-anticoagulant ratio in the specimen is required to maintain a suitable citrate concentration in the plasma. One convenient approach is to remove a specified volume of citrate from a coagulation tube, then fill the tube with blood to the usual fill volume. The volume to remove, as a function of hematocrit and tube size, For example, if a 3.0mL tube (0.3mL citrate and 2.7mL blood) is to be used and the patient has a hematocrit of 60%, first remove 0.08mL citrate, leaving 0.22mL citrate in the tube. Add enough blood to bring the total volume of blood and citrate to 3.0mL (i.e., add 2.78mL blood).It should be noted that CLSI's recommended hematocrit threshold of 0.55L/L was based on indirect data1; however, Marlar and colleagues validated this threshold for their laboratory methods by directly comparing paired specimens with adjusted and unadjusted citrateconcentration8. The effect was more clinically significant with PT andPTT tests than with fibrinogen, factor VIII, protein C activity, or protein C antigen tests. Given the difficulty, expense, and time delays involved in the preparation and use of citrate-reduced tubes, laboratories with a large number of high-hematocrit patients may wish to consider conducting a study to see whether a hematocrit threshold higher than0.55 L/L can be validated for the laboratory's test methods. (Sterling *et al*, 2007).

1.2.2.6.2 Thromboplastin

Thromboplastins were originally tissue extracts obtained from different species and different organs containing tissue factor and phospholipid. Because of the potential hazard of viral and other infections from handling human brain, it should no longer be used as a source of thromboplastin. The majority of animal thromboplastins now in use are extracts of rabbit brain or lung.

The introduction of recombinant thromboplastins has resulted in a move away from rabbit brain thromboplastin. They are manufactured using recombinant human tissue factor produced in *Escherichia coli* and synthetic phospholipids, which do not contain any other clotting factors such as prothrombin, factor VII, and factor X. Therefore they are highly sensitive to factor deficiencies and oral anticoagulant–treated patient plasma samples and have an International Sensitivity Index (ISI) close to 1.

Each preparation has a different sensitivity to clotting factor deficiencies and defects, in particular the defect induced by oral anticoagulants. For control of oral anticoagulation a preparation calibrated against the International Reference Thromboplastin should be used; calibrated commercially available thromboplastin has its ISI determined and clearly labeled. It is important to remember that some thromboplastins are not sensitive to an isolated factor VII deficiency and that use of animal thromboplastin for analysis of human samples may produce abnormalities solely as a result of species differences. If the manufacturer does not state in the accompanying literature that the reagent is sensitive to factor VII deficiency, it is advisable to check whether it is capable of detecting this deficiency by performing a PT on known factor VII deficient plasma. (Dacie and Lewis, 2006)

- **New Thromboplastins**

The new types of thromboplastins for measuring the PTare mixtures of phospholipids and recombinantlyderived human tissue factor. Because the new thromboplastinsare more sensitive (typical ISI, 1.0) than the traditional North American ones (ISIs, 1.8 to 3.0), the PTs for patients with inherited or

acquired deficiencies of coagulation factors will be much more prolonged with use of the new reagents,although normal values may change minimally. However, the therapeutic range (in seconds) of the PTs in patients receiving orally administered anticoagulant agents is wider with the sensitive thromboplastins than with the traditional ones. The INR, however, will be the same, as will the recommended ranges of the INR for intensity of anticoagulation.

Recombinant thromboplastin has the following advantages:

1. It is made from a human protein, not from the protein of a different species.

2. The material is pure, and the concentration can be readily, adjusted, unlike currently available rabbit brain thromboplastins. Adjustment will minimize variation between different lots of the reagent; thus, the normal and therapeutic ranges of the PT will remain the same.

3. The reagent is free of contamination with noxious viruses because it is a recombinant product.

4. When the ISI is approximately 1.0, the PTs will be the same as those obtained with use of the World Health organization reference thromboplastin. Therefore, the PT ratio (PT of patient/mean normal PT) will be the same as the INR.

5. The new reagents are more sensitive to mild deficiencies of coagulation factors than are the traditional thromboplastins. Patients with hemostatically adequate levels of coagulation factors II, V, VII, or X (30% to 40% of mean normal activity) will have INRs of 1.4 or less (Mary,2012).

1.2.2.6.3 Sampling

1.2.2.6.3.1 Sample collection

1. The sample should be collected as per standard guidelines.

2. The sample should preferably be collected near the laboratory to ensure quick transport.

3. Samples should be tested within four hours of collection.

4. Results of tests can change according to the sample storage conditions. Higher temperatures (>25°C)lead to loss of FVIII activity over time, whereas samplestorage in the cold (2-8°C) leads to cold activation. The sample should therefore be maintained at temperatures between 20°C and 25°Cwhere possible, but for no more than four hours.

5. Venipuncture must be clean and the sample collected within one minute of tourniquet application without prolonged venous stasis.

6. Blood should be withdrawn into a plastic syringeor an evacuated collection system. The needle should be 19-21 gauges for adults and 22-23 gauges for small children. Collection through peripheral venous catheters or non-heparinized central venous catheters can be successful for many tests of hemostasis.

7. Blood from an indwelling catheter should be avoided for coagulation tests.

8. Frothing of the blood sample should also be avoided. It is often useful to discard the first 2 mlof blood collected.

9. The sample should be collected in citrate tubes containing 0.105M–0.109M (c3.2%) aqueoustrisodium citrate dihydrate, maintaining theproportion of blood to citrate as 9:1. If the tube contains less than 80% of the target volume, results may be adversely affected. The higherstrength concentration of 3.8% trisodiumcitrate is no longer recommended.

10. Prompt and adequate mixing with citrate solution should be done by gentle inversion. (CLSI 2008)

1.2.2.6.3.2 Preparation of Platelet Poor Plasma

Most routine coagulation investigations are performed on platelet poor plasma (PPP), which is prepared by centrifugation at 2000 g for 15 min at

4°C (approximately 4000 rev/min in a standard bench cooling centrifuge). The sample should be kept at room temperature if it is to be used for PT tests, lupus anticoagulant (LAC), or factor VII assays, and it should be kept at 4°C for other assays; the testing should preferably be completed within 2 hours of collection. Care must be taken not to disturb the buffy coat layer when removing the PPP.

Samples for platelet function testing, LAC, and the activated PC resistance (APCR) test should not be centrifuged at 4°C. These samples should be prepared by centrifugation at room temperature to prevent activation of platelets and release of platelet contents such as phospholipid and factor V. For LAC testing and APCR it is very important that the number of platelets and platelet debris in the samples is minimised. The platelet count should be below $10^4/\mu l$. This is best achieved by double centrifugation or filtration of the plasma through a 0.2 μm filter.(Dacie and Lewis, 2006).

1.2.2.6.3.3 Storage of Plasma and Sample Thawing

Some tests such as the PT and APTT are carried out on fresh samples. Certain coagulation assays, unless urgently required, can be performed in batches at a later date on deep frozen plasma. Storage of small aliquots of samples in liquid nitrogen (–196°C) is the optimum, although samples may be frozen at -40°C or -80°C for several weeks without significant loss of most haemostatic activities. Gentle but thorough mixing of samples is essential after thawing and before testing. Once thawed the sample should never be refrozen. (Dacie and Lewis, 2006)

1.2.2.6.4 The preanalytical phase of test

The pre-analytical phase of testing is critical as sample integrity may be seriously and irreversibly impaired during this time. Attention to sample conditions is crucial in order to provide the highest quality hemostasis laboratory results Deviations from published guidelines may significantly impact sample integrity, leading to thepotential for patientmisdiagnosis and mismanagement. Unless local validation to challenge published procedures is per-formed, guidelines for proper sample collection, handling, transport,

and storage should be made widely available and strictly followed.(Kandice, 2012).

Specimen Collection variables that affect coagulation testing: SpecimenLabeling: The Clinical Laboratory Standards Institute (CLSI) recommends that specimens be collected, labeled, and stored in a manner that respects patient privacy in accordance with HIPAA. Positively identify the patient at the time of collection. Label the specimens in the patient's presence after the blood is drawn. Include on the label the patient's full name, a unique identifier, date and time of collection, and any other information required by your regulatory agency and your facility. Specimen tubes and devices: Use and Proper filling of tubes: It is critical that 3.2 percent citrated tubes are used and filled properly to maintain a ratio of nine parts blood to one part citrate (9:1). Under filled tubes will contain an excess of anticoagulant, causing erroneous testing results. It is never acceptable to pour partially filled tubes together tomake one full tube as this tube will contain too much anticoagulant.

Reject Specimens for the following reasons:

- •Clotted specimens.
- •Specimens with the wrong anticoagulant.
- •Under-filled tubes.
- •Over-filled tubes.
- •Mislabeled or unlabeled specimens.

Specimen Handling/Centrifugation: Check the blood specimen for gross clot formation prior to centrifugation. Centrifugation: Review the operator's manual for the coagulation analyzer and the reagent package insert to determine the optimal speed and time to process specimens. TheCLSI recommends that the capped specimen tube be centrifuged for sufficient time and speed (10 minutes at 1500g at room temperature) to consistently create platelet-poor plasma, since the presence of platelets in the specimen can shorten clotting times. Centrifuges such as "Stat spin", which spin at

higher rates and shorter duration, are acceptable.CLSI defines platelet poor plasma as plasma with a platelet count of less than 10,000/μL. This is crucial for specimens thatwill be frozen. However, for fresh plasma samples tested within 24 hours for PT or 4 hours for APTT, the samples are not affected by platelet counts as high as 200,000 /μL.In order to determine if the centrifuge time and speed can attain platelet poor plasma, centrifuge the specimen for the determined amount of time and then run the plasma portion of the sample through the hematology analyzer to determine theplatelet count. If the platelet count is higher than 10,000/μL, the sample should be centrifuged for a longer period of time.Once the laboratory establishes the optimum time and speed to process the specimen, a periodic check should be performedto ensure that the platelet count is still acceptable.

Specimen Storage: Specimens for PT testing may be stored at room temperature for up to 24 hours, provided that the collection tube remains unopened. If testing cannot be performed within this time period, the platelet-poor plasma should be frozen.SpecimensNew lot comparison studies: Laboratories must do comparison studies before switching to a new lot of PT reagent or changing methodology to confirm accuracy of the assigned ISI value. With each new lot number of PT reagent, there are certain CLIA requirements that must be met including:

•establish a new normal patient mean.

•Program the correct ISI (International Sensitivity Index) into the coagulation analyzer.

•comparison between the new and old lot numbers of PT reagent; anddocument the manual check of the INR calculation policies and Procedures: Review your laboratory's policies and procedures for performing coagulation testing and verifythat the specimen collection policy is up to date including specimen labeling, storage, preservation, processing, and rejectioncriteria.If the laboratory is using the coagulation analyzer operator's manual as the procedure, it must be signed and approved by thelaboratory director. The operator's guide must include specific quality control policies, calibration policies, and the laboratory's system for entering

patient results. If the operator's guide does not contain all of the necessary information, it is thelaboratory's responsibility to include this information in their procedures.Quality Control: The MTS/CLIA regulations state, "For all non manual coagulation test systems, the lab must include twolevels of control material each eight hours of operation and each time a reagent is changed."Test Requests-Standing Orders: Many patients on oral anticoagulant therapy have standing orders from their physiciansfor PT/INR testing. The laboratory should have a written policy clearly defining the use of standing orders, describing which tests may be covered by standing orders, and at what intervals standing orders should be reconfirmed with the physician.(Leonard,2013).

1.2.2.6.5 Unit in prothrombin time measurement

1.2.2.6.5.1Time (seconds)

Modern hand books of clinical chemistry give PT reference value only in seconds. Depending on the thromboplastinreagent, value of 10-13s(Henry, 2001)and 10-12s(recombinant thromboplastins)as well as 11-16s(Lewis, 2001) have been presented .These reference intervals for the quick method; when coagulation time are longer,since the relationship between sample and reagent is different. Thecorresponding reference interval for Owren reagent is 17.4-22.6s Using a second as unit there is no need for calibration may cause variation and errors in results .The disadvantage is that PTcoagulation time is vary accordingto thereagent source and from batch to batch.(Juha,2002).

1.2.2.6.5.2 Ratio and Percentage

Since the second unit is highly dependent on reagent PT unit, ratio(R) and Percentage (%) unit were introduce in clinicalpractice in order to reduce difference between different reagents and to harmonize the therapeutic range for OAT. Biggs and Denson (1967)proposed standardization towardsan international reference thromboplastin and the ratio method was accepted by WHO

(1977).Due to variability in results between reagents,however a revised ratio method was accepted by WHO (1983). (Juha, 2002).

1.2.2.6.5.3 INR

A revised international procedure for standardization of the prothrombin time (PT) in anticoagulant controlwas accepted by the international committees (Loeliger and Lewis, 1982)andapproved by the World Health Organization. (WHO, 1999). It isbased on reporting PT results as international normalized ratios (INR) derived from an international sensitivity (Poller*et al*, 1988).

The INR is the PT-ratio that would have been obtained had the patient's plasma been tested with the international standard instead of the working system. (Vanden*et al*, 1999).

Thisconcept of calibration leads to important considerations that apply to the international normalized ratio (INR) scale in expressing prothrombintime (PT) results:

1. International sensitivity index (ISI) values for working PT measuring systems are interrelated because they are determined against the same World Health Organization (WHO) international standard. As a consequence the INR measured with calibrated working systems minimizes system-related differences.

2. An ISI close to unity means that the working PT systems have the same responsiveness to the defect induced by vitamin K antagonists (VKA) as the international standard, whereas an ISI higher than one means a lower responsiveness.

3. It is important to realize that the calibration model is an approximate one and is intended only to minimize system-related differences, not to abolish them.

4. An ISI close to unity means that the working PT systems have the same responsiveness to the defect induced by vitamin K antagonists (VKA) as the

international standard, whereas an ISI higher than one means a lower responsiveness.

5. The ISI and the conversion of PT results into the INR scale are valid only for patients on VKA. This is because PTsfrom patients on VKA are inserted in the calibration plot (Tripodi*et al,*2003).

The INR has two major advantages: it allows comparison between results obtained from different laboratories, and it allows investigators to standardize anticoagulant therapy in clinical trials and scientific publications. (Reinhold *et al*, 2007).

Reliable determination of the International Normalized Ratio (INR) is mandatory for the control of oral anticoagulant therapy. Determination of the INR is based on a calibration model adopted by the WHO (WHO,1999) In a recent paper, Attermannargued that inaccuracy of the INR is due to faulty assumptions of the calibration model (Attermann J,2003). It should be realized that other factors are likely to influence INR reliability far more than faults with the established statistical method in the WHO model; the international sensitivity index (ISI) plays a central role. The ISI of the first international reference preparation (IRP) 67/40 is 1.0 by definition. Attermannarguedthat the ISI of all other PT systems, including all secondaryinternational standards, are not known but merely are estimated with inbuilt statistical error.

In the WHO guidelines,INRisdefinedasfollows: Forgiven plasma or whole blood specimen from a patient on long-term oral anticoagulant therapy, a value calculated from the prothrombin-time ratio using a prothrombin-time system with a known ISI according to the formula INR =(PT/MNPT) ISI.

IN therefore is not exact but an approximation that is sufficiently reliable in clinical terms. The above definition of INR is identical to the definition given by Kirkwood (Kirkwood,1983).

For monitoring oral anticoagulant control most routine coagulation laboratories in the United Kingdom now use an automated technique for the determination of the prothrombin time (PT). Results are reported as International NormalizedRatios (INR). (WHO, 1983).The desired INR for treatment of deep vein thrombosis is 2 to 3. For a patient with a thrombogenic mechanical heart valve, a higher INR is required (2.5 to 3.5 or 3 to 4.5).(William, 2002).

1.2.2.6.5.4 International sensitivity Index

This figure is provided by the manufacturer of the thromboplastin reagent. To obtain theINR, calculate the prothrombin ratio, log the ratio, multiplyby the ISI, and antilog the result. (Monica Cheesbrough 2006). At the beginning of the 1980's asystem of calibration was devised (Kirk Wood,1983)and endorsed bythe World Health Organization. (WHO,1983).

Accordingly,commercial PT systems (defined as the combination ofthromboplastin/coagulometer) are now calibrated bytheir manufacturers against one of the international standards for thromboplastin held by WHO. The system calls for the measurement of PT for plasmas from healthy subjects and patients stabilized on VKA. Paired values obtained with the system to be calibrated (working PT) and with the international standard are then plotted on a log-scale and the best-fit orthogonal regression line is drawn through the data points

The slope of the line, called international sensitivity index (ISI), is a measure of the responsiveness of the system to be calibrated relatively to the internationalstandard. The ISI can in turn be used to convert PT results obtained with any calibrated working system into the international scale called INR(.(Vanden*et al*, 1999).assay of coagulation factor deficiencies depends on the type of thromboplastinused because each thromboplastin has a different sensitivity. Therefore, the World Health Organization has proposed that thromboplastins be calibrated against an international reference preparation to derive an international sensivityindex (ISI).(Reinhold *et al*, 2007).

1.2.2.6.6 Calibration and Quality Control

- **Reference Standard (Calibrator)**

International (World Health Organization, or WHO) and national standards are available for a number of coagulation factors for diagnostic tests it is necessary to have a calibrated normal reference preparation tested alongside the patients' plasmas.

Because the concentration of some coagulation factors may vary as much as fourfold in different normal plasma samples, it is inadvisable to use plasma from any one person as representing 100% clotting activity. The larger the number of donors in the pool, the more likely the pool clotting activity will be 100% or 1.0 u/ml. A suggested minimum for the normal pool is 20 donors. It is preferable to use calibrated reference plasma for routine use with each assay. If this is not possible, then a locally prepared normal pool can be used provided it is itself calibrated against a reference preparation.

- **Calibration of Standard Pools and Suggested Calibration Procedure**

Whenever possible, the normal pool should be calibrated as described in the following against a freeze-dried reference material already calibrated against the international standard. The reference material may be a national standard (e.g., National Institute for Biological Standards and Control) or a commercial standard. In the absence of reference materials the laboratory should obtain as large a normal pool as possible and assign it a value of 100 u/dl (1.0 u/ml).

The most important principle of calibration is repetition to minimize possible errors at each stage of calibration. It is necessary to carry out at least four independent assays, and preferably six. An independent assay is an assay for which a new ampoule of standard is opened, or if a freeze-dried standard is not available, for which a new set of dilutions are prepared from frozen previous reference plasma. Each plasma must be tested in duplicate; two replicate assays should be carried out each day, and the procedure should be repeated on at least 4 days (four independent assays). Whenever possible more than one operator should be involved .

Comparison should always be made with the previous normal pool. The potency of the new normal pool is calculated for each replicate assay on each day and an overall mean value is calculated. This calibration also enables an assessment of the precision of the method used.

- **Control Plasma**

Controls are included alongside patient samples in a batch of tests. Inclusion of both normal and abnormal controls will enable detection of nonlinearity in the standard curve. Whereas a reference standard (calibrator) is used for accuracy, controls are used for precision. Precision control, the recording of the day-to-day variation in control values, is an important procedure in laboratory coagulation. Participation in an external assessment schemeis also important to ensure interlaboratory harmonization. The use of lyophilized reference standard and control plasmas has become widespread, whereas locally calibrated standard pools are used especially in under resourced countries. The results of participation in external quality-control schemes require careful attention. The large number of different reagents, substrate plasmas, reference preparations, and analyzers available makes comparison of like with like difficult. Ideally all combinations should give similar results, but this is often not the case and the results should be used to carefully choose the combination used.

A control must be stable and homogeneous; the exact potency is not important, although the approximate value should be known to select a preparation at the upper or lower limit of the normal reference range.

- **Variability of Coagulation Assays**

Within a laboratory, variability is most commonly the result of a dilution error, differences in the composition of reagents, failure to take the time-trend into account, and differences in experience and technique between operators. A coefficient of variation of 15–20% is not uncommon for factor VIII:C assays. Furthermore, the variability increases if like is not compared with like (e.g., if concentrate preparations are assayed against plasma).Variability between laboratories is much higher. Apart from the factors described for the within-laboratory variability, there is the major

effect of differences in methods and in the composition of reagents. Comparability between laboratories improves if standardized reagents are used.

The unavoidable variability associated with coagulation assays makes the use of reliable reference materials imperative. (Dacie and Lewis, 2006).

1.3 Previous study

Several researches have been done to evaluate the different new automated PT measuring in comparison with the manual method.

UdoBecker *et al*, 1983 assessment of the automated prothrombin time test with use of chromogenic peptide substrate and Centrifugal analyzer comparing with manual method, they analyzed 58samples from normal persons and from patients being treated with oral anticoagulant correlation between the manual and the Cobas Bio application is good (r=0.98) but the slop of 1, presumably because of the less precise temperature control in the manual method.

Another study was conducted in hemostasis research section,Nycomedas,Oslo,Norway.Gogstad.G.O*et al,**1986***they were used tow thromboplasitin reagent (Thrombotest and Normotest)were used in evaluation of an automated method for determination of prothrombin time based on turbid metric measurement of clot formation in a centrifugal analyzer they were used 76 plasma samples from patients with various disease or being treated with oral OAT. The result were correlated well with manual method (r=0.98-0.99).

1.4 Rationale

Prothrombin time is prolonged in patients with liver disease, DIC, Factor deficiencyetc. So it is a vital test to monitor treatment of those patients through INR and so follows up clinics.

Prothrombin time test isan important test and quality assurance must be applied.

SoPT will be tested in a variety of patientsand in two procedures to quality and precision of this test.

1.5 Objectives

General objectives

1-To evaluate Prothrombin time and international normalized ratio values onblood samples sent to the Khartoum Teaching Hospital laboratory by using the manual and semi automated method (DignosticaStago).

Specific Objectives

1-To measure the Prothrombin time values and Internationalnormalized ratioby usingthemanualandsemi automatedmethod (DignosticaStago).

2- To compare thedifference in the results of Prothrombin time and international normalized ratio obtained fromthe manual and semi automated methods.

Chapter TWO

2. Materials and Methods

2.1 Study design

This is descriptive cross sectional study to determine the difference in the measurement of the prothrombin time by using different techniques including manual and semi automated methods in Khartoum teaching hospital research and laboratory unit.

2.2 Study population

This research was performed on blood sample sent to the laboratory of Khartoum teaching hospital for determination of PTand INR value from individuals of different ages both males and females with different clinical manifestationwere tested.

2.3 Inclusion criteria

Patients requested coagulation profile of both sexes, were included in the study.

2.4 Exclusion criteria

The clotted and hemolysedsample.

2.5 Study area

Khartoum Teaching Hospital research and laboratory unitin Khartoum state.

2.6 Samplesize

100 samples both male and female are included with different ages.

2.7 Sampling technique

Simple random sample.

2.8 Methodology

2.8.1 Sample collection

Venous blood collected using sterile disposable plastic syringeafter cleaning the vein puncture area with 70% ethanol, without a pressure cuff, allowing the blood to enter the syringe by continuous free flow9 volume of blood were added to 1volume of anticoagulant, trisodium citrateA32g/l(109M)solution (i.e., 0.55 ml of anticoagulant for a 5 ml specimen).The blood is thoroughly mixed with the anticoagulant by inverting the container several times.(Dacie and Lewis)

The sample wascentrifuged at3000rpm for15 min to obtain plateletpoor plasma(ppp)and transfer plasma into clean test tubes which was caped the test tubes to prevent deterioration of samples.

2.8.2 Laboratory testing

PT was measuredby using manual and semi automated(Diagnosticastago) methods.

2.8.2.1 Manual method

Principle: Tissuethromboplastin in the presence of calcium activate the extrinsic path way of human blood coagulation mechanism .When reagent is added to normal anticoagulated plasma, the clotting mechanism is initiated ,forming asolid gel clot within aspecified period of time.The time required for clot formation

Would be prolonged if there is a deficiencyof factors/factor activity in the extrinsic pathway of the coagulation mechanism

- **Reagent and Material**

1. Water baths.

2-Centrifuge.

3-Prothrombinreagent (Spectrum).

4-Glass tubes.

5-Calibrated pipettes.

6-Pipettes tips.

7- Stop watch.

- **Procedure**

1- 0.2 ml of the thromboplastin/calcium reagent was pipetted into a small glass tube. Place in a 37°C water bath for 1–3 minutes.

2- Using a calibrated capillary or delivery pipette,0.1ml plasma was added, mixed, and start the stop-watch. Hold the tube in the water bath and tilt the mixture back and forth looking for clot formation. When a clot forms, stop the stop-watch and record the time in seconds.

3- The clotting time was converted to the INR using the table provided by the manufacturer. SeparateINR tables are provided for capillary blood and Plasma.

2.8.2.2 Automated Method (DiagnosticaStago)

- **Test principle**

The principles of the test consist of the use of calcium thromboplastinto measure the clotting time of the patient plasma and to compare it with that of normal standard.

- **Assay procedure**

Cuvette-strips were placed in the incubation area for prewarming at 37°C for at least 3 minutes.

Aball was dispensed to each cuvette.

In prewarmed cuvette (37°C):

- **Incubation area**

- Plasma was dispensed (standard, patientor control) 50µL.
- Timerstarted corresponding to the incubation column 60 sec.

- When the instrument starts to beep, the cuvettestransferred to the test column area.

-TheFinn pipette was primed once with start reagent.

- **Test column**

- The Finn pipette activatedby pressing the pipette key

- Reagent prewarmed at 37°C100 µL was dispensed.

-Result of PT test as whole,the activity of extrinsic coagulation factors.

2.8.3 Quality Control

Two levels of commercial controls are purchased from the manufacturer; manufacturer instructions must be adhered to. Control results are flagged when they fall out of range and can be displayed on Levey-Jennings charts to assess accuracy and precision

- **Controls are run:**

- To periodically monitor the performance of the testing system - see site procedure/handout for the control policy followed at your institution.

- For any test whenever reagents for that test are replaced .

- When troubleshooting an instrument problem or following maintenance procedures .

- **Maintenance**

Routine maintenance is necessary to ensure proper instrument functioning. All instruments need to be maintained according to manufacturer's recommendations. Daily checks may include temperature (reagents, reaction wells), disposables or other programmed checks. See site procedure or maintenance manual for daily instrument checks and scheduled weekly and monthly preventive maintenance requirements.

- **Troubleshooting**

Error messages will occur on the instruments. All persons operating the instruments should know the location of the Operator's manual so routine troubleshooting can be performed when necessary. In the event of an instrument malfunction requiring a service call, the operator should know the location of the technical service phone number.

- **Testing Variables**

Strict adherence to laboratory protocols is vital to prevent erroneous results caused by:

- Pre-analytical variables (sample collection and processing (Specimen integrity is critical to accurate coagulation results. Avoidance of tissue trauma and hemolysis is essential to minimize activation of the coagulation process prior to analysis.

- Analytical variables (reagents, instrumentation, equipment, technique(Due to the many variables that may cause instrument or reagent related errors, it is imperative that commercial control plasmas are used to monitor the performance of the testing system.

Reagent and/or control problems can arise if manufacturer recommendations for reconstitution and handling are not followed. The operator must be aware of instrument linearity limits.

- Post-analytical variables (reporting results) Patient values should be correlated with clinical information (if available) before reporting. All laboratories establish critical/panic values that must be verified and/or action taken per lab policy. Questionable results must be investigated before reporting, e.g., inconsistent values or results that fail a delta check with previous results .

Delta checks are designed to detect discrepancies in results before reporting by comparing current patient values to previous patient values.

Delta check limits define the allowable difference in consecutive results of a specific test for the same patient within a certain time period.

The delta limit should be set so that true changes in test results (i.e., correct results) are not flagged as delta check failures. When delta check limits are exceeded (i.e., fail delta), a potential error exists that may involve specimen collection or a change in the patient's clinical condition.

2.8.4 Data analysis

By computerized SPSS program (IndependentT.test).

2.8.5 Ethical consideration

Ethical approval from university ethical committee was obtained.

Permission from Khartoum Teaching Hospital research and laboratory unit administration as well as consent taken from taken from the patients.

Data were obtained with high confidentiality and sure data were used for research purpose only.

Chapter Three

3. Results

PT was measured using the manual method and semi-automated (Diagnosticastago) by the same rabbit thromboplastinreagent. The mean PT value in the manual method was (22.5±10.4) while in the semi automated the mean was (21.9±10.6) show figure (3-1).

Determination of PT value by manual and semi automated showed there was in significant variation P. value (≥0.05) as demonstrated in table (3-1).

INR was also measured from the manual and semi automated method (DiagnosticaStago).

The mean INR value in the manual method was to be found (1.8 ± 0.92) while by semi automated was (1.7 ± 0.93) show figure (3-2).

The INR value showed there was in significant variation P. value (≥0.05) as demonstrated in table (3-2).

Table (3-1) Correlation between the manual and semi automated methods for measurement of the Prothrombin time values

	Mean ± SD	P. value
semi automated method	21.9 ± 10.6	0.66
Manual method	22.5 ± 10.4	

In significant P. value ≥ 0.05

Table (3-2) Correlation between the manual and semi automated methods for measurement of the International normalized ratio values

	Mean ± SD	P. value
semi automated method	1.7 ± 0.93	0.55
Manual method	1.8 ± 0.92	

In significant P. value ≥ 0.05

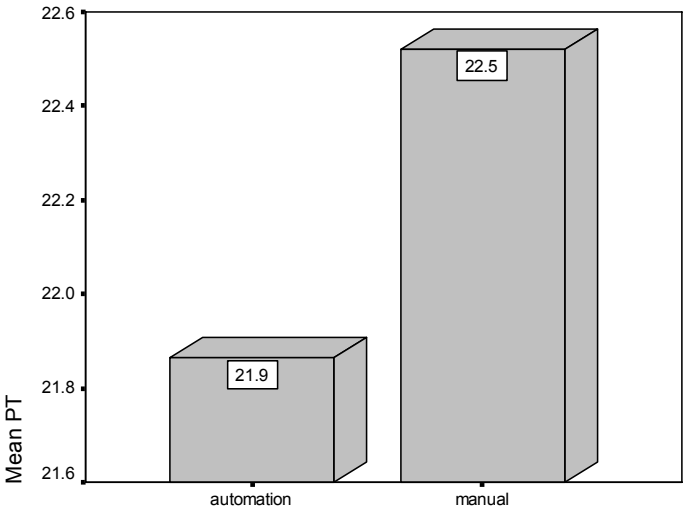

Figure (3-1): The mean Prothrombin time values by manual and semi automated methods.

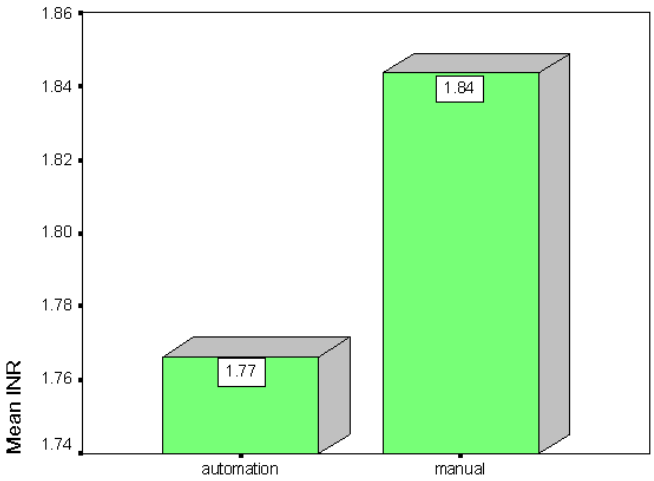

Figure (3-2): The mean International normalized ratio values by manual and semi automated methods.

Chapter Four

4. Discussion, Conclusion and recommendation

4.1 Discussion

Coagulation testing is an important part of every medical practice. Every laboratory is usually inundated with requests for prothrombin time (PT) and activated partial thromboplastin time (APTT). The prothrombin time is a one-stage test based upon the time required for a fibrin clot to form after the addition of tissue thromboplastin, phospholipid and calcium to decalcified platelet poor plasma. Ever since its successful description by Quick in the early twentieth century, the manual testing of PT has stood the test of time. But the place of the manual method in a large clinical laboratory of a tertiary care hospital; which caters to hundreds of samples each day has been eclipsed by automated methods. These instruments analyze the coagulation system through detection of clot formation (EnsGE and Jensen ,1993).

The result obtained from measuring the PT using the manual and semi automatedmethod (DiagnosticaStago) were compared with each other. The mean values using manual methods were found to be (22.5 sec) while the same samples the mean value were (21.9 sec) by using semi automated method. P.valuewas (0.66). There was no significant variation between the results.

And the result of INR value had mean of (1.8) by the manual method and (1.7) by the semi-automated method and P.value (0.55). There was also no significant variation between the results.

This is in agreement with the result of previous study obtained by UdoBeker,Helmut Jering,Knut Bartl,and Franz Jilek .1983done on 58 samples ,The correlation between the manual and COBAS BIO application is also good(r=0.98).

Another study conducted byGogstad.G.O*et al,* 1986 they were evaluating of an automated method for determination of PT based on turbid metric measurement of clot formation in centrifugal analyzer result were correlated with manual method (r=0.98-0.99).

The result is disagreement with Leon poller*et al,*2008 *study* they found most of the instruments gave shorter results PT than did the manual technique.

This study was in contrast with Angelo DA, *et al*, 1989 they are made comparison of two automated and manual tilt-tube method for the determination of Prothrombin time, they were found that is clotting times were longer with the manual than automated methods,presumably because of the less precise temperature in the manual method.

4.2 Conclusion

The semi automated(Diagnostic stago) is good alternative method for traditional manual tilt tube technique for PT determination we never the work load increase. The result for the prothrombin time test obtained by the semi automatedmethod (Diagnostic stago) is reliable and is statistically equivalent as those obtained by the manual method. Both methodologies can be relied upon while testing for coagulation.

Which indicate that instruments are well calibrated and have a system or internal quality control, records is maintained of the lot number of all reagents and disposable equipment used.

Periodic checks of the temperature, incubation bath and of the volume of pipette.

4.3 Recommendations

1-Semi automated method is recommended for the measurement of the PT.

2- Comparative studies involving the measurement of the PT by different method and estimation of INR can be performed to detect reliability of each method.

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Appendix No (1)

Questionnaire

Sudan University OF Science and Technology

College Of Graduate Studies

Department Of Hematology

Determination of the Prothrombin time and the International Normalized Ratio by using manual andsemi automated methods in Khartoum Teaching Hospital Laboratory

الموضوع جمع عينات لاجراء البحث التكميلى ٍ

Name:……

Age:……

Gender: Male ( ) Female ( )

Disease:……

Treatment:…….....

Receiving of anticoagulant therapy:
yes( ) No( )

Investigations:

PT:……

INR:……

Date: / /
Sig:…….

Appendix (2)

DiagnosticaStago