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

NOTE: This document has been prepared for the purpose of inviting comments on the proposals contained therein, and for the preparation of the materials to be considered by the Expert Committee on Biological Standardization at their next Meeting on 17-21 October 2011. **The text in its present form does not represent an agreed formulation of the Expert Committee. Comments proposing modifications to this text MUST be received by 20 September 2011** and should be addressed electronically to the attention of Dr Ana Padilla (e-mail address: padillaa@who.int) Blood Products and related Biologicals Programme, Quality Assurance and Safety: Medicines, World Health Organization, 1211 Geneva 27, Switzerland.

The outcome of the deliberations of the Expert Committee will be published in the WHO website (www.who.int/bloodproducts). The final agreed formulation of the document will be edited to be in conformity with the "WHO style guide" (WHO/KMS/WHP/09.1).

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68 INTRODUCTION

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

76
77 Various types of thromboplastin are prepared commercially and, in order to be able to interpret
78 the results of the prothrombin-time test, it is essential that each reagent is correctly calibrated.
79 This will ensure that the results of tests with different products and batches are reproducible and
80 can be compared. A procedure for the calibration of thromboplastins using a logarithmic plot of
81 prothrombin times has been developed (1) and was described in the forty-eighth report of the
82 WHO Expert Committee on Biological Standardization (2). With this procedure, the definition
83 of a calibration parameter called the International Sensitivity Index (ISI) became feasible. It is
84 possible to express prothrombin-time results on a common scale, i.e. the International
85 Normalized Ratio (INR), if the ISI of the thromboplastin used
86 is known.

87
88 There is now substantial evidence that coagulometers can have unpredictable and marked effects
89 on the ISI of thromboplastins (3-6). Because of these effects, some manufacturers provide a
90 "system ISI" for a particular thromboplastin/coagulometer combination. However, this
91 procedure appears to have limitations since variations in the system ISI with the same reagent
92 and coagulometer at different centres have been demonstrated in collaborative studies (7, 8).

93
94 In general, the calibration of a given thromboplastin is more precise if performed against an
95 international reference preparation¹ of similar composition and from the same species (9-11). A
96 system of coexisting international reference preparations has been established in which each of
97 these materials is related to the first primary international reference preparation, the first
98 International Reference Preparation of Thromboplastin (Human, Combined), coded 67/40 (see
99 Fig. 1). Two international reference preparations of thromboplastin are currently available from
100 the relevant WHO International Laboratory for Biological Standards: the fourth International
101 Standard for Thromboplastin, Rabbit, Plain (coded RBT/05) (13); and the fourth International
102 Standard for Thromboplastin, Human, Recombinant, Plain (coded rTF/09) (14). Other
103 international reference preparations have been discontinued. The development of these
104 preparations is described in section 2.

105
106 In theory, the ISI/INR system should ensure that the ISI value calculated for a given reagent is
107 independent of the species from which the international reference preparation is derived, because
108 all have been directly or indirectly calibrated against the first International Reference Preparation
109 of Thromboplastin, Human, Combined (coded 67/40). However, this is not always the case;
110 several recent observations have demonstrated that reagents calibrated against the second
111 International Reference Preparation of Thromboplastin, Human, Plain, a material coded
112 BCT/253 (the predecessor of rTF/95) (15), provide lower INR values than those calibrated
113 against RBT/79 (the predecessor of RBT/90) or OBT/79 (9, 11, 16). The extent of these

¹ 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

114 differences in INR is not usually large enough to cause serious concerns from a practical point of
115 view. The discrepancy is due to calibration errors that persist because the different international
116 reference preparations were not checked against each other in the original studies. A new
117 procedure has now been agreed upon: international thromboplastin reference preparations of
118 whatsoever origin and composition will be calibrated against all existing international reference
119 preparations in order to ensure consistency of results between different routes of calibration (17).

120

121 It is recommended that the international reference preparation of the same species or
122 composition should be used for calibration of secondary standards, e.g. working standards, by
123 manufacturers and national reference laboratories. Thus, plain rabbit thromboplastins should be
124 calibrated against RBT/05; plain human thromboplastins against the human recombinant
125 material rTF/09. It has been demonstrated that bovine or rabbit combined thromboplastins can be
126 calibrated with acceptable precision against RBT/05 (18). It is recommended that bovine or
127 rabbit combined thromboplastins should be calibrated against RBT/05.

128

129 The calibration of prothrombin-time systems is not an easy task. Furthermore, there is
130 considerable variation in results from different laboratories performing the same procedures, as
131 shown by published multicentre calibration studies (9-15, 19, 20). In these studies,
132 interlaboratory variation in ISI, expressed as a coefficient of variation, ranged from
133 approximately 1.7% to 8.1%. The preparation, certification, and use of deep-frozen or
134 lyophilized plasmas for ISI calibration and INR determination has been described as an
135 important adjunct to fresh-plasma ISI calibration (21). The purpose of these guidelines which
136 replace the requirements published in the forty-eighth report of the WHO Expert Committee on
137 Biological Standardization (2), now discontinued, is to take account of the above-mentioned
138 observations and to describe in detail the technical methods currently in use. These guidelines
139 represent the state of the art. Modifications to the methodology may give comparable results, but
140 must be validated against the methodology described in the guidelines.

141

142 1. DEFINITIONS

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

147

148 *Thromboplastin.* A reagent containing tissue factor and coagulant phospholipids. Many
149 commercial thromboplastins are crude extracts prepared from mammalian tissues, in which
150 tissue factor is only a minor component on a weight basis, and which also contain phospholipids.
151 A preparation of a thromboplastin consisting of a tissue extract alone, either with or without
152 added calcium chloride, is termed "plain". When the preparation contains adsorbed bovine
153 plasma as a source of additional factor V and fibrinogen it is termed "combined".

154 Thromboplastins may also be grouped into types, according to the tissue source from which they
155 are derived, e.g. human, bovine, rabbit brain or lung, or human placenta. The tissue-factor
156 component of recombinant human thromboplastin reagents is produced in *Escherichia coli* or
157 insect cells by recombinant DNA techniques and then lipidated in vitro.

158

159 *Prothrombin time (PT) (tissue-factor-induced coagulation time).* The clotting time of a plasma
160 (or whole blood) sample in the presence of a preparation of thromboplastin and the appropriate
161 amount of calcium ions. The time is reported in seconds (22).

162 *Prothrombin-time system.* A procedure by which the prothrombin time is determined using a
163 specific thromboplastin reagent and a particular method, which may be manual, e.g. a tilt-tube

164 method, or involve the use of an instrument that records the coagulation endpoint automatically.
165 The method should be described and the description should include all procedures and
166 equipment used, e.g. the pipettes and test-tubes.

167
168 *Mean normal prothrombin time (MNPT)*. The geometric mean of the prothrombin times of the
169 healthy adult population. For practical purposes, the geometric mean of the prothrombin time
170 calculated from at least 20 fresh samples from healthy individuals, including those of both sexes,
171 is a reliable approximation of MNPT. It is not necessary to collect and test all the individual
172 samples in one session. It is recommended that each laboratory should determine MNPT using
173 its own prothrombin-time system. Pooled normal plasma (either deep-frozen or freeze-dried)
174 may be suitable if the clotting time obtained is related to the MNPT value and its storage stability
175 is acceptable.

176
177 *Prothrombin-time ratio (tissue-factor-induced coagulation relative time)*. The prothrombin time
178 obtained with a test plasma or whole blood divided by the MNPT, all times having been
179 determined using the same prothrombin-time system.

180
181 *International Sensitivity Index (ISI)*. A quantitative measure, in terms of the first International
182 Reference Preparation of Thromboplastin, Human, Combined, coded 67/40, of the
183 responsiveness of a prothrombin-time system to the defect induced by oral anticoagulants (see
184 Appendix 1).

185
186 *International Normalized Ratio (INR)*. For a given plasma or whole blood specimen from a
187 patient on long-term oral anticoagulant therapy, a value calculated from the prothrombin-time
188 ratio using a prothrombin-time system with a valid ISI according to the formula $INR =$
189 $(PT/MNPT)^{ISI}$.

190

191 **2. INTERNATIONAL REFERENCE PREPARATIONS OF** 192 **THROMBOPLASTINS**

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

203

204 Current prothrombin-time systems are based on the use of three different species of
205 thromboplastin reagents: human, bovine and rabbit. Originally, the standardization of these
206 thromboplastin reagents likewise involved three different reference preparations, one for each of
207 the three species of plain thromboplastin reagents in use (Fig. 1).

208

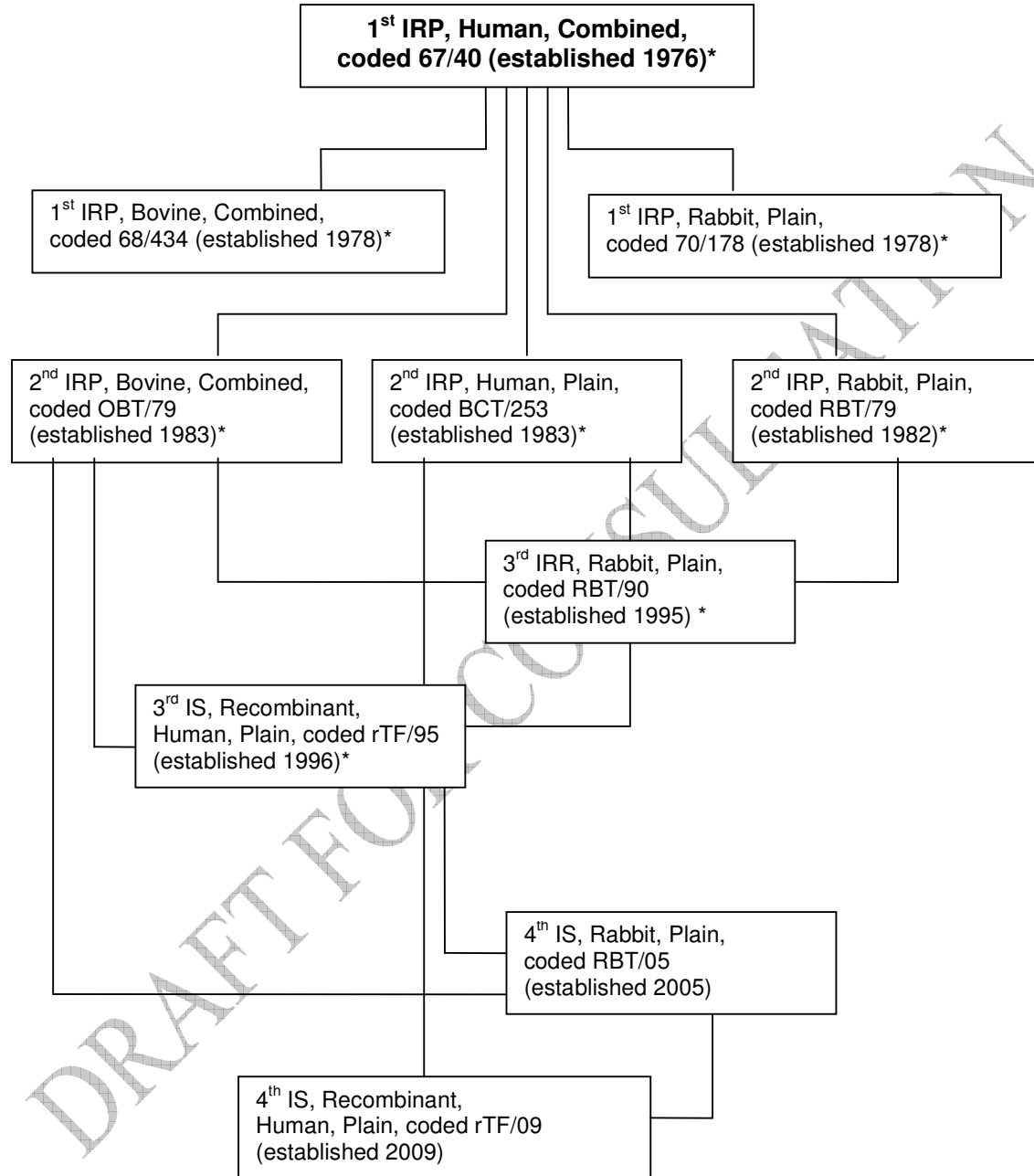
209 The first International Reference Preparation of Thromboplastin, Human, Combined (coded
210 67/40), was established by the WHO Expert Committee on Biological Standardization in 1976
211 (23). It was a freeze-dried preparation, filled in sealed glass ampoules, and contained a human
212 brain extract to which adsorbed bovine plasma had been added to optimize the content of non-
213 vitamin-K-dependent coagulation factors (i.e. factor V and fibrinogen). Its ISI value was set at

214 1.0 by definition. In 1983, this preparation was discontinued and replaced by the second
215 International Reference Preparation of Thromboplastin, Human, Plain (coded BCT/253), a
216 human brain extract with no added coagulation factors and an assigned ISI value of 1.1 (24).
217 When stocks of BCT/253 became exhausted, a new preparation of human recombinant
218 thromboplastin (coded rTF/95) was established in 1996 as the third International Standard for
219 Thromboplastin, Human, Recombinant, Plain, with an assigned ISI value of 0.94 (12,25). When
220 stocks of rTF/95 became exhausted, a new preparation of human recombinant thromboplastin
221 (coded rTF/09) was established in 2009 as the fourth International Standard for Thromboplastin,
222 Human, Recombinant. Plain, with an assigned ISI value of 1.082 (14).
223

224 The first International Reference Preparation of Thromboplastin, Bovine, Combined (coded
225 68/434) was established by the WHO Expert Committee on Biological Standardization in 1978
226 (26). It was calibrated using the first International Reference Preparation of Thromboplastin,
227 Human, Combined (67/40). Another material, also calibrated against 67/40, was established as
228 the second International Reference Preparation of Thromboplastin, Bovine, Combined (coded
229 OBT/79), in 1983 with an assigned ISI of 1.0 (27). This material (OBT/79), which was derived
230 from bovine brain and combined with factor V and fibrinogen, has been used to calibrate
231 thromboplastin materials of bovine origin and combined thromboplastins of whatever origin. When
232 OBT/79 became exhausted in 2004, it has not been replaced by a new international reference
233 preparation of bovine origin. For the calibration of thromboplastins of rabbit origin, a first
234 International Reference Preparation of Thromboplastin, Rabbit, Plain (coded 70/178), was
235 established in 1978. This material was calibrated against the first International Reference
236 Preparation of Thromboplastin, Human, Combined (coded 67/40), in an international
237 collaborative study which also included bovine thromboplastin combined (26). When stocks of
238 70/178 became exhausted, the second International Reference Preparation of Thromboplastin,
239 Rabbit, Plain (coded RBT/79), was established in 1982 with an ISI value of 1.4; this was also
240 calibrated against 67/40 (28). The third International Reference Reagent for Thromboplastin,
241 Rabbit, Plain (coded RBT/90), obtained from rabbit brain with no added factors, was calibrated
242 against each of the three species of thromboplastins and established by the WHO Expert
243 Committee on Biological Standardization in 1995 with an ISI of 1.0 (29). When stocks of
244 RBT/90 were exhausted, a new preparation of rabbit brain thromboplastin (coded RBT/05) was
245 established as the fourth International Standard for Thromboplastin, Rabbit, Plain, with an
246 assigned ISI value of 1.15 (13). This material should be used for the calibration of rabbit
247 thromboplastins as well as bovine thromboplastins.

248 **Figure 1**

249

250 **International reference preparations for thromboplastins and their**
251 **calibration relationships**
252

253 IRP International Reference Preparation

IRR International Reference Reagent

IS International Standard

* Now discontinued

254 The wide use of these international reference preparations for calibrating secondary standards
255 reflects the value placed on them by the scientific community responsible for the control of
256 thromboplastins. An independent control of a manufacturer's ISI assignments by a national
257 reference laboratory is also recommended. National control authorities should consider
258 designating an expert laboratory in the country for testing thromboplastin reagents and plasmas
259 used by clinical laboratories to control oral anticoagulant therapy to ensure that they are in
260 accordance with guidelines published by WHO.

261
262 The international reference materials for thromboplastins are in the custody of the National
263 Institute for Biological Standards and Control, Potters Bar, UK. Samples of these materials are
264 distributed to national reference laboratories or national control laboratories for biological
265 products and, with handling charges, to other organizations such as manufacturers, universities,
266 research institutes and hospital laboratories. The principle that international reference
267 preparations are distributed free of charge to national control authorities for the purpose of the
268 calibration of national standards has been adhered to since the establishment of international
269 biological standardization activities (30).

270

271 **3. PREPARATION OF THROMBOPLASTINS**

272 The method of preparation of thromboplastins should be such that there is consistency from
273 batch to batch and that the preparations are suitable for use in the control of oral anticoagulant
274 treatment. The thromboplastins shall comply with the specifications outlined in section 4, below.

275

276 All attempts should be made to use the least contaminated source material possible and to use a
277 manufacturing procedure that prevents further contamination and the growth of organisms during
278 manufacture. Thromboplastins of animal origin shall be prepared only from healthy animals.
279 Thromboplastins prepared from bovine brain shall be derived only from cattle from countries
280 that have not reported indigenous cases of bovine spongiform encephalopathy (BSE) and which
281 have a compulsory BSE notification system, compulsory clinical and laboratory verification of
282 suspected cases and a surveillance programme in place (31).

283

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

289

290 **4. TESTS ON THROMBOPLASTINS**

291 Each batch of thromboplastin shall satisfy the following criteria.

292

293 **4.1 Response to coumarin-induced coagulation defect**

294 The response to the coumarin-induced coagulation defect shall be measured by the prothrombin
295 time obtained using normal and coumarin plasmas. Thromboplastins with a manual ISI between
296 0.9 and 1.7 are acceptable. However, ISIs towards the lower end of this scale are desirable, since
297 some studies have shown that interlaboratory variation in ISI is greater for high than for low ISI
298 systems (20). It has been suggested that the INR is less accurate when prothrombin time is
299 determined with insensitive thromboplastins that have high ISI values (32).

300

301

302

303 **4.2 Content of haemoglobin and serum**

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

306

307 **4.3 Opacity and sediment volume**

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

311

312 **4.4 Containers**

313 International reference preparations for thromboplastins are freeze-dried in sealed glass
314 ampoules (33), but secondary standards may be freeze dried in ampoules or vials.

315

316 **4.5 Stability**

317 The method of manufacture shall be such that the thromboplastin preparations are stable. All
318 reagents eventually lose activity when stored at elevated temperatures, and stability shall be
319 checked by an accelerated degradation test (34, 35).

320

321 Accelerated degradation studies are considered to be only a useful rather than an absolute guide
322 to the stability of thromboplastins maintained at the storage temperatures recommended by the
323 manufacturer. Lyophilized standard thromboplastins are routinely stored at low temperatures to
324 maintain their stability. A small part of the standard material may be stored at an even lower
325 temperature ('ultra-low temperature stock'). Under the assumption that the rate of degradation is
326 different in the two storage conditions, a comparison of the results of samples of the routine
327 storage to those of the 'ultra-low-temperature stock' can be used to assess the stability status of
328 the standard material (36). The stability of the thromboplastins must also be determined for the
329 conditions under which they are stored, i.e. in a real-time stability study (37, 38).

330

331 **5. CALIBRATION OF PROTHROMBIN-TIME SYSTEMS**

332 Four types of calibration should be distinguished:

333

a) calibration of international reference preparations;

334

b) calibration of secondary standards, e.g. national reference preparations and
335 manufacturers' working standards;

336

c) calibration of manufacturers' commercial preparations against the corresponding working
337 standard ("lot-to-lot" calibration);

338

d) local-system calibration.

339

340 In general, the results of calibrations are used by laboratories other than the calibrating
341 laboratories. The clinical laboratories should therefore be aware of the interlaboratory variation
342 in ISI values for the thromboplastin reagent. Type (d) calibration involves the use of deep-frozen
343 or freeze-dried plasmas with assigned INR or prothrombin-time values which are described
344 below. Type (a) and (b) calibrations should be carried out with a large number of fresh plasma or
345 whole blood samples. Several studies suggest that, under certain circumstances, fresh plasmas
346 for type (c) calibrations can be reliably replaced by frozen, freeze-dried, pooled plasma or
347 plasmas artificially depleted of vitamin-K-dependent coagulation factors (41-43). Manufacturers
348 should validate this procedure by means of fresh plasmas.

349

350 Prothrombin-time systems shall be calibrated in terms of the appropriate international reference
351 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

352 preparations are limited, and it is not possible to use these materials in routine tests to calibrate
353 each batch of the many thromboplastins produced by different manufacturers. Calibration of
354 individual batches of thromboplastin shall be carried out by comparison with a secondary
355 standard, which shall be a batch of the same or a similar thromboplastin calibrated against the
356 appropriate international reference preparation.

357

358 The basis of the thromboplastin calibration model is necessarily an empirical one. While there is
359 good evidence that the calibration relationship defined in a double-logarithmic plot of
360 prothrombin times is usually linear, and that the same line represents data points for both patients
361 and healthy subjects, the possibility of departure from these assumptions cannot be ruled out.
362 Statistical methods to test deviations from the latter assumption have been described (44, 45). In
363 the case of marked deviation, the assignment of an ISI would not be meaningful. For practical
364 purposes, the assignment of an ISI is acceptable if INRs calculated with the ISI derived from the
365 overall regression line (i.e. for patients plus healthy subjects) do not differ by more than 10%, in
366 the INR range 2-4.5, from INRs calculated with the equation describing the regression line for
367 patients only (see Appendix 1).

368

369 **5.1 The calibration of international reference preparations**

370 The calibration of the international reference preparations for thromboplastins, and their future
371 replacements, should be carried out in international multicentre collaborative studies using fresh
372 coumarin, normal plasma and manual techniques. Each collaborative study for replacement of an
373 international reference preparation should include the testing of all existing international
374 reference preparations. The ISI assigned to the replacement material should be the mean of the
375 ISIs obtained by calibration with all existing international reference preparations (17).

376

377 **5.2 The calibration of secondary standards**

378 Secondary standards of human origin should be calibrated against the current International
379 Standard, i.e. the fourth International Standard for Thromboplastin, Human, Recombinant, Plain
380 (coded rTF/09); plain thromboplastins of rabbit brain and rabbit lung should be calibrated against
381 the fourth International Standard for Thromboplastin, Rabbit, Plain (coded RBT/05).
382 Thromboplastins of bovine or rabbit brain combined with adsorbed bovine plasma should also be
383 calibrated against RBT/05.

384

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

388

389 **5.3 The calibration of individual batches of thromboplastins**

390 The precision of calibration is greatest when similar materials and methods are compared. For
391 this reason, a national reference preparation or manufacturer's working standard used for the
392 calibration of individual batches of thromboplastin should be a thromboplastin of similar
393 characteristics to these batches (i.e. derived from the same tissue of the same species, using a
394 similar manufacturing process). Batch-to-batch calibration should be performed by the
395 manufacturer before release of the reagent and consistency of ISI values should be shown.

396

397 **6. THE CALIBRATION PROCEDURE**

398 The calibration procedure entails the determination of a series of prothrombin times, using
399 normal and abnormal plasmas or whole blood samples, with both the reference and the test
400 thromboplastin. The tests are performed using either fresh samples from individual subjects
401 (procedure 1) or freeze-dried or frozen plasmas (procedure 2 and 3). Abnormal plasmas for

402 procedure 1 are obtained from patients undergoing long-term oral anticoagulant treatment.
403 Freeze-dried or frozen plasmas for procedure 2 may be pooled plasmas from healthy subjects and
404 from patients undergoing long-term anticoagulant treatment.
405

406 Procedure 1 is recommended for the calibration of secondary standards or any other
407 prothrombin- time system against the appropriate international reference preparation and for the
408 calibration of whole-blood coagulometers. Procedure 1 can also be used for the calibration of
409 individual batches of thromboplastin against the corresponding secondary standard (i.e. lot-to-
410 lotcalibration), but may be replaced by procedure 2 if the same results are obtained.
411

412 The precision of the calibration relationship depends on the number of plasmas and on a
413 balanced distribution of normal and abnormal plasmas over the "therapeutic" range of INR
414 values. The recommended number of abnormal plasmas is three times the number of normal
415 plasmas.
416

417 **6.1 Procedure 1. Calibration of a secondary standard using individual fresh** 418 **plasma or blood samples**

419 This procedure consists of a set of tests using freshly opened or reconstituted thromboplastins
420 and different individual samples of fresh plasma or whole blood. The procedure should be
421 repeated on at least five separate occasions using fresh reagents on each occasion (see section
422 6.1.4). The procedure need not be repeated on consecutive days but should be completed as soon
423 as possible. The tests in any one laboratory on any one day should be performed by the same
424 person.
425

426 **6.1.1 Blood samples**

427 Blood samples from healthy subjects and patients who have been on oral anticoagulants for at
428 least 6 weeks should be selected. Samples from patients treated with heparin should not be used.
429 It is recommended that patients' samples with INR values in the range 1.5-4.5 should be selected.
430

431 Blood should be obtained by venepuncture, avoiding haemolysis and contamination with tissue
432 fluids. It should be drawn either with a plastic syringe and transferred to a plastic tube, or with
433 other non-contact activation equipment. Nine volumes of blood should be decalcified with one
434 volume of 109 mmol/l trisodium citrate solution (22). A mixture of trisodium citrate and citric
435 acid is also acceptable if the total citrate plus citric acid concentration is 109 mmol/l and the pH
436 is no lower than 5. The same procedure and materials should be used for all the samples in a
437 given calibration.
438

439 If evacuated tubes are used for blood collection, their lot number should be noted, as there may
440 be lot-to-lot variation. If evacuated tubes are made of glass, they must be properly siliconized
441 internally and the pH of the trisodium citrate plus citric acid solution must be in the range 5-6
442 (46). The sample should be centrifuged as soon as received but no later than 2 hours after blood
443 collection. The centrifugation should be such that the plasma is rendered poor in platelets (i.e. at
444 least 2500g for 10 minutes at a controlled room temperature). The plasma should be taken off the
445 red-cell layer with a plastic pipette, stored undisturbed in a narrow, stoppered, non-contact tube
446 at room temperature and tested within 5 hours after blood collection.
447

448 Some techniques or instruments require the use of non-citrated capillary blood (47). Capillary
449 blood can be obtained by finger or heel puncture. The capillary blood should be obtained without
450 squeezing and tested immediately with the technique or instrument to be calibrated. Venous
451 blood should be obtained from the same subjects (healthy subjects and patients) within 5 minutes

452 of taking the capillary sample, for preparation of citrated plasma as described above and testing
453 with the most appropriate international reference preparation.
454

455 **6.1.2 Reference thromboplastins**

456 The appropriate international reference preparation of thromboplastin (human, or rabbit) should
457 be reconstituted as instructed and the contents of the ampoules transferred to a container in
458 sufficient volume for all tests to be performed in a single calibration session. Specific
459 instructions for use should be supplied by the custodian of these materials.
460

461 **6.1.3 The prothrombin-time test**

462 The prothrombin-time test is performed either by mixing equal volumes of citrated plasma,
463 thromboplastin and calcium chloride solution (25 mmol/l), or by adding a volume of plasma to
464 the required volume of thromboplastin premixed with calcium, and therefore available as a single
465 reagent. The time (in seconds) taken for the mixture to clot when maintained at a temperature of
466 between 36.5 °C and 37.5 °C is recorded. Test instructions for commercial thromboplastins shall
467 be provided by the manufacturers.
468

469 The coagulation end-point for international reference preparations of thromboplastin should be
470 detected by a manual (tilt -tube) technique. The coagulation end-point for other thromboplastins
471 should e detected by a manual technique or with the aid of an automatic endpoint recorder. The
472 same technique should be used throughout the series of tests with a given thromboplastin.
473 Each laboratory should have a system for internal quality control. Records should be maintained
474 of the lot number of all reagents and disposable equipment used. Periodic checks of the
475 temperature of incubation baths or heating blocks and of the volumes of pipettes or pumps
476 should be made and recorded.
477

478 A suggested protocol for reporting the procedure is shown in Appendix 1.
479

480 **6.1.4 Statistical evaluation**

481 The suggested procedure for calculation of the ISI is given in Appendix 1.
482 Before the final orthogonal regression line for the ISI is calculated, it is important to detect
483 blunders and any samples beyond the therapeutic range. Blunders may be due to technical or
484 clerical errors and may seriously influence the estimated ISI. Blunders may be detected as points
485 with a perpendicular distance greater than 3 residual standard deviations from the preliminary
486 orthogonal regression line calculated with all data included (48). It is suggested to detect and
487 remove blunders in a single step. In the next step any patient samples beyond the therapeutic
488 range ($INR < 1.5$ or $INR > 4.5$) should be removed. In this procedure it is important to assess each
489 patient's INR as the mean INR determined with international standard and with the system being
490 calibrated using the ISI obtained after the removal of blunders. Using the INR determined solely
491 with the international standard could induce a bias in the orthogonal regression line and should
492 be avoided (49).
493

494 It is not necessary to replace the removed blunders and non-therapeutic patient samples by new
495 samples, provided that the number of remaining patient samples is at least 55. In any case, the
496 within-laboratory coefficient of variation of the slope of the orthogonal regression line for
497 normals + patients samples should be 3% or less. The number of normal samples should be at
498 least 20 for the calculation of the MNPT. After removal of blunders and non-therapeutic patient
499 samples, the adequacy of the ISI model should be assessed. If the deviation of the INR with the
500 ISI model is greater than 10%, it is suggested to use a different model according to Tomenson
501 (45).

- 502 The sequence of steps in the statistical evaluation is as follows:
- 503 1. Calculate preliminary orthogonal regression line (20 normals + 60 patients).
 - 504 2. Detect blunders defined as points with a perpendicular distance greater than 3 residual
 - 505 standard deviations from the preliminary line.
 - 506 3. Remove blunders in one step and recalculate the orthogonal regression line (normals +
 - 507 patients) and ISI.
 - 508 4. Calculate each patient's INR using the PT determined with the international standard.
 - 509 5. Calculate each patient's INR using the PT determined with the system being calibrated
 - 510 and the ISI from step 3.
 - 511 6. Calculate each patient's mean INR from step 4 and 5.
 - 512 7. Remove patients with mean INR<1.5 or mean INR>4.5
 - 513 8. Recalculate the orthogonal regression line (normals + patients) and ISI.
 - 514 9. To assess the adequacy of the ISI model, calculate the deviation D of the INR
 - 515 determined with the ISI model from the true INR for INR=2.0 and for INR=4.5. If $D <$
 - 516 10 %, the ISI model is deemed to be adequate. If $D > 10\%$, use Tomenson's formula for
 - 517 INR calculation (see Appendix 1).
 - 518

519 **6.2 Procedure 2. Calibration of individual batches of thromboplastin**

520 Calibration of individual batches of thromboplastin may be carried out with pooled normal
521 plasmas and pooled coumarin plasmas or plasmas artificially depleted of vitamin-K-dependent
522 coagulation factors (41, 42). The number of plasma pools required for precise calibration is, in
523 general, much smaller than the number of fresh individual plasma samples required for
524 procedure 1. The scatter of data points about the regression line is relatively small because the
525 batch to be calibrated is very similar to the working reference preparation and/or because the
526 biological variation caused by individual samples is reduced by the pooling of plasmas. It has
527 been reported that lot-to-lot calibration of bovine and rabbit thromboplastins could be performed
528 with as few as three plasma pools (41, 42), but the accuracy of such a simplified procedure may
529 depend on the quality of the pooled plasmas and the thromboplastin being calibrated. It is
530 recommended that any procedure using pooled or artificially depleted plasmas be validated
531 against the fresh plasma procedure (procedure 1).

532 **6.2.1 Pooled plasma**

534 *Properties of pooled normal plasma*

535 Plasma should be obtained from healthy adults and should comply with the appropriate section
536 of Requirements for the Collection, Processing and Quality Control of Blood, Blood
537 Components and Plasma Derivatives (50). The normal plasmas for pooling should be obtained
538 from at least 20 different donors. Nine volumes of blood should be decalcified with one volume
539 of 109 mmol/l trisodium citrate solution. The packed-cell volume-fraction should be between
540 0.35 and 0.45.

541 The final preparation should be platelet-poor plasma, which has been freeze-dried or frozen (at -
542 40°C or below) in suitable containers. The stability of deep-frozen plasma should be monitored
543 regularly by testing the prothrombin time. After reconstitution or thawing, the pH should not be
544 lower than 7.3 and should not exceed 7.9, and the plasma should not show any shortening or
545 prolongation of clotting times for at least 2 hours when held at ambient temperature (51). The
546 stability of freeze-dried normal plasma should be checked by accelerated degradation tests. Such
547 plasma should not show a prolongation of prothrombin time of over 5% after storage for 4 weeks
548 at 37°C. The factor V content should be between 60% and 140% of the average content of fresh
549 normal plasma (52).

550
551

552 *Properties of pooled coumarin plasma*

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

556
557 Plasma should not be obtained from donors with a history of jaundice or from those with plasma-
558 lipid abnormalities. The collection of plasma, the properties of the final preparation and the
559 stability of the freeze-dried pools are the same as for pooled normal plasma described above.

560

561 The INR of the pooled plasma should be stated, as should the thromboplastins used for its
562 assignment. It should be noted that the INR value of a freeze-dried plasma usually depends on
563 the thromboplastin used for its assignment (53-55). At least two different plasma pools, having
564 an INR between 1.5 and 4.5 and with a difference of at least 1.0 in their INRs, in combination
565 with one normal plasma pool are necessary for the calibration procedure.

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

568

569 *Freedom from infectious agents*

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

572

573 **6.2.2 The test**

574 The test should be carried out by the same procedure as described for procedure 1 (see section
575 6.1.3). An example of the protocol for the recording of the results is given in Appendix 2. The
576 procedure should be repeated on at least four separate occasions (43), with fresh reagents used on
577 each occasion. At least three plasma pools should be used to permit the testing of linearity.

578

579 Freeze-dried plasma pools should be reconstituted at least 15 minutes before the actual test.

580 Plasma that has been frozen and subsequently thawed, or reconstituted freeze-dried plasma,
581 should not be centrifuged, and unused reconstituted or thawed material should be discarded after
582 2 hours.

583

584 **6.2.3 Statistical evaluation**

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

592

593 To define the ISI of a batch of thromboplastin, a sufficient number of tests should be carried out
594 to obtain a within-laboratory coefficient of variation for the slope of the orthogonal regression
595 line of 3% or less. The recommended procedure for calculation of the ISI is given in Appendix 2.

596

597 **6.3 Procedure 3. Local system calibration using certified plasmas**

598 Laboratories may calibrate their own local system (i.e. instrument/thromboplastin combination)
599 using certified plasmas supplied by manufacturers or reference laboratories. A certified plasma is

600 a deep-frozen or lyophilized plasma with an assigned prothrombin time or INR value. Two
601 procedures using certified plasmas have been described:

602
603 One procedure is a modification of the WHO method for ISI determination. In a set of plasmas
604 each plasma is assigned a manual PT value by the manufacturer or reference centre using an
605 international standard for thromboplastin. In the local laboratory PT's of each plasma are
606 measured with the local instrument/reagent combination, and the two sets of PT's are plotted on
607 a log/log plot. The slope of the orthogonal regression line is used to determine the local ISI (See
608 Appendix 1), which can then be used for subsequent determination of INR's from the local PT's
609 and MNPT (7, 8, 40, 57, 58). An underlying assumption of the WHO orthogonal regression
610 model is that a single line describes the relationship between log(PT) of abnormal and normal
611 plasmas. If there is a significant deviation of the two calibration lines (i.e. abnormal-only and
612 normal/abnormal combined), a correction according to Tomenson should be applied (45, 59).

613
614 The other procedure which has been proposed involves assignment of INR values to a set of
615 plasmas with the manual method and an international thromboplastin standard by the
616 manufacturer or reference centre. The PT's of these plasmas are measured locally using the local
617 instrument/reagent combination, and the local test system PT's are plotted against the reference
618 INR's on a log/log plot. An orthogonal regression line is calculated, and INR's of patients'
619 plasmas can be interpolated directly from local PT's using this line, without the need for ISI or
620 MNPT determination. Although many studies of direct INR determination were performed with
621 linear rather than orthogonal regression, the latter is preferable (see later) (39, 55, 60-65).

622
623 The primary aim of the above procedures is to improve INR reliability.
624 A number of studies have shown that use of either of these procedures can considerably reduce
625 inter-laboratory imprecision in INR determination (8, 60-62, 66, 67). For example, in one study
626 the mean deviation of 95 local systems from the "true" INR was +14.4% with the manufacturers'
627 ISI, but reduced to +1.04% with the local ISI (8). In another study, the inter-laboratory CV of the
628 INR was reduced from 12% with the manufacturers' ISI to 6% using direct INR determination
629 with a certified plasma procedure (60).

630
631 It should be recognised that there are a number of different ways in which plasmas can be
632 prepared and certified. The following sections describe the various methods of preparation,
633 certification and use and their advantages and disadvantages.

634

635

636 **6.3.1 Preparation of Certified Plasmas**

637 **6.3.1.1. Type of plasma – AVK (from patients on Anti-Vitamin K therapy) or artificially** 638 **depleted of prothrombin complex factors (ART)**

639 The intention is for certified plasmas to be as similar as possible to fresh plasmas from patients,
640 thus on theoretical grounds AVK plasmas might be preferred, although for practical reasons
641 these have to be pooled rather than individual donations. In some studies where the two types of
642 plasmas have been compared, AVK plasmas give closer agreement with fresh plasmas and better
643 inter-laboratory agreement than artificially depleted plasmas (53, 68). Artificially depleted
644 plasmas have several advantages over plasmas from patients on oral anticoagulants, including
645 availability of larger volumes, wider selection of PT values across the therapeutic interval, and
646 the possible reduced risk of virus transmission (69). It can be argued that larger volumes of AVK
647 plasmas could be obtained by pooling donations from patients on anti-vitamin K therapy, but this

648 procedure would make a spectrum of INR values more difficult to obtain because of the
649 averaging in such a pool.

650
651 The European Concerted Action on Anticoagulation (ECAA) has prepared depleted plasmas by
652 artificial depletion of normal human plasma by selective adsorption of vitamin K-dependent
653 clotting factors with barium sulfate to provide a range of values which spanned the therapeutic
654 interval (40). The ECAA has found that there is a small difference between the results with
655 ECAA lyophilized artificially depleted plasmas and lyophilized AVK plasmas in ISI value
656 assignment, but both of these differed by a similar amount from a conventional fresh plasma ISI
657 calibration (40). The mean calibration slopes with both types of lyophilized plasma were
658 generally higher than that with fresh AVK plasmas but the differences were not great in clinical
659 terms. It should be noted that the ECAA study was performed with one combination of a human
660 brain IRP and recombinant thromboplastin and the manual technique, and that the conclusions
661 may not be applicable to all other reagent-instrument combinations.

662 The reliability of artificially depleted plasmas and AVK plasmas depends on the method of
663 preparation and certification.

664

665 **6.3.1.2 . Method of preparation – frozen or freeze-dried**

666 Although lyophilization seems a simple solution to the difficulties associated with storage and
667 shipment of certified plasmas, there are problems associated with lyophilized materials.

668 Studies have shown that the INR of fresh plasmas is largely unchanged on freezing, whereas on
669 freeze drying the INR may change significantly depending on the method of freeze-drying and
670 the thromboplastin/instrument combination used (52, 70-72). Buffering of plasmas shortly after
671 blood collection can reduce but not eliminate changes after freeze-drying. The magnitude of the
672 changes is not the same for all reagents or instruments. The measured INR of lyophilized
673 certified plasmas depends on the thromboplastin reagent and instrument used. The use of
674 RBT/90 presents problems relating to its poor endpoint particularly with lyophilized plasmas
675 giving long prothrombin times.

676 The widespread use of frozen plasmas presents logistical difficulties due to the potential
677 instability of frozen plasmas, although in some countries frozen certified plasmas have been used
678 to a limited extent with success regarding the reduction of the interlaboratory imprecision (64,
679 66)..

680
681 Freeze-dried plasmas represent the most practical approach in general laboratories and their use
682 has been associated with reduced interlaboratory imprecision in several studies.

683 **6.3.1.3. Citrate Concentration**

684 It is well known that citrate concentration can affect the PT, especially of high INR plasmas (73,
685 74). Furthermore, citrate concentration has a variable effect on the ISI, but the magnitude of the
686 effect is not the same for all reagents and instruments (74-77). The recommended citrate
687 concentration for the collection of blood (1 volume of citrate solution + 9 volumes of blood) for
688 PT is 0.109 M (3.2%), although concentrations in the range 0.105 – 0.11M can be accepted (76),
689 and the citrate concentration of certified plasmas should be as close as possible to that in fresh
690 plasma collected in the above anticoagulant (69). Citrate concentrations of 0.129 M (3.8%)
691 should not be used for PT tests.

692

693 **6.3.1.4. Number of Plasmas**

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

695 a) Local test system ISI calibration

696 According to the WHO Guidelines, to define the ISI of a working thromboplastin, a sufficient
697 number of separate tests should be carried out to obtain a within-laboratory coefficient of
698 variation (CV) for the slope of the orthogonal regression line of 3% or less (see par.6.1.4). In an
699 ECAA study of lyophilized depleted and individual AVK plasmas, it has been shown that the
700 number of 60 lyophilized abnormal samples required for a full WHO calibration can be reduced
701 to 20 if combined with results from 7 lyophilized normal plasmas; further reductions below this
702 number were associated with decreased precision of the calibration line and hence increased
703 variability of the INR (78). However, the use of pooled AVK plasmas may reduce the scatter of
704 individual plasmas about the line (79), and with pooled plasmas and repeat testing it is possible
705 that a lower number could be used, e.g. acceptable precision has been achieved with 6 pooled
706 AVK plasmas containing at least 50 patient samples in each pool and 2 pooled normal plasmas if
707 these were analyzed on at least 3 days (43).

708

709 b) "Direct" INR determination

710 For "direct" INR determination a smaller number of pooled plasmas can be used. Studies have
711 shown improved inter-laboratory variability with as few as 6 (64), 5 (61, 55), 3 (39), or 2 (60)
712 plasmas, but considering that one of the plasmas should be a normal and that at least 3 plasmas
713 are required to define a line, a set of one normal and at least 3 abnormal is recommended. One
714 study documented the within-laboratory imprecision of the slope of a calibration line (one
715 normal + 3 abnormal plasmas): the CV ranged from 0.1 to 4.6% (65). The number of donations
716 in each pool should be at least 10 but higher numbers are preferable to ensure normal levels of
717 factor V.

718

719 For both procedures it is important that the abnormal plasmas be chosen to cover the range of 1.5
720 - 4.5 INR. The fibrinogen and factor V content should be between 60% and 140% of the average
721 content of fresh normal plasma (52).

722

723 **6.3.2 Certification (Value Assignment) of Plasmas**

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

725

726 **6.3.2.1 Thromboplastins for certification**

727 WHO standard or European Reference thromboplastins should be used directly if possible.
728 Assuming that the certified plasmas are intended for use with the various types and species of
729 thromboplastin, the two types of WHO standard preparations should be used (human and
730 rabbit). If insufficient WHO or European standards are available, national or secondary
731 standards can be used provided these have been calibrated against the appropriate WHO or
732 European thromboplastin standards in a multi-centre study. If the plasmas are intended for use
733 with only one type of thromboplastin (e.g. human), the appropriate thromboplastin standard
734 preparation should be used. Several studies have shown that the INR value for some lyophilized
735 plasmas obtained with the previous rabbit standard thromboplastin (RBT/90) was greater than
736 the INR obtained with the human and bovine standard preparations (11, 53, 68, 80), especially
737 for artificially depleted plasmas (54). For use with one manufacturer's thromboplastin reagent
738 only, certification with the manufacturer's calibrated reagent is acceptable; such "reagent

739 specific” value assignments have been shown to be reliable in recent collaborative studies (55,
740 65). The manufacturer’s thromboplastin reagent used for reagent-specific certification of plasmas
741 should be calibrated by at least two independent laboratories using the original WHO procedure
742 (see par. 6.1).

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

746

747 **6.3.2.2 Number of laboratories**

748 It is recommended that 3-5 laboratories should be involved in the certification process for each
749 set of plasmas. An individual laboratory’s mean value should differ by no more than $\pm 10\%$ of the
750 overall mean (in terms of INR) obtained with a given thromboplastin reagent. If the difference is
751 greater than 10%, the divergent individual laboratory’s value should not be used.

752

753 **6.3.2.3. Manual technique or instruments**

754 The manual tilt tube method should be used for international standard preparations for
755 thromboplastin, as described in par. 6.1.3. Once certified, the plasmas should be tested for their
756 suitability with various reagent/instrument combinations. Where certification of plasmas is made
757 with one manufacturer’s reagent only, an instrument may be used. In this case the
758 reagent/instrument combination must have been calibrated using the original WHO procedure
759 (see par. 6.1).

760

761 **6.3.2.4. Single or multiple values**

762 For the local test system ISI calibration, the actual values of the PT’s of the certified plasmas
763 will differ according to the species of the standard thromboplastin used, and therefore PT values
764 must be independently certified for the different species. For the direct INR determination
765 approach, the INR values of the plasmas should theoretically be the same whichever reference
766 thromboplastin reagent is used. In practice, differences in INR’s using different thromboplastins
767 have been observed with some freeze-dried plasmas; averaging into a single INR should not be
768 performed if the INR’s with individual standard reagents differ by more than 10% from the mean.
769 Large discrepancies between INR’s with different thromboplastins may indicate that the
770 plasmas are unsuitable for use with thromboplastins of all types. It should be noted that the
771 manufacturer or supplier of the certified plasmas should clearly specify the set of
772 reagent/instrument combinations for which their materials may be reliably used (81) (see
773 Validation of certified plasmas).

774

775 **6.3.2.5. Orthogonal regression**

776 Orthogonal regression is used if each coordinate is subject to independent random error of
777 constant variance (44, 82), e.g. PT measurements with two different reagents by the same
778 instrument or operator. Linear regression is used when one of the values is fixed, i.e. essentially
779 without error. The use of certified plasmas does not conform completely to either of these
780 models, but it is important to recognise that apparently “fixed” values of these plasmas are
781 themselves subject to error. Therefore, orthogonal regression should be used for both procedures,
782 i.e. local system ISI calibration and direct INR determination. The equations for orthogonal
783 regression are given in Appendix 1.

784

785 6.3.2.6. International Reference Plasmas

786 At present there are no established International Reference Plasmas. Work has been initiated
787 towards the development of reference plasmas for “direct” INR assignment (39, 80). These could
788 then be used for direct certification of batches of commercial plasmas, in the same way as for
789 coagulation factor assays. One difficulty, as mentioned above, is that of preparing lyophilized
790 plasmas with the same properties as fresh plasmas, and it may be that frozen plasmas have to be
791 used. Furthermore, for long-term use, the stability of such reference plasmas would need to be
792 carefully checked. In the meantime, commercial plasmas will continue to have their values
793 assigned as described above.

794

795 For local system ISI calibration, ECAA artificially depleted lyophilised plasmas may be used.
796 Sets of 20 are FDA approved to provide calibration of local prothrombin time systems to accord
797 with the WHO PT standardization scheme as substantially equivalent to the latter.

798 For direct INR determination, a set of EU Certified Reference Materials (CRM’s), which
799 consists of a panel of 3 lyophilised plasmas with assigned INR’s, is available from EU Institute
800 for Reference Materials and Measurements, Geel, Belgium.

801

802 It should be realized that the validity of ECAA artificially depleted plasmas and EU CRM’s may
803 be limited to certain combinations of thromboplastins and coagulometers, and may not be
804 generalized to all other reagent-instrument combinations.

805

806

807 6.3.3 Validation of certified plasmas

808 Each set or batch of certified plasmas intended for either local test system ISI calibration or
809 direct INR determination must be validated before release. The validation should be the
810 responsibility of the manufacturer or supplier who may seek help from expert laboratories. The
811 validation should go through the following process. (i) Ten or more fresh plasmas from patients
812 on long-term oral anticoagulation are selected to represent the full therapeutic range of
813 anticoagulation. (ii) The INR of these fresh plasmas shall be determined with an appropriate
814 international standard for thromboplastin, and the mean value (INR_R) shall be calculated. (iii)
815 The INR of the same fresh plasmas shall also be determined with a variety of commercial
816 reagent/instrument combinations following the certified plasma procedure (either ISI calibration
817 or direct INR determination). The mean value (INR_C) shall be calculated. (iv) Finally, paired
818 INR values obtained with the international standard and with the local system are compared to
819 assess their agreement using Bland and Altman’s procedure (83). If the relative difference
820 between the mean values INR_R and INR_C , i.e. $2(INR_R - INR_C)/(INR_R + INR_C)$, is 0.1 or less, the
821 set of certified plasmas are considered as acceptable and may be released for local ISI calibration
822 or direct INR determination. New batches of the same type of preparation should be validated
823 according to the above procedure.

824

825 6.3.4 Use of Certified Plasmas in Clinical Laboratories**826 6.3.4.1 Quality Assessment**

827 An important use of certified plasmas is to perform internal or external quality assessment, ie to
828 determine whether or not corrective action is necessary (81, 84). For quality assessment, a set of
829 3 to 5 certified plasmas with INR in the range 1.5 – 4.5 would be required. The INR’s of the
830 certified plasmas should be calculated from local PT’s and routine ISI, and compared with the
831 certified INR values. If the differences between routine INR and certified INR are greater than

832 15 %, local ISI calibration or direct INR correction should be performed. In addition, the
833 manufacturer of the reagent and certified plasmas should be informed about the discrepant
834 results. Quality assessment with certified plasmas should be performed regularly at intervals of
835 no more than 6 months and should be repeated whenever there is a change in reagent batch or
836 instrument (e.g. servicing, modification, or new instrument). It should be realized that errors
837 caused by local pre-analytical factors (e.g. divergent citrate concentration or contamination of
838 citrate with divalent cations) cannot be corrected by certified plasma procedures (85).
839

840 **6.3.4.2. To determine local ISI**

841 PT's should be measured in quadruplicate in the same working session, with the local
842 instrument/reagent combination for the full set of normal and abnormal plasmas. It is
843 recommended to repeat the measurements on 3 sessions or days to control day-to-day variation.
844 Mean local PT's should be plotted on the horizontal axis against the certified PT values on the
845 vertical axis (log scales). Tomenson's test should be performed to test the hypothesis that the
846 mean log(PT) of the certified normal plasmas lies on the same line as the log(PT) of the certified
847 abnormal plasmas (45, 58, 59). If the hypothesis is not confirmed, Tomenson's correction
848 formula should be applied (45, 58, 59, Appendix 1). Like-to-like comparison should be used
849 wherever possible, i.e. if the local reagent is a human thromboplastin the certified values should
850 be those determined with a human reference reagent. If the INR difference between the routine
851 ISI and the local ISI calibration procedure is greater than 10%, the calibration should be repeated.
852 If the discrepancy persists, the manufacturer or supplier of the local thromboplastin reagent and
853 coagulometer and certified plasmas should be informed. After consultation with the
854 manufacturer of the certified plasmas and, if possible, an expert laboratory, the clinical
855 laboratory should decide which materials and methods for local ISI calibration should be used.
856

857 **6.3.4.3 For Direct INR Measurements**

858 This method is simpler to use as it does not require local ISI or MNPT determinations. PT's
859 should be measured in duplicate with the local instrument/reagent combination for each certified
860 plasma. To allow for day to day variation the measurements should be repeated on at least three
861 separate days. Mean PT's should be plotted on the horizontal axis against the certified INR
862 values on the vertical axis (log scales), and an orthogonal regression line derived. The INR's of
863 patients' plasmas should be calculated from the measured PT's. If the INR difference between
864 the routine ISI procedure and the direct determination is greater than 10%, the certified plasma
865 procedure should be repeated. If the discrepancy persists, the manufacturer or supplier of the
866 local thromboplastin reagent and coagulometer and certified plasmas should be informed. After
867 consultation with the manufacturer of the certified plasmas and, if possible, an expert laboratory,
868 the clinical laboratory should decide which materials and methods for direct INR measurement
869 should be used.
870

871 **7. THE USE OF CALIBRATED THROMBOPLASTINS IN** 872 **CLINICAL PRACTICE**

873 It is possible to express prothrombin-time results on a common scale, i.e. the International
874 Normalized Ratio (INR), provided that the ISI of the thromboplastin and the method used are
875 known. The following formula is used:
876

$$877 \text{INR} = (\text{PT}/\text{MNPT})^{\text{ISI}}$$

878

879 where PT is the patient's prothrombin time and MNPT is the mean normal prothrombin time
880 determined with the same thromboplastin and method. The use of the INR enables comparisons
881 to be made between results obtained using different thromboplastins and methods. It is a
882 misconception, however, that for an individual patient's plasma the INR will always be identical
883 with different thromboplastins and methods (45, 56). Different thromboplastins vary greatly in
884 their responsiveness to individual vitamin-K-dependent clotting factors, i.e. factors II, VII and X,
885 as well as to some non-vitamin-K- dependent factors, e.g. factor V. Discrepancies between INRs
886 determined with different thromboplastins arising from these biological variations and from
887 additional technical errors are therefore not unexpected.

888
889 All medical staff and health auxiliaries involved in controlling oral anticoagulant treatment
890 should be encouraged to use the INR system. It should be appreciated, however, that this system
891 can be accurate only in the INR range explored by the calibration procedure, i.e. 1.5-4.5.

892
893 Manufacturers of commercial reagents should state on the package insert the ISI of the relevant
894 batch of thromboplastin together with the reference preparation against which it has been
895 determined and the instrument for which it is valid.

896

897

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899

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905

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907

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911

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1105 **Appendix 1**1106 **Example of the use of the suggested method for reporting the data for the**
1107 **calibration of any system or a secondary standard of thromboplastin against**
1108 **an international standard preparation**
1109

1110 Thromboplastins: 1. Recombinant human thromboplastin secondary standard
1111 2. Third international standard (IS) for Thromboplastin, Human,
1112 Recombinant, Plain (rTF/95) with an established ISI = 0.94

1113

1114 End-point recording: 1. Automated photoelectric coagulometer for secondary standard
1115 2. Manual (tilt-tube) technique for rTF/95

1116

1117 The tests were conducted on 5 different days. On each day, fresh samples from 4 healthy subjects
1118 and 12 patients were tested (plasma samples from healthy subjects are referred to as “normal”).
1119 On each day, different subjects were selected. The automated coagulometer and manual
1120 determinations were performed more-or-less simultaneously.

1121

1122 **Table 1**1123 **Prothrombin times for the calibration of a secondary standard of**
1124 **recombinant human thromboplastin**

1125 Date	1125 Plasma	1125 rTF/95	1125 Secondary standard
1126 23 February 2009	1126 Normal 1	1126 12.8	1126 10.8
1127	1127 Normal 2	1127 13.2	1127 10.5
1128	1128 Patient 1	1128 33.0	1128 18.5
1129	1129 Patient 2	1129 50.8	1129 27.8
1130	1130 Patient 3	1130 32.0	1130 18.7
1131	1131 Patient 4	1131 46.2	1131 25.4
1132	1132 Patient 5	1132 35.6	1132 21.7
1133	1133 Patient 6	1133 59.8	1133 34.4
1134	1134 Patient 7	1134 45.8	1134 24.9
1135	1135 Patient 8	1135 31.5	1135 18.6
1136	1136 Patient 9	1136 40.4	1136 22.1
1137	1137 Patient 10	1137 49.8	1137 28.2
1138	1138 Patient 11	1138 56.4	1138 27.0
1139	1139 Patient 12	1139 47.8	1139 24.4
1140	1140 Normal 3	1140 14.9	1140 11.6
1141	1141 Normal 4	1141 14.0	1141 10.6
1142 24 February 2009	1142 Normal 5	1142 16.8	1142 12.9
1143	1143 Normal 6	1143 12.8	1143 11.2
1144	1144 Patient 13	1144 37.4	1144 21.2
1145	1145 Patient 14	1145 29.4	1145 17.7
1146	1146 Patient 15	1146 37.2	1146 21.8
1147	1147 Patient 16	1147 49.4	1147 25.9
1148	1148 Patient 17	1148 35.4	1148 20.0
1149	1149 Patient 18	1149 58.8	1149 32.4

1150	Date	Plasma	rTF/95	Secondary standard
1151				
1152		Patient 19	41.6	23.9
1153		Patient 20	35.2	19.3
1154		Patient 21	44.7	25.5
1155		Patient 22	47.4	58.1
1156		Patient 23	38.8	21.3
1157		Patient 24	45.0	25.2
1158		Normal 7	13.3	11.0
1159		Normal 8	14.8	11.3
1160	26 February 2009	Normal 9	13.1	10.9
1161		Normal 10	13.0	10.9
1162		Patient 25	42.0	22.1
1163		Patient 26	31.0	17.7
1164		Patient 27	39.3	20.4
1165		Patient 28	59.0	31.0
1166		Patient 29	35.3	19.4
1167		Patient 30	48.3	25.2
1168		Patient 31	46.5	23.5
1169		Patient 32	52.0	26.4
1170		Patient 33	42.3	22.6
1171		Patient 34	45.7	23.1
1172		Patient 35	50.7	26.3
1173		Patient 36	46.3	22.5
1174		Normal 11	13.2	11.0
1175		Normal 12	13.4	11.1
1176	27 February 2009	Normal 13	13.2	10.3
1177		Normal 14	11.4	10.4
1178		Patient 37	39.0	21.6
1179		Patient 38	32.0	18.7
1180		Patient 39	45.2	24.6
1181		Patient 40	35.8	20.8
1182		Patient 41	40.0	22.3
1183		Patient 42	25.8	16.3
1184		Patient 43	64.0	33.2
1185		Patient 44	51.0	28.1
1186		Patient 45	41.4	23.3
1187		Patient 46	38.4	20.9
1188		Patient 47	48.4	25.9
1189		Patient 48	33.0	19.0
1190		Normal 15	12.9	11.2
1191		Normal 16	13.8	11.1
1192	2 March 2009	Normal 17	15.0	11.9
1193		Normal 18	12.8	10.0
1194		Patient 49	35.8	19.2
1195		Patient 50	43.0	23.1
1196		Patient 51	44.3	24.4
1197		Patient 52	32.3	18.5
1198		Patient 53	43.3	23.9
1199	Date	Plasma	rTF/95	Secondary standard

1200	Patient 54	30.0	17.6
1201	Patient 55	50.0	27.9
1202	Patient 56	43.0	22.8
1203	Patient 57	28.6	18.0
1204	Patient 58	41.6	23.1
1205	Patient 59	39.0	21.4
1206	Patient 60	38.8	22.9
1207	Normal 19	13.1	10.9

1208

1209 **Calculations**

1210

1211 The International Sensitivity Index of the secondary standard (ISI_w) is obtained by plotting the
 1212 prothrombin times of the two thromboplastins on logarithmic scales as shown in Fig. 2, fitting a
 1213 straight line of the form

1214

$$1215 \quad Y = A + BX \quad (1)$$

1216

1217 and estimating the slope B. The recommended method involves estimation of a linear structural
 1218 relation (also called an “orthogonal regression equation”). With this technique, the slope B can
 1219 be estimated as follows.

1220 Consider a set of N independent observations (x_i, y_i) , where $i = 1, 2, 3, \dots, N$; for N paired tests, y_i
 1221 represents the natural logarithm of the measured prothrombin time of the international standard,
 1222 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 ,
 1223 respectively. Write Q_1, Q_2 , for the sums of the squares of $(x_i - x_0)$ and $(y_i - y_0)$, respectively, and
 1224 P for the sum of their products $(x_i - x_0)(y_i - y_0)$. These quantities are all that is necessary for
 1225 computing a and b , the least-squares estimators for the parameters A and B of equation (1). Now
 1226 define:

1227

$$1228 \quad E = (Q_2 - Q_1)^2 + 4P^2. \quad (2)$$

1229

1230 Then

$$1231 \quad b = (Q_2 - Q_1 + \sqrt{E})/2P \quad (3)$$

1232

1233 and

$$1234 \quad a = y_0 - bx_0 \quad (4)$$

1235

1236 are the estimators that minimize the sum of the squares of the perpendicular distances of the N
 1237 points from the line represented by equation (1). The variance of b is given by:

1238

$$1239 \quad \text{Var}(b) = \{(1 + b^2)P + NbV\}bV/P^2 \quad (5)$$

1240 where V is defined as

1241

$$1242 \quad V = (Q_2 - bP)/(N - 2) \quad (6)$$

1243

1244 The standard error of b (s_b) is the square root of $\text{Var}(b)$. The coefficient of variation of b is $CV(b)$
 1245 $= 100 \times (s_b/b)$.

1246 If t is a deviate from the t -distribution, with $(N - 2)$ degrees of freedom and at a chosen
 1247 probability, approximate confidence limits for B can be obtained by setting an interval $t \times s_b$ on
 1248 either side of b .

1249 The residual standard deviation is the square root of V . Outlying points should be rejected if their
 1250 vertical (i.e. perpendicular) distance from the calibration line is greater than $3 \times \sqrt{V}$.
 1251 The ISI_w for the secondary standard is calculated as follows:

$$1252$$

$$1253 \quad ISI_w = ISI_{IS} \times b \quad (7)$$

1254
 1255 where ISI_{IS} is the ISI of the International Standard.

1256 The prothrombin-time ratio for a given patient (i) with the secondary standard can be estimated
 1257 according to the equation

$$1258$$

$$1259 \quad R_{w,i} = \exp(x_i - x_n) \quad (8)$$

1260
 1261 where x_n is the mean natural logarithm of the prothrombin times of the normals. Likewise, the
 1262 prothrombin-time ratio with the international standard can be estimated according to the equation

$$1263$$

$$1264 \quad R_{IS,i} = \exp(y_i - y_n) \quad (9)$$

1265
 1266 where y_n is the mean natural logarithm of the prothrombin times of the normals.

1267 If the same linear structural relation is valid for patients and normals it can be shown that the
 1268 calibration model implies a relationship between prothrombin-time ratios of the form

$$1269$$

$$1270 \quad R_{IS} = (R_w)^b \quad (10)$$

1271
 1272 where R_w is the prothrombin-time ratio obtained with the secondary standard, and R_{IS} is the
 1273 prothrombin-time ratio for the international standard. A similar equation can be written for the
 1274 prothrombin-ratio of first international standard coded 67/40:

$$1275$$

$$1276$$

$$1277 \quad R_{67/40} = (R_{IS})^{ISI_{IS}} \quad (11)$$

1278
 1279 Equations (7), (10) and (11) are the base for calculation of the INR according to the ISI
 1280 calibration model:

$$1281$$

$$1282 \quad INR_w = (R_w)^{ISI_w} \quad (12)$$

1283 One of the underlying assumptions of the ISI calibration model is that a single line describes the
 1284 relationship between logarithms of prothrombin times of both normal and patient plasmas. Thus
 1285 the line describing the relationship between logarithms of patient prothrombin times should
 1286 ideally pass through the mean of the logarithms of normal prothrombin times. In the case of
 1287 marked deviation, the assignment of an ISI would not be meaningful. The natural way to
 1288 overcome this problem is to introduce a scale parameter and use a model for prothrombin ratios
 1289 of the form

$$1290$$

$$1291 \quad R_{IS} = e^d \times (R_w)^b \quad (13)$$

1292

1293 The above model leads to the following equation for calculation of the corrected $INR_{w,p}$:

1294

$$1295 \quad INR_{w,p} = \{e^d \times (R_w)^{b'}\}^{ISI_{IS}} \quad (14)$$

1296

1297 Clearly equation (10) is a particular case of equation (13) but the generalized model will also
 1298 cope with data sets for which the mean logarithms of the prothrombin times of normals do not lie
 1299 on the linear structural relation of the patients. It can be shown that d in equations (13) and (14)
 1300 is estimated as

1301

$$1302 \quad d = a' + b'x_n - y_n \quad (15)$$

1303

1304 where x_n and y_n are the mean natural logarithms of the prothrombin times of normals determined
 1305 with the secondary standard and the international standard, and a' and b' the intercept and slope
 1306 of the “orthogonal regression line” calculated using only the patient data.

1307

1308 Example

1309 For the full set of data shown in Table 1, the various parameters were calculated according to
 1310 equations (3), (4), (5), (6), (7) and (15). The results are shown in Table 2. The next step is to
 1311 detect any outliers. In this example there was one data pair (patient number 22) for which the
 1312 distance to the line was greater than $3 \times \sqrt{V}$. This data pair was excluded. The parameters
 1313 calculated for the remaining 79 data pairs are shown in Table 2. The ISI_w calculated for the
 1314 remaining 79 data pairs was 4.8 % greater than the preliminary ISI calculated with the one
 1315 outlier included. Each patient's INR can be calculated in two ways. The first is to calculate INR
 1316 from the PT measured with the international standard:

1317

$$1318 \quad INR_{IS,i} = (R_{IS,i})^{ISI_{IS}} \quad (16)$$

1319

1320 The second way of calculating each patient's INR is by using the PT measured with the
 1321 secondary standard:

1322

$$1323 \quad INR_{w,i} = (R_{w,i})^{ISI_w} \quad (17)$$

1324

1325 Now it is possible to calculate the mean $INR_{m,i}$ for each patient's sample:

1326

$$1327 \quad INR_{m,i} = (INR_{IS,i} + INR_{w,i})/2 \quad (18)$$

1328

1329 For example, the INR for patient number 43 is 4.32 with the International Standard and 4.70 with
 1330 the secondary standard. The mean INR is 4.51 which is just at the limit of the therapeutic range.
 1331 There are no other patients for which the mean INR is outside the 1.5-4.5 interval.

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1335

Table 2

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

	20 normals + 60 patients (full data set)	20 normals + 59 patients (outlier excluded)	59 patients
Intercept	- 0.7598	- 0.9432	- 0.0386
Slope	1.4216	1.4889	1.2024
CV of slope	3.3 %	1.6 %	3.4 %
ISI _{IS}	0.94	0.94	0.94
ISI _w	1.336	1.400	-
\sqrt{V}	0.0865	0.0396	0.0322
d	-	-	0.2431
y_n	2,602	2,602	2,602
x_n	2,399	2,399	2,399

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The relative difference D between the INR calculated according to equation (12) and equation (14) is given by:

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$$D = 100 \times (\exp(\text{ISI}_w \times (((y_n + (\ln(\text{INR})/\text{ISI}_{IS})) - a')/b') - x_n)) - \text{INR}) / \text{INR} \quad (19)$$

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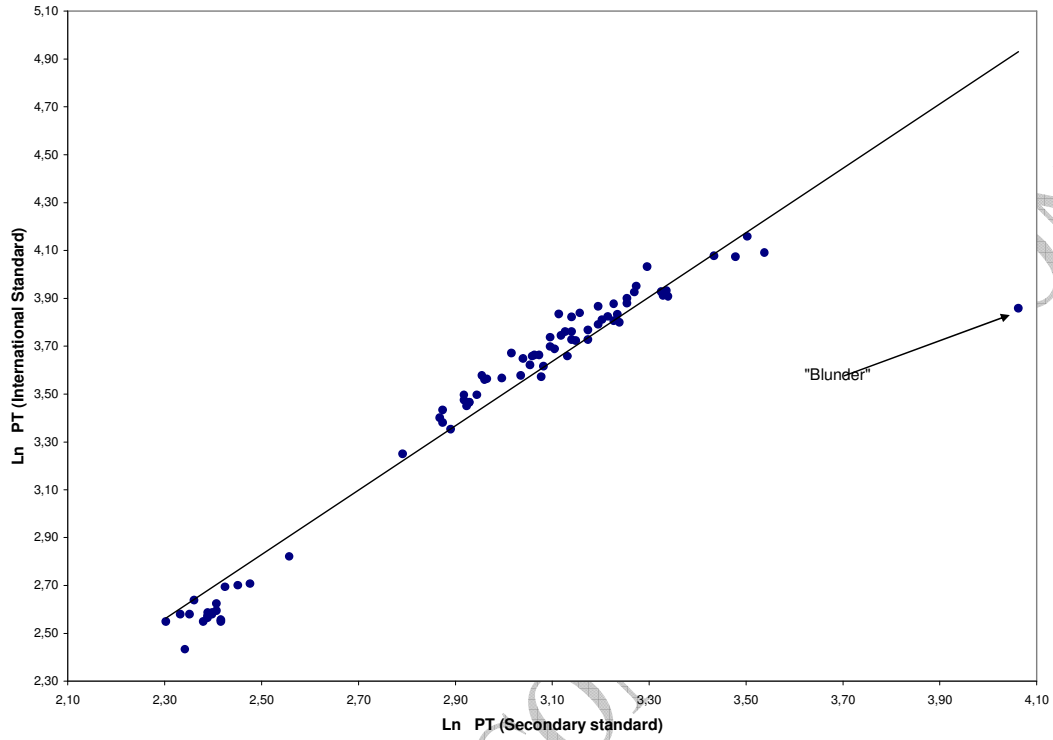
In this example the orthogonal regression line calculated for 59 patient data pairs did not pass through the mean of the normal data pairs (see figure 2). The difference D calculated at INR = 2 is -11% and at INR = 4.5 is 7.8%. It is therefore important to consider the alternative calibration model according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

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$$\text{INR}_{w,p,i} = \{e^{0.2431} \times (R_{w,i})^{1.2024}\}^{0.94} \quad (20)$$

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Figure 2.
Log-log plot of prothrombin times for determination of ISI.



1357

1358 **Appendix 2**1359 **Example of the use of the suggested method for reporting the data for the**
1360 **calibration of individual batches of thromboplastin**

1361

1362 Thromboplastins: 1. Rabbit brain thromboplastin secondary standard
1363 2. New batch of rabbit brain thromboplastin

1364

1365 End-point recording: Automated photoelectric coagulometer

1366

1367 Pooled coumarin plasmas: lot 960606, 1-5 (deep-frozen)

1368

1369 Pooled normal plasma: lot 900423 (deep-frozen)

1370

1371 The ISI and MNPT of the rabbit brain thromboplastin secondary standard used with this
1372 automated photoelectric coagulometer are 1.31 and 12.7 seconds, respectively.

1373

1374 The tests were conducted in four separate runs. For each run, thromboplastins were reconstituted
1375 freshly and deep-frozen plasmas were thawed freshly. Since the secondary standard and the new
1376 batch were both timed with the same photoelectric coagulometer, the order in which the two
1377 reagents were tested was alternated from one run to the next. This was done to avoid any bias
1378 due to possible instability of the thromboplastins and pooled plasmas.

1379

1380 **Calculation**1381 The ISI of the new batch (ISI_b) is calculated as $ISI_b = ISI_w \times b$, where b is the slope of the
1382 straight line fitted to a double-logarithmic plot of the prothrombin times in Table 3, with the
1383 prothrombin times for the secondary standard and the new batch being shown on the vertical and
1384 horizontal axes, respectively. The formula for b is given by equation (3) in Appendix 1. The
1385 standard error of b is obtained from equation (5) in Appendix 1. The coefficient of variation (%)
1386 of b is $100 \times (s_b/b)$.

1387

1388 **Example**1389 For the data from Table 3, the calculated residual standard deviation is 0.02482. One pair of
1390 determinations for plasma lot no. 960606-5 (run no. 3) has a perpendicular distance from the line
1391 greater than three residual standard deviations. When this pair is excluded, the calculated value
1392 for b is 0.9538. The ISI for the secondary standard is given as 1.31. Thus, the ISI for the new
1393 batch is estimated as $1.31 \times 0.9538 = 1.25$. The standard error for b is calculated as 0.0130. The
1394 coefficient of variation for b is $100 \times (0.0130/0.9538) = 1.36\%$.

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1405 **Table 3**
 1406 **Prothrombin times (PT) for the calibration of a new batch of rabbit**
 1407 **thromboplastin**

		Secondary standard			New batch	
Run No.	Plasma Lot no.	Order of testing (within-run)	PT	Order of testing (within-run)	PT	
1413	1 900423	1	14.0	7	15.1	
1414	960606-1	2	20.5	8	21.5	
1415	960606-2	3	29.1	9	31.5	
1416	960606-3	4	32.9	10	36.4	
1417	960606-4	5	36.2	11	41.0	
1418	960605-5	6	39.7	12	44.6	
1419						
1420	2 900423	7	14.1	1	15.4	
1421	960606-1	8	20.3	2	22.6	
1422	960606-2	9	29.5	3	31.2	
1423	960606-3	10	32.8	4	37.6	
1424	960606-4	11	37.3	5	40.8	
1425	960605-5	12	39.8	6	44.5	
1426						
1427	3 900423	1	14.0	7	15.0	
1428	960606-1	2	20.0	8	21.5	
1429	960606-2	3	28.1	9	32.1	
1430	960606-3	4	31.8	10	34.2	
1431	960606-4	5	35.9	11	40.7	
1432	960605-5	6	37.2	12	47.7	
1433						
1434	4 900423	7	13.9	1	15.0	
1435	960606-1	8	20.0	2	21.9	
1436	960606-2	9	27.9	3	30.9	
1437	960606-3	10	31.5	4	35.7	
1438	960606-4	11	34.6	5	39.2	
1439	960605-5	12	37.6	6	44.4	

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