THE PROTHROMBIN TIME*

Prepared on behalf of the World Health Organization

by

L. Poller, DSc., European Concerted Action on Anticoagulation (ECAA)
Department of Pathological Sciences, The University of Manchester
Manchester, UK

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1. THE PT TEST

It is over 60 years since the prothrombin time (PT)* test (scientific name - tissue factor induced coagulation time) was first introduced by Quick (1). It was designed to measure a coagulation defect before the introduction of oral anticoagulants, and later adapted for monitoring their dosage. The PT measures changes in factors II, VII and X, three of the principal clotting factors depressed by coumarin drugs. The PT also reflects changes in factor V which is not reduced by oral anticoagulation. Although specific methods were subsequently developed for anticoagulant control, e.g. Prothrombin and Proconvertin test (2) Thrombotest (3) and native prothrombin antigen (4), Quick’s prothrombin time has continued in most countries to be the exclusive control method. In the PT the clotting time of recalcified citrated plasma is accelerated by a thromboplastin which is an extract of tissue factor (TF). The test depends on the activation of factor X in the presence of factor VII by TF and the bypassing of the intrinsic clotting pathway. The speed of the reaction and the responsiveness of the PT to deficiencies of clotting factors depend upon the properties and concentration of the TF as well on the clotting factor concentrations.

2. TISSUE FACTOR

TF extracts vary in their procoagulant properties and their ability to detect alterations in individual clotting factors or systems. Responsiveness of the PT to depression of the extrinsic clotting factors depends on the source and type of tissue factor (TF). TF is a combination of an apoprotein and phospholipid. It has been purified and its chemical structure has been defined (5,6).

3. SOURCE OF TISSUE FACTOR

Many modifications of the PT test have been introduced since the original description. Other tissues than rabbit brain have been used as a source of TF including human brain, ox, monkey brain, rabbit lung and human placenta. Recombinant human preparations have been produced in the last few years, consisting of pure TF relipidated with natural or synthetic phospholipids (7-9). TF from animals may be less responsive than human and human recombinant TF to deficiencies of human coumarin-dependent clotting factors, due to “species” effects, or contamination by serum products in processing the small rabbit brains.

3.1 Risks of transmission of infections

In recent years the dangers of virus transmission from human tissue led to the most widely used preparations being derived from rabbit brain but there is now increasing demand for human recombinant preparations. The revised WHO protocol states that thromboplastin of animal origin shall be prepared only from healthy animals (10). Thromboplastins from bovine brain shall be derived only from cattle from countries with no recent cases of BSE and where there is a compulsory BSE notification scheme, compulsory clinical and laboratory verification of suspected cases and a surveillance programme. Human brain tissue should not be used because of the possibility of transmission of Creutzfeldt-Jakob Disease. Thromboplastin derived from human placenta shall be prepared only from persons in whom there is no evidence of systemic microbiological infection or localised infections. Further specifications for thromboplastin apart from the absence of haemoglobin, include low opacity, freedom from infectious agents with particular reference for human placenta to be free of hepatitis B surface antigen, antibodies to human immuno-deficiency viruses HIV 1 and 2, and antibodies to hepatitis C virus (HCV).
4. PRE-ANALYTICAL VARIABLES AND SOURCES OF ERROR

Although the PT is relatively simple, a large number of variables affect its reliability. These include faulty blood collection, haemolysis, the use of siliconised or plastic instead of borosilicate test tubes, speed of centrifugation, the length and bore of glass tubes and the angle and speed of the manual tube tilting. The effects of coagulometers are discussed later.

Further causes of erroneous results include incorrect citrate anticoagulant concentration, presence of heparin, addition of an incorrect volume of blood to anticoagulant (overfilling = undercitration, underfilling = overcitration), partial clotting during venesection or blood collection and the contamination of a blood sample direct from indwelling catheters or intravenous lines.

4.1 Blood volume

Severe anaemia or considerable polycythaemia may give an incorrect result due to changes in the ratio of blood sample to citrate anticoagulant. The same may occur if an incorrect volume of blood is added to the citrate.

4.2 Trisodium citrate anticoagulant

The importance of the correct concentration of citrate has been emphasized recently. A marked difference has been observed on PT results expressed from the use of the two widely employed concentrations i.e. 109 mmol/L (3.2%) and 129 mmol/L (3.8%) (11). The revised WHO recommendation is to use only 109 mmol/L trisodium citrate (10).

4.3 Blood containers

Specimens should be collected and stored in special containers which do not induce contact activation e.g. plastic or siliconised glass tubes (12,13).

4.4 Storage of specimens

Blood should be tested as soon as convenient after collection. An optimum time of 4 hours’ storage of whole blood specimens is suggested for routine work, although it has been shown that citrated plasma stored at room temperature for 6 hours is satisfactory (14) and even longer in moderate ambient temperatures when the INR was shown not to be affected (15). Storage at 4°C may be undesirable owing to cold activation of factor VII which affects some samples. In the presence of high ambient temperatures however, a maximum of 4 hours storage at room temperature seems preferable although 4°C may in these circumstances be advantageous and outweigh risks of cold activation. Plasma should be maintained in a stoppered polystyrene container at room temperature before testing.
5. PREPARATION OF A TISSUE THROMBOPLASTIN EXTRACT

Method of extraction of TF

The following is a method for preparation of thromboplastin from rabbit brain [modified from (16)]

- Obtain fresh tissue (within 4 hours of death at high ambient temperatures, but within 12 hours in temperate climates. The tissue should not be frozen). Remove the cerebellum and peel off the meninges under running tap water. Cut the remaining tissue into slices.
- Large blood vessels and clots should be removed from the surface of the brain.
- Macerate the sliced tissue with a pestle and mortar to a coarse, lumpy consistency. The use of an electric mixer at a slow speed with a whisk attachment for a few seconds simplifies and expedites this stage.
- Add warm (40°C) phenol saline (between 0.5 to 2.0% of phenol in normal saline in a concentration of 90 g/L) and continue to macerate the tissue to a consistent suspension. The duration of maceration is dependent on the type of tissue.
- Incubate the suspension at 37°C for 30 minutes.
- Decant into a sterile glass container and leave at 4°C for 24 hours.
- Centrifuge at 2-8°C. The speed and duration of spinning will depend upon the degree of homogenization (after this the material should form clearly visible layers of sediment). Remove the supernatant and measure its volume. Remove the top layer of the sediment and add one part of this to 5-10 parts of supernatant. Discard the remaining sediment.
- Store the extract at 2-8°C in sterile containers. It is also advisable to add as preservatives an antibiotic cocktail and an antifungal preparation to prevent contamination.

6. TECHNIQUE OF PT TEST

6.1 Manual technique

Reagents

1. Calcium chloride 0.025 mol/L. Prepare from a 1 mole per litre solution. Store at 2-8 °C and the solution will be stable at this temperature indefinitely if not contaminated.
2. Thromboplastin. Store at 2-8°C. It should not be frozen as it will inactivate the TF.
3. Test plasma citrated with 109 mmol/L (3.2%) trisodium citrate.

As cleaning of test tubes for the PT is difficult and may cause problems, e.g. from residual detergent or chemicals, it is advised that new test tubes should be used once only and after this replaced.

Method for manual (tilt-tube) technique

Warm test tubes for performance of the test in a 37°C water bath (tolerance limits 37.0 ± 0.2°C). Chemically clean glass tubes should be used.

1. Resuspend the TF extract by gentle inversion and using an accurate manual or automatic 0.1 mL pipette transfer 0.1 mL into glass test tube in the water bath. Allow to warm for 1-2 min. Thromboplastin should not be allowed to pre-warm in the test tubes for > 1 h.
2. Add 0.1 mL plasma using an automatic pipette or well calibrated manual pipette. New pipetting tips must be used for each plasma with an automatic pipette.

3. After approximately 1 minute add 0.1 mL pre-warmed (37°C) calcium chloride (0.025 mol/L) and start stopwatch.

Tilt gently, keeping the tube under water as much as possible to maintain temperature. The speed and angle of tilting the test tube must be standardised to control glass activation and minimise cooling (three tilts through 90° every 5 sec is recommended). The endpoint, the appearance of the fibrin clot, is recorded.

Tests should be performed in duplicate. If the discrepancy between duplicate tests is more than 5% from the mean, the test should be repeated, e.g. if the mean clotting time is 30 sec, duplicates should range between 28.5 and 31.5 sec.

6.2 **Technique with coagulometers**

Modifications of the above technique with automated and semi-automated procedures have been introduced. The coagulometer manufacturer’s handbook should be consulted for details.

7. **THE NORMAL (REFERENCE) RANGE**

The normal range for the PT test varies with the different laboratory techniques. It is affected by pre-test and analytical variables including the species of TF origin, the potency of the extract as well as the method of endpoint detection and possible ethnic/geographical variations (17). The normal range should be established on a minimum of 20 healthy adults including both sexes over a wide age range of 20-80 years. Each time a brand of thromboplastin or a batch of reagent is changed, this should be re-assessed. The same applies when a coagulometer is replaced or adjusted. The mean normal prothrombin time (MNPT) which is the geometric mean of the 20 healthy subjects is then calculated. A larger number than 20 will give a more reliable normal mean but 20 is the conventional number. For a more dependable range 50-100 healthy subjects is recommended e.g. for a manufacturer’s stated normal values. Normal PT with some coagulometers may be appreciably shorter than with the manual technique (18).

8. **LABORATORY VARIATION IN PT TESTING**

The responsiveness of the PT test to a specific coagulation defect is dependent on the source and type of tissue thromboplastin. When the prothrombin time is used as a measure of the depression of vitamin K-dependent clotting factors, e.g. following oral anticoagulant administration or in liver function testing, PT results vary greatly with different thromboplastins. Using the WHO international system of PT standardisation the overall sensitivity to the depression of factors II, VII and X during oral anticoagulant treatment is quantified numerically as the International Sensitivity Index (ISI) (19-21). The ISI depends on a calibration of the individual thromboplastin extract against the WHO International Reference Preparation (IRP) for thromboplastin or a secondary IRP reagent calibrated in terms of the first primary WHO IRP. The ISI of the first WHO IRP was by definition 1.0. The slope of the orthogonal regression line obtained when the PT with the local thromboplastin of 20 normal and 60 coumarin-treated patients samples are plotted on a double logarithmic scale against the PT with an IRP, is the measure of the responsiveness of the thromboplastin. The orthogonal regression calibration slope (b) of a local reagent against the IRP with which it is calibrated is used to calculate the ISI as follows:
ISI = (b x ISI of the IRP)

For further details of the ISI calculation see the appendix.

A PT with a lower ISI thromboplastin and by definition improved responsiveness to factors II, VII and X also provides a more sensitive assessment of liver function. Not only anticoagulant control but also the severity of patients’ liver disease, liver impairment secondary to other disease and drug over dosages (e.g. paracetamol, aspirin, etc.) may thus be monitored more reliably.

9. THE PT IN DIFFERENT CLINICAL STATES

The PT is prolonged by oral anticoagulation, impaired liver function, biliary obstruction and congenital deficiencies of factors II, V, VII and X. The PT with many reagents may also be prolonged by heparin administration. The PT is considerably prolonged in the neonatal period and to a lesser extent throughout childhood. Some patients with lupus anticoagulants give long PT with some thromboplastin reagents (22). On the other hand, PT may be accelerated in certain pre-thrombotic states, e.g. after administration of high-dose oestrogen oral contraceptives, after surgical operations and post-partum.

9.1 Heparin resistant thromboplastins

Heparin may prolong the PT but this depends on the thromboplastin as well as on the concentration of heparin. Some commercial thromboplastins therefore have an added anti-heparin agent, e.g. polybrene, making them unresponsive to heparin at therapeutic levels. These are for use with combined heparin and warfarin therapy.

10. STANDARDIZATION OF THE PROTHROMBIN TIME

It has long been recognized that PT results show poor agreement when the same blood specimens are tested at different centres. This is mainly due to the differences in the performance of constituent thromboplastins. The problem is aggravated by different methods of reporting results, e.g. as prothrombin activity %, index (the reciprocal of the activity %) or ratio (i.e. patient’s PT/MNPT).

The lack of uniformity has contributed to continuing considerable differences in the intensity of anticoagulation for case-matched patients on a world-wide scale (23). Thus the result on a given plasma may signify overdosage at one hospital and underdosage at another, dependent entirely on the thromboplastin (TF) incorporated in the PT test.
10.1 The WHO standardization scheme

The establishment of a primary reference thromboplastin by the World Health Organization (WHO) provided the basis for the subsequent international standardization of the PT (24-26). In a PT standardization statistical model developed by Charcot (19), the logarithms of PT (sec) with different thromboplastins are related linearly, and this relationship is used to calibrate the thromboplastins. The relationship between the logarithms of PT with the two reagents is estimated using a symmetric procedure of least squares estimation of a functional relationship described as orthogonal regression (27) (Fig. 1). This method differs from linear regression analysis in that the perpendicular or orthogonal distance between point and line is minimized. In the linear regression analysis the vertical distance between point and line is minimized (see Fig. 2). Log PT with the IRP reference thromboplastin are plotted on the horizontal axis and with the local reagent on the vertical. In the orthogonal regression log PT with the reference thromboplastin are plotted on the vertical axis with the local reagent on the horizontal.

The orthogonal regression analysis allows for the fact that individual patients’ prothrombin times determined using both thromboplastins (the IRP and test reagent) show biological and experimental variation about the calibration line. Conventional linear regression analysis, which only takes account of error on one axis (the local reagent axis), is not appropriate for ISI calibration and has been shown to be unreliable in a large field study with coagulometers. The details of the orthogonal regression calibration procedure and calculation are given in the Appendix.

![Orthogonal regression calibration](image)

**Fig. 1 - Orthogonal regression calibration:** measuring the perpendicular distance of the observations from the line. The log10 PT (sec) of the certified IRP values on the vertical (y) and the log10 PT (sec) of the locally determined values for ECAA human thromboplastin on the horizontal (x) axis.
Fig. 2 - *Linear regression calibration:* measuring the vertical distance of the observations to the line. The log 10 PT (sec) of the locally determined values for ECAA human thromboplastin on the vertical (y) and certified IRP log 10 INR values on the horizontal (x) axis.

10.2 **International sensitivity index (ISI)**

For any given thromboplastin this is derived from the slope of the calibration line when the logarithms of PT with the IRP are plotted on the vertical axis against the logarithms of PT obtained with the test thromboplastin on the same plasmas from 20 healthy subjects and 60 stabilized long-term anticoagulated patients on the horizontal. This number of plasmas should provide sufficient numbers of patients to achieve the desirable minimum precision expressed as a CV of the calibration slope of 3% or less.

10.3 **International normalized ratio (INR)**

This is the prothrombin ratio which it is calculated would have been obtained if the first primary WHO reference thromboplastin (67/40 human combined) had been used to perform the test on the blood sample with the manual technique. It is calculated as follows:

\[ \text{INR} = (\text{observed ratio})^{\text{ISI}} \]

Where the ISI is derived from calibration of the local reagent against the IRP.

For example, a working thromboplastin with an ISI of 2.0 gives with a prothrombin ratio of 1.5 an INR of 2.25, i.e.:

\[ \text{INR} = 1.5^{2.0} = 2.25 \]
11. SIGNIFICANCE OF ISI

The ISI represents the average responsiveness of a reagent to the depression of factors II, VII and X of patients during oral anticoagulant treatment as quantified using the WHO international system of PT standardization (19-21,28).

12. DERIVATION OF ISI AND RELATIONSHIP TO IRP

ISI derivation depends on calibration of the individual thromboplastin extract in terms of the first primary WHO international reference preparation (IRP) for thromboplastin (human combined 67/40) or a secondary reference preparation which has been previously calibrated in terms of the first primary WHO. The ISI of the first primary WHO IRP was by definition 1.0. The calibration slope (b) of the orthogonal regression line from which ISI are derived is normally obtained by plotting the log PT of 20 healthy subjects and of 60 long term stabilised coumarin-treated patients’ samples with the local thromboplastin against the log PT of the same plasma samples with the relevant IRP (see Fig. 3 and Section 15). Responsive thromboplastins have an ISI close to 1.0, whereas less responsive reagents have higher values. As a result, anticoagulant dosage to achieve a simple target prothrombin ratio of 2.5 with a high ISI thromboplastin needs to be twice or more the dose required to achieve the same ratio with a low ISI, responsive, reagent. Clinicians were largely unaware of the problem with resulting increased bleeding with the higher doses.

![Graph showing calibration exercise with log PT](image)

*Fig. 3 - Example of a calibration exercise with log PT with the test thromboplastin plotted against the log PT of the IRP. NB. The PT results with the IRP are always on the vertical axis which means that paradoxically increased responsiveness gives a lower ISI.*
13. CONVERSION OF PT TO INR

Manufacturers of thromboplastin are expected to provide the ISI of their reagent. Each package should contain a table or graph to convert results expressed as prothrombin times obtained with the local thromboplastin into INR. An example of a conversion chart is given in Table 1, which shows the relationship between the PT and INR with a working thromboplastin calibrated in terms of the human plain IRP (BCT/441) (see section 14.3).

Table 1 - Relationship between prothrombin times and INR with a low ISI reagent

<table>
<thead>
<tr>
<th>Clotting Time (sec)</th>
<th>INR</th>
<th>Mean normal prothrombin time</th>
<th>Normal range</th>
<th>International Sensitivity Index (ISI) = 1.10</th>
</tr>
</thead>
<tbody>
<tr>
<td>16.0</td>
<td>1.0</td>
<td>15.9 sec</td>
<td>13.9 - 17.9 sec</td>
<td></td>
</tr>
<tr>
<td>17.0</td>
<td>1.1</td>
<td></td>
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<td>19.0</td>
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<td>65.0</td>
<td>4.7</td>
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14. THE HIERARCHICAL RELATIONSHIP OF THE THROMBOPLASTINS

**WHO Biological Standards**

The WHO calibration scheme is hierarchical (see Table 2). Starting with the first WHO primary IRP (ISI = 1.0), the calibration model proceeds successive generations of IRP down to house standards and manufacturers’ production lots. Manufacturers of thromboplastin are expected to produce their own house standard which should be a lyophilized batch of thromboplastin of similar material to the IRP. The house standard is to be set aside for the calibration of production batches and should be produced in sufficient volume to last for several years. The WHO IRP\(^1\) are in very limited supply and are intended only for the calibration of national reference preparations or working reference preparations at “national control laboratories”. With the finite sizes of batches and recommendations that working reagents should be calibrated against IRP of similar composition and of the same species (25), a series of inter-related IRP of human, rabbit and bovine origin have been introduced by WHO and the European Commission Bureau Communautaire de Référence (BCR). For example, a rabbit plain routine preparation should be calibrated in terms of the WHO rabbit plain IRP (RBT/90 or CRM rabbit plain 149S).

**Table 2 - Hierarchical relationships of thromboplastin IRP**

<table>
<thead>
<tr>
<th>WHO 1st primary IRP</th>
<th>WHO 2nd primary IRP</th>
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<tbody>
<tr>
<td>67/40* (ISI = 1.0)</td>
<td>BCT/253* (ISI = 1.085)</td>
</tr>
</tbody>
</table>

Additional primary WHO IRP

| RBT/79* rabbit plain (ISI = 1.4) | OBT/79 bovine combined (ISI = 1.0) | human recombinant rTF/95 (ISI = 0.94) |

\[\text{Discontinued or restricted}\]

\(^1\) They can be obtained from the Central Laboratory for Blood Transfusion, Plesmanlaan 125, 1066CX, Amsterdam, Netherlands
Table 3 - BCR reference thromboplastin²

<table>
<thead>
<tr>
<th>OBT/79</th>
<th>RBT/79</th>
<th>BCT/099*</th>
</tr>
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<tbody>
<tr>
<td>bovine combined</td>
<td>↓</td>
<td>human plain</td>
</tr>
<tr>
<td>(ISI = 1.0)</td>
<td>CRM/149 rabbit plain</td>
<td>(ISI = 1.05)</td>
</tr>
<tr>
<td></td>
<td>↓</td>
<td></td>
</tr>
<tr>
<td>CRM/149R*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(ISI = 1.27)</td>
<td></td>
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<tr>
<td></td>
<td>↓</td>
<td></td>
</tr>
<tr>
<td>CRM/149S</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(ISI = 1.21)</td>
<td></td>
<td></td>
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</tbody>
</table>

* Discontinued or restricted

BCR currently provides on a small scale certified reference thromboplastins CRM/149S (rabbit plain) (29) and OBT/79 (bovine combined) (30). The CRM human plain reagent (BCT/099) has been discontinued and not yet replaced. The CRM thromboplastins are intended to be available to manufacturers of commercial thromboplastins and are of relatively high cost. The International Council for Standardization in Haematology (ICSH) made available to national control laboratories an additional human plain IRP (BCT/441) to bridge the gap in human IRP supplies (31). The present WHO rabbit plain replacement for RBT/79 is RBT/90 and a new WHO human plain IRP (recombinant) has now been approved by WHO and designated rTF/95 (32).

In 1996, the WHO Expert Committee on Biological Standardization decreed that it was no longer necessary for international reference preparations or international reference reagents with defined units of activity to constitute a separate category and that all such materials could be considered functionally to be international standards. The question of whether it would be desirable to rename the international biological reference materials already established was discussed, and it was agreed that to do so would probably cause confusion because of the extensive scientific literature in which their existing names had been used. The recognised established names and designations of the international reference preparations for thromboplastin are therefore retained unchanged in this publication.

15. ISI CALIBRATION

A calibration exercise consists of a sequence of parallel PT with the respective thromboplastins on fresh plasma samples from healthy subjects and from long term stabilized coumarin-treated patients. The number of plasma test samples included in a calibration should be as large as necessary to ensure that the precision of the slope of the orthogonal regression calibration line (b), expressed as a coefficient of

²Available from Bureau Communautaire de Référence, 200 rue de la Loi, B-1049 Brussels, Belgium
variation (CV), is less than 3% (CV of slope (b) = 100 × SD(b)/b 10). In practice the arbitrary number of blood samples from 20 normals and 60 stabilized patients receiving oral anticoagulants has invariably achieved this precision by a wide margin at experienced centres. This arbitrary fixed number of constituent plasma samples has therefore been a constant feature of multicentre exercises in IRP calibration and allows better advance planning of a calibration exercise. It is customarily referred to as a “full fresh calibration”. Testing on at least five days is advised to reduce the effects of the day-to-day technical (intra-laboratory) variation in the PT determination. The coagulation end-point may be determined by a manual technique or with the aid of a semi-automated end-point recorder. With recognition of the effect on PT results by coagulometers even of the same brand it has become accepted that only manual PT calibrations be used for ISI calibration. The manual PT technique has been used in all multicentre calibrations of the thromboplastin IRP and the reference INR has become accepted universally as the equivalent of the manual PT result with the first WHO IRP.

Figure 3 illustrates a single centre calibration exercise with PT expressed as log values. Figure 4 gives the range of orthogonal regression slopes from 14 centres in the multicentre calibration of the human plain WHO IRP in a very precise calibration (low within and between-centre CVs).

Fig. 4 - Slopes and intercepts of calibration lines for the 14 centres for ECAA rabbit versus the WHO rabbit plain IRP RBT/90
16. "LIKE TO LIKE" CALIBRATIONS

The precision of a calibration is greater when similar reagents and techniques are compared. Each step of the calibration should be made whenever possible, against areference preparation which is most similar in terms of the species of tissue extract. It is also important to compare "plain" reagents, i.e. used with a prothrombin time technique with reference reagents that are also plain. Similarly combined reagents (e.g. Thrombotest with added factor V and fibrinogen) should be calibrated with a combined reference preparation (33).

17. CRITERIA FOR ISI CALIBRATIONS

Recommended specifications in ISI calculation include the following:

17.1 the relationship between the logarithms of the PT values should be linear;
17.2 a line drawn through the logarithms of PT values of the patients should intersect the logarithms of PT values from healthy normal subjects (for good example see figure 3, for example of non-conformity see figure 5);
17.3 the variability of points about the orthogonal regression line should be constant over its whole length;
17.4 outlying points (more than 3SD from the line) should be removed (10).

18. INTER-LABORATORY VARIABILITY OF ISI

It should be emphasised that the ISI calibration exercise is not an easy task. There is a considerable variation between laboratories performing the same procedure as observed in the published multicentre ISI calibration studies (20,29,32,34). In these calibrations, inter-laboratory variation has varied between 1.7% to 8.1%. A single centre’s calibration of a reagent, even if done with a full WHO specified procedure, is therefore of limited reliability.

19. NON-LINEARITY OF PATIENTS AND NORMALS

The logarithms of the PT values of the normals may lie significantly away from the calibration line estimated using only patients’ plasma samples (Fig. 5). In the event of this marked non-linearity, the assignment of an ISI would not be meaningful but if some description of the calibration relationship is essential, it should be expressed in terms of the observed non-linear double-logarithmic plot. Tomenson (33) developed a formula for identifying and correction of non-linearity.

20. NOMOGRAM FOR INR DERIVATION

Figure 6 is a nomogram for correcting prothrombin ratios to INR and can be used for any thromboplastin reagent where the ISI is known, without the need for calculations (35).
21. **BETWEEN-REAGENT DIFFERENCES**

It is a common misconception that for an individual patient’s plasma sample the INR will be identical with a range of thromboplastins. There is necessarily a degree of imprecision in ISI calibration as it is based on comparison of the average PT responses of the two different thromboplastin reagents of plasma from patients with anticoagulant-induced coagulation defects. These defects are of varying intensity and clotting factor composition. The ISI calibration is based on this average response used in the empirically demonstrated linear relationship between the logarithms of PT and individual samples. These samples may show a marked degree of difference due to biological and technical variables. A good correlation does not necessarily imply the interchangeability of two methods of measurement (36). A demonstration of such variability, based on average response, is that there is a strong correlation between height and shoe size but most people would find it extremely uncomfortable wearing shoes predicted on the basis of their height! When it is appreciated that different thromboplastins also vary greatly in responsiveness to the individual coumarin-dependent clotting factors, i.e. II, VII and X, as well as some non-coumarin dependent clotting factors which affect the PT, e.g. factor V, discrepancies between INR with different working thromboplastins arising from these biological variations and additional technical errors are therefore to be expected.
Fig. 6 - Nomogram providing the equivalent INR for a given ratio where the ISI of the thromboplastin has been established. Example: Observed ratio 2.65; with thromboplastin ISI 1.3, INR = 4.
22. WITHIN-LABORATORY PRECISION

The WHO recommended an upper limit of within-centre precision. This is a CV of the orthogonal regression slope of less than 3%. This differs from the CV of the ISI defined as CV of

\[ \text{ISI} = 100 \times \frac{\text{SD(ISI)}}{\text{ISI}} \]

where

\[ \text{SD(ISI)} = \sqrt{(\text{ISI}_{\text{ref}} - \text{SD(b)})^2 + (b \times \text{SD(ISI}_{\text{ref}}))^2} \]

is also used in calibration programmes and takes into account also the imprecision of the earlier calibration(s) of the IRP and in-house reference preparations against which a new reagent is being calibrated. The CV of the ISI is therefore greater than the CV of the slope. A desirable limit of a within-centre CV of the slope of 3% with the absolute limit of 5% is recommended in the WHO protocol(10).

23. BETWEEN-LABORATORY PRECISION

The inter-laboratory CV of the ISI of an IRP calibrated in a multicentre study is a different term from the CV of the slope and, is a measure of the variability in calibration relationships obtained at different centres.

At a single laboratory the precision of the estimated ISI can only be a measure of the variability about the average relationship relevant to the one centre. There is quite a degree of inter-laboratory variation even between expert centres reported in multicentre calibrations. This can be seen in figure 4 showing the calibration relationships between the 14 participants in the international collaborative exercise performed to calibrate the ECAA rabbit reagent in terms of the WHO rabbit plain IRP, RBT/90.

24. COAGULOMETER EFFECTS

The WHO scheme made no allowance for instrumentation effects in calibration, manual and semi-automated PT results being regarded as interchangeable (25). There is now unequivocal data on the unpredictable and marked effects of coagulometers on the ISI of thromboplastins and hence INR (37-41).

25. COAGULOMETER SYSTEM ISI

Coagulometers have now largely replaced the manual PT technique in most countries. Some instruments cause a disproportionate shortening of PT results from healthy subjects compared to their effects on patients’ plasmas thus artificially increasing the prothrombin ratio and reducing ISI. This appears to be the explanation of the disturbance of the ISI of thromboplastins by coagulometers which has been observed by various authors listed above (18). In view of this some manufacturers provide a "system ISI" for a particular thromboplastin/coagulometer combination. A "system ISI" may assist but its limitations have been demonstrated by the variability of "system ISI" with the same reagent and coagulometer at different centres found in collaborative studies (41,42).
26. **LOCAL COAGULOMETER ISI CALIBRATION**

The need for local system ISI at individual laboratories has been demonstrated as well as the need for separate calibrations for the different instruments where more than one coagulometer is in use in a single laboratory. As conventional ISI calibration necessitates the performance of the almost universally discarded manual PT technique with the thromboplastin IRP it is not usually feasible at the local level. The use of lyophilized plasma calibrants certified with manual PT values in terms of the relevant IRP (rabbit, human or bovine) has been recommended as an alternative for local ISI determination (41-44). The reliability of the procedure and the minimum requirement of numbers of certified lyophilized plasmas for a calibration is currently being studied. Lyophilized plasmas certified with PT values using a thromboplastin IRP when tested with the manual PT technique give a reasonable approximation to the calibration slopes to fresh plasmas. However, there was a demonstrable but small difference (mean deviation of 4.2%) from the conventional fresh plasma calibrations with a very responsive (low ISI) human recombinant reagent. Lyophilized artificially depleted plasmas could be substituted for lyophilized coumarin plasmas from coumarin treated patients making lyophilized plasma calibration a more practical and probably safer procedure. A further European Concerted Action on Anticoagulation report (45) showed that a minimum of 20 certified lyophilized depleted plasmas, with a proportion of normal samples from healthy subjects comparable to a full calibration (i.e. 7 normals to 20 abnormal plasma samples in place of 20 fresh normal and 60 fresh coumarin plasmas), give reliable ISI.

27. **CATEGORIES OF CALIBRATORS**

The revised WHO protocol lists four different categories of calibrators(10):

27.1 **International reference preparations (IRP)**

Calibration of IRP shall be carried out with large numbers of fresh plasmas as indicated in the WHO conventional procedure. IRP calibrations have always been performed on a multicentre basis with a minimum of seven centres and with most calibrations involving between ten and twenty laboratories. All three types of established IRP (rabbit, human and bovine) should be used to provide the ISI of a new IRP based on the average of the three.

27.2 **National reference preparations and manufacturer’s house standards**

Calibrations of the above should be against the relevant current IRP and should be the average of a calibration performed by at least two individual laboratories.

27.3 **Manufacturer’s lot-to-lot calibrators**

Testing for inter-batch variation of successive lots of the same manufacturer’s reagent is less demanding from the standpoint of precision because very similar material is being calibrated. Sufficient plasmas need to be included however to check against possible drift. A full calibration with fresh plasmas as described earlier is preferable. A more limited calibration procedure using pooled plasmas, coumarin or artificially depleted, needs to have been validated against the full WHO type calibration on fresh plasmas before being used for the inter-batch monitoring.
27.4 Local ISI calibrators

For local calibration with lyophilized plasmas, it has been shown that 20 artificially depleted abnormal plasmas certified with the manual technique PT values with the relevant IRP (human or rabbit) combined with the PT of 7 lyophilized normals give a reliable local ISI calibration (45). In the absence of lyophilized normals the local MNPT of fresh plasmas should be substituted. The certified PT values for the abnormal plasmas and the 7 lyophilized normals or the local MNPT of fresh plasmas with the reference reagent are marked on the vertical axis and the results with the corresponding local PT test system on the same lyophilized plasmas, on the horizontal axis. The log PTs of the two sets of values are used and the ISI is derived either from the calibration calculation or by entering into a computer disc programme. The use of PT certified plasmas avoids the need for the performance of the manual technique using an IRP in parallel with the local PT system on the 60 fresh plasmas and 20 normals as required in a conventional fresh plasma calibration (41,42). Sets of lyophilized plasmas certified with manual PT values with the IRP are now being commercially produced.

28. THE NEED FOR LOW ISI THROMBOPLASTIN

Reference has been made to the importance of a low ISI thromboplastin in maintaining good laboratory precision when PR are expressed as INR. A major clinical advantage of highly responsive reagents, i.e. ISI ranging from 0.9 to 1.2 in terms of oral anticoagulant dosage, is shown in figure 7. With higher ISI, the width of the therapeutic equivalents expressed as PT ratios increases progressively and the difference from the normal diminishes, i.e. the therapeutic window becomes progressively narrower (35). Dosage control with a poorly responsive (high ISI) preparation thus spans a very narrow PT (sec) or prothrombin ratio (PR) range compared with those observed with low-ISI reagents. It should be recognized that the responsiveness of a thromboplastin and its ISI are not related linearly. In other words, a reagent with an ISI of 2.0 has far less than half the responsiveness to the coumarin-induced defect of a thromboplastin with an ISI of 1.0. This results from the logarithmic relationship of INR to PT ratios in orthogonal regression analysis.

A further reason for favouring low ISI reagents is that the precision of the PT test is at least partly dependent on the ISI. Higher ISI reagents give an increased CV of the INR (less precision) i.e. \( CV(INR) = CV \) of the PT ratio multiplied by the ISI.

PT measurements based on low ISI reagents not only are more precise but give a wider therapeutic interval in PT terms which should be safer for the patient. The 4th ACCP Consensus Conference (46) recommended that thromboplastins should have an ISI of less than 1.5 and preferably less than 1.2. The revised WHO protocol (10) makes similar recommendations, allowing the use of reagents with ISI between 0.9 and 1.7 with a preference for thromboplastins towards the lower end of this scale.
29. COMPUTERIZED CALIBRATION PROGRAMMES

The mathematics of the orthogonal regression ISI calculation described in the Appendix have proved too complex for most physicians and hospital laboratories and in some instances have been incorrectly reproduced in papers and books. The calculation has therefore been computerised in several centres and specialist laboratories have developed their own programmes. “National Control Laboratories” should be able to provide to other laboratories a formatted disk with a recommended calibration programme and its method of use. The European Concerted Action on Anticoagulation has made such a programme available, which is now being provided to WHO for distribution to national control laboratories.³

30. COMPUTERIZED ORAL ANTICOAGULANT DOSAGE

Computerized systems for clinical dosage control have been developed mainly for warfarin but also the other anticoagulant drugs e.g. phenprocoumon, nicoumalone. Most of the published reports of computer-assisted dosage have been uncontrolled or non-randomised studies. A small randomised trial showed that three different computerized dosage regimes compared favourably with the conventional (manual) dosage by experienced specialist medical staff in an anticoagulant clinic (47). The preliminary results show considerable benefit from computerized dosage assessed by success in achieving the target therapeutic intervals (therapeutic quality control) combined with reduced requirements for laboratory PT testing. Computer-assisted dosage programmes may therefore be advantageous particularly as they may also incorporate administrative (record keeping, clinic attendance, etc) as well as dosage facilities.

³Available upon request, at a cost of Sfr.10.- excluding package and posting, from the Unit of Health Laboratory Technology (LAB), World Health Organization, 1211 Geneva 27, Switzerland.
31. IMPORTANCE OF NORMAL PLASMAS IN ISI CALIBRATIONS

The importance of the entry of reliable PT data from fresh plasma from healthy individuals in the ISI calibration needs to be emphasised as the normal values have an important influence on the slope of the calibration line and hence the ISI. The use of alternatives, e.g. a single lyophilized "normal", a single MNPT, a single pooled "normal" may result in unreliable ISI as error in the local normal value may give an erroneous ISI. The importance of the use of 20 fresh normal plasmas to determine a MNPT or the minimum comparable number of normals to abnormals is stressed in the European Concerted Action on Anticoagulation study (45).

32. LYOPHILIZED PLASMAS FOR ISI CALIBRATION

Lyophilized plasmas and artificially depleted plasmas can be used for local calibration of thromboplastins and of PT coagulometer systems (see 27.4)(41,42). Artificially depleted plasmas have safety advantages over lyophilized plasmas from coumarin treated patients in that they are prepared from blood donations from healthy subjects and are available in larger amounts. It is thus possible to obtain a more controlled INR spread over the therapeutic interval than with donations from coumarin-treated patients who tend to have lower INR values. Pooling of plasmas from coumarin treated patients originally proposed to produce sufficient volume of plasma must be performed with circumspection because of possible unreliability of HIV testing of plasma pools and greater danger of transmission of other viruses.

33. MINIMUM NUMBER OF LYOPHILIZED PLASMAS FOR ISI CALIBRATIONS

A minimum of 20 lyophilized plasmas combined with 7 lyophilized plasmas from healthy subjects was found to give a satisfactory CV of the slope with a low ISI reagent. The results were also satisfactory at most centres in terms of INR deviations (45).

34. EFFECT OF ROUTE OF CALIBRATION ON INR

In theory the INR of test plasmas should be similar with the different thromboplastin IRP apart from technical and biological variation differences, whichever IRP for thromboplastin is chosen to calibrate PT.

Differences in INR were however noted when either fresh or lyophilized coumarin test plasmas were tested with the WHO rabbit plain and human plain IRP. The INR were lower with the human IRP. The differences were on average between 5.7% and 9.5% although greater at higher INR (29, 48-50). To resolve this problem, Tripodi et al recommended that new batches of IRP be calibrated in terms of the average ISI of all three IRP [human, rabbit and bovine (51)]. This approach had been adopted for the calibration of a new batch of the WHO rabbit plain IRP [RBT/90](29). It was also used in the calibration of the replacement WHO human plain IRP (32). This procedure is now recommended for all new IRPs (see 27.1).
35. INR FOR OTHER CLINICAL STATES

INR were intended solely for control of long-term oral anticoagulant treatment. However, they are also widely used during the induction phase of oral anticoagulants; although less reliable during this period they are still an advance on the use of non-standardised prothrombin ratios or simple PT values (46). As in many centres it is difficult to discriminate between specimens intended for oral anticoagulant control and PT in other clinical indications e.g. liver function tests, some laboratories report INR for all PT specimens. The decision which unit to use, must be a local one, as there are arguments for and against the universal use of INR for all types of PT indications.
(A) RECOMMENDED PROTOCOL FOR THROMBOPLASTIN CALIBRATION

1. Study protocol

Blood samples from healthy individuals and patients stabilized on coumarin treatment are required for the conventional fresh plasma thromboplastin calibration. The total number of plasmas recommended is 20 normals and 60 samples from coumarin treated patients. Testing is performed on separate days but these need not be consecutive.

A recommended plan for a working day is as follows:

PT tests on:
- 8 fresh plasmas from long-term stabilized coumarin patients (single tests)
- 2 fresh plasmas from healthy subjects (duplicate tests)
- 1 lyophilized plasma from a healthy subject (duplicate test, tested on one day only)

The following reagents are to be tested:

1. IRP
2. Local thromboplastin

Number of tests per day: 50 (suggested)
Number of test days: 8 (suggested)

The schedule of a single exercise could be as follows:

1.1 Collection of blood samples. Blood drawn from healthy subjects and from patients stabilized on oral anticoagulants should be centrifuged immediately after collection and the plasma transferred to a non-wettable, stoppered container. Maintain at room temperature until tested.

1.2 Testing of the plasma samples with the thromboplastins is described in section 6.

Note:
(a) Single tests only should be performed on the samples from coumarin patients.
(b) Tests on each plasma should be performed with each thromboplastin in turn before proceeding to the next plasma.

2. Selection of healthy subjects and patients

2.1 Normals. These samples should be obtained from healthy adults of both sexes with a reasonable age spread. Select different normal subjects on each day of testing. Test each sample in duplicate.

2.2 Plasmas from coumarin treated patients. The patients should have been on oral anticoagulants for at least six weeks. Participant centres should include patient samples displaying a variety of levels of anticoagulation within the therapeutic range. Perform single tests only.
3. **Sample collection**

3.1 The blood is collected by clean venepuncture. 1 part of sterile trisodium citrate 109 mmol/L for 9 parts of sample is recommended. If an evacuated tube is employed it should be siliconized (see section 5.2).

3.2 The blood is centrifuged immediately after collection (approx. 800 x g) for 5 min, at room temperature.

3.3 The plasma is transferred by siliconised or plastic pipette to a stoppered, non-wettable container.

3.4 The stoppered plasma container is to be kept at room temperature until testing.

4. **Reconstitution of international reference preparation (IRP) for thromboplastin**

4.1 Extreme care should be taken to avoid wastage of IRP.

4.2 Reconstitute each ampoule of IRP with requisite volume of reconstitution fluid. Leave thromboplastin in ampoule (or vial). Keep at room temperature.

4.3 Ensure that the thromboplastin is completely resuspended before use. Gentle tapping on the side of the ampoule with a finger will facilitate resuspension. It is not necessary or desirable to shake the suspension vigorously.

5. **Other reagents and equipment**

5.1 Calcium chloride 0.025 mol/L; for recalcification of plasma/thromboplastin mixture. Store at 4°C. Use fresh aliquots each day.

5.2 Equipment. Syringes and evacuated tubes, must be silicone-coated or made of good quality plastic material to prevent contact activation of coagulation factors. Plastic or siliconised pipettes for transfer of plasma into glass test tubes at the time of testing. Use non-siliconised disposable glass test tubes for the actual testing. Water bath at 37°C (tolerance limits: 37.0°C ±0.2°C). Use calibrated thermometer. New pipetting tips must be used for each test. Accurate stop-watch.

6. **Testing procedure**

6.1 The WHO recommended procedure for calibration of thromboplastin is based on the manual tilt tube (or hook) technique. This method must be used for all calibrations of thromboplastins but for coagulometer system ISI the parallel manual procedure with the IRP only is required.

6.2 The sequence of thromboplastin testing should be alternated on each day of testing. The exercise should take no more than ten days to complete. The order of testing should be changed on each day. A specimen workplan is given below.
Specimen Work Sheet

<table>
<thead>
<tr>
<th>Number</th>
<th>Fresh plasma PT (sec)</th>
<th>Reagents</th>
<th>IRP</th>
<th>Local Reagent</th>
</tr>
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<tbody>
<tr>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Fresh normal 1</td>
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<td></td>
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<tr>
<td>Coumarin 1</td>
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<tr>
<td>Coumarin 6</td>
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<tr>
<td>Fresh normal 2</td>
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</tbody>
</table>

(Coumarin = plasma from oral anticoagulated treated patient)

NB. Test all “normal” plasmas in duplicate but perform single tests only on coumarin plasma treated samples.

6.3 The coagulation endpoint should be determined by the recommended tilt-tube technique. Test tubes should be kept under water at 37°C as much as possible in order to maintain optimal temperature. Proceed as follows: pre-warm glass test tubes in the water bath prior to testing. The testing procedure for rabbit and human plain IRP is as follows:

(a) transfer 0.1 mL thromboplastin to a test tube;
(b) incubate for 2 min;
(c) add 0.1 mL plasma;
(d) mix gently with the thromboplastin;
(e) wait 1 min for incubation to reach the optimal reaction temperature;
(f) recalcify with 0.1 mL pre-warmed Calcium Chloride 0.025 mol/L;
(g) tilt test-tube manually 3 times in 5 seconds through an angle of 90°. Record clotting time (sec).

For bovine IRP the volumes are different.

6.4 Prothrombin times must be recorded to one decimal place and must not be rounded to the nearest second or half-second. For example, if a PT of 34.1 seconds is observed on the stop watch this time must be recorded, the time should not be rounded to 34 seconds.
(B) ISI CALCULATION

The prothrombin times of all plasma specimens (20 healthy subjects and 60 patients) are converted to the corresponding logarithms. Let \( y \) be the logarithm of a prothrombin time/s determined with the IRP and \( x \) be the logarithm of prothrombin time/s determined with the local reagent to be calibrated. The relationship \( y = a_i + b_ix \) is calculated by the following formulae, in which \( a_i \) and \( b_i \) are the orthogonal regression line parameters representing the intercept and the slope respectively.

\[
b_i = m + \sqrt{n^2 + 1} \quad \text{where} \quad m = \frac{\sum (x - \bar{x})^2 - \sum (y - \bar{y})^2}{2\sum (x - \bar{x})(y - \bar{y})} = \frac{1}{2r} \left[ \frac{s_y}{s_x} - \frac{s_x}{s_y} \right]
\]

and \( a_i = \bar{y} - b_i\bar{x} \);
\( \bar{x} \) is the arithmetic mean of \( x \) and \( y \) the mean of \( y \), \( s_x \) and \( s_y \) are the standard deviations of the \( x \) and \( y \) values and \( r \) the correlation coefficient.

This orthogonal regression slope estimates the relationship between log prothrombin time/s of IRP and local thromboplastin. The ISI of the newly calibrated reagent is determined as follows:

\[
\text{ISI} = b_i \times \text{ISI of the reference reagent}
\]

where \( b_i \) is the newly determined calibration slope.

To transform the logarithmic value into the value that would have been obtained with the first WHO IRP human combined (67/40) the following equation is used:

\[
PT_{67/40} = \text{antilog} (a_i + b_i x)
\]

where \( a_i \) and \( b_i \) are the certified parameters and \( x \) is the log PT.
REFERENCES

161-3.


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Gratitude is expressed to the following members of the Co-ordinating Group of the WHO IEQAS in Blood Coagulation for their advice and assistance: