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**INTERNATIONAL COLLABORATIVE STUDY FOR THE CALIBRATION OF A
PROPOSED INTERNATIONAL STANDARD FOR THROMBOPLASTIN, RABBIT,
PLAIN**

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SUMMARY

A Preparation of rabbit brain thromboplastin, provisionally coded 04/162, is proposed as a candidate for the International Standard (IS) for thromboplastin (rabbit, plain), meant to replace the IS coded RBT/90 (rabbit, plain) whose stocks are now exhausted. The preparation was calibrated in an international collaborative study organized and carried out under the auspices of the Subcommittee on Control of Anticoagulation of the Scientific and Standardization Committee (SSC) of the International Society on Thrombosis and Haemostasis (ISTH). The study involved 22 laboratories from 13 countries and the calibration was performed against the existing WHO ISs (i.e., rTF/95 and OBT/79) and other certified reference materials from the Institute for Reference Materials and Measurements (IRMM) of the European Commission (i.e., CRM 148 and CRM149 S) and from the European Action on Anticoagulation (EAA) (i.e., EUTHR-01). An additional candidate rabbit brain thromboplastin coded as 04/106 was also included in the study. On the basis of predetermined criteria which included (i) the within-laboratory precision of calibration; (ii) the between-laboratory precision of the calibration and (iii) the conformity to the calibration model, the Subcommittee on Control of Anticoagulation of the SSC of the ISTH deliberated unanimously that 04/162 was to be preferred as the candidate IS. Twenty-one of 22 participants eventually submitted data for statistical analysis. Within-laboratory precision of the calibration of 04/162, expressed as the Coefficient of Variation (CV) for the estimation of the slope was below 3% in the vast majority of the cases (94%). The mean International Sensitivity Index (ISI) value of the candidate 04/162 calibrated against the five existing ISs was 1.15 and the interlaboratory agreement was good (between-laboratory CV of the ISI was 4.9%). Accelerated degradation studies performed in one laboratory indicated adequate long-term stability (prothrombin time ratio did not change significantly after exposure of 04/162 at temperature as high as 45 °C for 2 months). The homogeneity of the preparation 04/162 and the precision of the prothrombin time measurement were satisfactory (inter-vial variation, expressed as the CV of the prothrombin time measurement was 1.31% and 1.27 % for normal and coumarin plasma, respectively). Overall, the preparation coded 04/162 proved to be a suitable candidate to serve as the replacement to the IS coded RBT/90.

INTRODUCTION

According to the recommendation issued by the World Health Organization (WHO), working thromboplastins used in the prothrombin time (PT) test for the laboratory control of oral anticoagulant treatment must be calibrated against International Standards (IS) to determine the International Sensitivity Index (ISI) necessary to convert PT results into International Normalized Ratio (INR) (1). The observation that the calibration of a given thromboplastin is in general more precise when it is performed against an IS of similar composition and from the same species, supports the recommendation made by the WHO Expert Committee on Biological Standardization (ECBS) that like vs. like calibration should be performed and is one of the reasons to maintain ISs from different species (1). Another reason to maintain more than one IS is that it permits to assess periodically the relative stability of the ISI (2, 3). The first IS coded 67/40, was a human brain extract to which it was added adsorbed bovine plasma (combined reagent). In 1984, 67/40 was replaced by BCT/253 (4), a human brain extract (plain reagent). This was in turn replaced in 1996 by rTF/95 (human recombinant, plain) (5). Until recently, there were two additional IS available

from WHO: OBT/79 (bovine, combined) (6) and RBT/90 (rabbit, plain) (7). Reference Materials for thromboplastins are also available from other agencies: CRM 149S (rabbit, plain) and CRM 148 (bovine, combined) from the Institute for Reference Materials and Measurements (IRMM) of the European Commission and EUTHR-01 (rabbit, plain) from the European Action on Anticoagulation (EAA). Each of the above IS and Reference Materials is characterized by a value of the ISI, which is the slope of relationship of the logPT values derived directly or indirectly with the primary IS coded 67/40. The ISI of each of the above-mentioned IS and Reference Materials were established in multicenter collaborative studies using methods recommended by the WHO ECBS.

Stocks of the WHO IS from rabbit origin coded RBT/90 (7) are no longer available and must be replaced to maintain continuity of the rabbit route. The present report deals with the results of an international multicenter collaborative study organized under the auspices of the Subcommittee on Control of Anticoagulation of the Scientific and Standardization Committee (SSC) of the International Society on Thrombosis and Haemostasis (ISTH) for the calibration of the replacement candidates.

Since RBT/90 was no longer available, it could not be used for the calibration of the replacement candidates. It was justified to use other Reference Materials for rabbit thromboplastins instead. Both CRM 149S and EUTHR-01 were used because both had been calibrated against RBT/90 in a multicenter study, and because the amount of either one was not sufficient to cover the needs of all centers in the collaborative study.

DESIGN OF THE COLLABORATIVE STUDY

Two candidates (04/106 and 04/162) and the current ISs rTF/95 (human), OBT/79 or CRM148 (bovine), and CRM149S or EUTHR-01 (rabbit) were tested in each center by an expert operator using the manual (tilt tube) technique. Test plasmas were freshly prepared from healthy subjects and patients stabilized on long term anticoagulant therapy. Participants were instructed to select patient plasmas with PT corresponding to an interval of INR from 1.5 to 4.5. Also included in the series of measurements were four lyophilized plasmas for quality control purposes. To account for the effect of inter-day variation, PT measurements were performed in each laboratory on 10 different days (not necessarily consecutive). Participants were instructed to include on each day plasmas from 2 healthy individuals and 6 anticoagulated patients. Healthy individuals and patients had to be different on each working day. To minimize the effect of plasma instability on the relationship between the thromboplastins, the order of testing was changed each day. Plasmas were tested on each day according to the order specified in the data-collection form.

MATERIALS PROVIDED

Participants received the following material:

1. Study protocol with detailed instructions on how to collect and store fresh plasmas, to reconstitute lyophilized plasmas and thromboplastins and to do actual testing.
2. Lyophilized plasmas (coded A, B, C and D).
3. ISs and Reference Materials presently available from different agencies and belonging to the three different species, rTF/95 (human), OBT/79 or CRM 148 (bovine), CRM 149S or EUTHR-01 (rabbit). The amount of OBT/79 was not sufficient for all centers. Therefore one center used CRM 148 instead of OBT/79.

4. Candidate replacement thromboplastins provisionally coded as 04/106 and 04/162.
5. Blood collection tubes containing 0.106 M sodium citrate.
6. Appropriate aqueous solutions to reconstitute rTF/95 (calcium chloride solution attached to the IS), EUTHR-01; OBT/79 or CRM 148 (calcium chloride 3.2 mM) and candidate 04/162 (water plus phenoxyethanol attached to the candidate and coded 04/210).
7. Sterile redistilled water to reconstitute CRM 149S, candidate 04/106 and lyophilized plasmas.
8. Sterile 25 mM calcium chloride to recalcify plasma/thromboplastin mixtures for CRM 149S, EUTHR-01 and candidate 04/162.

STATISTICAL METHODS

The statistical methods were those employed for the calibration of previous ISs (4, 5, 6, 7) and recommended by WHO (1). In particular, orthogonal regression was used to estimate the slope of the relationship between the log-transformed PTs obtained with the candidates and the ISs. Values exceeding the interval 1.5-4.5 INR as measured with the ISs or Reference Materials were excluded and outliers were rejected if their orthogonal distance was more than 3 times the Standard Deviation (SD) from the orthogonal regression line (calculated with all points included).

The ISI value was calculated as the product of the slope of the orthogonal regression line and the ISI value of the IS or Reference Material. The following established ISI values were used: 0.94 (rTF/95) (5); 1.011 (OBT/79 and CRM148) (6); 1.257 (CRM149S) (8); 1.67 (EUTHR-01) (9). ISI values within each route of calibration and each IS or Reference Material were calculated as the mean of the separate regression lines calculated for the different laboratories. The final ISI value assigned to the candidates was the overall mean value of ISIs obtained with the three different routes of calibration and ISs after exclusion of outliers. These were detected within each route of calibration by the algorithm described previously (10) and used for the calibration of RBT/90 (7) and rTF/95 (5).

INR values for fresh patients' samples were calculated by dividing PT from patients by the geometric mean PT of normals and raising this ratio to the ISI value of the reagent (1, 11).

The statistical analysis was performed by V. Chantarangkul (Angelo Bianchi Bonomi Haemophilia and Thrombosis Centre, Milano, Italy). The same software was used as for the calibration of rTF/95.

RESULTS

Twenty-one of 22 laboratories eventually provided results. The results reported in this section are those obtained with the candidate 04/162. Results of the calibration of 04/106 and their comparison vs. 04/162 are reported in Appendix IV and V. The geometric means of the PTs from fresh normal plasmas are shown in Table 1. The values obtained with the ISs and Reference Materials are in agreement with those obtained at the time when they were calibrated against their predecessors (5, 6, 8, 9). Slopes and their coefficients of variation (CV) for calibration of 04/162 vs. different ISs are shown in Table 2. The CV values which estimate the within-laboratory precision of the calibration were within acceptable limits (i.e., 3% or less) (1) in the vast majority of laboratories. The individual centers' ISI values obtained with calibration of 04/162 against the ISs are shown in Table 3. There was one outlier value in one center when the calibration was performed against OBT/79. The results of one center (nr. 1) which used CRM 148 instead of OBT/79 were not included for the calculation of the final ISI value. The between-laboratory CV values ranged from 3.4% to 4.6%. The final mean ISI value, i.e., the average value of all ISI values generated with the IS and Reference Materials after exclusion of one outlier, was 1.146 with a Standard Deviation of 0.057 (Table 3). Table 4 shows the geometric mean values of the INRs calculated for fresh patients' plasmas by using the mean ISI of all laboratories. They were in good agreement and ranged from 2.56 with rTF/95 to 2.76 for EUTHR-01.

DISCUSSION

The study included all the existing ISs. Following recommendations issued by the SSC of the ISTH (12) and WHO (1) the ISI of the candidate replacement has been calculated as the average value of all calibrations against the existing WHO ISs. Furthermore, it was decided to include in the calibration all other Reference Materials available from other agencies and used worldwide to calibrate thromboplastins used to monitor oral anticoagulant treatment. This was deemed necessary in order to minimize small differences that have been observed between the routes of calibration (2, 3). This strategy had already been implemented for the calibration of RBT/90 (7) and rTF/95 (5). This scheme does not substantially infringe the rule of like-vs.-like calibration (1). This rule will still remain in place for commercial reagents, thus reducing the variability of the ISI value, particularly when small numbers of laboratories perform the calibration. Whereas, the rule of like-vs.-like calibration will be broken only for calibration of the ISs. In this case the risk of increasing the interlaboratory standard deviation of the ISI may be kept at bay by increasing the numbers of laboratories (at least twenty) and by well planned study designs.

Manufacturers of rabbit brain thromboplastin reagents were invited to provide potential candidates (13). Additional requirements were that the material should have an ISI within the range of 1.1 – 1.3 (manual technique) and have to be provided in glass sealed ampoules. A suitable number of them would be donated to WHO after the evaluation was completed and the final choice of the most suitable candidate was made. Two candidates that met the above requirements were eventually submitted for evaluation. They were lyophilized and ampouled at the National Institute for Biological Standard and Controls (NIBSC, Potters Bar, Hertfordshire, UK) following instructions from the manufacturers. Suitable numbers of ampoules of the two candidates were coded as 04/162 and 04/106 by people neither involved in the calibration exercise nor in the statistical analysis and were calibrated in an international collaborative study.

Twenty-two laboratories from Europe, North America, South America and Asia were invited to participate in the study. The vast majority of them had already participated in the collaborative exercise to calibrate RBT/90 (7) and rTF/95 (5) and had experience with the manual (tilt tube) technique for PT testing. All except one laboratories eventually provided results.

The criteria used to judge the calibration of the two candidates were the within- and between-laboratory precision of the calibration and the conformity to the model of calibration. They were derived from the procedure recommended by WHO (1) and the literature that appeared on this topic over the last 25 years (4, 5, 6, 7). The within-laboratory precision of calibration was better for 04/162 than for 04/106 because the percentage of laboratories which scored a CV value below the recommended 3% (1) were 94% vs. 84%. The between-laboratory precision was equivalent for both candidates (5.0%). Finally, 04/162 was more adequate to fulfill the requirements of the calibration model. These require that in a given calibration the overall regression line describes patient and normal data points adequately. According to Tomenson (14) this can be achieved by testing the assumption that the mean log PT of the normals lies on the orthogonal regression line drawn through patient data points. We tested this assumption in all calibration plots generated with the two candidates and found that 04/162 deviated from the assumption in 11.1% of the cases, whereas 04/106 in 14.3% of the cases.

On the basis of these results the Subcommittee on Control of Anticoagulation of the SSC of the ISTH during its Annual Meeting (Sydney, Australia, August 7, 2005) deliberated unanimously that 04/162 was the preferred candidate IS to be submitted to the ECBS of the WHO (15). The material is presently stored at -20° C under the care of the NIBSC. The manufacturer of the candidate thromboplastin (South Manchester University Hospital Trust, Manchester, UK) accepted to offer free of charge 8200 ampoules (plus an equivalent number of ampoules containing the diluent for reconstitution) to WHO. As soon as the material will be established as an IS for thromboplastin (rabbit, plain), ampoules will be labelled by NIBSC according to the instructions of WHO (suggested code RBT/05) and transferred to the custodian laboratory.

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Table 1. Mean prothrombin times (seconds) of the normal subjects.

Laboratory	rTF/95	OBT/79	CRM 149S	EUTHR-1	04/106	04/162
1 ^a	12.80	33.09	14.96		15.81	15.78
2	15.15	37.46		17.03	17.25	16.43
3	12.64	35.44		15.72	15.89	15.41
4	13.27	37.55		17.37	17.40	17.39
5	15.35	37.20	16.45		19.07	18.30
6	14.69	37.58	16.30		18.11	16.96
7	13.40	37.63		16.60	16.47	16.68
8	15.82	41.62		18.00	19.27	18.05
9	15.60	38.53	15.56		18.08	17.45
10	15.73	42.25	16.56		18.94	18.87
11	13.25	35.46		16.78	15.50	16.10
12	14.21	36.17		17.90	17.55	17.94
13	13.13	37.84		16.94	16.36	15.96
14	13.99	38.21		16.90	16.79	16.06
15	12.40	34.42	15.11		15.28	15.52
16	12.12	33.44		17.41	15.10	16.20
17	16.73	38.74		18.28	20.95	18.67
18	14.25	35.25	15.37		16.93	17.61
19	13.65	34.75	15.30		16.72	15.73
20	12.59	37.50		15.61	16.75	16.59
21	14.78	39.10	15.00		18.37	16.29
Overall Mean	14.07	37.11	15.62	17.04	17.27	16.86
Min	12.12	33.09	14.96	15.61	15.10	15.41
Max	16.73	42.25	16.56	18.28	20.95	18.87

^aThis lab used CRM 148 instead of OBT/79

Table 2. Slopes and coefficients of variation (CV) for calibration of 04/162 vs. ISs.

Laboratory	Vs. rTF/95		Vs. OBT/79		Vs. CRM149S		Vs. EUTHR-1	
	Slope	CV	Slope	CV	Slope	CV	Slope	CV
1 ^a	1.225	1.6	1.193	1.5	0.938	1.4		
2	1.128	2.7	1.188	2.3			0.674	2.9
3	1.189	2.0	1.140	2.3			0.713	2.2
4	1.204	2.6	1.102*	3.3			0.713	3.9
5	1.143	1.4	1.170	2.0	0.979*	1.3		
6	1.101*	2.3	1.117	2.4	0.956*	1.6		
7	1.181	1.6	1.146	1.8			0.688	1.9
8	1.169	1.5	1.163	2.1			0.714*	2.1
9	1.079	2.7	1.032	3.8	0.911	2.6		
10	1.173*	2.4	1.076	2.2	0.897	2.0		
11	1.159	1.7	1.146	2.5			0.687	1.9
12	1.191	2.3	1.199	2.3			0.674*	2.8
13	1.234	1.9	1.188	2.0			0.700	2.2
14	1.263	2.0	1.223	1.8			0.746	2.0
15	1.184	1.5	1.150	1.7	0.905	0.9		
16	1.265	2.4	1.232	1.9			0.697	2.8
17	1.112	2.6	1.158	2.2			0.717	2.7
18	1.107	2.4	1.123	2.9	0.873	2.4		
19	1.114	1.4	1.138	1.8	0.938	1.2		
20	1.221	2.2	1.182	2.2			0.771	3.2
21	1.134	2.0	1.127	2.2	0.884	2.0		

^aThis lab used CRM 148 instead of OBT/79

* Statistically significant deviation of normals from patients line

Table 3. ISI values for 04/162 vs. ISs and Reference Materials.

Laboratory	rTF/95	OBT/79	CRM 149S	EUTHR-01	Overall
1 ^a	1.152	1.206	1.179		
2	1.060	1.201		1.125	
3	1.118	1.153		1.190	
4	1.132	1.114		1.190	
5	1.074	1.183	1.231		
6	1.035	1.130	1.202		
7	1.110	1.159		1.149	
8	1.099	1.176		1.193	
9	1.014	1.043 ^b	1.145		
10	1.103	1.087	1.128		
11	1.090	1.159		1.148	
12	1.120	1.212		1.125	
13	1.160	1.201		1.169	
14	1.188	1.236		1.246	
15	1.113	1.162	1.137		
16	1.190	1.246		1.164	
17	1.045	1.171		1.198	
18	1.040	1.136	1.097		
19	1.047	1.151	1.179		
20	1.148	1.195		1.288	
21	1.066	1.140	1.112		
Mean	1.100	1.165	1.157	1.182	
N.	21	21	9	12	
SD	0.050	0.048	0.044	0.048	
CV	4.6	4.1	3.8	4.0	
Mean ^c		1.171			
N.		20			
SD		0.040			
CV		3.4			
Overall Mean ^d					1.146
N.					61
SD					0.057
CV					4.9

^aThis lab used CRM 148 instead of OBT/79; the ISI was not included in the overall mean value.

^bidentified as outlier. ^cMean value after exclusion of outliers. ^dOverall mean value of all calibrations.

Table 4. Means of the patients' INR values.

Laboratory	RTF/95	OBT/79	CRM 149 S	EUTHR-1	04/106	04/162
1 ^a	2.96	3.09	2.98		3.07	2.93
2	2.25	2.57		2.38	2.46	2.46
3	2.88	3.01		3.16	3.12	3.04
4	2.65	2.56		2.71	2.62	2.76
5	2.80	3.12	3.25		2.94	3.02
6	2.05	2.21	2.32		2.22	2.30
7	2.67	2.79		2.70	2.89	2.74
8	2.56	2.71		2.70	2.61	2.69
9	2.06	2.07	2.27		2.21	2.29
10	2.53	2.44	2.52		2.57	2.58
11	2.97	3.23		3.16	3.15	3.16
12	2.25	2.41		2.16	2.31	2.30
13	2.35	2.43		2.36	2.47	2.38
14	2.79	2.93		2.90	2.75	2.67
15	2.56	2.71	2.62		2.69	2.66
16	2.63	2.74		2.50	2.44	2.52
17	2.71	3.08		3.09	2.90	2.93
18	2.31	2.53	2.45		2.58	2.56
19	2.63	2.88	2.92		2.75	2.88
20	2.72	2.86		3.25	2.71	2.79
21	2.49	2.87	2.83		2.97	2.95
Overall Mean	2.56	2.73	2.69	2.76	2.69	2.70
Min	2.05	2.07	2.27	2.16	2.21	2.29
Max	2.97	3.23	3.25	3.25	3.15	3.16

^aThis lab used CRM 148 instead of OBT/79

Appendix I

Participants (in alphabetical order) of the international collaborative study for the calibration of a proposed reference preparation for thromboplastin, rabbit, plain.

- P. Angchaisuksiri/K. Aryurachai. Hemostasis and Thrombosis Laboratory, Division of Hematology, Department of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok, Thailand.
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- K. Denson. Thame Thrombosis and Haemostasis Research Foundation, Thame, Oxon, UK.
- T. Gago. Serviço Patologia Clínica, Hospital de Santa Cruz, Carnaxide, Portugal.
- P. Herbel/A. Jünschke/W. Plesch. Roche Diagnostics, Mannheim, Germany
- M. Johnston. Hemostasis Reference Laboratory, Henderson Research Centre, Hamilton, Canada.
- S. Kitchen. Dept of Coagulation, Sheffield Haemophilia and Thrombosis Centre, Royal Hallamshire Hospital, Sheffield, UK.
- C. Legnani. U.O. Angiologia e Malattie della Coagulazione "Marino Golinelli", Azienda Ospedaliera di Bologna, Policlinico S. Orsola - Malpighi, Bologna, Italy.
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Appendix II

Inter-ampoule variation and precision of PT measurements with the proposed standard.

Introduction

The precision of PT measurements depends on the skill of the operator, in particular when the manual technique is used, and/or on the instrument used. If PT measurements are used to determine inter-vial variation of the thromboplastin, the precision of the PT measurement should be known. The aim of the present study is to estimate the precision of the PT measurement with two plasma samples, i.e. a pooled normal plasma and a pooled coumarin plasma. At the same time, these plasmas were used to estimate the inter-ampoule variation of the proposed standard. In order to complete the measurements in a relatively short time, a semi-automatic coagulation instrument according to Schnitger & Gross was used (1, 2). It is based on electro-mechanical detection of fibrin formation, similar to the detection used in the Fibrometer (3).

Materials and Methods

Two deep-frozen pooled plasmas (one derived from healthy donors and the other from patients treated with anti-vitamin K drugs) were used for testing of the proposed standard coded 04/162. Each plasma had been frozen in 0.75 ml aliquots at -70°C , and was thawed in a waterbath at 37°C for 4 minutes. In order to minimize any effect of plasma instability on the PT test, each plasma was used between 30 and 120 minutes after thawing.

Thirty ampoules of the proposed standard coded 04/162 were tested in two separate sessions on the same day. In each session, 15 ampoules were reconstituted with the fluid coded 04/210 at room temperature, and then tested with freshly thawed plasmas. Each ampoule was tested in quadruplicate using the coagulation instrument according to Schnitger and Gross (manufactured by H. Amelung GmbH, Lemgo, Germany). Each ampoule was tested between 60 and 90 minutes after reconstitution. For each ampoule, the mean PT and standard deviation (SD) was calculated from the 4 repeat measurements. The average imprecision of the PT measurement was calculated as the mean of 30 SD values. The inter-ampoule variation was calculated from the 30 mean PT values.

Results

Individual PT results are shown in Table 1. The mean imprecision of PT measurements and the apparent inter-ampoule variation are shown in Table 2. The apparent inter-ampoule variation was not significantly (at 5% level) greater than the mean imprecision of the measurement. The true inter-ampoule variation may be obtained by subtracting the variance of imprecision from the variance of the apparent inter-ampoule variation. It is inferred that the true inter-ampoule coefficient of variation (i.e., corrected for imprecision of the measurement) is less than 1.3%.

Conclusion

The magnitude of the inter-ampoule variation is very small in comparison with the total error of the normalized PT (i.e., on the INR scale) (4). Therefore, the proposed standard was judged to be sufficiently homogeneous, also keeping in mind that the contents of three ampoules should be pooled on each testing occasion in practice.

References

1. Schnitger H, Gross R. Über ein Universalgerät zur automatischen Registrierung von Gerinnungszeiten. *Klin Wschr* 1954; 32: 1011-2.
2. Van den Besselaar AMHP, Bertina RM. Multi-Center calibration of the second reference material for thromboplastin, rabbit, plain, coded CRM 149R. *Thromb Haemost* 1991; 65:263-7.
3. Koepke JA, Klee GG. Automated coagulation detection systems. *Clin lab Haemat* 1979; 1:75-86.
4. Van den Besselaar AMHP. Precision and accuracy of the international normalized ratio in oral anticoagulant control. *Haemostasis* 1996; 26(Suppl 4): 248-265.

Table 1. Results of inter-ampoule and imprecision testing. Prothrombin times (in seconds) were determined with 30 different ampoules of the proposed standard coded 04/162 using two pooled plasmas (NP010507 and AP040802). In the columns headed by "Ampoule", the times (h = hour) of reconstitution and measurement of each ampoule are given.

Ampoule	NP010507	AP040802	Ampoule	NP010507	AP040802	Ampoule	NP010507	AP040802
1	16,6	34,7	11	16,4	34,6	21	15,7	34,9
9:00 h	16,5	34,3	9:40 h	15,8	34,8	13:20 h	15,8	35,1
10:00h	16,6	34,4	11:00 h	16,2	34,7	14:30 h	15,9	34,1
	16,8	34,4		15,9	34,1		15,8	35,0
2	16,3	34,3	12	16,1	34,4	22	16,1	34,8
9:04 h	16,1	34,3	9:44 h	15,6	34,4	13:24 h	16,2	34,7
10:06 h	16,4	34,8	11:06 h	15,4	34,2	14:36 h	15,8	35,0
	15,9	34,3		16,1	33,4		15,6	34,5
3	16,2	33,5	13	15,9	34,3	23	16,1	35,3
9:08 h	16,0	33,5	9:48 h	15,5	34,3	13:28 h	16,0	34,3
10:12 h	16,0	32,9	11:12 h	15,7	34,1	14:42 h	15,7	34,0
	15,7	32,7		15,9	33,8		15,9	34,4
4	16,5	33,6	14	16,2	35,3	24	15,6	35,0
9:12 h	16,3	34,1	9:52 h	16,0	34,1	13:32 h	15,6	33,3
10:24 h	16,2	34,2	11:18 h	15,9	34,3	14:48 h	15,7	34,6
	16,2	33,6		16,4	34,8		15,9	34,3
5	16,3	33,9	15	16,1	34,9	25	16,0	34,7
9:16 h	15,8	33,8	9:56 h	16,1	35,9	13:36 h	15,9	34,6
10:30 h	16,2	34,3	11:24 h	15,9	36,1	14:54 h	16,0	34,5
	16,1	33,8		15,8	35,0		15,6	34,5
6	15,9	34,6	16	15,8	34,1	26	16,1	35,1
9:20 h	16,0	34,3	13:00 h	15,7	33,7	13:40 h	15,7	34,6
10:36 h	16,1	34,9	14:00 h	16,0	34,2	15:00 h	15,7	35,1
	16,3	34,1		15,8	34,4		15,6	34,6
7	15,8	34,5	17	15,7	33,7	27	15,8	33,9
9:24 h	16,1	35,1	13:04 h	15,7	33,6	13:44 h	15,8	34,5
10:36 h	15,9	34,6	14:06 h	15,7	33,7	15:06 h	16,0	33,7
	16,1	34,4		15,4	34,4		15,9	34,4
8	16,0	34,6	18	16,3	35,4	28	15,8	35,2
9:28 h	15,6	34,2	13:08 h	15,9	35,2	13:48 h	15,4	34,0
10:42 h	15,8	34,4	14:12 h	16,4	34,8	15:12 h	15,7	34,1
	15,9	34,5		15,5	34,7		15,5	34,9
9	16,6	35,0	19	15,9	34,6	29	16,0	35,3
9:32 h	15,7	34,5	13:12 h	16,4	34,2	13:52 h	15,6	34,9
10:48 h	15,6	35,0	14:18 h	15,8	34,4	15:18 h	15,8	34,9
	15,7	34,7		15,9	34,2		15,8	35,0
10	15,9	34,4	20	15,8	33,9	30	16,1	35,2
9:36 h	16,0	34,3	13:16 h	15,9	33,8	13:56h	15,5	33,9
10:54 h	16,1	35,0	14:24 h	15,9	34,3	15:24h	15,7	33,6
	15,8	34,3		15,8	34,4		15,5	34,4

Table 2. Average imprecision of PT measurement and inter-ampoule variation calculated for the proposed standard coded 04/162, using two pooled plasmas (NP010507 and AP040802).

	Average imprecision		Inter-ampoule variation	
	NP010507	AP040802	NP010507	AP040802
Standard deviation (s)	0.20	0.36	0.21	0.44
Coefficient of variation (%)	1.26	1.03	1.31	1.27

Appendix III

Accelerated degradation test of two candidates international standard for thromboplastin, rabbit, plain.

Introduction

Tissue factor is a glycoprotein that needs the association with phospholipids for full expression of its procoagulant activity. Lyophilized tissue factor reagents (thromboplastins) for prothrombin time (PT) assays usually contain many other components such as residual water which may affect the stability of the reagent. In general, the long-term stability of lyophilized tissue factor from human or animal brain stored at low temperature is excellent.

The stability of biological materials may be predicted from accelerated degradation tests. The purpose of an accelerated degradation test is to measure the relative rates of potency loss at several temperatures and to extrapolate the rate to the desired temperature of storage. Only few investigators have attempted to predict the stability of tissue factor from accelerated degradation studies. One reason for this may be that thromboplastins are not easily assayed for potency in the usual sense. Furthermore, complex kinetics of the deterioration process are expected for tissue factor as it is a lipoprotein. Although the accelerated degradation test may not be used to predict the stability of lyophilized tissue factor at low temperature, it may be useful to assess the relative stability under transportation conditions at ambient temperatures. The accelerated degradation test is a standard procedure to check the stability of thromboplastins.

Materials and methods

Following shipment of the candidates (coded 04/106 and 04/162) from NIBSC to Leiden, they were stored at -20°C. The reconstitution fluid for candidate 04/162 was stored at 4°C. For the accelerated degradation test, a number of ampoules of 04/106 and 04/162 were stored at 4°C, 30°C, 37°C, and 45°C, for different time intervals. At 4°C, 30°C, 37°C, the storage times were 3, 12, 26, 40, 54, 68, 82, 96, 110, 124, 138, 152 days. At 45°C, the storage times were 3, 7, 14, 21, 28, 35, 42, 49, 56, 63, 70 days. After storage of the candidates at these temperatures, they were reconstituted and tested with two deep-frozen pooled plasmas (one normal and one coumarin). The tests were performed with a semi-automatic, electro-mechanical coagulometer according to Schnitger & Gross manufactured by Amelung GmbH (Lemgo, Germany). All ampoules stored at a given temperature were tested in the same session. The time between reconstitution and testing was 2 hours. The time interval of each testing session was 2-2.5 hours. For each storage temperature and time, 3 ampoules were used. Each ampoule was tested in single PT determination. The mean values of the 3 PTs were used for statistical analysis. For each storage temperature and time, a clotting time ratio (PT ratio) was calculated as the mean PT of the coumarin plasma divided by the mean PT of the normal plasma.

Statistical analysis

Linear regression lines were calculated for PT or PT ratio on storage time. Spearman's rank correlation coefficient was used to determine whether there was a significant association between PT (or PT ratio) and storage time.

Results

The slopes of the linear regression lines for the PT with normal and cumarin plasma are shown in Tables 1 and 2, respectively. In several cases, there was a significant ($p < 0.05$) increase of the PT on storage time. The slope of the regression lines tended to increase with increasing storage temperature. The magnitudes of the slopes for the two candidates were practically the same. The slopes of the linear regression lines for the PT ratio are shown in Table 3. There were 3 cases in which a significant ($p < 0.05$) increase of the PT ratio on storage time was observed. For candidate 04/106, the slope of the regression line tended to increase with temperature.

Discussion

The accelerated degradation test showed that there was a minor increase of the PT after storage of the candidates at elevated temperatures. The magnitude of the increase was of the same order as reported previously for another reference material for thromboplastin, rabbit, plain (1). There was no significant change of the PT ratio of the candidates stored at 4°C and 37°C. With candidate 04/162, no significant change of the PT ratio was observed at 45°C. The absence of a change in the PT ratio suggests that there is neither change in the ISI.

Conclusion

Both candidates show acceptable stability at elevated temperatures, similar to the stability of previous reference materials for thromboplastin, rabbit, plain. Candidate 04/162 showed marginally better stability of the PT ratio at 45°C.

Reference

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Table 1

Linear regression lines of PT of pooled normal plasma. n is the number of storage times. Significance is given by the p value.

		Candidate 04/106		Candidate 04/162		
Temperature	n	slope (s/day)	p	n	slope (s/day)	p
4°	13	0.005	0.001	13	0.002	0.080
30°	13	0.003	0.004	13	0.003	0.002
37°	13	0.004	0.029	13	0.008	0.000
45°	12	0.010	0.002	12	0.010	0.062

Table 2

Linear regression lines of PT of pooled coumarin plasma. n is the number of storage times. Significance is given by the p value.

		Candidate 04/106		Candidate 04/162		
Temperature	n	slope (s/day)	p	n	slope (s/day)	p
4°	13	0.012	0.000	13	0.010	0.007
30°	13	0.012	0.000	13	0.015	0.000
37°	13	0.014	0.002	13	0.026	0.000
45°	12	0.052	0.000	12	0.026	0.290