Annex 6

Guidelines for thromboplastins and plasma used to control oral anticoagulant therapy with vitamin K antagonists

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1. Introduction

Oral anticoagulant drugs derived from 4-hydroxycoumarin (and sometimes from indandiones) are widely used in the treatment and prophylaxis of thrombotic disorders. Coumarin drugs inhibit the biosynthesis of vitamin K-dependent coagulation factors by the liver. For each patient, the dose of these drugs must be adjusted periodically to ensure that an adequate, but not excessive, degree of anticoagulation is achieved. The adjustments are made on the basis of the results of the prothrombin-time or a similar test on the patient’s blood. The test, which requires reagents called thromboplastins, is controlled by the use of calibrated thromboplastins and plasmas.

Various types of thromboplastin are prepared commercially and, in order to be able to interpret the results of the prothrombin-time test, it is essential that each reagent is correctly calibrated. This will ensure that the results of tests with different products and batches are reproducible and can be compared. A procedure for the calibration of thromboplastins using a logarithmic plot of prothrombin times (PTs) has been developed (1) and was described in the report of the forty-eighth meeting of the WHO Expert Committee on Biological Standardization (2). With this procedure, the definition of a calibration parameter called the International Sensitivity Index (ISI) became feasible. It is possible to express prothrombin-time results on a common scale, i.e. the International Normalized Ratio (INR), if the ISI of the thromboplastin used is known.

Many routine laboratories use automated coagulometers for detection of the clotting end-point. There is now substantial evidence that coagulometers can have unpredictable and marked effects on the ISI of thromboplastins (3–6). Because of these effects, some manufacturers provide a “system ISI” for a particular thromboplastin/coagulometer combination. However, this procedure appears to have limitations since variations in the system ISI with the same reagent and coagulometer at different centres have been demonstrated in collaborative studies (7, 8).

In general, the calibration of a given thromboplastin is more precise if performed against an International Reference Preparation of similar composition and from the same species (9–11). A system of coexisting International Reference Preparations has been established in which each of these materials is related to the first primary International Reference Preparation – the First WHO International

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1 International reference materials established by the WHO Expert Committee on Biological Standardization have been denoted, variously, as International Reference Preparations, International Reference Reagents and International Standards. These Guidelines refer to all thromboplastin reference materials established by the WHO Expert Committee, independent of the nomenclature. International reference materials so established are by definition “primary” Reference Preparations, secondary Reference Preparations being calibrated in relation to them.
Reference Preparation of thromboplastin (human, combined), coded 67/40 (see Figure A6.1). Two International Reference Preparations of thromboplastin are currently available from the relevant WHO International Laboratory for Biological Standards: the Fourth WHO International Standard for thromboplastin (rabbit, plain) (coded RBT/05) (12) and the Fourth WHO International Standard for thromboplastin (human, recombinant, plain) (coded rTF/09) (13). Other International Reference Preparations have been discontinued. The development of these preparations is described in section 3.

In theory, the ISI/INR system should ensure that the ISI value calculated for a given reagent is independent of the species from which the International Reference Preparation is derived, because all have been directly or indirectly calibrated against the First WHO International Reference Preparation of thromboplastin (human, combined) (coded 67/40). However, this is not always the case; several observations have demonstrated that reagents calibrated against the Second WHO International Reference Preparation of thromboplastin (human, plain) – a material coded BCT/253 (the predecessor of rTF/95) (14) – provide lower INR values than those calibrated against RBT/79 (the predecessor of RBT/90) or OBT/79 (9, 11, 15). The extent of these differences in INR is not usually large enough to cause serious concerns from a practical point of view. The discrepancy is due to calibration errors that persist because the different International Reference Preparations were not checked against each other in the original studies. A new procedure has now been agreed upon: international thromboplastin Reference Preparations, whatever their origin and composition, will be calibrated against all existing International Reference Preparations in order to ensure consistency of results between different routes of calibration (16).

It is recommended that the International Reference Preparation of the same species or composition should be used for calibration of secondary standards, e.g. working standards, by manufacturers and national reference laboratories. Thus, plain rabbit thromboplastins should be calibrated against RBT/05 and plain human thromboplastins against the human recombinant material rTF/09. It has been demonstrated that bovine or rabbit combined thromboplastins can be calibrated with acceptable precision against RBT/05 (17). Thus, it is recommended that bovine or rabbit combined thromboplastins should be calibrated against RBT/05.

The calibration of prothrombin-time systems is not easy. Furthermore, there is considerable variation in results from different laboratories performing the same procedures, as shown by published multicentre calibration studies (9–14, 18–20). In these studies, interlaboratory variation in ISI, expressed as a coefficient of variation, ranged from approximately 1.7% to 8.1%.

The preparation, certification, and use of deep-frozen or lyophilized plasmas for ISI calibration and INR determination has been described as an important adjunct to fresh-plasma ISI calibration (21). The purpose of these
Guidelines, which replace the Requirements published in the forty-eighth report of the WHO Expert Committee on Biological Standardization (2), now discontinued, is to take account of the above-mentioned observations and to describe in detail the technical methods currently in use. Modifications to the methodology may give comparable results, but must be validated against the methodology described in these Guidelines.

How to use these Guidelines
Both manufacturers and clinical laboratories should be informed of the definitions used for the control of oral anticoagulant therapy with vitamin K-antagonists (section 2). Manufacturers of thromboplastins and certified plasmas should be informed of the current Guidelines. These Guidelines contain information for manufacturers of thromboplastins on the methods for calibration of their reagents against International Standards and for calibration of consecutive lots of each type of reagent (section 6). Calibration with International Standards is generally not to be performed by clinical laboratories.

These Guidelines contain information for clinical laboratories on the methods for simplified local-system calibration as described in section 7.3. The use of certified plasmas in clinical laboratories is described in section 7.3.4. Appendix 1 contains criteria for clinical laboratories that may assist with the choice of a commercial thromboplastin reagent or a set of certified plasmas.

2. Definitions

**International Normalized Ratio (INR):** for a given plasma or whole blood specimen from a patient on long-term oral anticoagulant therapy, a value calculated from the prothrombin-time ratio using a prothrombin-time system with a valid ISI according to the formula \( \text{INR} = \frac{\text{PT}}{\text{MNPT}}^{\text{ISI}} \).

**International Sensitivity Index (ISI):** a quantitative measure, in terms of the First WHO International Reference Preparation of thromboplastin (human, combined), coded 67/40, of the responsiveness of a prothrombin-time system to the defect induced by oral anticoagulants (see Appendix 2).

**Mean normal prothrombin time (MNPT):** the geometric mean of the PTs of the healthy adult population. For practical purposes, the geometric mean of the PT calculated from at least 20 fresh samples from healthy individuals, including both sexes, is a reliable approximation of MNPT. It is recommended that individual samples should be collected and tested over at least three working days in order to include inter-assay variation. It is also recommended that each laboratory should determine MNPT using its own prothrombin-time system. Pooled normal plasma (either deep-frozen or freeze-dried) may be suitable if the clotting time obtained is related to the MNPT value and its storage stability is acceptable.
Prothrombin time (PT) (tissue-factor-induced coagulation time): the clotting time of a plasma (or whole blood) sample in the presence of a preparation of thromboplastin and the appropriate amount of calcium ions. The time is reported in seconds (22).

Prothrombin-time ratio (tissue-factor-induced coagulation relative time): the PT obtained with a test plasma or whole blood divided by the MNPT, all times having been determined using the same prothrombin-time system.

Prothrombin-time system: a procedure by which the PT is determined using a specific thromboplastin reagent and a particular method, which may be manual, e.g. a tilt-tube method, or involve the use of an instrument that records the coagulation end-point automatically. The method should be described and the description should include all procedures and equipment used, e.g. the pipettes and test-tubes.

Thromboplastin: a reagent containing tissue factor and coagulant phospholipids. Many commercial thromboplastins are crude extracts prepared from mammalian tissues, in which tissue factor is only a minor component on a weight basis, and which also contain phospholipids. A preparation of a thromboplastin consisting of a tissue extract alone, either with or without added calcium chloride, is termed “plain”. When the preparation contains adsorbed bovine plasma as a source of additional factor V and fibrinogen it is termed “combined”. Thromboplastins may also be grouped into types, according to the tissue source from which they are derived, e.g. human, bovine, rabbit brain or lung, or human placenta. The tissue-factor component of recombinant human thromboplastin reagents is produced in Escherichia coli or insect cells by recombinant DNA techniques and then lipidated in vitro.

Tissue factor: an integral transmembrane protein functioning as a cofactor enhancing the proteolytic activity of factor VIIa towards factor X and factor IX in the blood. Tissue factor needs to be associated with coagulant phospholipids for the full expression of its cofactor function.

3. International Reference Preparations of thromboplastins

International Reference Preparations, International Standards and International Reference Reagents are intended to serve throughout the world as sources of defined biological activity quantitatively expressed in International Units or in terms of a suitable property or characteristic defining the biological activity. These preparations are used to calibrate secondary standards, which include regional, national and manufacturers’ working standards. Normally, working standards are used for routine calibration of individual batches of thromboplastin, and
working standards should have been calibrated with the appropriate International Reference Preparation. If secondary standards are developed using procedures that involve multiple calibration steps, there is a risk that unnecessary variability and discontinuity will occur in relation to the primary International Reference Preparation because of cumulative serial calibration errors.

Current prothrombin-time systems are based on the use of three different species of thromboplastin reagents: human, bovine and rabbit. Originally, the standardization of these thromboplastin reagents likewise involved three different Reference Preparations, one for each of the three species of plain thromboplastin reagents in use (Figure A6.1).

The First WHO International Reference Preparation of thromboplastin (human, combined) (coded 67/40), was established by the WHO Expert Committee on Biological Standardization in 1976 (23). It was a freeze-dried preparation, filled in sealed glass ampoules, and contained a human brain extract to which adsorbed bovine plasma had been added to optimize the content of non-vitamin-K-dependent coagulation factors (i.e. factor V and fibrinogen). Its ISI value was set at 1.0 by definition. In 1983, this preparation was discontinued and replaced by the Second WHO International Reference Preparation of thromboplastin (human, plain) (coded BCT/253), a human brain extract with no added coagulation factors and an assigned ISI value of 1.1 (24). When stocks of BCT/253 became exhausted, a new preparation of human recombinant thromboplastin (coded rTF/95) was established in 1996 as the Third WHO International Standard for thromboplastin (human, recombinant, plain) with an assigned ISI value of 0.94 (18, 25). When stocks of rTF/95 became exhausted, a new preparation of human recombinant thromboplastin (coded rTF/09) was established in 2009 as the Fourth WHO International Standard for thromboplastin (human, recombinant, plain), calibrated against rTF/95 and RBT/05, with an assigned ISI value of 1.082 (13).

The First WHO International Reference Preparation of thromboplastin (bovine, combined) (coded 68/434) was established by the WHO Expert Committee on Biological Standardization in 1978 (26). It was calibrated using the First WHO International Reference Preparation of thromboplastin (human, combined) (67/40). Another material, also calibrated against 67/40, was established as the Second WHO International Reference Preparation of thromboplastin (bovine, combined) (coded OBT/79), in 1983 with an assigned ISI of 1.0 (27). This material (OBT/79), which was derived from bovine brain and combined with factor V and fibrinogen, was used to calibrate thromboplastin materials of bovine origin and combined thromboplastins of whatever origin. When stocks of OBT/79 became exhausted in 2004, it was not replaced by a new International Reference Preparation of bovine origin.
For the calibration of thromboplastins of rabbit origin, the First WHO International Reference Preparation of thromboplastin (rabbit, plain) (coded 70/178), was established in 1978. This material was calibrated against the First WHO International Reference Preparation of thromboplastin (human, combined) (coded 67/40), in an international collaborative study which also included the First WHO International Reference Preparation of thromboplastin (bovine, combined) (26). When stocks of 70/178 became exhausted, the Second WHO International
Reference Preparation of thromboplastin (rabbit, plain) (coded RBT/79), was established in 1982 with an ISI value of 1.4; this was also calibrated against 67/40 (27). The Third WHO International Reference Reagent for thromboplastin (rabbit, plain) (coded RBT/90), obtained from rabbit brain with no added factors, was calibrated against each of the three species of thromboplastins and established by the WHO Expert Committee on Biological Standardization in 1995 with an ISI of 1.0 (28). When stocks of RBT/90 were exhausted, a new preparation of rabbit brain thromboplastin (coded RBT/05) was established as the Fourth WHO International Standard for thromboplastin (rabbit, plain), calibrated against rTF/95 and OBT/79, with an assigned ISI value of 1.15 (12). This material should be used for the calibration of rabbit thromboplastins as well as bovine thromboplastins.

The widespread use of these International Reference Preparations for calibrating secondary standards reflects the value placed on them by the scientific community responsible for the control of thromboplastins. An independent control of a manufacturer’s ISI assignments by a national reference laboratory is also recommended. National control authorities should consider designating an expert laboratory in their country for testing thromboplastin reagents and plasmas used by clinical laboratories to control oral anticoagulant therapy to ensure that they are in accordance with guidelines published by WHO.

The international reference materials for thromboplastins are in the custody of the National Institute for Biological Standards and Control, Potters Bar, England. Samples of these materials are distributed to national reference laboratories or national control laboratories for biological products and, upon payment of handling charges, to other organizations such as manufacturers, universities, research institutes and hospital laboratories. The principle that WHO International Reference Preparations are distributed free of charge to national control authorities for the purpose of the calibration of national standards has been adhered to since the establishment of international biological standardization activities (29).

4. Preparation of thromboplastins

The method of preparation of thromboplastins should be such that there is consistency from batch to batch and that the preparations are suitable for use in the control of oral anticoagulant treatment. The thromboplastins should comply with the specifications outlined in section 5.

Every attempt should be made to use the least contaminated source material possible and to use a manufacturing procedure that prevents further contamination and the growth of organisms during manufacture. Thromboplastins of animal origin should be prepared only from healthy animals. Thromboplastins prepared from bovine brain should be derived only from cattle from countries
that have not reported indigenous cases of bovine spongiform encephalopathy (BSE) and which have a compulsory BSE notification system, compulsory clinical and laboratory verification of suspected cases and a surveillance programme in place (30).

Human brain tissue should not be used because of the risk of transmission of Creutzfeldt–Jakob disease. Thromboplastins derived from human placenta should be prepared from donors in whom there is no evidence of systemic microbiological infection or localized infection and who have been shown to be free from hepatitis B surface antigen, antibodies to human immunodeficiency viruses (HIV-1 and HIV-2) and antibodies to hepatitis C virus.

5. Tests on thromboplastins

Each batch of thromboplastin should satisfy the following criteria.

5.1 **Response to coumarin-induced coagulation defect**

The response to the coumarin-induced coagulation defect should be measured by the PT obtained using normal and coumarin plasmas. Thromboplastins with a manual ISI between 0.9 and 1.7 are acceptable. However, ISIs towards the lower end of this scale are desirable, since some studies have shown that interlaboratory variation in ISI is greater for high than for low ISI systems (20). It has been suggested that the INR is less accurate when PT is determined with insensitive thromboplastins that have high ISI values (31).

5.2 **Content of haemoglobin and serum**

To prevent contamination of the product with (activated) clotting factors, the thromboplastin preparation should be free from serum and show no detectable haemoglobin.

5.3 **Opacity and sediment volume**

The method of manufacture, particularly the method of breaking up the tissue, has a marked effect on the activity, opacity and sediment volume of the thromboplastin. The opacity of preparations intended for use in photoelectric instruments should be suitably low, as specified by the manufacturer.

5.4 **Containers**

International Reference Preparations for thromboplastins are freeze-dried in sealed glass ampoules (32), but secondary standards may be freeze-dried in ampoules or vials.
5.5 Stability
The method of manufacture should be such that the thromboplastin preparations are stable for at least one year. All reagents eventually lose activity when stored at elevated temperatures, and stability should be checked by an accelerated degradation test (33, 34).

Accelerated degradation studies are considered to be only an indicative rather than an absolute guide to the stability of thromboplastins maintained at the storage temperatures recommended by the manufacturer. Lyophilized standard thromboplastins are routinely stored at low temperatures to maintain their stability. A small part of the standard material may be stored at an even lower temperature (“ultra-low temperature stock”). Under the assumption that the rate of degradation is different under the two storage conditions, a comparison of the results of samples of stock kept under the routine storage conditions with those of the ultra-low-temperature stock can be used to assess the stability status of the standard material (35). The stability of the thromboplastins must also be determined for the conditions under which they are stored, i.e. in a real-time stability study (36, 37).

6. Calibration of prothrombin-time systems

Four types of calibration should be distinguished:

a. calibration of International Reference Preparations;

b. calibration of secondary standards, e.g. national Reference Preparations and manufacturers’ working standards;

c. calibration of manufacturers’ commercial preparations against the corresponding working standard (“lot-to-lot” calibration);

d. local-system calibration.

In general, the results of calibrations are used by laboratories other than the calibrating laboratories. The clinical laboratories should therefore be aware of the interlaboratory variation in ISI values for the thromboplastin reagent. Type (d) calibration involves the use of deep-frozen or freeze-dried plasmas with assigned INR or prothrombin-time values which are described below. Type (a) and (b) calibrations should be carried out with a large number of fresh plasma or whole blood samples. Several studies suggest that, under certain circumstances, fresh plasmas for type (c) calibrations can be reliably replaced by frozen, freeze-dried, pooled plasma or plasmas artificially depleted of vitamin K-dependent coagulation factors (38–40). Manufacturers should validate this procedure by means of fresh plasmas.

Prothrombin-time systems should be calibrated in terms of the appropriate International Reference Preparation of thromboplastin, and the response to the
The basis of the thromboplastin calibration model is necessarily an empirical one. While there is good evidence that the calibration relationship defined in a double-logarithmic plot of PTs is usually linear, and that the same line represents data points for both patients and healthy subjects, the possibility of departure from these assumptions cannot be ruled out. Statistical methods to test deviations from the above-mentioned assumption have been described (41, 42). In the case of marked deviation, the assignment of an ISI would not be meaningful. For practical purposes, the assignment of an ISI is acceptable if INRs calculated with the ISI derived from the overall regression line (i.e. for patients plus healthy subjects) do not differ by more than 10%, in the INR range 2–4.5, from INRs calculated with the equation describing the regression line for patients only (see Appendix 2). A difference of 10% is considered as a critical difference according to the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis (SSC/ISTH) Guidelines on preparation, certification, and use of certified plasmas for ISI calibration and INR determination (21).

### 6.1 Calibration of International Reference Preparations

The calibration of the International Reference Preparations for thromboplastins, and their future replacements, should be carried out as part of international multicentre collaborative studies using fresh coumarin, normal plasma and manual techniques. In recent studies for the calibration of replacement International Standards, approximately 20 centres participated. These centres were located in North and South America, Asia, and Europe (12, 13). Each collaborative study for replacement of an International Reference Preparation should include the testing of all existing International Reference Preparations. The ISI assigned to the replacement material should be the mean of the ISIs obtained by calibration with all existing International Reference Preparations (16).

### 6.2 Calibration of secondary standards

Secondary standards of human origin should be calibrated against the current International Standard, i.e. the Fourth WHO International Standard
for thromboplastin (human, recombinant, plain) (coded rTF/09); plain thromboplastins of rabbit brain and rabbit lung should be calibrated against the Fourth WHO International Standard for thromboplastin (rabbit, plain) (coded RBT/05). Thromboplastins of bovine or rabbit brain combined with adsorbed bovine plasma should also be calibrated against RBT/05.

In view of the interlaboratory variation observed in multicentre calibration studies, it is recommended that calibration of national reference materials or manufacturer’s working standards should be performed by at least two laboratories. The ISI assigned to the national reference material or the manufacturer’s working standard should be the mean of ISIs obtained by the individual laboratories.

6.3 **Calibration of individual batches of thromboplastins**

The precision of calibration is greatest when similar materials and methods are compared. For this reason, a national Reference Preparation or manufacturer’s working standard used for the calibration of individual batches of thromboplastin should be a thromboplastin with similar characteristics to the batches being calibrated (i.e. it should be derived from the same tissue of the same species, using a similar manufacturing process). Batch-to-batch calibration should be performed by the manufacturer before release of the reagent, and consistency of ISI values should be shown. Manufacturers should state the applicable end-point detection systems including any relevant coagulometer lines alongside any stated ISI values.

**7. Calibration procedure**

The calibration procedure entails the determination of a series of PTs, using normal and abnormal plasmas or whole blood samples, with both the reference and the test thromboplastin. The tests are performed using either fresh samples from individual subjects (procedure 1) or freeze-dried or frozen plasmas (procedures 2 and 3). Abnormal plasmas for procedure 1 are obtained from patients undergoing long-term oral anticoagulant treatment. Freeze-dried or frozen plasmas for procedure 2 may be pooled plasmas from healthy subjects and from patients undergoing long-term anticoagulant treatment.

Procedure 1 is recommended for the calibration of secondary standards or any other prothrombin-time system against the appropriate International Reference Preparation and for the calibration of whole-blood coagulometers. Procedure 1 can also be used for the calibration of individual batches of thromboplastin against the corresponding secondary standard (i.e. lot-to-lot-calibration), but may be replaced by procedure 2 if the same results are obtained.
The precision of the calibration relationship depends on the number of plasmas and on a balanced distribution of normal and abnormal plasmas over the “therapeutic” range of INR values. The recommended number of abnormal plasmas is three times the number of normal plasmas.

7.1 **Procedure 1: Calibration of a secondary standard using individual fresh plasma or blood samples**

This procedure consists of a set of tests using freshly opened or reconstituted thromboplastins and a number of different individual samples of fresh plasma or whole blood. The procedure should be repeated on at least five separate occasions using fresh reagents on each occasion (see section 7.1.4). The procedure need not be repeated on consecutive days but should be completed as soon as possible. The tests in any one laboratory on any one day should be performed by the same person.

7.1.1 **Blood samples**

Blood samples from healthy subjects and patients who have been on oral anticoagulants for at least 6 weeks should be selected. Samples from patients treated with heparin should not be used. Patients’ samples with INR values in the range 1.5–4.5 should be selected.

Blood should be obtained by venipuncture, avoiding haemolysis and contamination with tissue fluids. It should be drawn either with a plastic syringe and transferred to a plastic tube, or with other non-contact activation equipment. Nine volumes of blood should be decalcified with one volume of 109 mmol/l trisodium citrate solution (22). A mixture of trisodium citrate and citric acid is also acceptable if the total citrate plus citric acid concentration is 109 mmol/l and the pH is no lower than 5. The same procedure and materials should be used for all the samples in a given calibration.

The lot number of tubes used for blood collection should be noted, as there may be lot-to-lot variation. If tubes are made of glass, they must be properly siliconized internally and the pH of the trisodium citrate plus citric acid solution must be in the range 5–6 (43). The sample should be centrifuged as soon as it is received but, in any case, no later than 2 hours after blood collection. The centrifugation should render the plasma poor in platelets (i.e. at least 2500 g for 10 minutes at a controlled room temperature, or at a speed and for a time that allow a platelet count of the platelet-poor plasma lower than $10 \times 10^9$/l). The plasma should be taken off the red-cell layer with a plastic pipette, stored undisturbed in a narrow, stoppered, non-contact tube at room temperature and tested within 5 hours after blood collection.

Some techniques or instruments require the use of non-citrated capillary blood (44). Capillary blood can be obtained by finger or heel puncture. The
capillary blood should be obtained without squeezing of the finger or heel and tested immediately with the technique or instrument to be calibrated. Venous blood should be obtained from the same subjects (healthy subjects and patients) within 5 minutes of taking the capillary sample, for preparation of citrated plasma as described above and testing with the most appropriate International Reference Preparation.

7.1.2 **Reference thromboplastins**
The appropriate International Reference Preparation of thromboplastin (human or rabbit) should be reconstituted as instructed and the contents of the ampoules transferred to a container in sufficient volume for all tests to be performed in a single calibration session. Specific instructions for use should be supplied by the custodian of these materials.

7.1.3 **Prothrombin-time test**
The prothrombin-time test is performed either by mixing equal volumes of citrated plasma, thromboplastin and calcium chloride solution (25 mmol/l), or by adding a volume of plasma to the required volume of thromboplastin premixed with calcium, and therefore available as a single reagent. The time (in seconds) taken for the mixture to clot when maintained at a temperature of between 36.5 °C and 37.5 °C is recorded. Test instructions for commercial thromboplastins should be provided by the manufacturers.

The coagulation end-point for International Reference Preparations of thromboplastin must be detected by a manual (tilt-tube) technique because the manual technique has been used for the establishment of the ISI for the International Reference Preparations and International Standards. The angle and speed of tilting the test-tube must be standardized (through 90 °C three times every 5 seconds) to control glass activation and minimize cooling (45).

The coagulation end-point for other thromboplastins (e.g. commercial preparations) may be detected with the aid of an automatic or semi-automatic end-point recorder. The same technique should be used throughout the series of tests with a given thromboplastin.

Each laboratory should have a system for internal quality control. Records should be maintained of the lot number of all reagents and disposable equipment used. Periodic checks of the temperature of incubation baths or heating blocks and of the volumes of pipettes or pumps should be made and recorded.

An example of results obtained with procedure 1 is provided in Appendix 2.

7.1.4 **Statistical evaluation**
The suggested procedure for calculation of the ISI is given in Appendix 2.
Before the final orthogonal regression line for the ISI is calculated, it is important to detect outliers and any samples beyond the therapeutic range. Outliers may result from technical or clerical errors and may strongly influence the estimated ISI. Outliers may be detected as points with a perpendicular distance greater than 3 residual standard deviations from the preliminary orthogonal regression line calculated with all data included (46). It is suggested that outliers be detected and removed in a single step. In the next step any patient samples beyond the therapeutic range (INR < 1.5 or INR > 4.5) should be removed. In this procedure it is important to assess each patient’s INR as the mean INR determined with the International Standard and with the system being calibrated using the ISI obtained after the removal of outliers. Using the INR determined solely with the International Standard could introduce a bias in the orthogonal regression line and should be avoided (47).

It is not necessary to replace the removed outliers and non-therapeutic patient samples with new samples, provided that the number of patient samples remaining is at least 55. In any case, the within-laboratory coefficient of variation of the slope of the orthogonal regression line for normal samples + patient samples should be 3% or less. The number of normal samples should be at least 20 for the calculation of the MNPT. After removal of outliers and non-therapeutic patient samples, the adequacy of the ISI model should be assessed. The ISI model is deemed adequate if the deviation D of the INR calculated with the ISI model from the INR calculated with the International Standard is not greater than 10% (see equation 19 in Appendix 2). If the deviation of the INR calculated with the ISI model is greater than 10%, it is advisable to use a different model according to Tomenson (42).

The sequence of steps in the statistical evaluation is as follows.

1. Calculate preliminary orthogonal regression line (20 normal samples + 60 patient samples).
2. Detect outliers defined as points with a perpendicular distance greater than 3 residual standard deviations from the preliminary line.
3. Remove outliers in one step and recalculate the orthogonal regression line (normal samples + patient samples) and ISI.
4. Calculate each patient’s INR using the PT determined with the International Standard.
5. Calculate each patient’s INR using the PT determined with the system being calibrated and the ISI from step 3.
6. Calculate each patient’s mean INR from steps 4 and 5.
7. Remove patients with mean INR < 1.5 or mean INR > 4.5.
8. Recalculate the orthogonal regression line (normal samples + patient samples) and ISI.
To assess the adequacy of the ISI model, calculate the deviation $D$ of the INR determined with the ISI model from the true INR for $\text{INR} = 2.0$ and for $\text{INR} = 4.5$. If $D < 10\%$, the ISI model is deemed to be adequate. If $D > 10\%$, use Tomenson’s formula for INR calculation (see Appendix 2).

7.2 **Procedure 2: Calibration of individual batches of thromboplastin**

Calibration of individual batches of thromboplastin may be carried out with pooled normal plasmas and pooled coumarin plasmas or plasmas artificially depleted of vitamin K-dependent coagulation factors (38, 39). The number of plasma pools required for precise calibration is, in general, much smaller than the number of fresh individual plasma samples required for procedure 1. The scatter of data points about the regression line is relatively small because the batch to be calibrated is very similar to the working Reference Preparation and/or because the biological variation caused by individual samples is reduced by the pooling of plasmas. It has been reported that lot-to-lot calibration of bovine and rabbit thromboplastins could be performed with as few as three plasma pools (38, 39), but the accuracy of such a simplified procedure may depend on the quality of the pooled plasmas and the thromboplastin being calibrated. It is recommended that any procedure using pooled or artificially depleted plasmas be validated against the fresh plasma procedure (procedure 1).

7.2.1 **Pooled plasma**

7.2.1.1 **Properties of pooled normal plasma**

Plasma should be obtained from healthy adults and should comply with the appropriate section of the *Requirements for the collection, processing and quality control of blood, blood components and plasma derivatives* (48). The normal plasmas for pooling should be obtained from at least 20 different donors with an approximately equal number of males and females. Nine volumes of blood should be decalcified with one volume of 109 mmol/l trisodium citrate solution. The packed-cell volume-fraction should be between 0.35 and 0.45.

The final preparation should be platelet-poor plasma, which has been freeze-dried or frozen (at −40 °C or below) in suitable containers. The stability of deep-frozen plasma should be monitored regularly by testing the PT. Thawing of deep-frozen plasma should be done in a water bath at 37 °C for a fixed time depending on the volume in each container. After reconstitution or thawing, the pH should not be lower than 7.3 and should not exceed 7.9, and the plasma should not show any shortening or prolongation of clotting times for at least 2 hours when held at ambient temperature (49). The stability of freeze-dried normal plasma should be checked by accelerated degradation tests. Such plasma
should not show a prolongation of PT of over 5% after storage for 4 weeks at 37 °C. The factor V content should be between 60% (or 60 IU/dl) and 140% (or 140 IU/dl) of the average content of fresh normal plasma (50).

7.2.1.2 Properties of pooled coumarin plasma

Pooled coumarin plasma is obtained from patients who have been on oral anticoagulant therapy for at least 6 weeks. Coumarin plasmas for pooling should be obtained from at least 20 different donors.

Plasma should not be obtained from donors with a history of jaundice or from those with plasma-lipid abnormalities. The collection of plasma, the properties of the final preparation and the stability of the freeze-dried pools are the same as described above for pooled normal plasma.

The INR of the pooled plasma should be stated, as should the thromboplastins used for its assignment. It should be noted that the INR value of a freeze-dried plasma usually depends on the thromboplastin used for its assignment (51–53). At least two different plasma pools, having an INR between 1.5 and 4.5 and with a difference of at least 1.0 in their INRs, in combination with one normal plasma pool are necessary for the calibration procedure.

The factor V content, opacity and citrate concentration for blood decalcification should comply with the requirements for normal plasma (see above).

7.2.1.3 Freedom from infectious agents

The plasma should be shown to be free from hepatitis B surface antigen, antibodies to human immunodeficiency viruses (HIV-1 and HIV-2) and antibodies to hepatitis C virus.

7.2.2 The test

The test should be carried out using the same procedure as described for procedure 1 (see section 7.1.3). An example of the protocol for the recording of the results is given in Appendix 3. The procedure should be repeated on at least four separate occasions (40), with fresh reagents used on each occasion. At least three plasma pools should be used to permit the testing of linearity.

Freeze-dried plasma pools should be reconstituted at least 15 minutes before the actual test. Plasma that has been frozen and subsequently thawed, or reconstituted freeze-dried plasma, should not be centrifuged, and unused reconstituted or thawed material should be discarded after 2 hours.

7.2.3 Statistical evaluation

An orthogonal regression line should be calculated on the basis of the ln PT value of the pooled plasmas. Individual determinations should be entered
when multiple determinations for each plasma pool are available. Ln PT for the working reference thromboplastin system is plotted on the vertical axis and Ln PT for the test batch of thromboplastin on the horizontal axis. Any samples with a perpendicular distance greater than 3 residual standard deviations from the regression line should be removed. After removal of such samples, the final orthogonal regression line is calculated.

To define the ISI of a batch of thromboplastin, a sufficient number of tests should be carried out to obtain a within-laboratory coefficient of variation for the slope of the orthogonal regression line of 3% or less. The recommended procedure for calculation of the ISI is given in Appendix 3.

7.3 Procedure 3: Local system calibration using certified plasmas

Laboratories may calibrate their own local system (i.e. instrument/thromboplastin combination) using certified plasmas supplied by manufacturers or reference laboratories. A certified plasma is a deep-frozen or lyophilized plasma with an assigned PT or INR value. Two procedures using certified plasmas have been described and are summarized below.

Local test system ISI calibration – this procedure is a modification of the WHO method for ISI determination. In a set of plasmas, each plasma is assigned a manual PT value by the manufacturer or reference centre using an International Standard for thromboplastin. In the local laboratory, the PTs of each plasma are measured with the local instrument/reagent combination, and the two sets of PTs are plotted on a log/log plot. The slope of the orthogonal regression line is used to determine the local ISI (see Appendix 2), which can then be used for subsequent determination of INRs from the local PTs and MNPT (7, 8, 54–56). An underlying assumption of the WHO orthogonal regression model is that a single line describes the relationship between log(PT) of abnormal and normal plasmas. If there is a significant deviation of the two calibration lines (i.e. abnormal-only and normal/abnormal combined), a correction according to Tomenson should be applied (42, 57).

“Direct” INR determination – this procedure involves assignment of INR values to a set of plasmas with the manual method and an international thromboplastin standard by the manufacturer or reference centre. The PTs of these plasmas are measured locally using the local instrument/reagent combination, and the local test system PTs are plotted against the reference INRs on a log/log plot. An orthogonal regression line is calculated, and the INRs of patients’ plasmas can be interpolated directly from local PTs using this line, without the need for ISI or MNPT determination. Although many studies of direct INR determination were performed with linear rather than orthogonal regression, the latter is preferable from a theoretical point of view (see section 7.3.2.5) (53, 58–66).
A number of studies have shown that use of either of these procedures can considerably reduce inter-laboratory imprecision in INR determination (8, 59–61, 67, 68). For example, in one study the mean deviation of 95 local systems from the “true” INR was +14.4% with the manufacturers’ ISI, but was reduced to +1.04% with the local ISI (8). In another study, the inter-laboratory coefficient of variation of the INR was reduced from 12% with the manufacturers’ ISI to 6% using direct INR determination with a certified plasma procedure (59).

It should be recognized that there are a number of different ways in which plasmas can be prepared and certified. The following sections describe the various methods of preparation, certification and use, and their advantages and disadvantages.

7.3.1 Preparation of certified plasmas

7.3.1.1 Type of plasma – AVK (from patients on anti-vitamin K therapy) or artificially depleted of prothrombin complex factors (ART)

The intention is for certified plasmas to be as similar as possible to fresh plasmas from patients, thus on theoretical grounds, AVK plasmas might be preferred, although for practical reasons these have to be pooled, rather than individual, donations. In some studies where the two types of plasmas have been compared, AVK plasmas give closer agreement with fresh plasmas and better inter-laboratory agreement than artificially depleted plasmas (51, 69). Artificially depleted plasmas have several advantages over plasmas from patients on oral anticoagulants, including availability of larger volumes, wider selection of PT values across the therapeutic interval, and the possibly reduced risk of virus transmission (70). It can be argued that larger volumes of AVK plasmas could be obtained by pooling donations from patients on anti-vitamin K therapy, but this procedure would make a spectrum of INR values more difficult to obtain because of averaging of individual INRs in such a pool.

The European Concerted Action on Anticoagulation (ECAA) prepared depleted plasmas using artificial depletion of normal human plasma by selective adsorption of vitamin K-dependent clotting factors with barium sulfate to provide a range of values which spanned the therapeutic interval (54). The ECAA found that there is a small difference between the results obtained with ECAA lyophilized artificially depleted plasmas and lyophilized AVK plasmas in ISI value assignment, but both of these lyophilized plasmas differed by a similar amount from a conventional fresh plasma ISI calibration (54). The mean calibration slopes with both types of lyophilized plasma were generally higher than with fresh AVK plasmas but the differences were not great in clinical terms. It should be noted that the ECAA study was performed with one combination of a human brain International Reference Preparation and recombinant thromboplastin and the manual technique, and that the conclusions may not be applicable to all other reagent/instrument combinations.
The reliability of artificially depleted plasmas and AVK plasmas depends on the method of preparation and certification.

7.3.1.2 Method of preparation – frozen or freeze-dried
Although lyophilization seems a simple solution to the difficulties associated with storage and shipment of certified plasmas, there are problems associated with lyophilized materials.

Studies have shown that the INR of fresh plasmas is largely unchanged on freezing, whereas freeze-drying may change the INR significantly, depending on the method of freeze-drying and the thromboplastin/instrument combination used (50, 71–73). Buffering of plasmas shortly after blood collection can reduce but not eliminate changes after freeze-drying. The magnitude of the changes is not the same for all reagents or instruments. The measured INR of lyophilized certified plasmas depends on the thromboplastin reagent and instrument used. The use of RBT/90 presented problems relating to its poor end-point particularly with lyophilized plasmas giving long PTs.

The widespread use of frozen plasmas presents logistical difficulties due to their potential instability, although in some countries frozen certified plasmas have been used with success regarding the reduction of interlaboratory imprecision (63, 67).

Freeze-dried plasmas represent the most practical option for general laboratories and their use has been associated with reduced interlaboratory imprecision in several studies.

7.3.1.3 Citrate concentration
It is well known that citrate concentration can affect the PT, especially of high INR plasmas (74, 75). Furthermore, citrate concentration has a variable effect on the ISI, but the magnitude of the effect is not the same for all reagents and instruments (75–78). The recommended citrate concentration for the collection of blood (1 volume of citrate solution + 9 volumes of blood) for PT is 0.109 mol/l (3.2%), although concentrations in the range 0.105–0.11 mol/l can be accepted (77), and the citrate concentration of certified plasmas should be as close as possible to that in fresh plasma collected in the above anticoagulant (70). Citrate concentrations of 0.129 mol/l (3.8%) should not be used for PT tests.

7.3.1.4 Number of plasmas
The number of plasmas depends on the purpose for which they are used.

Local test system ISI calibration – according to the present Guidelines, to define the ISI of a working thromboplastin, the number of tests carried out should be sufficient to obtain a within-laboratory coefficient of variation (CV) for the slope...
of the orthogonal regression line of 3% or less (see section 7.1.4). In an ECAA study of lyophilized artificially depleted and individual AVK plasmas, it has been shown that the requirement for 60 lyophilized abnormal samples for a full WHO calibration can be reduced to 20 if combined with results from seven lyophilized normal plasmas; reductions below this number were associated with decreased precision of the calibration line and hence increased variability of the INR (79). However, the use of pooled AVK plasmas may reduce the scatter of individual plasmas about the line (80), and with pooled plasmas and repeat testing it is possible that a lower number could be used, e.g. acceptable precision has been achieved with six pooled AVK plasmas containing at least 50 patient samples in each pool and two pooled normal plasmas if these were analysed on at least 3 days (40).

“Direct” INR determination – for “direct” INR determination a smaller number of pooled plasmas can be used. Studies have shown improved inter-laboratory variability with as few as six (63), five (53, 60, 61, 65), four (66), three (58), or two (59, 81) plasmas, but considering that one of the plasmas should be a normal plasma and that at least three plasmas are required to define a line, a set of one normal and at least three abnormal plasmas is recommended. One study documented the within-laboratory imprecision of the slope of a calibration line (one normal + three abnormal plasmas): the CV ranged from 0.1% to 4.6% (64).

The number of donations in each pool should be at least 10, but higher numbers are preferable to ensure normal levels of factor V.

For both procedures it is important that the abnormal plasmas be chosen to cover the range of 1.5–4.5 INR. The fibrinogen and factor V content should be between 60% and 140% of the average content of fresh normal plasma (50).

7.3.2 Certification (value assignment) of plasmas

Manufacturers or suppliers are responsible for certification, i.e. value assignment to the plasmas.

7.3.2.1 Thromboplastins for certification

WHO standard or European reference thromboplastins should be used directly if possible. Assuming that the certified plasmas are intended for use with the various types and species of thromboplastin, the two types of WHO standard preparations should be used (human and rabbit). If insufficient supplies of WHO or European standards are available, national or secondary standards can be used provided they have been calibrated against the appropriate WHO or European thromboplastin standards in a multicentre study. If the plasmas are intended for use with only one type of thromboplastin (e.g. human), the
appropriate thromboplastin standard preparation should be used. Several studies have shown that the INR value for some lyophilized plasmas obtained with the previous rabbit standard thromboplastin (RBT/90) was greater than the INR obtained with the human and bovine standard preparations (11, 51, 69, 82), especially for artificially depleted plasmas (52). Certified INRs for lyophilized artificially depleted plasmas determined with the ECAA rabbit plain reference thromboplastin were approximately 15% greater than those determined with the recombinant human International Standard and approximately 30% greater than those determined with the bovine combined International Standard (65).

For use with one manufacturer’s thromboplastin reagent only, certification with the manufacturer’s calibrated reagent is acceptable; such “reagent-specific” value assignments have been shown to be reliable in recent collaborative studies (53, 64). The manufacturer’s thromboplastin reagent used for reagent-specific certification of plasmas should be calibrated by at least two independent laboratories using the original WHO procedure (see section 7.1).

Although thromboplastin standards should be used for the assignment of values, the certified plasmas should be tested for suitability with a variety of commercial thromboplastins before release for general use (see section 7.3.3).

7.3.2.2 Number of laboratories

It is recommended that three to five laboratories should be involved in the certification process for each set of plasmas. An individual laboratory’s mean value should differ by no more than ± 10% of the overall mean (in terms of INR) obtained with a given thromboplastin reagent. If the difference is greater than 10%, the divergent individual laboratory’s value should not be used.

7.3.2.3 Manual technique or instruments

The manual tilt-tube method must be used for International Standard preparations, as described in section 7.1.3. Once certified, the plasmas should be tested for their suitability with various reagent/instrument combinations. Where certification of plasmas is done with one manufacturer’s reagent only, an instrument may be used. In this case the reagent/instrument combination must have been calibrated using the original WHO procedure (see section 7.1).

7.3.2.4 Single or multiple values

For the local test system ISI calibration, the actual values of the PTs of the certified plasmas will differ according to the species of the standard thromboplastin used, and therefore PT values must be independently certified for the different species. For the direct INR determination approach, the INR values of the plasmas should theoretically be the same whichever reference thromboplastin reagent is used.
In practice, differences in INRs obtained using different thromboplastins have been observed with some freeze-dried plasmas; results should not be averaged into a single INR if the INRs obtained with individual standard reagents differ by more than 10% from the mean. Large discrepancies between INRs obtained with different thromboplastins may indicate that the plasmas are unsuitable for use with thromboplastins of all types. It should be noted that the manufacturer or supplier of the certified plasmas should clearly specify the set of reagent/instrument combinations with which their materials may be reliably used (83) (see section 7.3.3).

7.3.2.5 Orthogonal regression

Orthogonal regression is used if each coordinate is subject to independent random error of constant variance (41, 84), e.g. PT measurements with two different reagents recorded by the same instrument or operator. Linear regression is used when one of the values is fixed, i.e. essentially without error. The use of certified plasmas does not conform completely to either of these models, but it is important to recognize that apparently “fixed” values of these plasmas are themselves subject to error. Therefore, orthogonal regression should be used for both procedures, i.e. local system ISI calibration and direct INR determination. The equations for orthogonal regression are given in Appendix 2.

7.3.2.6 International reference plasmas

At present there are no established international reference plasmas. Work has begun on the development of reference plasmas for “direct” INR assignment (58, 82). These could then be used for direct certification of batches of commercial plasmas, in the same way as for coagulation factor assays. One difficulty, as mentioned above, is that of preparing lyophilized plasmas with the same properties as fresh plasmas, and it may be that frozen plasmas have to be used. Furthermore, for long-term use, the stability of such reference plasmas would need to be carefully checked. In the meantime, commercial plasmas will continue to have their values assigned as described above.

It should be noted that the validity of lyophilized certified plasmas may be limited to certain combinations of thromboplastins and coagulometers, and may not be generalizable to all other reagent/instrument combinations.

7.3.3 Validation of certified plasmas

Each set or batch of certified plasmas intended for either local test system ISI calibration or direct INR determination must be validated before release (21). The validation should be the responsibility of the manufacturer or supplier who
may seek help from expert laboratories. The validation process should go through the following steps.

1. Ten or more fresh plasmas from patients on long-term oral anticoagulants are selected to represent the full therapeutic range of anticoagulation.

2. The INR of these fresh plasmas is determined with an appropriate International Standard for thromboplastin, and the mean value ($\text{INR}_R$) is calculated.

3. The INR of the same fresh plasmas is also determined with a variety of commercial reagent/instrument combinations following the certified plasma procedure (either ISI calibration or direct INR determination). The mean value ($\text{INR}_C$) is calculated.

4. Finally, paired INR values obtained with the International Standard and with the local system are compared to assess their agreement using Bland and Altman’s procedure (85).

If the relative difference between the mean values $\text{INR}_R$ and $\text{INR}_C$, i.e. $2(\text{INR}_R - \text{INR}_C)/(\text{INR}_R + \text{INR}_C)$, is 0.1 or less, the set of certified plasmas is considered acceptable and may be released for local ISI calibration or direct INR determination. New batches of the same type of preparation should be validated according to the above procedure.

Studies on simplified local calibration with certified plasmas have been published (65, 81), but the value of the studies is limited if the sets of plasmas have not been validated with fresh plasmas from patients as described in this section.

7.3.4 Use of certified plasmas in clinical laboratories

7.3.4.1 Quality assessment

An important use of certified plasmas is to perform internal or external quality assessment, i.e. to determine whether or not corrective action is necessary (83, 86). For quality assessment, a set of three to five certified plasmas with INRs in the range 1.5–4.5 would be required. The INRs of the certified plasmas should be calculated from local PTs and routine ISI, and compared with the certified INR values. If the differences between routine INR and certified INR are greater than 15%, local ISI calibration or direct INR correction should be performed. In addition, the manufacturer of the reagent and certified plasmas should be informed about the discrepant results. Quality assessment with certified plasmas should be performed regularly at intervals of no more than 6 months and should be repeated whenever there is a change in reagent batch or instrument (e.g. servicing, modification, or new instrument). It should be realized that errors
caused by local pre-analytical factors (e.g. divergent citrate concentration or contamination of citrate with divalent cations) cannot be corrected by certified plasma procedures (87).

7.3.4.2 Method for local ISI determination

PTs should be measured in quadruplicate in the same working session, with the local instrument/reagent combination for the full set of normal and abnormal plasmas. Duplicate PT measurements are permitted if the imprecision of the PT system is not greater than 2% CV. It is recommended to repeat the measurements over three sessions or on 3 days to control for day-to-day variation. Mean local PTs should be plotted on the horizontal axis against the certified PT values on the vertical axis (log scales). Tomenson’s test should be performed to test the hypothesis that the mean log(PT) of the certified normal plasmas lies on the same line as the log(PT) of the certified abnormal plasmas (42, 56, 57). If the hypothesis is not confirmed, Tomenson’s correction formula should be applied (42, 56, 57, Appendix 2). Like-to-like comparison should be used wherever possible, i.e. if the local reagent is a human thromboplastin, the certified values should be those determined with a human Reference Reagent. If the INR difference between the routine ISI and the local ISI calibration procedure is greater than 10%, the calibration should be repeated. If the discrepancy persists, the manufacturer or supplier of the local thromboplastin reagent and coagulometer and certified plasmas should be informed. After consultation with the manufacturer of the certified plasmas and, if possible, an expert laboratory, the clinical laboratory should decide which materials and methods should be used for local ISI calibration.

7.3.4.3 Method for direct INR measurements

This method is simpler to use than the one described above as it does not require local ISI and MNPT determinations. PTs should be measured in duplicate with the local instrument/reagent combination for each certified plasma. To allow for day-to-day variation, the measurements should be repeated on at least three different days. Mean PTs should be plotted on the horizontal axis against the certified INR values on the vertical axis (log scales), and an orthogonal regression line derived. Manufacturers of certified plasmas should state for which thromboplastin brands the certified values are valid and provide instructions on how to calculate the calibration line. The INRs of patients’ plasmas should be calculated from the measured PTs. If the INR difference between the routine ISI procedure and the direct determination is greater than 10%, the certified plasma procedure should be repeated. If the discrepancy persists, the manufacturer or supplier of the local thromboplastin reagent and coagulometer and certified plasmas should be informed. After consultation with the manufacturer of the certified plasmas
and, if possible, an expert laboratory, the clinical laboratory should decide which materials and methods should be used for direct INR measurement.

8. The use of calibrated thromboplastins in clinical practice

It is possible to express prothrombin-time results on a common scale, i.e. the International Normalized Ratio (INR), provided that the ISI of the thromboplastin and the method used are known. The following formula is used:

\[
\text{INR} = \left( \frac{\text{PT}}{\text{MNPT}} \right)^{\text{ISI}}
\]

where PT is the patient’s PT and MNPT is the mean normal PT determined with the same thromboplastin and method. The use of the INR enables comparisons to be made between results obtained using different thromboplastins and methods. It is a misconception, however, that for an individual patient’s plasma the INR will always be identical when determined with different thromboplastins and methods (42, 88). Different thromboplastins vary greatly in their responsiveness to individual vitamin K-dependent clotting factors, i.e. factors II, VII and X, as well as to some factors that are not dependent on vitamin K, e.g. factor V. Discrepancies between INRs determined with different thromboplastins arising from these biological variations and from additional technical errors are therefore not unexpected.

All medical staff and health auxiliaries involved in managing oral anticoagulant treatment should be encouraged to use the INR system. It should be appreciated, however, that this system can be accurate only in the INR range explored by the calibration procedure, i.e. 1.5–4.5.

Manufacturers of commercial reagents should state on the package insert the ISI of the relevant batch of thromboplastin together with the Reference Preparation against which it has been determined and the instrument for which it is valid.

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References


Appendix 1

Criteria which may assist clinical laboratories in the choice of a reagent

The purpose of this appendix is to provide criteria that are useful for a clinical laboratory to apply when choosing a thromboplastin reagent. These criteria relate to the manufacturer of the reagent providing standard information to the user on the following:

- instrument-specific International Sensitivity Index (ISI) values for the reagent;
- which International Standard has been used for the ISI calibration;
- whether the adequacy of the ISI model has been checked;
- local-system calibration (if an instrument-specific ISI is not available).

The manufacturer’s provision of the following may assist with the choice of a set of certified plasmas:

- information on the International Standards and other thromboplastin reagents that have been used for the certification and the validation of the set of plasmas;
- a statement that the set of certified plasmas has been validated;
- a value of the relative mean International Normalized Ratio (INR) difference obtained in the validation procedure (according to section 7.3.3 of the main text);
- a list of the thromboplastin reagent brands for which the set of plasmas can be used (for both quality assessment and local-system calibration);
- instructions for calculation of local-system ISI or direct INR measurement;
- a spreadsheet for performing the calculations.
Appendix 2

Example of the use of the suggested method for reporting the data for the calibration of any system or a secondary standard of thromboplastin against an International Standard preparation

Thromboplastins:
1. Recombinant human thromboplastin secondary standard
2. Third WHO International Standard for thromboplastin (human, recombinant, plain) (rTF/95) with an established ISI = 0.94.

End-point recording:
1. Automated photoelectric coagulometer for secondary standard

The tests were conducted on five different days (Table 1). On each day, fresh samples from four healthy subjects and 12 patients were tested (plasma samples from healthy subjects are referred to as “normal”). On each day, different subjects were selected. The automated coagulometer and manual determinations were performed more or less simultaneously.

Table 1
Prothrombin times for the calibration of a secondary standard of recombinant human thromboplastin

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<th>Plasma</th>
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Calculations

The International Sensitivity Index of the secondary standard ($ISI_w$) is obtained by plotting the prothrombin times of the two thromboplastins on logarithmic scales as shown in Figure A6.2, fitting a straight line of the form:

$$Y = A + BX$$

and estimating the slope $B$. The recommended method involves estimation of a linear structural relation (also called an “orthogonal regression equation”). With this technique, the slope $B$ can be estimated as follows.

Consider a set of $N$ independent observations $(x_i, y_i)$, where $i = 1, 2, 3, \ldots, N$; for $N$ paired tests, $y_i$ represents the natural logarithm of the measured prothrombin time of the International Standard, and $x_i$ that of the secondary standard. Write $x_0, y_0$, for the arithmetic means of the $N$ values of $x_i, y_i$, respectively. Write $Q_1, Q_2$, for the sums of the squares of $(x_i - x_0)$ and $(y_i - y_0)$, respectively, and $P$ for the sum of their products $(x_i - x_0)(y_i - y_0)$. These quantities are all that is necessary for computing $a$ and $b$, the least-squares estimators for the parameters $A$ and $B$ of equation (1). Now define:

$$E = (Q_2 - Q_1)^2 + 4P^2$$

Then

$$b = (Q_2 - Q_1 + \sqrt{E})/2P$$

and

$$a = y_0 - bx_0$$

are the estimators that minimize the sum of the squares of the perpendicular distances of the $N$ points from the line represented by equation (1). The variance of $b$ is given by:

$$\text{Var}(b) = \{(1 + b^2)P + NbV\}bV/P^2$$

where $V$ is defined as

$$V = (Q_2 - bP)/(N - 2)$$

The standard error of $b$ ($s_b$) is the square root of $\text{Var}(b)$. The coefficient of variation of $b$ is $CV(b) = 100 \times (s_b/b)$.

If $t$ is a deviate from the $t$-distribution, with $(N - 2)$ degrees of freedom and at a chosen probability, approximate confidence limits for $B$ can be obtained by setting an interval $t \times s_b$ on either side of $b$.

The residual standard deviation is the square root of $V$. Outlying points should be rejected if their vertical (i.e. perpendicular) distance from the calibration line is greater than $3 \times \sqrt{V}$. 
The ISI\textsubscript{w} for the secondary standard is calculated as follows:

\[ \text{ISI}_{\text{w}} = \text{ISI}_{\text{IS}} \times b \]  

(7)

where ISI\textsubscript{IS} is the ISI of the International Standard.

The prothrombin-time ratio for a given patient (i) with the secondary standard can be estimated according to the equation

\[ \text{R}_{\text{w},i} = \exp(x_i - x_n) \]  

(8)

where \( x_n \) is the mean natural logarithm of the prothrombin times of the normals. Likewise, the prothrombin-time ratio with the International Standard can be estimated according to the equation

\[ \text{R}_{\text{IS},i} = \exp(y_i - y_n) \]  

(9)

where \( y_n \) is the mean natural logarithm of the prothrombin times of the normals.

If the same linear structural relation is valid for patients and normals it can be shown that the calibration model implies a relationship between prothrombin-time ratios of the form

\[ \text{R}_{\text{IS}} = (\text{R}_{\text{w}})^b \]  

(10)

where \( \text{R}_{\text{w}} \) is the prothrombin-time ratio obtained with the secondary standard, and \( \text{R}_{\text{IS}} \) is the prothrombin-time ratio for the International Standard. A similar equation can be written for the prothrombin-time ratio of the First WHO International Reference Preparation of thromboplastin (human, combined) coded 67/40:

\[ \text{R}_{67/40} = (\text{R}_{\text{IS}})^{\text{ISI}_{\text{IS}}} \]  

(11)

Equations (7), (10) and (11) are the base for calculation of the INR according to the ISI calibration model:

\[ \text{INR}_{\text{w}} = (\text{R}_{\text{w}})^{\text{ISI}_{\text{w}}} \]  

(12)

One of the underlying assumptions of the ISI calibration model is that a single line describes the relationship between logarithms of prothrombin times of both normal and patient plasmas. Thus the line describing the relationship between logarithms of patient prothrombin times should ideally pass through the mean of the logarithms of normal prothrombin times. In the case of marked deviation, the assignment of an ISI would not be meaningful. The natural way to overcome this problem is to introduce a scale parameter and use a model for prothrombin ratios of the form

\[ \text{R}_{\text{IS}} = e^d \times (\text{R}_{\text{w}})^{b'} \]  

(13)

The above model is referred to as Tomenson’s (1).
Tomenson’s model leads to the following equation for calculation of the corrected INR<sub>wp</sub>:

\[
\text{INR}_{IS} = \text{INR}_{wp} = \left\{ e^{d} \times (R_w)^{b'} \right\}^{ISI}_{IS}
\]  \hspace{1cm} (14)

Clearly equation (10) is a particular case of equation (13) but the generalized model will also cope with data sets for which the line describing the relationship between logarithms of patient prothrombin times does not pass through the mean of the logarithms of normal prothrombin times. It can be shown that \( d \) in equations (13) and (14) is estimated as

\[
d = a' + b' x_n - y_n
\]  \hspace{1cm} (15)

where \( x_n \) and \( y_n \) are the mean natural logarithms of the prothrombin times of normals determined with the secondary standard and the International Standard, and \( a' \) and \( b' \) the intercept and slope of the “orthogonal regression line” calculated using only the patient data.

Example

For the full set of data shown in Table 1, the various parameters were calculated according to equations (3), (4), (5), (6), (7) and (15). The results are shown in Table 2. The next step is to detect any outliers. In this example there was one data pair (patient number 22) for which the distance to the line was greater than 3×√V. This data pair was excluded. The parameters calculated for the remaining 79 data pairs are shown in Table 2. The ISI<sub>w</sub> calculated for the remaining 79 data pairs was 4.8% greater than the preliminary ISI calculated with the one outlier included. Each patient’s INR can be calculated in two ways. The first is to calculate INR from the PT measured with the International Standard:

\[
\text{INR}_{IS,i} = (R_{IS,i})^{ISI}_{IS}
\]  \hspace{1cm} (16)

The second way of calculating each patient’s INR is by using the PT measured with the secondary standard:

\[
\text{INR}_{w,i} = (R_{w,i})^{ISIw}
\]  \hspace{1cm} (17)

Now it is possible to calculate the mean INR<sub>m,i</sub> for each patient’s sample:

\[
\text{INR}_{m,i} = \frac{\text{INR}_{IS,i} + \text{INR}_{w,i}}{2}
\]  \hspace{1cm} (18)

For example, the INR for patient number 43 is 4.32 with the International Standard and 4.70 with the secondary standard. The mean INR is 4.51 which is just at the limit of the therapeutic range. There are no other patients for whom the mean INR is outside the 1.5–4.5 interval.
The relative difference $D$ between the INR calculated according to equation (12) and equation (14) is given by:

$$D = 100 \times \left( \exp(ISI_w \times (((y_n + (\ln(INR_{IS})/ISI_{IS})) - a')/b' - x_n)) - INR_{IS}\right)/INR_{IS} \quad (19)$$

In this example the orthogonal regression line calculated for 59 patient data pairs did not pass through the mean of the normal data pairs (see Figure 1). The difference $D$ calculated at $INR_{IS} = 2$ is $-11\%$ and at $INR_{IS} = 4.5$ it is $7.8\%$. It is therefore important to consider the alternative calibration model according to equation (13). By substituting the values from Table 2 in equation (14) the following formula is obtained:

$$INR_{wp} = \left\{ e^{0.2431 \times (R_w)^{1.2024}} \right\}^{0.94} \quad (20)$$

### Table 2
**Parameters calculated for the calibration of a secondary standard (see Table 1)**

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<th>20 normal samples + 60 patient samples (full data set)</th>
<th>20 normal samples + 59 patient samples (outlier excluded)</th>
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Figure 1
Log-log plot of prothrombin times for determination of ISI

Reference
Appendix 3

Example of the use of the suggested method for reporting the data for the calibration of individual batches of thromboplastin

Thromboplastins: 1. Rabbit brain thromboplastin secondary standard  
2. New batch of rabbit brain thromboplastin

End-point recording: Automated photoelectric coagulometer

Pooled coumarin plasmas: lot 960606, 1–5 (deep-frozen)

Pooled normal plasma: lot 900423 (deep-frozen)

The International Sensitivity Index (ISI) and mean normal prothrombin time (MNPT) of the rabbit brain thromboplastin secondary standard used with this automated photoelectric coagulometer are 1.31 and 12.7 seconds, respectively.

The tests were conducted in four separate runs. For each run, thromboplastins were freshly reconstituted and deep-frozen plasmas were freshly thawed. Since the secondary standard and the new batch were both timed with the same photoelectric coagulometer, the order in which the two reagents were tested was alternated from one run to the next. This was done to avoid any bias due to possible instability of the thromboplastins and pooled plasmas.

Calculation

The ISI of the new batch (ISI\( b \)) is calculated as ISI\( b = ISI_w \times b \), where \( b \) is the slope of the straight line fitted to a double-logarithmic plot of the prothrombin times in Table 1, with the prothrombin times for the secondary standard and the new batch being shown on the vertical and horizontal axes, respectively. The formula for \( b \) is given by equation (3) in Appendix 2. The standard error of \( b \) is obtained from equation (5) in Appendix 2. The coefficient of variation (%) of \( b \) is 100 × (s\( b / b \)).

Example

For the data from Table 1, the calculated residual standard deviation is 0.02482. One pair of determinations for plasma lot no. 960606-5 (run no. 3)
has a perpendicular distance from the line greater than three residual standard deviations. When this pair is excluded, the calculated value for $b$ is 0.9538. The ISI for the secondary standard is given as 1.31. Thus, the ISI for the new batch is estimated as $1.31 \times 0.9538 = 1.25$. The standard error for $b$ is calculated as 0.0130. The coefficient of variation for $b$ is $100 \times (0.0130/0.9538) = 1.36\%$.

Table 1
Prothrombin times (PT) for the calibration of a new batch of rabbit thromboplastin

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<td>6</td>
<td>44.4</td>
</tr>
</tbody>
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