JUHA HORSTI

Prothrombin Time

Evaluation of Determination Methods

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“Blessing and honour and glory and power Be to Him who sits on the throne, And to the Lamb, forever and ever !” (Rev.5:13)

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, referred to in the text by their roman numerals **I – V.**

I. Horsti J (2000): Measurement of Prothrombin Time in EDTA Plasma with Combined Thromboplastin Reagent. Clin Chem 46: 1844–1846.

II. Horsti J (2001): Use of EDTA samples for prothrombin time measurement in patients receiving oral anticoagulants. Haematologica 86: 851–855.

III. Horsti J (2001): EDTA Samples are Stable for Prothrombin Time Measurement by Combined Thromboplastin Reagent. Clin Chem 47: 1731–1733.

IV. Horsti J (2001): Agreement of Owren and Quick Prothrombin Times: Effects of Citrate and Calcium Concentrations and International Sensitivity Index Correction. Clin Chem 47: 940–943.

V. Horsti J (2002): Comparison of Quick and Owren Prothrombin Time with Regard to the Harmonisation of the International Normalised Ratio (INR) system. Clin Chem Lab Med 40: 399–403.

ABBREVIATIONS

a	Activated
A.D.	Anno Domini
APTT	Activated partial thromboplastin time
B.C.	Before Christ
Ca^{++}	Ionised calcium, Factor IV
CV	Coefficient of variation
EDTA	Ethylenediaminetetraacetic acid
FNPP	Fresh normal pooled plasma
F I	Fibrinogen
Fibrin i	Insoluble fibrin
Fibrin s	Soluble fibrin
FSF	Fibrin stabilising factor
ICSH	The International Council for Standardisation in Haematology
INR	The International Normalised Ratio
IRP	The International Reference Thromboplastin Preparation
ISI	The International Sensitivity Index
ISTH	The International Society for Thrombosis and Hemostasis
MNPT	Mean normal prothrombin time
NaCl	Sodium chloride
NCCLS	National Committee for Clinical Laboratory Standards
OAT	Oral anticoagulant therapy
PIVKA	Protein Induced by Vitamin K Absence or Antagonists
PL	Phospholipids
POCT	Point-of–Care Testing
PT	Prothrombin time
R	Ratio
S.E.	Standard error
TT	Thromboplastin time
WHO	World Health Organisation
WRP	Working reference preparation

1. INTRODUCTION

1.1 Historical perspective

The traditions of medicine go back to the dawn of human history and are interwoven into all civilisations past, present and future, because health is a matter of concern to every human being.

Scientific observations have been a part of diagnostic medicine from the beginning of history, more recently giving rise to formal clinical chemistry. The priest-physicians of Egypt and Babylon were already making observations on patients. Likewise millennia later, perhaps more systematically, the Greek physician Hippocrates (ca. 460–ca. 377 B.C.). He is regarded as the Father of Medicine. The first reference to blood coagulation seems to have been made by Hippocrates. Also Aristotle (384–322 B.C.), Celsus (25 B.C.–A.D.50), and Galen (A.D. 130–200) observed that freshly drawn blood usually clots within minutes. They also described in detail various internal and superficial bleeding tendencies, as recently reviewed (Owen 2001). These early observations were based on whole blood coagulation.

It might be concluded that clinical chemistry findings in body fluids have walked together hand in hand with medicine in general to confirm diagnosis, to establish a prognosis and to monitor therapy.

1.2 Current model of blood coagulation

The arrest of bleeding involves a complicated process in the body. The characterisation of coagulation pathways and coagulation factors has constituted an enormous undertaking, lasting over two hundred years since Hewson (1780) made the first observations in fibrinogen (coagulation factor I). For haemostasis two major components are necessary: a platelet plug and a stable fibrin clot. The latter can be formed in vivo through an "intrinsic" or an "extrinsic" pathway. The final outcome of both pathways is the same, i.e. the reactions from coagulation Factor X to insoluble fibrin. The biochemical structure of coagulation proteins as well as the structure and code inheritance of the respective genes are for the most part known.

SCHEMATIC ILLUSTRATION OF THE COAGULATION PATHWAY

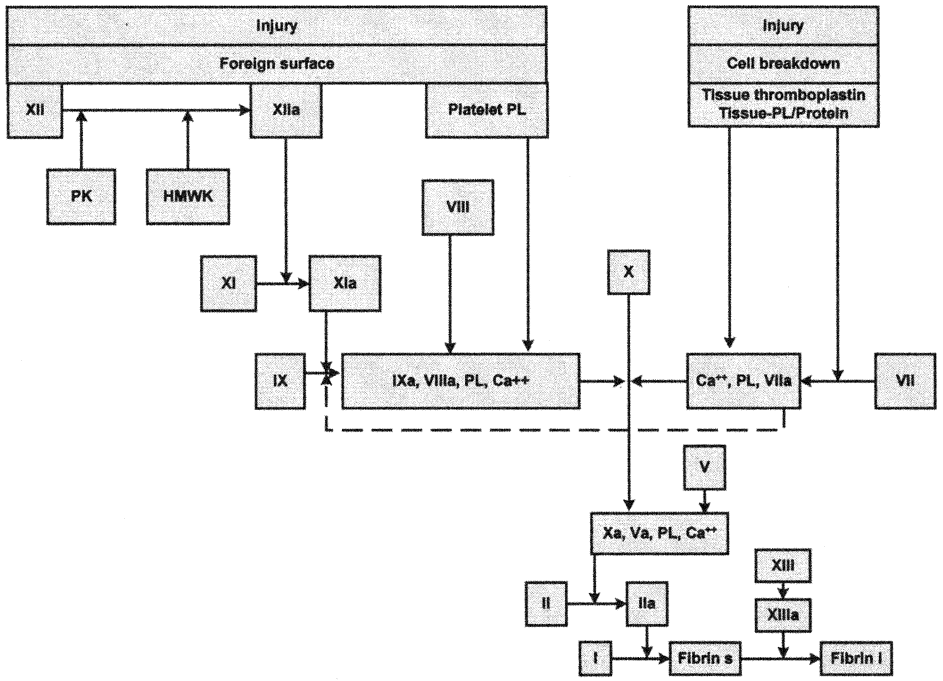

Figure 1. Schematic illustration of the "intrinsic pathway" and the "extrinsic coagulation pathway".

Injury and exposure to foreign surfaces under vascular endothelial cells (surface activator) with exogenous phospholipid and Ca^{++} starts the "intrinsic coagulation pathway" and the subsequent reactions produce an insoluble fibrin clot. The activated partial thromboplastin time (APTT) test is sensitive to decreased blood levels of coagulation factors in the "intrinsic pathway".

Abbreviations: XII, Factor XII; PK, prekallikrein; HMWK, high-molecular-weight kininogen; PL, phospholipids; XI, Factor XI; IX, Factor IX; VIII, Factor VIII; a, activated

Injury, cell breakdown (tissue thromboplastin, exogenous phospholipid, Ca^{++}) initiates the "extrinsic pathway" reactions and the end product is an insoluble fibrin clot.

Abbreviations: X, Factor X; IV, Factor IV, Ca^{++}; PL, phospholipids; VII, Factor VII; V, Factor V; II, Factor II; XIII, Factor XIII; I, Factor I, fibrinogen; fibrin s, soluble fibrin; fibrin i, insoluble fibrin; a, activated (Henry 1996, Lutze et al. 2000).

Factor IXa can bind to the cofactor factor VIIIa bound on membrane surfaces in the presence of calcium ions to generate a complex with enzymatic activity known as tenase. This compex converts the proenzyme factor X to its enzyme form, factor Xa.

In a parallel series of interactions, factor Xa binds to the cofactor factor Va, bound on membrane surfaces, in the presence of calcium ions to generate a complex with enzymatic activity known as prothrombinase (Beutler 1995).

1.3 Development of modern PT analysis

Warner and colleagues (1934) and Quick (1935) developed independently and published at almost the same time the first techniques for measurement of certain plasma coagulation factors. They designated the test prothrombin time, (PT). In the Finnish terminology the term 'thromboplastin time' (TT) is used instead of PT, and is employed throughout the present work.

In the method developed by Warner and associates also called the Iowa Two-Stage Test, the two phases of clotting (prothrombin to thrombin and fibrinogen to fibrin) are separated and the reaction of fibrinogen to fibrin measured (Warner et al. 1934). The estimation was technically more complicated than that for Quick's prothrombin time and is no longer in clinical use.

In the Quick prothrombin time test an excess of thromboplastin and calcium is added to oxalate-anticoagulated plasma. The clotting time was considered to be a direct measure of the prothrombin concentration of the blood (Quick 1935).

The Quick reagents currently used are based on this principle. The test is easy to perform both manually and automatically. The principle basis of the PT test for laboratory and Point-of –Care Testing (POCT) methods is presented in Figure 2.

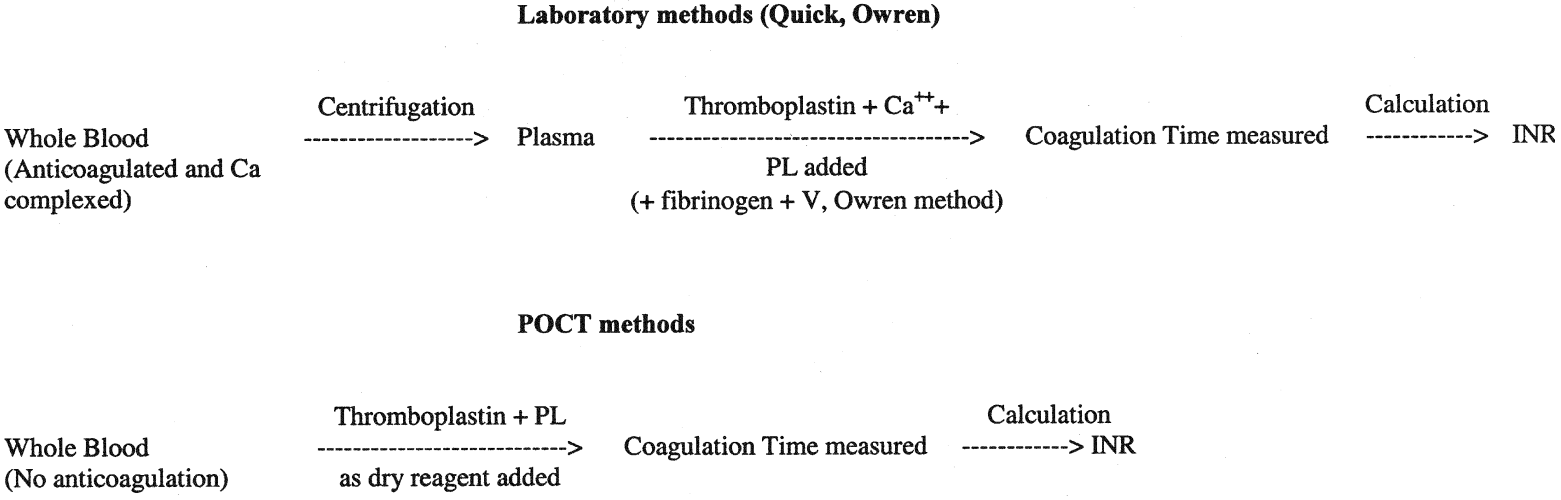

Figure 2. Basic model for PT measurement using laboratory or POCT method.

Some 20 years after the first accounts of PT measurement, Owren (1959) developed a new principle for PT estimation using a combined thromboplastin reagent (fibrinogen and factor V added to the reagent). This was subsequently developed as a modification of the P & P method (Owren and Aas 1951) to overcome the drawbacks of the Quick method.

PT and APTT measurements have also recently been adopted in Point-of–Care Testing (POCT) technology. These methods are now wide in clinical use and are targets of general interest. In this case laboratory technology has come close to the patient, offering new possibilities for patient care also in anticoagulant therapy monitoring. Patient can perform by the test at home (Price and Hicks 1999).

2. PROTHROMBIN TIME

The prothrombin time test measures coagulation factors of the "extrinsic pathway" illustrated in Figure 1. It is carried out by adding thromboplastin (containing tissue factor), phospholipid and an excess of calcium to anticoagulated plasma and measuring the clotting time. PT is the most commonly used coagulation test in routine laboratories.

The PT test is used for assessment in pre-operative detection of bleeding tendencies in risk groups, the monitoring of anticoagulant therapy; used for prevention and treatment of venous thromboembolism, prosthetic heart valves, artrial fibrillation and other indications (Hirsh et al. 1998). The most markedly increasing use of PT is now seen in oral anticoagulant therapy (OAT) control. This means for example in Finland an annual increase of 10% in the number of tests. The prevalence of OAT in the Finnish population is 0.6 – 0.7% (Syrjälä 2000). The need for PT tests is constantly increasing as the population ages and more thrombotic diseases are encountered. PT determinations during OAT are needed at regular intervals since the therapeutic range of oral anticoagulants is narrow. Aged patients are in greater danger of bleeding; over-medication increases the bleeding risk and under-medication entails the possibility of thrombosis.

2.1 Sampling

2.1.1 Anticoagulants in sampling for coagulation tests

Many anticoagulants are available for blood sampling for clinical chemistry tests. One group of them bind and inactivate calcium. These include, for example, citrate, EDTA and oxalate.

Ionised calcium (Factor IV) is in key position in both the intrinsic and the extrinsic coagulation pathway. When anticoagulants bind (inactivate) calcium in blood sampling, they simultaneously inhibit coagulation reactions in the test tube and preserve coagulation factors for further analyses. In the coagulation activity measurements, excess calcium is added to set of the coagulation reaction.

For anticoagulation of blood samples for coagulation analysis the World Health Organisation (WHO) and the National Committee for Clinical

Laboratory Standards (NCCLS) recommend the use of 3.2 % (0.109 mol/l) citrate and a dilution of 1 part of citrate with 9 parts of blood for coagulation test sampling (WHO 1999). Unfortunately this recommendation is not necessarily carefully followed and the citrate concentration used may thus vary. At least two types of vacuum tubes are currently available with different citrate concentrations (0.129 mol/l, 3.8 %, w/v ; 0.109 mol/l, 3.2 %, w/v). This causes problems in analytical accuracy and precision, resulting in variability of PT results between different laboratories (Horsti 2001a, Horsti 2002).

The Quick PT method is highly sensitive to differences in sample citrate concentration and this may cause analytical errors both in INR results and in ISI calibration (Adcock et al. 1997a, Chantarangkul et al. 1998, Duncan et al. 1994) as discussed in greater detail below.

The proportion of citrate in the final sample is not constant; although constant in the test tube, it is distributed only in the plasma phase of the blood sample. Blood haematocrit has an important effect on the citrate concentration: high haematocrit means a high citrate concentration (small volume of plasma) and low haematocrit means a lower citrate concentration (in a larger volume of plasma). Owing to the relatively large proportion of citrate in the final analysis, the Quick PT method is sensitive to sample citrate concentration, and this constitutes one important source of analytical variation.

Two citrate molecules bind three calcium molecules in a complex ($C_{12} H_{10} Ca_3 O_{14} \times 4 H_2 O$). Citrate buffered to pH 5.5 should be used since this stabilises pH in the sample close to the physiological pH. The use of unbuffered citrate for blood collection is not recommended (Guder 1997, Narayanan 1995).

The International Council for Standardisation in Haematology (ISTH) recommends the use of Hepes buffer for all investigations of haemostastic function (Polack 1996), but tubes with Hepes buffered citrate are not widely used and are not available from manufacturers.

Oxalic acid binds calcium, forming a complex $Ca C_2 O_4$, and this anticoagulant has previously been used for coagulation test sampling; it is no longer recommended.

2.1.2 Sampling and other preanalytical preparations for PT

The accuracy of the prothrombin time result, as in all other coagulation tests, depends greatly on the quality of sampling. Venous compression before vein puncture should be no more than 60 seconds (Lutze 2000).

Underfilling of specimen tubes causes a higher citrate concentration in the sample (plasma) and this comprises a source of error. Tubes should be

filled to at least 90 % of capacity to avoid falsely elevated PT coagulation times (Reneke et al. 1998).

Tissue contamination of the sample should be minimal, and a previous standard practice has been to draw a discard tube or a tube for other tests before filling a coagulation tube. NCCLS (1984) has also recommended this order of blood drawing. Several recent studies have however evaluated the necessity of drawing a discard tube and the results showed no statistically significant differences between the first or second tubes for PT (Adcock et al. 1997b, Gottfried 1997, McGlasson et al. 1999). The practice is well come to routine laboratories since it makes sampling faster and saves material.

Platelet-poor plasma is obtained by a centrifugation step, and at least three recommendations have been published: centrifugation at 1000 - 2000 x g for 10 min. (Guder 1997), 2500 x g for 15 min. (NCCLS 1991), 1500 - 2000 x g (Lutze 2000) at room temperature. The supernatant material, normally called "plasma", is used for a large majority of coagulation tests. It still contains small numbers of platelets, but these do not significantly interfere with PT tests. Preparation of tinly platelet-free plasma needs a higher g value and longer centrifugation time, i.e. 2000 - 3000 x g for 15 - 30 min. (Guder 1997).

In POCT instruments capillary blood from the fingertip without exogenous anticoagulant is usually used for analysing PT. Plasma or whole blood collected with citrate, oxalate or EDTA as anticoagulants are incompatible with most POCT instruments (Hasenkam 1997, Solomon 1998).

2.1.3 Sample stability

All published works on sample stability have been concluded using the Quick PT method, but they are obviously also relevant for the Owren PT, since more coagulation factors are measured with Quick PT (Factors I, II, V, VII and X) than Owren PT (Factors II, VII and X).

Sample tubes should be stored at room temperature, the reason being that Factor VII activated and the PT becomes shorter at 4° C in both whole blood and plasma when stored in borosilicate or siliconised borosilicate tubes (Palmer and Gralnick 1982, Palmer and Gralnick 1985).

Recommendations in the literature are variable regarding the maximum duration of plasma storage before PT measurement. PT should be estimated within two hours at room temperature or within four hours at 4° C from sample collection (Tietz 1995, Dacie and Lewis 1995, Ravel 1995) or eight hours at room temperature (Guder 1997).

According to some studies polypropylene material and unopened vacuum tubes extend PT stability at room temperature or at 4° C to 24h (Ridyard et al. 1998, Heil et al. 1998, Vadher et al. 1997).

Baglin and Luddington (1997) reported that plasma is highly stable in unopened sample tubes; the overall mean difference in INR after three days was only 0.05 INR units as compared to a fresh sample. This conclusion is not, however, necessarily justified; individual INR values were not reported and the reader cannot exclude the possibility that INR values could change too much, from the clinical point of view, during the long storage period.

Leeming and associates (1998) noted that the mean PT change after 24 h was relatively small, but that some samples changed markedly, namely over 0.5 INR units, which is too much for clinical purposes. The authors recommend a plasma sample storage period of no longer than 24 hours at room temperature.

Zawta and colleagues (2001) and Lutze (2000) have used different kinds of methods in their sample preservation studies. They investigated the stability of some PT coagulation factors as a function of time and temperature. If the preservation temperature was 37° C, coagulation factors evinced markedly reduced stability. Sample temperature should be controlled, since in summer time the temperature in laboratories and during transport may be much higher than the ordinary "room temperature". This may cause error in results. Thus, Zawta's group (2001) recommend for PT a maximum preservation of six hours at room temperature for PT samples. This recommendation would appear to be based on reliable assessments for routine laboratories.

2.2 Units in prothrombin time measurement

2.2.1 Time (seconds)

Modern handbooks of clinical chemistry give PT reference values only in seconds. Depending on the thromboplastin reagent, values of 10 - 13 s (Henry 2001) and 10 - 12 s (recombinant thromboplastins) as well as 11 - 16 s (Lewis 2001) have been presented. These reference intervals are for the Quick method; Owren coagulation times are longer, since the relationship between sample and reagent is different. The corresponding reference interval for Owren reagents is 17.4 - 22.6 s (Table 3).

The therapeutic range of Quick PT for oral anticoagulant therapy was two to three times the normal clotting time. With this range satisfactory anticoagulation was achieved and few bleeding problems occurred (Quick

1937, Talstad 2000). Values in seconds values were used before the adoption of ratio units.

Using a second as unit there is no need for calibration, which is an advantage, since calibration may cause variation and errors in results. The disadvantage is that PT coagulation times vary according to the reagent source and even from batch to batch. Furthermore, the coagulation time is highly dependent on the source of thromboplastin raw material, and the therapeutic time range for OAT depends on the reagent.

When seconds are used as unit, only one measurement and no calibration is required. This automatically results a smaller variation and better accuracy as compared with ratio and INR strategies.

2.2.2 Ratio and percentage

Since the second as unit is highly dependent on reagent PT units, ratio (R) and percentage (%) units were adopted for clinical use according to the following formulas:

Eq. 2.2.2.a $R = \frac{\text{sample}_{sec}}{\text{normal plasma}_{sec}}$ Eq. 2.2.2.b $\% = \frac{\text{sample}_{sec}}{\text{normal plasma}_{sec}} \times 100$

It is obvious that R and % values were introduced in clinical practice in order to reduce differences between different reagents and to harmonise the therapeutic range for OAT. Biggs and Denson (1967) proposed standardisation towards an 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).

According to WHO (1977) and Kirkwood (1984) the regression line between ratios of reference thromboplastin (y) and any other PT reagent (x) is: $y - 1 = b (x - 1)$.

The value of b was calculated for any reagent using reference thromboplastin, normal plasma and samples from patients on OAT.

The revised ratio method (WHO 1983, Anderson 1976) recommended orthogonal calibration between log clotting times of abnormal and normal plasmas: $\log PT_y = c \log PT_x + d$

PT_y = international reference thromboplastin, seconds
PT_x = any reagent, seconds
NPT = normal plasma
c and d are calculated for the reagent using reference thromboplastin.

Simplified conversion from clotting times into ratios by presuming identical regression lines between clotting times of abnormal and normal plasmas proceeds as follows (Kirkwood 1984, Talstad 2000):

Eq. 2.2.2.c $$y = x^c,\ y = \frac{PT_y}{NPT_y},\ x = \frac{PT_x}{NPT_x}$$

In routine laboratories ratio or percentage values were used before the adoption of the INR strategy. Percentages and ratios were not corrected by calibration with international reference thromboplastin; instead, calibration curves were obtained by diluting a normal human plasma pool (100 % or R = 1) with buffer or physiological salt solution (NaCl 0.15 mol/l), for example, to calibrators 50 % (R = 0.5) and 25 % (R = 0.25). Automatic coagulation analysers carried out these dilutions from normal plasma. A calibration curve was prepared automatically for the instrument's memory. The standard curve was prepared by plotting clotting time, s, (y) versus coagulation activity, % or R (x) and sample seconds were changed to % or R values using this curve.

The calibration method based on normal plasma pool as main standard was very useful for routine work. It was practical and easy to perform in laboratories of different sizes. The normal plasma coagulation time did not vary much between different thromboplastin reagent lots and normal plasma was easily available in clinical laboratories. For this method of PT determination two time measurements are required: one for analysing sample seconds and another for normal plasma seconds. The error of measurement by ratio or percentage is mathematically determined by deriving the equation:

Eq. 2.2.2.d $$|\ dR/R\ | = |\ d\ sample_{sec} / sample_{sec}\ | + |\ d\ normal_{sec} / normal_{sec}\ |$$

In the equation $|\ |$ means absolute values and the absolute error can be calculated:

Eq. 2.2.2.e $$(d\ R/R) \times R = d\ R$$

(Lähteenmäki I and Minni E 1986)

International reference thromboplastin correction makes estimation more complex. Variation increases, since several measurements and regressions have to be used. Ratio and percentage as units are still dependent on the reagent, and accordingly, the therapeutic range of PTs for OAT are reagent-dependent. For this reason the same reagent has been used in the whole of a central hospital district in Finland and the therapeutic range is same in this area, but is not necessarily the same in other districts; different reagents and different therapeutic ranges of PT have been used and this may well confuse both patients and physicians when patients move from district to another. Therapeutic ranges for patient care and scientific literature are likewise not globally comparable.

2.2.3 INR (the International Normalised Ratio)

WHO has supported the use of INR units and reference thromboplastins globally. This was done in order to harmonise PT results in OAT monitoring and to facilitate the comparability of related scientific work. WHO furthermore recommends editors and reviewers of scientific papers not to accept manuscripts expressing their PT results in traditional terms without INR values (ICSH/ICTH 1985, WHO 1983, Kirkwood 1983, Hermans et al. 1983). Recommendations for therapeutic ranges for different patient groups are now globally uniform as INR units (Hirsh et al. 1998).

INR units were originally not recommended for screening and follow-up of patients with liver diseases. However, Kovacs and associates (1994) have since demonstrated that INR can also be used to monitor patients with severe form of a liver disease.

The recommendation for the use of INR was put forward already in the 1980s. However, adoption of this unit globally in clinical practice has taken decades and has not yet been completed. The slow progress with INR has perhaps been a consequence of the practical problems involved in its use as well as of unacceptable levels of inter-laboratory variation. In the Nordic countries, Norway and Sweden (Egberg et al. 1999) changed to the use INR units some months before Finland. Since the beginning of the year 2001, INR has been the only unit for PT in OAT control in Finland (Syrjälä 2000, Horsti 2001b). The practice of reporting PT results in INR units has now been implemented in over 90 % of North American laboratories (College of American Pathologists 1994).

The use of INR has encountered many unpredictable difficulties and much remains to be done in order to harmonise results globally, as is necessary to increase the safety of patients on OAT. However, Hirsh and colleagues (1998)

claim that the INR system is much more reliable and clinically safer than PT in seconds or the unconverted PT ratio for monitoring OAT.

A computer simulation study of serial INR measurements has been conducted within the most widely used therapeutic range (INR 2.0 - 3.0); the authors concluded that analytical imprecision should be < 5% and analytical bias < $\pm$ 0.2 INR units (Kjeldsen et al. 1997).

In view of the preanalytical and analytical factors involved this is a very demanding target for clinical laboratories anywhere.

2.2.3.1 Mathematics of INR calculation

The following formula is used for INR calculation:

Eq. 2.2.3.1 a $$INR = (sample_{sec} / normal_{sec})^{ISI}$$

The equation for calculation of PT INR values is designed to eliminate the variance caused by reagents and instrumentation. The INR calculation formula is based on the previously used ratio formula ($R = sample_{sec} / normal_{sec}$). However, in order to minimise the dependence on different reagents and instrumentation, the International Sensitivity Index, or ISI, is added to the formula as a power function. ISI is individual for each reagent and instrument pair. It stands in relation to reagent (thromboplastin) sensitivity. Being a power function ISI causes a mathematical unlinearity if it diverges from the value 1.00. When ISI parts from 1.00, its impact grows and the unlinearity of the function increases. If the equation were $INR = (sample_{sec} / normal_{sec}) \times ISI$, then the sensitivity correlation between reagents would be linear.

For the evaluation of variation in INR results it is convenient to use the logarithm from the formula:

Eq. 2.2.3.1 b $$Log_{10} INR = ISI \times Log_{10} (sample_{sec} / normal_{sec})$$

When errors are $d\,sample_{sec,}$ $d\,normal_{sec}$ and d ISI, the equation is derived and the relative error for INR may be obtained:

Eq. 2.2.3.1 c $$d\,INR/INR = d\,ISI \times |Log_{10} (sample_{sec} / normal_{sec})| + |(ISI / sample_{sec})| \times d\,sample_{sec} + |ISI / normal_{sec}| \times d\,normal_{sec}$$

In the equation | | means absolute values and we can calculate the absolute error:

Eq. 2.2.3.1 d (d INR/INR) x INR = d INR

(Lähteenmäki I and Minni E 1986)

d ISI x | Log_{10} ($sample_{sec}$ / $normal_{sec}$) | has an important role in calculating analytical errors. Accuracy is very good and variation small for today's automatic coagulation analysers when sample and normal plasma coagulation times are measured in seconds. This emphasises the importance of ISI as a source of error. The establishment of ISI on the International Reference Preparation (IRP) causes variation in the ISI of routine reagents. The use of responsive reagents (ISI < 1.7) significantly improves the accuracy and precision of INR results (Hirsh et al. 1998). Introcaso and associates (2000) applied mathematical derivatives using 10 different ISI values (from 1.1 to 2) and showed the same result, namely that the analytical variation increases with higher ISI values.

2.2.3.2 ISI calibration using international reference preparation

The aim in the original recommendation (WHO 1983, Hermans 1983) was to harmonise INR results by calibrating reagent ISI values with Human Combined, which was the primary reference preparation (IRP coded 67/40). For this purpose, their mathematical relationship was used. Thromboplastins from different sources (human brain, rabbit brain, rabbit lung and ox brain) yield quite different levels of PT. There was thus a need for secondary international reference preparations: Rabbit (plain, brain and lung, coded RBT/79) and Bovine Combined (OBT/79). The hierarchy of reference thromboplastin preparations is presented in Figure 3 (van den Besselaar 1993).

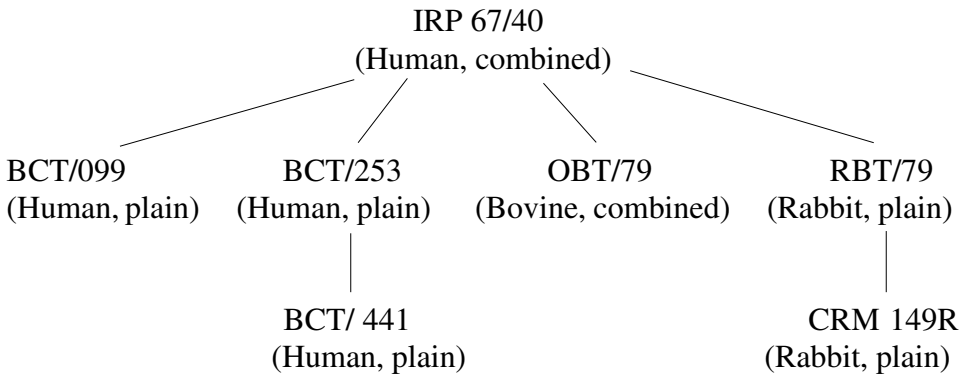

Figure 3. International reference thromboplastins for ISI calibration. The primary standard is IRP 67/40. Secondary reference standards were calibrated directly against the primary IRP. Working thromboplastins are calibrated against secondary or tertiary reference preparations of the same type.

Manufacturers and "local" ISI standards are calibrated against these international reference standards. Routine laboratories use manufacturer's or "local" ISI calibrators to estimate the ISI value for own reagent with their own instrument combination. Alternatively, they use the reagent manufacturer's ISI value determined specifically for their own instrument.

Estimation of the ISI value (WHO 1983) for "working reference preparation, WRP" (manufacturer's or "local" ISI calibration sets for ISI calibration) is done by using, for example, the International Reference Thromboplastin Preparation, Rabbit Plain, RBT/79.

There are two models (A and B) for calculating $ISI_{wrp.}$:
(A) Fitting of straight line

Table 1. An example of a regression strategy used for establishing the values of WRP on the basis of IRP. Six patient samples and two normal plasmas were used. The regression line and equation are given in Figure 4.

Plasma	Working reference preparation, WRP		International ref.preparaton RBT/79	
	Clotting time, s	log s	Clotting time, s	log s
Normal 1	13,8	1,140	15,9	1,201
Patient 2	19,6	1,292	21,4	1,330
Patient 3	30,8	1,489	35,6	1,551
Patient 4	28,6	1,456	29,1	1,464
Patient 5	19,6	1,292	21,8	1,338
Patient 6	33,6	1,526	41,1	1,614
Patient 7	38,1	1,581	37,9	1,579
Normal 2	15,2	1,182	17,1	1,233

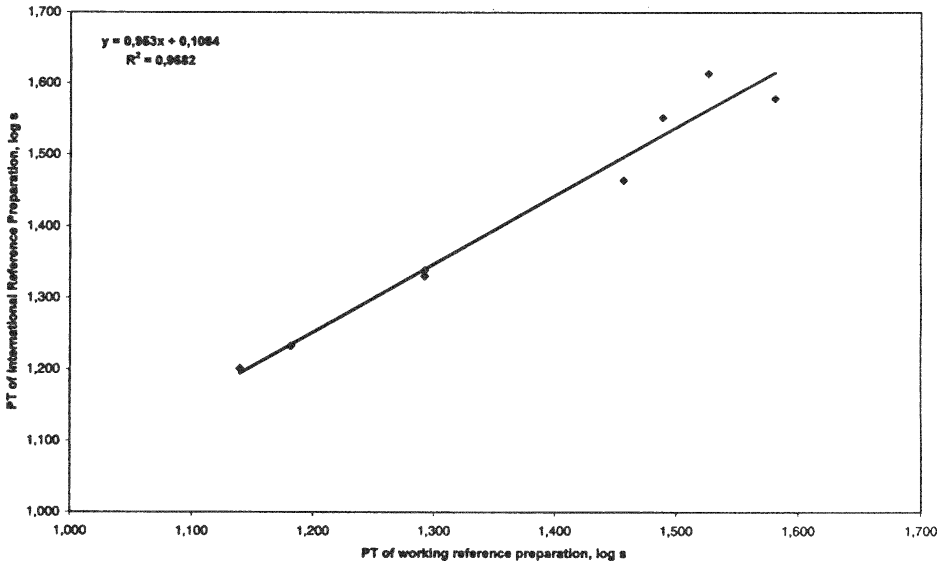

Figure 4. Double-logarithmic plot for determination of ISI

The slope in Figure 4 is $C_{irp, wrp}$ (0.953) for ISI_{wrp} calculation

(B) By a more laborious means using orthogonal regression according to the following equation:

Eq. 2.2.3.2 a

$$m = \frac{\Sigma(LPT_{irp} - LPT_{irpm})^2 - \Sigma(LPT_{wrp} - LPT_{wrpm})^2}{2\,\Sigma(LPT_{irp} - LPT_{irpm})\,(LPT_{wrp} - LPT_{wrpm})}$$

LPT_{irp} = logarithm of individual PT using IRP
LPT_{irpm} = mean of logarithms of individual PT using IRP
LPT_{wrp} = logarithm of individual PT using WRP
LPT_{wrpm} = mean of logarithms of individual PT using WRP
Σ = denotes summation of terms for all plasmas

Eq. 2.2.3.2 b $\quad C_{irp,\,wrp} = m + \sqrt{m^2 + 1}$

The ISI for the working reference preparation is calculated using the linear or orthogonal regression equation according to the values of an example given in Table 1:

Eq. 2.2.3.2 c $\quad ISI_{wrp} = ISI_{irp} \times C_{irp,\,wrp}$

ISI_{irp} = RBT ISI known as 1.4
$C_{irp,\,wrp}$ = 0.953 using linear regression (ISI_{wrp} = 1.334)
$C_{irp,\,wrp}$ = 0.9695 using orthogonal regression (ISI_{wrp} = 1.357)

The difference between linear and orthogonal regression calculation is 0.0165 in $C_{irp,\,wrp.}$ WHO has published the above equations for the calculation of ISI_{wrp}, but also to calculate S.E. of ISI_{wrp} (WHO 1983).

In a routine laboratory the reagent ISI is calculated by using, in principle, the same linear or orthogonal equations as above. The working reference preparation can be used to produce or the reagent manufacturer can provide the ISI value for a certain reagent batch and instrument pair.

The WHO calibration procedure is complex and demanding, although it may be simplified using certified lyophilised plasmas (Clarke et al. 1992, Poller et al. 1995, Poller et al. 1998a)

By reason of the mathematical complexity of orthogonal regression calibration, the procedure based on linear regression has been used as an alternative. In one study, linear regression gave as good results as orthogonal regression for local INR (Poller et al. 1997).

It has recently been reported that the conventional orthogonal regression gives good results for local ISI, while results based on linear regression are inappropriate (Poller et al. 1998b). The authors are inclined to use orthogonal regression, instead of improving the INR system.

The use of certified plasma calibrators is recommended for reducing INR variation. The need for consensus guidelines on the use of plasma calibrators for INR standardisation has been recognised (Adcock and Duff 2000).

The alternative means of determining the International Sensitivity Index is the use of freshly pooled plasmas from 20 normal individuals and 60 patients on OAT. These numbers of samples are necessary to obtain a precise calibration line for ISI calculation. Freshly pooled plasma can be used to determine reagent/instrument ISI with acceptable precision, or as good as with the WHO calibration model. Using pooled plasma is however too laborious for smaller laboratories. The calibration model using pooled plasma allows the possibility to consider preanalytical variables, for example, the anticoagulant (EDTA) of the sample (van den Besselaar et al. 1998).

2.2.3.3 ISI calibration in a routine laboratory

Routine laboratories can use the reagent manufacturer's ISI value for the instrument they have. Owing to the large number of different instruments on the market, however, it is not always available from manufacturers. ISI calibration sets for estimation of the ISI value are available from reagent manufacturers and "local calibrators", for example, from Labquality in Finland and Equalis in Sweden. In calibration sets there are two or three PT calibrators with known INR value. One standard is often near the normal plasma value (pooled normal plasma). Coagulation time in seconds is measured in every standard by several estimations (3 - 5) and the mean in seconds is calculated for the equation.

Table 2 and Figure 5 give an example for calculation of the ISI value.

Table 2. Example for estimation reagent ISI value in a routine laboratory. Two calibrators with known ISI values (0.95 INR and 4.03 INR) are measured with reagent (unknown ISI) to obtain clotting time for calculation.

	The mean in seconds	log seconds	INR	log INR
Calibrator 1	20,0	1,301	0,95	-0,022
Calibrator 2	92,0	1,964	4,03	0,605

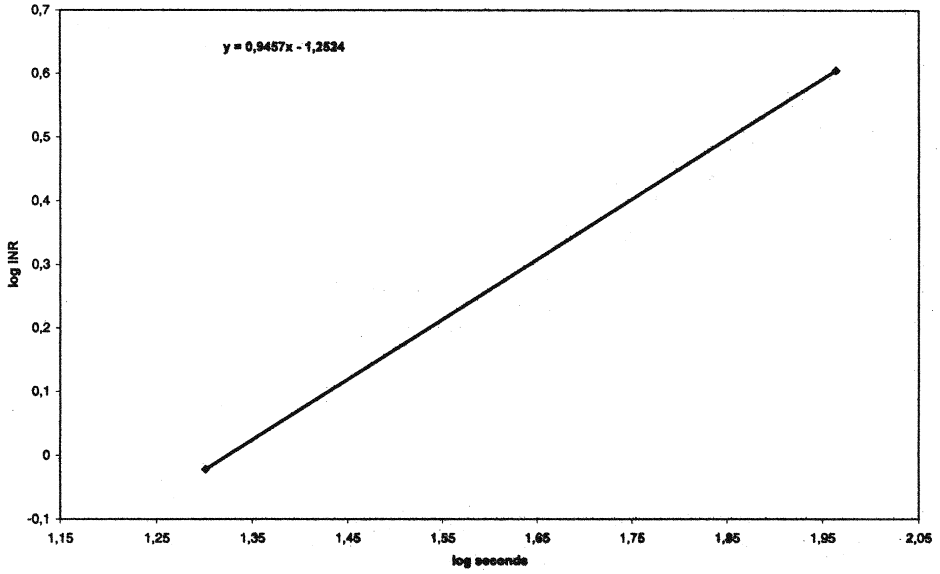

Figure 5. ISI calibration for a reagent using two standards.

The slope of the line presented in Figure 5 is the reagent ISI (0.946). The calibration set does not always include a standard of exactly 1.0 INR, which is the value for normal plasma. The value in seconds for normal plasma can be calculated from the linear equation, when log INR = y = 0 (ratio = 1.0) is x = 1.324, which is exponent to 10 and the normal coagulation time is 21.1 s.

2.2.3.4 Normal plasma calibration

Better precision and accuracy in PT reporting are needed for the INR system as compared with the second or ratio units. One potential source of error is the mean normal prothrombin time (MNPT). This has a major influence in the INR calculation as the denominator. A laboratory can estimate the MNPT from a minimum of 20 healthy individuals with a relatively equal mix of both sexes over a range of age groups. Estimation of a geometric mean is to be preferred to the arithmetic mean, since PT values do not follow the Gaussian distribution. MNPT samples must be fresh. The mean of a laboratory normal control is not an acceptable substitute for the MNPT, since control samples may differ excessively from each other, particularly in the case of less responsive reagents. The MNPT should be determined with each new lot of PT reagent (Adcock and Duff 2000, van den Besselaar et al. 1993, Hirsh et al. 1998).

D'Angelo and associates (1997) made a comparison for mean normal PT (MNPT), fresh normal pooled plasma (FNPP) and for lyophilised normal pooled plasma (R82A). MNPT and FNPP samples were obtained from the same 30 healthy subjects. Mean PT ratios did not differ from 1.00 (mean 1.00, range 1.00 - 1.01) with the use of the MNPT as denominator in the INR calculation equation. Using FNPP as the denominator the difference was significantly > 1.00 (1.02, range 0.96 - 1.05) and the difference was also significant < 1.00 (0.98, range 0.91 - 1.06) when R82A normal plasma was used as denominator. The conclusion was that the system-specific MNPT is the best alternative for normal plasma PT in calculation of INR values.

ISI calibration sets have one standard "normal pooled plasma". This pool is not acceptable for estimation of the MNPT value for a specific reagent and instrument combination (Adcock and Duff 2000, van den Besselaar et al. 1993, Hirsh et al. 1998, D'Angelo et al. 1997). This entails problems for routine laboratories. The MNPT should be estimated using fresh normal plasmas (minimum 20) and the geometric mean should be used. This is too laborious a task for most routine laboratories.

The mathematical calculation for normal plasma coagulation time in seconds might possibly offer a good solution for routine laboratories to cope with MNPT estimation. This is presented in the previous chapter (2.2.3.3).

2.2.3.5 Some other concerns with the INR system

Lack of reliability of the INR system at the onset of warfarin therapy

PT is responsive to warfarin-induced reduction of coagulation Factors II, VII, IX and X. PT reagents vary in their sensitivities to any decrease in these factors, since their plasma half lives are not similar. During the first two to five days from commencement of OAT, PT in INR units does not give the real picture of the situation in vivo, and in this regard the situation is similar to ratio, % and second units. Prolongation of PT is mainly the result of a reduction in Factor VII, with some contribution from a decrease in Factor X levels. In long-term therapy, INR values reflect well the decrease in all three Factors II, VII and X, which are dependent on K-vitamin (Poller 1964, Zucker et al. 1970, McKernan et al. 1988).

Manufacturer ISI and effect of instrumentation

Manual and automated coagulation instruments have varying technical solutions for measurement. Clot detection can be mechanical, static electrical or optical. This causes variability between instruments in clotting point (time) detection and also in ISI values.

Reagents and instruments do not behave in the same way and manufacturer ISI values may be inaccurate (Cunningham et al. 1994, van Rijn et al. 1989). Lot-to-lot variation from the same reagent manufacturer combined with local ISI calibration has been found to yield inconsistent INR results in 84 out of 280 paired samples (30 %) (Ng 1993). These studies were carried out using Quick methods. The College of American Pathologists has stated that laboratories should use reagent/instrument combinations for which the ISI has been established (Fairweather et al. 1998).

2.3 PT methods and modifications

2.3.1 The Quick prothrombin time

Armand Quick (1894–1978) was a pioneer in developing a modern prothrombin time method(Quick 1935, Quick 1937). The "Quick method" is clearly the most widely used PT principle in the world today and most studies reported in the literature have used this method. The method principle is rarely emphasised in the literature, since the Owren method covers perhaps only about 10 % of the markets.

The Quick method measures fibrinogen and Factors II, V, VII and X. It has the advantage in the diagnosis of coagulation factor deficiencies since, it measures Factor V. This, however, is a drawback in oral anticoagulant therapy follow-up, since oral anticoagulant medication does not affect Factor V synthesis. The method can be used to reveal Factor V deficiency.

In the Quick method 50 µl plasma is added to 100 µl reagent and the coagulation time is measured. The reagent volume is 40 % smaller than in the Owren method (140 µl), which means smaller expenses per test. The test can be done by manual method or automated instruments. Sample seconds are converted to INR units. The normal plasma coagulation time is about 10 s. Fibrinogen can be estimated in the same measurement with additional reagents. The proportion of sample in the final coagulation reaction mixture is large, i.e. 33 %. This makes the coagulation reaction in the Quick method more sensitive to preanalytical variables such as heparin and some drugs

than in the Owren method. The sample-derived molecules which may affect coagulation reactions, for example citrate, heparin, drugs and others, are in a six-fold concentration in Quick's PT reaction as compared to that of the Owren method (2.3.2).

PT reagents contain tissue factors extracted from natural sources such as bovine brain, human placenta and brain, and rabbit brain or lung (van den Besselaar 1993). Each of these sources has limitations: rabbit brain thromboplastin shows some seasonal variability and lot-to-lot variability, and even the raw material can be variable. Human tissue factor, again can be a source of HIV and other human viral diseases. Natural sources may contain other coagulation factors as contaminants. Recombinant thromboplastins, of which rabbit tissue and human tissue factor are currently commercially available, were developed to eliminate the drawbacks inherent in thromboplastins obtained from natural sources.

2.3.2 The Owren prothrombin time

Paul Owren (1905–1990) studied Factor V (Owren 1944, Owren 1947) and began to develop a Thrombotest (combined thromboplastin reagent) making long series of experiments with OAT patients. He attempted to overcome the drawbacks of the Quick method (Factor I and V added to reagent) (Owren and Aas 1951, Owren 1959). The Owren method is predominant in the Nordic countries, Benelux and Japan, but is also used on a lesser scale in other countries, for example, elsewhere in Europe (Hepato Quick, Roche). This method measures coagulation Factors II, VII and X. Bovine plasma (adsorbed with $BaSO_4$) is used as source to add fibrinogen (Factor I) and Factor V to the thromboplastin reagent. This method has advantages over the Quick method since it does not measure Factor V, which is not decreased in OAT. The method cannot thus be used to detect Factor V deficiency. In theory, it is the only PT method for OAT if the patient has a specific Factor V deficiency. In the Owren method 10 µl of plasma and 50 µl of diluent are added to 140 µl of reagent, where after the coagulation time is measured. Sample seconds are converted to INR units. The normal plasma coagulation time is about 20 s, which is twice as long as the Quick normal coagulation time. The small sample volume (5 %) in the reaction mixture makes Owren PT less sensitive to preanalytical variables such as citrate and heparin in the sample. This low sample/reagent relation may also affect PIVKA inhibitors (Protein Induced by Vitamin K Absence or Antagonists; see Chapter 3 below). The reagents used contain tissue factor extracted from natural sources such as bovine, monkey or rabbit brain.

2.3.3 Point-of-care testing (POCT) measurement

Point-of-care (POCT) technology offers a new sampling mode and measurement principle for monitoring patients on OAT. The instruments are suitable for self-testing at home. They use non-anticoagulated capillary whole blood as sample. POCT instruments measure the whole blood clotting time with thromboplastin activation and seconds are converted to the equivalent plasma PT and the results are expressed as a ratio, percentage or INR.

PTs measured by POCT technology has be compared to laboratory methods in a number of investigations, many of which report good agreement. Correlation coefficients such as 0.96, 0.95, 0.91, 0.96 and 0.91 have been published (Lucas et al. 1987, Yano et al. 1992, Weibert and Adler 1989, Oberhardt et al. 1991, Ansell et al. 1995).

Some investigators have found poorer concordance between POCT and traditional methods; Jennings (1991) and Rose (1993), for example, arrived at a correlation coefficient of 0.86 in comparison between the POCT and the reference laboratory methods.

Patient self-testing was studied randomised when 163 patients tested PT in INR units at home and venous samples from 162 patients were analysed in a laboratory. Over a 6-month period investigators recorded a major haemorrhage rate of 12 % in the latter group vs 5.7% in the self-testing group. Patient self-testing gave good results in this study (Beyth and Landefeld 1997).

Criticism may be made of correlation studies between POCT and standard laboratory methods in that only correlation summaries are presented. Individual INR results and their clinical acceptability have not been provided, in contrast to the recommendation given by Bland and Altman (1986).

In all comparisons between POCT and standard laboratory methods Quick's PT method has been used as reference. The Owren and Quick method results do not spesify well, and this variability may cause problems if patients who use Quick PT-based POCT are tested exceptionally in laboratories operating with the Owren method (Monsen 2000, Horsti 2001b).

Poller and associates (2002) studied the possibility of making ISI calibration easier and using fresh plasma instead of using fresh whole blood samples for POCT instrument calibration. PT plasma could be used for accurate ISI calibration with two of three POCT (whole blood) instruments.

The use of POCT measurements for OAT control is on the increase and there is an urgent need for guidance and recommendations for clinicians and patients. Fitzmaurice and Machin (2001) have published a guide for clinicians, based on self-management, in which patients themselves measure INR values and interpret results for medication.

2.4 Single or duplicate analysis for automated PT determination

PT measuring techniques have been developed from manual to automated during the last few decades. In smaller laboratories and world-wide, automated instruments are not necessarily available. For coagulation tests an old tradition has been to use manual methods to estimate the mean of duplicate analyses in order to confirm reliable results. This practice is still recommended for manual or partly manual techniques. Ivey and colleagues (1997) studied 4152 PTs using a coagulation analyser and found statistical agreement between the traditional mean duplicate result and the single test. In practice, many laboratories equipped with automated coagulation analysers have adopted a single determination strategy.

3. ORAL ANTICOAGULANT THERAPY AND PIVKA INHIBITION

The discovery of dicoumarol made it possible to inhibit thrombosis and to study anticoagulation therapy for humans in the early 1940s (Butt et al. 1941, Townsend and Mills 1942, Marple and Wright 1950). Dicoumarol, the first drug for OAT, was subsequently found to have drawbacks by reason of its long half-life. Link (1959) synthesised more than 150 anticoagulant compounds and found one particularly active molecule which was named warfarin (a 4-hydroxy compound). Warfarin is now the most widely used drug world-wide for OAT, and it has a predictable onset and duration of action and excellent bioavailability. It is rapidly adsorbed from the gastrointestinal tract (within 90 min) and its half-life is 36-42 h. In the circulation warfarin is bound to plasma proteins (mainly albumin). It accumulates rapidly in the liver, where it is metabolised (Duxbury and Poller 2001, Hirsh et al. 1998).

Warfarin interferes with the cyclic interconversion of vitamin K and its 2,3epoxide (vitamin K epoxide) and is thus a vitamin K antagonist. Vitamin K is a cofactor in carboxylation of vitamin K-dependent coagulation factors and is necessary for protein synthesis (coagulation factors). OAT inhibits the synthesis of coagulation Factors II, VII, IX and X in the liver. In addition, the vitamin K antagonists limit the carboxylation of the protein C and S and impair their function (Hirsh et al. 1998). In Finland Marevan (Pharmacal AB) is the only drug available for OAT.

As mentioned above, most PT determinations are used for monitoring the efficiency of OAT.

The history of PIVKA (Protein Induced by Vitamin K Absence or Antagonists) has been involved with that of the Thrombotest (PT reagent of Nycomed, Oslo, Norway). This test showed very high sensitivity to the coumarin-induced defect in the coagulation system. Originally this could not be measured by any other thromboplastin method (Gilhuus–Moe 1984), and the phenomenon was a target of many investigations.

Hemker and colleagues were the first to characterise the role of PIVKA proteins. They reported the important discovery that coumarin therapy is associated with the occurrence of an endogenous inhibitor, which they later named PIVKA (Hemker et al. 1963, Hemker 1964). The proteins in question were pre-stages of the vitamin K-dependent four factors. They inhibited the

prothrombin-converting complex, presumably against Factors X - Xa (Hemker 1968).

PIVKA factors have a structural defect. They lack gamma carboxyglutamic acid, which is necessary for calcium binding and thereby for "adsorption" of these factors to phospholipid surfaces (Stenflo 1977).

The exact mechanism whereby PIVKA molecules inhibit bovine brain thromboplastin (Thrombotest) more forcibly than other thromboplastins is unknown. Rabbit and human brain thromboplastins and most PT reagents have a low sensitivity to the PIVKA inhibitors, as compared with the bovine brain thromboplastins. The activity of PIVKA inhibitors can be measured indirectly using sensitive and non-sensitive reagents and calculating their difference. The inhibition varies individually in patients on stabilised anticoagulant therapy.

It can be concluded that PIVKA inhibition is important for both bleeding complications and for the antithrombotic effect of oral anticoagulants.

PT methods should measure the total effect of anticoagulant drugs on blood coagulation, including reduction of K-dependent clotting factors and PIVKA inhibition (Owren 1971).

PIVKA inhibition causes problems in INR (ISI) calibration when plasmas from patients on OAT are used as standards. Talstad has developed a method using Thrombotest estimation and an equation for correcting any other PT assay to obtain PIVKA-corrected coagulation activities (Talstad 1996): $PT_y = c\ PT_x + d$

4. AIMS OF THE STUDY

I primarily became interested in the question whether EDTA samples can be used for erythrocyte sedimentation rate measurement, and this led me to investigate the suitability of EDTA samples also for PT determination. Living in the Nordic countries, where the Owren method was developed and is almost the only one used today, I could not resist the temptation to compare the Quick and Owren PT methods in greater detail. The specific objectives in this present work were:

4.1 Using EDTA samples for PT

- To establish whether an EDTA sample is suitable for PT measurement by the Owren method.
- To investigate Ca^{++} level and / or effect in reaction mixtures (citrate and EDTA samples) **(I)**.
- To establish the agreement of INR and time (second) results in EDTA and citrate anticoagulated clinical samples (Owren's PT) obtained from patients on OAT.
- To investigate the correlation between INR results of citrate and EDTA samples.
- To compare ISI values obtained with citrate and EDTA samples **(II)**.
- To ascertain the stability of EDTA plasma at room temperature for PT measurement **(III)**.

4.2 Comparison of the Owren and Quick methods from patient samples on OAT

- To compare one Owren and one Quick method using two anticoagulant concentrations (0.129 mol/l and 0.109 mol/l).
- To study the effect of sample citrate concentrations on the ionised Ca concentration in reaction mixtures by both Owren and Quick methods.
- To compare the PT INR results from clinical samples with two different Owren reagents **(IV)**.
- To compare PTs from patients on OAT using two sensitive Quick and two sensitive Owren reagents.

- To compare the influence of different reagents on patient INR results obtained by the Owren and Quick methods, respectively.
- To establish the superiority of the Quick or the Owren method using different reagents for INR result harmonisation **(V)**.

5. MATERIALS AND METHODS

All procedures were approved by our institution's responsible committee in accordance with the Helsinki Declaration of 1975 **(I -V)**.

5.1 Patients and sampling

We studied the difference of PTs between 123 paired patient samples in both coagulation tube (citrate) and EDTA tube. PT was measured and reported in seconds, per cent and INR units. Sample tubes were centrifuged at 1560 g for 10 min. at 12° C to separate plasma. For 31 of the same patients, ionised Ca^{++} concentrations were measured in the reaction mixtures for both sample types **(I)**.

We studied 107 patients on oral anticoagulant therapy, using paired samples. In both the coagulation tube (citrate) and the EDTA tube, PT was measured and reported in seconds and INR. Sample tubes were centrifuged at 1560 g for 10 min. at 20° C to separate plasma **(II)**.

We studied 62 (glass tubes) paired EDTA samples from patients on OAT. The baseline measurement commenced as soon as possible within 2 hours of blood collection. Sample tube 1 (fresh) was opened within 2 h of collection and analysed. The same sample tube 1 was analysed 4 h after sampling from the opened tube at room temperature. Sample tube 2 was opened 6 h after sampling and analysed. Sample tubes were centrifuged at 1560 g for 10 min. at 20° C to separate plasma **(III)**.

A total of 145 patients were sampled with citrate coagulation tubes containing 0.129 mol/l (3.8 %) citrate solution and 157 patients were sampled with citrate coagulation tubes containing 0.109 mol/l (3.2 %) citrate solution. In both cases PT was measured and reported in INR units. Sample tubes were centrifuged at 1560 g for 10 min. at 20° C to separate plasma.

In 30 of the same patients, ionised Ca^{++} concentrations were measured in the reaction mixtures for both sample types (0.129 mol/l and 0.109 mol/l) and for both methods **(IV)**.

We studied 179 patients for Quick PT and 137 patients for Owren PT, all of them receiving oral anticoagulant therapy. PT was measured and reported in seconds and INR. Sample tubes were centrifuged at 1560 g for 10 min. at 20° C to separate plasma. **(V)**.

5.2 Instruments, reagents and methods

The instruments and methods used in prothrombin time and calcium determinations are summarised in Table 3.

Table 3. Different research topics and methods used in their investigation

Original article[a]	Topic of investigation	Equipment	Reagents			Reaction mixture
			Reagent	Diluent	Others	
(I)	Suitability of EDTA sample for PT determination	ACL 1000 (Instrumentation Laboratory, Milano, Italy	Nycotest PT (ISI, 1.21), Nycomed Pharma As, Oslo, Norway	Citrate-barbiturate buffer (Nycomed)	Normal plasma (Normal kontroll-plasma 160), Global Hemostasis Institute, Linköping, Sweden	Reagent 100 µl, diluent 42 µl, sample 8 µl
(I)	Follow up of Ca^{++} concentration during the coagulation reaction	IL1640 blood gas analyser (Instrumentation Laboratory)	Nycotest PT (ISI, 1.21; Nycomed)	Citrate-barbiturate buffer (Nycomed)	Controls: Test Cal 1 and Test Cal 2 (Instrumentation Laboratory)	Reagent 300 µl, diluent 126 µ, sample 24 µl
(II)	Comparison of ISI values from EDTA- versus citrate-anticoagulated samples	ACL 7000 (Instrumentation Laboratory)	Owren's PT (ISI 1.22; Global Hemostasis Institute)	Owren's buffer, (Global Hemostasis Institute)	ISI calibrator Etaloquick (Diagnostica Stago, Asnieres, France). In-house normal plasma calibrator (pool from 8 donors)	Reagent 140 µl, diluent 50 µ, sample 10 µl
(III)	Stability of EDTA plasma at room temperature	ACL 1000	Nycotest PT (ISI, 1.16)	Citrate-barbiturate buffer	Calibrator (Calibration Reference Plasma 100%, Nycomed). Normal plasma (Normal kontroll-	Reagent 100 µl, diluent 42 µl, sample 8 µl

					plasma 160, NKP 160)	
(IV)	Comparison of Owren and Quick PT methods using same calibrators	ACL 1000, ACL 7000	Nycotest PT (ISI, 1.006), Owren's PT (ISI, 1.22), PT Fibrinogen Recombinant (ISI, 1.12; Instrumentation Laboratory, Lexington, USA)	Citrate-barbiturate buffer (for Nycotest PT), Owren's buffer (for Owren's PT)	ISI calibrator Etaloquick, calibration plasma (Instrumentation Laboratory)	Reagent 100 µl, diluent 42 µl, sample 8 µl (ACL 1000); reagent 140 µl, diluent 50 µ, sample 10 µl (ACL 7000)
(IV)	Effect of two different sample citrate concentrations on Ca^{++} in Owren and Quick PT reaction mixtures	IL1640 blood gas analyser	Nycotest PT (ISI, 1.006), Owren's PT (ISI, 1.22), PT Fibrinogen Recombinant (ISI, 1.12; Instrumentation Laboratory)	Citrate-barbiturate buffer (for Nycotest PT), Owren's buffer (for Owren's PT)	Controls: Test Cal 1 and Test Cal 2	Reagent 280 µl, diluent 100 µ, sample 20 µl (Owren's PT); reagent 200 µl, sample 100 µl (Quick's PT)
(V)	Effect of different reagents on Quick's and Owren's PTs	ACL 7000	PT Fibrinogen Recombinant, Dade Innovin (Dade Behring, Marburg, Germany), Nycotest PT, SPA 20 (Stago)	Citrate-barbiturate buffer (for Nycotest PT), SPA buffer (Stago)	ISI calibrator Etaloquick, NKP160, abnormal plasma (OKP 165, Global Hemostasis Institute)	Reagent 100 µl, sample 50 µl (Quick's PT) reagent 140 µl, diluent 50 µl, sample 10 µl (Owren's PT);

[a] For more detail, see the original article

5.3 Statistics

Correlation analyses were made using either the linear least-squares regression method or the power functions. MS Excel 5.0 algorithms were used for distribution fitting. Difference plots and justification for clinical use were prepared according to Bland and Altman (1986):

x-axis represents the average of results and y-axis represents the difference of the results. The bias and 2 SD limits were calculated from differences.

6. RESULTS

6.1 EDTA samples for PT determination **(I)**

Owren PTs were determined from 123 patient samples, anticoagulated with either citrate or EDTA. A similar PT range was obtained from both types of samples: citrate samples from 15.8 sec (0.89 INR) to 110.0 sec (9.34 INR) and EDTA samples from 15.4 sec (0.86 INR) to 95.6 sec (7.85 INR). A very close linear correlation was observed in PTs when the two types of samples were compared. The regression equations for PT estimations from citrate plasma (y) and EDTA plasma (x) were:

$y = 1.17\ x - 2.37$ seconds, $R^2 = 0.998$
$y = 1.20\ x - 0.162$ INR, $R^2 = 0.998$

The mean Ca^{++} concentration in the reaction mixture with citrate samples was 1.52 mmol/l (S.D.0.07) and with EDTA samples 1.71 mmol/l (S.D.0.07). The reaction mixture Ca^{++} concentration was always higher with the EDTA than with the citrate sample. On average, the difference was 0.183 mmol/l or 12%.

6.2 Comparison of ISI values using citrate and EDTA plasmas **(II)**

Owren PTs were measured with 107 paired samples (EDTA and citrate) for comparison of the ISI values of each sample type. Samples represented a large variety of PTs from normal to therapeutic and even to highly prolonged: PT results with citrate samples were between 0.83 INR (17.2 sec) and 5.48 INR (81,0 sec). As in the previous case **(I)**, a very good correlation of PTs was observed with both sample types. The regression equations for PT estimations with Owren's PT from citrate plasma (y) and EDTA plasma (x) were:

$y = 1.11\ x - 0.24$ INR, $R^2 = 0.99$
$y = 1.10\ x - 3.88$ sec, $R^2 = 0.99$

Interestingly, different regression parameters were obtained depending not only on dilution and anticoagulant but to a greater extent on the PT reagent. INR results from citrate and EDTA samples were similar enough for clinical purposes as justified using the statistical criteria proposed by Bland and Altman (1986). This indicates that EDTA samples are as reliable as citrate samples for PT determination provided that citrate calibrators and correlation equation (INR) are used. Only 4 out of 107 results were out of range (± 2 SDs). The similar suitability of citrate and EDTA samples was also demonstrable when seconds (time) instead of INR units were used. EDTA samples were also clinically acceptable when seconds instead of INR units were evaluated by Bland and Altman (1986) criteria.

ISI values were determined for citrate and EDTA samples from the same 107 patients. A power function correlation ($y = 0.98\ x^{1.333}$, $R^2 = 1.00$) was observed between citrate INR (y) and EDTA PTs when expressed as ratios (x) ($sample_{sec}$ / $normal_{sec}$). Surprisingly, the ISI values for citrate and EDTA samples were different. The ISI for citrate samples was 1.216 and that for EDTA samples 1.333, a difference of 0.117 (10 %).

The clinical significance of this difference in INR values obtained from citrate versus EDTA samples was evaluated using the Bland-Altman criteria. With ISI 1.33 ($0.98\ x^{1.333} \sim 1.0\ x^{1.333}$) the mean difference was -0.06 INR-units (±2 SD; from –0.20 to 0.08 INR). Thus, according to the criteria, INR results from citrate versus EDTA samples showed no statistically significant difference ($p = 0.65$).

6.3 Stability of EDTA samples for PT determination **(III)**

The stability of EDTA-anticoagulated whole blood samples was investigated in 63 patients on oral anticoagulation therapy. To this end, Owren's PT method was used. The mean INR value changed very little, if at all, during the six hour's observation period. The mean baseline PT value, representing analyses performed within two hours from the sampling, was 2.32 INR. Those after four and six hours were 2.30 INR and 2.29 INR, respectively. The mean difference (bias) four hours after sampling was +0.023 INR units (± 2 SD from -0.085 to 0.131 INR units). One sample was out of this range, the difference being 0.22 INR (5 %). The Student t-test showed no statistically significant change ($p= 0.89$). This is in agreement with the Bland-Altman interpretation (1986); there is no clinically significant difference between results after four hours.

The mean difference seemed to increase with time; six hours after sampling the difference from the base-line value was +0.032 INR units (± 2 SD

from -0.138 to 0.202 INR units). Four samples were out of range and differences between results were 0.28 (10 %), 0.22 (8 %), 0.21 (6 %) and -0.15 (4 %) INR units. The mean difference was not statistically significant when analysed by Student t test (p= 0.85). According to the Bland-Altman interpretation there is a probability of 93.5% that the difference is not clinically significant from the base-line value if the sample is stored for six hours at room temperature.

6.4 Comparison of Owren and Quick PT methods **(IV)**

We first compared whether two different Owren reagents yield similar PT times. For this purpose the PTs of 145 patients were determined. The regression equations for PTs determined from citrate plasma (0.129 mol/l) revealed a very close correlation: Nycotest PT (x), Owren PT (y), INR, $y = 1.08\ x - 0.15$ ($R^2 = 0.99$).

No such a good correlation was observed between the Owren and Quick methods; in the same samples, the correlation between Owren and Quick PTs was not strictly linear. In fact, an exponential fitting gave the best correlation, the corresponding equation being: Owren PT (x), and Quick PT (y), INR, $y = 0.98\ x^{1.20}$ ($R^2 = 0.95$)

The comparison of the two PT methods was carried out on three different INR levels, namely < 2 INR units (mild anticoagulation and normal coagulation capacity), 2-4 INR units (effective anticoagulation), > 4 INR units (excess anticoagulation). Owren (Nycotest PT) and Quick PT results were in very good agreement if the INR value was less than 2, and the sample citrate concentration (0.129 mol/l and 0.109 mol/l citrate samples) did not significantly affect the results. However, if the INR was larger than 2 INR units, the PT means for Owren and Quick methods were different and the difference increased exponentially as the INR became larger using with both citrate concentrations. This difference was also clinically significant when assessed with the Bland-Altman plot. The difference in PT values obtained by the Owren versus the Quick method was larger than 0.5 INR units in a considerable number of cases. For example, within the therapeutic range or PT = 2 - 4 INR units, the proportion of cases evincing this marked difference was 31/83 (37 %), (citrate 0.129 mol/l); 28/103 (27 %), (citrate 0.109 mol/l). INR results using Quick method were higher.

As described above, the results obtained by the two commonly used PT methods, Owren and Quick, were very different. Both are in routine clinical use. In order to improve agreement a mathematical solution was sought. Because most clinical treatment recommendations have been based on the

Quick method, the Owren results were corrected to correspond to Quick PT values. This was first done with samples containing the higher citrate concentration (0.129 mol/l). The best agreement in PTs was obtained if ISI was corrected according to the correlation equation, INR = $(sample_{sec} / normal_{sec})^{1.21}$. A change in Owren ISI from 1.006 to 1.21 notably improved the correlation of the results. After correction, the number of outliers (2 SD) was 8/145 (mean difference 0.07 INR; ±2 SD limits from -0.55 INR to 0.69 INR). Hence, 137/145 (94%) of the PT values obtained by the two methods were in agreement (after correction of Owren PTs). This is very close to the Bland-Altman requirement (95%) for clinical applicability of both of these methods.

A similar correction of Owren PT times was made using samples with lower citrate concentration (0.109 mol/l). The regression equation was: for the Owren PT (Nycotest PT) (x) and Quick PT (y), INR, $y = 1.007\ x^{1.14}$ ($R^2 = 0.97$). From the Bland-Altman difference plot it could be determined that the mean difference between Owren versus Quick PTs was -0.33 INR units, (±2 SD from -0.99 to 0.33 INR units).

The agreement of the two methods improved significantly when the ISI of the Owren method was corrected according to the correlation equation, INR = $(sample_{sec} / normal_{sec})^{1.15}$. After ISI correction from 1.006 to 1.15 only nine PTs were outside the ±2 SD range. After ISI correction the mean difference between Owren and Quick PTs was -0.01 INR units (±2 SD from -0.49 to 0.47 INR units). According to the Bland-Altman approach, 8/157 results were outside these limits. With a probability of 94% (149/157) the results of the two methods were thus in clinical agreement

6.5 Ca^{++} concentration in coagulation reaction mixture: comparison of Owren and Quick methods **(IV)**

The Ca^{++} concentrations were measured in a coagulation reaction mixture of 30 patient samples. This took place after clot formation. A significant difference was observed between Owren's versus Quick's methods. Furthermore, the sample citrate concentration affected the Ca^{++} concentration in Quick's reaction mixture ($p<0.0001$) but not in the Owren mixture ($p=0.79$). The respective Ca^{++} concentrations were: Owren (citrate 0.129 mol/l) 2.43 mmol/l (SD=0.12 mmol/l), Owren (citrate 0.109 mol/l) 2.44 mmol/l (SD=0.07 mmol/l), Quick (citrate 0.129 mol/l) 1.48 mmol/l (SD=0.20 mmol/l), Quick (citrate 0.109 mol/l) 1.69 mmol/l (SD=0.16 mmol/l).

6.6 Effect of different reagents on Quick and Owren PTs (V)

The agreement of results tested by two Quick reagents and two Owren reagents was assessed; 179 samples were tested with Quick and 137 with Owren reagents. Smaller pairwise differences were observed in PT determined with Owren reagents as compared to those determined with Quick reagents. In order to bring the results to the same arbitrary level, the same calibrators were used for each of the four reagents. This procedure succeeded very well, as indicated by similar mean INR values in both patient groups investigated: Quick; PT-Fibrinogen Recombinant 2.55 INR and Dade Innovin 2.49 INR. Owren; Nycotest PT 2.32 INR and SPA 20 2.31 INR.

The Bland-Altman evaluation revealed a very large scatter of results when Quick methods were used: mean difference was 0.05 INR units (±2 SD from –0.53 to 0.63 INR units). A much smaller scatter was observed when Owren methods were used: mean difference was 0.01 INR unit (±2 SD from –0.11 to 0.11).

7. DISCUSSION

7.1 EDTA samples for PT determination **(I-III)**

EDTA is a versatile anticoagulant for haematology. The central areas have been cell counting and morphology. More recently, the use of EDTA plasma has been introduced in blood compatibility testing. From the point of view of sample logistics and economy it would be beneficial if as many analyses as possible could be performed from the same sample; and *vice versa*, the use of citrate samples is limited to coagulation tests only.

We have previously shown that EDTA samples are suitable for erythrocyte sedimentation rate determinations (Horsti and Kovanen 2000, Horsti 2001c). In the present series of investigations the suitability of EDTA samples for PT determinations was assessed. Direct comparison of EDTA and citrate anticoagulation demonstrated an excellent correlation between the PT results obtained with both sample types. Furthermore, the clinical applicability, as assessed using the method of Bland and Altman (1986), was equally good with both sample types. We also demonstrated that EDTA sample stability did not differ from that of citrate samples. This information is important for larger units and regional services where sample transportation is required.

To the best of our knowledge, EDTA samples have not previously been used for PT determinations. The implication is that necessary EDTA-based standards are not commercially or otherwise available. We here demonstrated that ordinary citrate calibrators can be used to calibrate PT determinations for EDTA samples. As shown, a correlation equation between citrate and EDTA samples has to be calculated. Owing to the linear correlation, however, this is an easy task. The ISI was not same for EDTA and citrate samples.

7.2 Comparison of Owren and Quick PT methods **(IV-V)**

Prothrombin time is the most widely used and predominantly mode of coagulation analysis. The main clinical indication is the follow-up of oral anticoagulation therapy. According to the generally accepted recommendation, PTs are reported as INR values. It is generally assumed that, independently of reagents and instruments, INR results are globally comparable. From this point of view it is surprising that the similarity of various methods has not

been confirmed in practical investigations. None has been reported in the literature.

The present investigation demonstrated unequivocally that PT results differ substantially depending on the methods and reagents used. Striking differences (Quick INR results were higher than Owren INR results) were observed in PT values which were at a commonly used treatment level, i.e., from 2 to 4 INR units. The differences were even larger when the values exceeded 4 INR units. The aim of the present work was not to assess the accuracy of the results. In principle, this could have been done using the primary standard IRP. We demonstrated, however, that the agreement of different methods can be markedly improved by an ISI correction. We used Quick PT as "basic" PT method and calculated the ISI correction accordingly for the Owren PT results. The reason was that Quick's PT is in much wider use and that most clinical anticoagulation investigations have used precisely Quick's PTs.

We confirmed the previously known fact that Quick's PT is sensitive to citrate concentration (Adcock et al. 1997a, Chantarangkul et al. 1998, Duncan et al. 1994). The relatively large proportion of citrate sample (33%) in the coagulation reaction mixture may alter the final calcium concentration in an unpredictable way and thus affect the PT and cause variation. The variation in Ca^{++} concentration in Quick's reaction mixture was demonstrated here for the first time. In contrast, the Owren method is free of these disadvantages; the final concentration of citrate sample in the reaction mixture is only 5%.

The INR system is highly dependent on the standardisation of reagents and sample. The results of the present investigation revealed the unfortunate situation that different reagents may cause unacceptable variation in PTs. This proved to be the case when two Quick reagents were compared. The two investigated Owren methods, in contrast, were in very good agreement and had less differences in INR results than Quick methods.

In conclusion, the EDTA sample is a good alternative for the traditional citrate sample for PT determinations. Surprising and clinically unacceptable differences in INR values were observed when different methods (Quick vs Owren) were compared. This indicates that more rigorous harmonisation is required to correct ISI value for Owren reagents. Our results give new support to the view that Owren's PT is less sensitive to interfering sample factors that Quick's PT. The present work furthermore suggests that Owren's PT may show less variation between individual reagents.

8. SUMMARY AND CONCLUSIONS

Prothrombin time determination (PT) is widely used for oral anticoagulant therapy monitoring. Three major aspects of PT were investigated in this work. First, I it was assessed whether a non-traditional anticoagulant (EDTA) can be used for PT. Secondly, the concentration of ionised calcium (Ca^{++}) in the coagulation reaction mixture was investigated, for the first time, using two different anticoagulants and Owren and Quick PT methods. Thirdly, the agreement of PTs measured by Owren and Quick methods was compared.

EDTA samples were found suitable for PT determination (Owren method). This was confirmed using pairwise coagulometric PT determinations; samples from the same patients were anticoagulated either with citrate or with EDTA. A very good linear correlation between PT results from EDTA versus citrate samples was demonstrated. EDTA sample calibrators are not currently available. Hence, citrate calibrators and the correlation equation, as described in this work, must be used. The clinical validation of the EDTA method was further confirmed by the statistic approach described by Bland and Altman (1986). EDTA samples could safely be maintained at room temperature for six hours without changes in the PT. This makes EDTA a suitable anticoagulant for transported samples.

The Ca^{++} concentration in the coagulation reaction mixture was measured with ion-selective electrodes. EDTA samples resulted in higher Ca^{++} concentrations than citrate samples. This is obviously a benefit, since Ca^{++} is less likely to be a limiting factor in the coagulation reaction. The Ca^{++} determination approach, as described here, may be useful in scientific investigation of the role of various coagulation factors. Furthermore, it remains to be investigated whether very low blood Ca^{++} concentrations in exceptional patients might result in suboptimal coagulation reactions in vitro.

The reaction mixture Ca^{++} concentrations were also compared in Owren and Quick PT reaction mixtures. Two sample citrate concentrations were used. With this approach it was possible, for the first time, to prove that the Owren method is not dependent on the sample citrate concentration. In contrast, the results of the present investigation confirmed that the Quick method might be affected by the citrate concentration in the sample. It is noteworthy, however, that this is the first time that Ca^{++} determination has been used for this purpose.

The Owren and Quick methods were compared by measuring PTs from the same samples from patients on oral anticoagulant therapy. The same

calibrators had to been used in order to harmonise the results to a common level. A poor correlation was observed between Owren and Quick PT results. This information is novel, since no large-scale direct comparisons have previously been made. The data-fitting approach demonstrated that the best correlation was obtained using a power function.

Since the Quick PT is "a world standard" it seemed reasonable to attempt to calibrate Owren results to the same level as Quick results. To this end an ISI correction had to be developed. By means of this correction and the Bland-Altman approach (1986) in evaluation of the clinical acceptability of the Owren versus Quick results, a 94% agreement level was observed. This comes very close to the 95% level the recommended limit for justification for the clinical use of the two methods.

Comparison of Quick and Owren methods was further made using two Quick and two Owren reagents. The same calibration was used for all methods. This should eliminate calibration-based bias when the results are expressed as INR units. The PTs, as determined by the two Quick methods, were different, and could not be accepted for clinical use when evaluated by the Bland-Altman approach. In contrast, the two Owren assays gave very similar results. On this basis, the Owren method seems to be more suitable than the Quick for INR harmonisation.

The main practical implications:

- EDTA samples are suitable for Owren PT method and samples could be preserved six hours at room temperature.
- The Quick PT method is dependent on sample citrate concentration while the Owren PT is not.
- Owren PT results should be calibrated to the same level as Quick results by changing Owren ISI value.
- The Owren method is more suitable than the Quick method for INR result harmonisation when different reagents are used.

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