Annex 3

Guidelines for thromboplastins and plasma used to control oral anticoagulant therapy¹

Introduction

Oral anticoagulant drugs derived from coumarin (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 has been developed (1) and was described in the thirty-third report 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.

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

¹ These guidelines replace Requirements for Thromboplastins and Plasma Used to Control Oral Anticoagulant Therapy (Requirements for Biological Substances No. 30), which are discontinued (see WHO Technical Report Series, No. 889, 1999:7–8).
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 International Reference Preparation of Thromboplastin (Human, Combined), coded 67/40 (see Fig. 1, p. 69). Three international reference preparations of thromboplastin are currently available from the relevant WHO International Laboratory for Biological Standards: the second International Reference Preparation of Thromboplastin, Bovine, Combined (coded OBT/79) (9); the third International Reference Reagent for Thromboplastin, Rabbit, Plain (coded RBT/90) (11); and the third International Standard for Thromboplastin, Human, Recombinant, Plain (coded rTF/95) (12). Other international reference preparations have been discontinued. The development of these preparations is described in section 2 (pp. 68–70).

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 International Reference Preparation of Thromboplastin, Human, Combined (coded 67/40). However, this is not always the case; several recent observations have demonstrated that reagents calibrated against the second International Reference Preparation of Thromboplastin, Human, Plain, a material coded BCT/253 (the predecessor of rTF/95) (13), provide lower INR values than those calibrated against RBT/79 (the predecessor of RBT/90) or OBT/79 (9, 11, 14). 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 minor 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 of whatsoever origin

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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.
and composition will be calibrated against all existing international reference preparations in order to ensure consistency of results between different routes of calibration (15).

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/90; plain human thromboplastins against the human recombinant material rTF/95; and bovine or combined thromboplastins (of whatever species) against OBT/79.

The calibration of prothrombin-time systems is not an easy task. Furthermore, there is considerable variation in results from different laboratories performing the same procedures, as shown by published multicentre calibration studies (9–13, 16, 17). In these studies, inter-laboratory variation in ISI, expressed as a coefficient of variation, ranged from approximately 1.7% to 8.1%. The purpose of these guidelines, which replace the requirements published in the thirty-third 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. These guidelines represent the state of the art. Modifications to the methodology may give comparable results, but must be validated against the methodology described in the guidelines.

1. **Definitions**

_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.

_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 lipitated *in vitro*.

**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 (18).

**Prothrombin-time system.** A procedure by which the prothrombin time 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 endpoint automatically. The method should be described and the description should include all procedures and equipment used, e.g. the pipettes and test-tubes.

**Mean normal prothrombin time (MNPT).** The geometric mean of the prothrombin times of the healthy adult population. For practical purposes, the geometric mean of the prothrombin time calculated from at least 20 fresh samples from healthy individuals, including those of both sexes, is a reliable approximation of MNPT. It is not necessary to collect and test all the individual samples in one session. It is 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 (see p. 78).

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

**International Sensitivity Index (ISI).** A quantitative measure, in terms of the first 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 1, p. 86).

**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 known ISI according to the formula

\[
INR = \left( \frac{PT}{MNPT} \right)^{ISI}
\]
2. **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. The standardization of these thromboplastin reagents likewise involves three different reference preparations, one for each of the three species of plain thromboplastin reagents in use (Fig. 1).

The first International Reference Preparation of Thromboplastin, Human, Combined (coded 67/40), was established by the WHO Expert Committee on Biological Standardization in 1976 (19). 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 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 (20). When stocks of BCT/253 became exhausted, a new preparation of human recombinant thromboplastin (coded rTF/95) was established in 1996 as the third International Standard for Thromboplastin, Human, Recombinant, Plain, with an assigned ISI value of 0.94 (12, 21).

The first International Reference Preparation of Thromboplastin, Bovine, Combined (coded 68/434) was established by the WHO Expert Committee on Biological Standardization in 1978 (22). It was calibrated using the first International Reference Preparation of Thromboplastin, Human, Combined (67/40). Another material, also
calibrated against 67/40, was established as the second International Reference Preparation of Thromboplastin. Bovine, Combined (coded OBT/79), in 1983 with an assigned ISI of 1.0 (23), in accordance with the revised Requirements for Thromboplastins and Plasma used to Control Oral Anticoagulant Therapy (2). This material (OBT/79), which was derived from bovine brain and combined with factor V and fibrinogen, should be used to calibrate thrombo-
plastin materials of bovine origin and combined thromboplastins of whatever origin.

Finally, for the calibration of thromboplastins of rabbit origin, a first International Reference Preparation of Thromboplastin, Rabbit, Plain (coded 70/178), was established in 1978. This material was calibrated against the first International Reference Preparation of Thromboplastin, Human, Combined (coded 67/40), in an international collaborative study which also included bovine thromboplastin, combined (22). When stocks of 70/178 became exhausted, the second 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 (24). The third 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 (25).

The wide 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 the 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 Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam. Samples of these materials are distributed free of charge, on request, to national reference laboratories or national control laboratories for biological products and, with small handling charges, to other organizations such as manufacturers, universities, research institutes and hospital laboratories. The principle that 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 (26).

3. **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 shall comply with the specifications outlined in section 4, below.

All attempts 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 shall be prepared only from healthy animals. Thromboplastins prepared from bovine brain shall 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 (27).

Human brain tissue should not be used because of the risk of transmission of Creutzfeldt-Jakob disease. Thromboplastins derived from human placenta shall 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.

4. **Tests on thromboplastins**

Each batch of thromboplastin shall satisfy the following criteria.

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

The response to the coumarin-induced coagulation defect shall be measured by the prothrombin time 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 (17). It has been suggested that the INR is less accurate when prothrombin time is determined with insensitive thromboplastins that have high ISI values (28).

4.2 **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.

4.3 **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.

4.4 Containers

International reference preparations for thromboplastins are freeze-dried in sealed glass ampoules (29), but secondary standards may be freeze dried in ampoules or vials.

4.5 Stability

The method of manufacture shall be such that the thromboplastin preparations are stable. All reagents eventually lose activity when stored at elevated temperatures, and stability shall be checked by an accelerated degradation test. When a thromboplastin reagent is exposed to accelerated degradation at 37°C, the initial change in the prothrombin time of normal plasma should not exceed 0.05% per day (30). With regard to the response to the effect of coumarin, the reagent when stored at 37°C should maintain activity as measured by the prothrombin-time ratio of coumarin plasma for 6 months (31).

Accelerated degradation studies are considered to be only a useful rather than an absolute guide to the stability of thromboplastins maintained at the storage temperatures recommended by the manufacturer. Therefore, 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 (32, 33).

5. 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. The possibility of correcting for local-thromboplastin/instrument-combination effects (i.e. prothrombin-time system) by means of type (d) calibration is currently under study (34, 35). Type (d) calibration involves the use of freeze-dried plasmas
with assigned INR or prothrombin-time values which are not described in these guidelines. 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 (36–38). Manufacturers should validate this procedure by means of fresh plasmas.

Prothrombin-time systems shall be calibrated in terms of the appropriate international reference preparation of thromboplastin, and the response to the coumarin-induced coagulation defect shall be defined by the ISI obtained in the calibration procedure. Supplies of international reference preparations are limited, and it is not possible to use these materials in routine tests to calibrate each batch of the many thromboplastins produced by different manufacturers. Calibration of individual batches of thromboplastin shall be carried out by comparison with a secondary standard, which shall be a batch of the same or a similar thromboplastin calibrated against the appropriate international reference preparation.

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 prothrombin times 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 latter assumption have been described (39, 40). 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 1, p. 86).

5.1 The calibration of international reference preparations

The calibration of the international reference preparations for thromboplastins, and their future replacements, should be carried out in international multicentre collaborative studies using fresh coumarin, normal plasma and manual techniques. 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 (15).

5.2 **The calibration of secondary standards**

Secondary standards of human origin should be calibrated against the current International Standard, i.e. the third International Standard for Thromboplastin, Human, Recombinant, Plain (coded rTF/95); plain thromboplastins of rabbit brain and rabbit lung should be calibrated against the third International Reference Reagent for Thromboplastin, Rabbit, Plain (coded RBT/90); and thromboplastins of bovine origin should be calibrated against the second International Reference Preparation of Thromboplastin, Bovine, Combined (coded OBT/79). Thromboplastins of rabbit brain combined with adsorbed bovine plasma should also be calibrated against OBT/79.

In view of the interlaboratory variation observed in multicentre calibration studies, it is recommended that calibration of national reference materials or manufacturers' working standards should be performed by at least two laboratories.

5.3 **The 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 of similar characteristics to these batches (i.e. 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.

6. **The calibration procedure**

The calibration procedure entails the determination of a series of prothrombin times, 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 (procedure 2). 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 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.

6.1 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 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 6.1.4, p. 77). 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.

6.1.1 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. It is recommended that patients' samples with INR values in the range 1.5–4.5 should be selected.

Blood should be obtained by venepuncture, 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 (18). 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.

If evacuated tubes are used for blood collection, their lot number should be noted, as there may be lot-to-lot variation. If evacuated 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 (41). The sample should be centrifuged as soon as received but no later than 2 hours after blood collection. The centrifugation should be such that the plasma is rendered poor in platelets (i.e. at least 2500g for 10 minutes at a controlled room temperature). 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 (42). Capillary blood can be obtained by finger or heel puncture. The capillary blood should be obtained without squeezing 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.

6.1.2 Reference thromboplastins

The appropriate international reference preparation of thromboplastin (human, rabbit or bovine) 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.

6.1.3 The prothrombin-time test

The prothrombin-time test is performed either by mixing equal volumes of citrated plasma, thromboplastin and calcium chloride solution (25mmol/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 shall be provided by the manufacturers.

The coagulation end-point for international reference preparations of thromboplastin should be detected by a manual (tilt-tube) technique. The coagulation end-point for other thromboplastins should be detected by a manual technique or with the aid of an 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.

A suggested protocol for reporting the procedure is shown in Appendix 1 (p. 86).

6.1.4 Statistical evaluation

Samples with INRs outside the 1.5–4.5 range shall be excluded. An orthogonal regression line should be calculated on the basis of the natural logarithm1 of the prothrombin time (PT) of 20 healthy subjects plus 60 patients stabilized on long-term anticoagulant therapy. LnPT for the international reference preparation is plotted on the vertical axis and lnPT for the working reference material on the horizontal. Orthogonal regression analysis provides an estimate of the standard deviation of data points about the regression line. Any samples with a perpendicular distance greater than 3 residual standard deviations from the regression line should be excluded. After removal of such samples, the final orthogonal regression line should be calculated. The suggested procedure for calculation of the ISI is given in Appendix 1.

To define the ISI of the working reference material, a sufficient number of separate 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.

6.2 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 (36, 37). 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 (36, 37), but the accuracy of such a simplified procedure may depend on the quality of the pooled

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1 The abbreviation “ln” is used henceforth for the natural, also known as the Napierian, logarithm.
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).

6.2.1 Pooled plasma

Properties of pooled normal plasma

Plasma should be obtained from healthy adults and should comply with the appropriate section of Requirements for the Collection, Processing and Quality Control of Blood, Blood Components and Plasma Derivatives (43). The normal plasmas for pooling should be obtained from at least 20 different donors. 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 prothrombin time. 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 (44). The stability of freeze-dried normal plasma should be checked by accelerated degradation tests. Such plasma should not show a prolongation of prothrombin time of over 5% after storage for 4 weeks at 37 °C.

The factor V content should be between 60% and 140% of the average content of fresh normal plasma (45).

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 for pooled normal plasma described above.

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 (46–48). 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).

*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.

6.2.2 The test

The test should be carried out by the same procedure as described for procedure 1 (see section 6.1.3, p. 76). An example of the protocol for the recording of the results is given in Appendix 2 (p. 92). The procedure should be repeated on at least four separate occasions (38), 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.

6.2.3 Statistical evaluation

An orthogonal regression line should be calculated on the basis of the ln PT value of the pooled plasmas. Individual determinations shall 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. 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 2, p. 92.

7. 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 prothrombin time and MNPT is the mean normal prothrombin time 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 with different thromboplastins and methods (40, 49). 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 non-vitamin-K-dependent factors, 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 controlling 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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The text prepared following this Consultation was circulated among the members of the ISTH Standardization Subcommittee on Control of Anticoagulation and discussed during the Subcommittee meeting in Florence, 7 June 1997.

Acknowledgements

Acknowledgements are due to the members of the ISTH Subcommittee for their expert advice and for supplying additional relevant comments, and to Dr D. Finney and Dr D. Houghton for their valuable statistical advice.

References


20. **WHO Expert Committee on Biological Standardization. Thirty-fourth report.**

21. **WHO Expert Committee on Biological Standardization. Forty-seventh report.**

22. **WHO Expert Committee on Biological Standardization. Thirty-sixth report.**

23. **WHO Expert Committee on Biological Standardization. Thirty-fourth report.**

24. **WHO Expert Committee on Biological Standardization. Thirty-third report.**

25. **WHO Expert Committee on Biological Standardization. Forty-sixth report.**


Appendix 1
Example of the use of the suggested method for reporting the data for the calibration of a secondary standard of thromboplastin against an international reference preparation

Thromboplastins:
1. Rabbit brain thromboplastin secondary standard
2. Third International Reference Reagent for Thromboplastin, Rabbit, Plain (RBT/90)

End-point recording:
1. Automated photoelectric coagulometer for secondary standard
2. Manual (tilt-tube) technique for RBT/90

The tests were conducted on 5 different days. On each day, fresh samples from 4 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 rabbit thromboplastin

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12 March 1997

13 March 1997

14 March 1997

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**Calculations**

The International Sensitivity Index of the secondary standard (ISI<sub>s</sub>) is obtained by plotting the prothrombin times of the two thromboplastins on logarithmic axes as shown in Fig. 2 (p. 90), fitting a straight line of the form

\[ Y = A + BX \]  

(1)

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 reference preparation, and \( x_i \) that of the secondary standard. Write \( x_n, y_n \) 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 \( \Sigma(x_i - x_0)(y_i - y_0) \). 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:

\[ E = (Q_2 - Q_1)^2 + 4P^2. \]  

(2)

Then

\[ b = \frac{Q_2 - Q_1 + EP}{2P} \]  

(3)

and

\[ a = y_0 - bx_0 \]  

(4)
are the estimators that minimize the sum of the squares of the per-

cpendicular distances of the \( N \) points from the line represented by
equation (1). The variance of \( b \) is given by:

\[
\text{Var}(b) = \frac{\{(1+b^2)^p + NbV\}bV}{P^2},
\]

(5)

where \( V \) is defined as

\[
V = \frac{Q^2 - bP}{N-2}
\]

(6)

The standard error of \( b \) \((s_b)\) is the square root of \( \text{Var}(b) \). 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 distance from the calibration line is greater
than \( 3\sqrt{V} \).

The \( \text{ISI}_{\omega} \) is calculated as follows:

\[
\text{ISI}_{\omega} = \text{ISI}_{\text{IRP}} \times b,
\]

(7)

where \( \text{ISI}_{\text{IRP}} \) is the ISI of the international reference preparation.

The coefficient of variation (\%) of \( b \) is \( 100 \times (s_b/b) \).

An ingenious alternative has been suggested for calculating confi-
dence limits for the slope \( B \), appropriate here if the frequency dis-
tribution of the random errors of \( x_i \) and \( y_i \) can be assumed to be
approximately normal \((I)\). In practice, these confidence limits are
likely to be trustworthy unless \( N \) is so small as to introduce problems
due to non-normality \((2)\). With \( t \) as above, define:

\[
D = \frac{4r^2(QQ_2 - P^2)}{E(N-2)}
\]

(8)

and calculate

\[
b_\omega = \tan^{-1}(b)
\]

(9)

\[
c_\omega = \tan^{-1}\left(D/(1-D)^{\frac{P}{2}}\right)
\]

(10)

Confidence limits then are:

\[
\tan(b_\omega - c_\omega) \text{ and } \tan(b_\omega + c_\omega).
\]

(11)

In most cases, when \( t \) is chosen for probability = 0.95, \( D < 1.0 \).
However, at higher probabilities an unusual conjunction of data
can make the calculation of confidence limits impossible if \( D \) exceeds
Figure 2
Log-log plot of prothrombin times for determination of ISI

The continuous line represents the structural relation for both normal samples (dots) and samples from patients (circles). The dashed line represents the relation for samples from patients only.
1.0, since the square root of a negative number is not defined. There is no simple way of detecting this problem until D has been calculated.

Example

For the data from Table 1, the calculated MNPT value for RBT/90 is 17.7 seconds. The ISI of RBT/90 is 1.0. There were three patients with INRs beyond the 1.5–4.5 range (Patient 21, 33 and 37). These values were excluded from the calculation of b. The calculated value for b, based on the remaining 77 samples, is 1.3138. Thus, the ISI for the secondary standard is estimated as 1.0 × 1.3138 = 1.3138.

The standard error for b is calculated as 0.0151. The coefficient of variation for b is 100 × (0.0151 × 1.3138) = 1.1%. Confidence limits for b are calculated as in reference (7) at probability = 0.95, i.e. 1.2841 (lower limit) and 1.3444 (upper limit).

In this example, there was a deviation from linearity. This can be shown by calculating the structural relation for patients’ samples only. The latter does not pass through the mean of the normal samples (see Fig. 2). The equation for the line for patients only is characterized by b = 1.4083 and a = −0.8028. For this equation, a clotting time of 35.4 seconds with RBT/90 (i.e. INR = 2.0) corresponds to a clotting time of 22.26 seconds with the rabbit-brain secondary standard. With the ISI (i.e. 1.3138) and the MNPT (12.73 seconds) for this secondary standard, a clotting time of 22.26 seconds can be transformed to an INR of 2.08 (see formula, p. 80). In this example, the assignment of an ISI based on all samples is acceptable because INRs calculated with the ISI do not differ by more than 10% from INRs calculated with the equation describing the line for patients only.

References


Appendix 2

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

Thromboplastins: 1. Rabbit brain thromboplastin secondary standard
                2. 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 ISI and 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 test batch were both timed with the same photoelectric coagulometer, the order in which the two preparations 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 batch (ISI_b) is calculated as \( \text{ISI}_b = \text{ISI}_w \times b \), where \( b \) is the slope of the straight line fitted to a double-logarithmic plot (similar to Fig. 2; see Appendix 1, p. 90) of the prothrombin times in Table 2, with the prothrombin times for the secondary standard and the test batch being shown on the vertical and horizontal axes, respectively. The formula for \( b \) is given by equation (3) in Appendix 1, p. 88. The standard error of \( b \) is obtained from equation (5) in Appendix 1, p. 89. The coefficient of variation (%) of \( b \) is \( 100 \times \left( \frac{s_b}{b} \right) \).

Example

For the data from Table 2, 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
Table 2
Prothrombin times for the calibration of an individual batch of rabbit thromboplastin

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<tr>
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<td>37.6</td>
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given as 1.31. Thus, the ISI for the test 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 $100 \times (0.0130/0.9538) = 1.36\%$. The confidence limits for $b$ at probability $p = 0.95$ are calculated as in Appendix 1, p. 89, i.e. 0.9271 (lower limit) and 0.9812 (upper limit).