Summary

Stocks of the International Reference Preparation (IRP) for thromboplastin, human, plain, coded BCT/253 and held by the World Health Organization (WHO) are nearly exhausted and must be replaced. For practical reasons the choice of the replacement candidate was restricted to two available human recombinant preparations which were coded as X/95 and Y/95 and calibrated in an international collaborative study involving 19 laboratories from Europe, Australia, Canada and Argentina. To minimize the differences between routes of calibration, the two candidates were calibrated against the existing WHO-IRP from human, rabbit and bovine origin and the final ISI was the resultant average value. On the basis of predefined criteria (i.e., within- and between-laboratory precision of the calibration and the conformity to the calibration model), X/95 was the preferred candidate. The assigned ISI (SE of the mean) value is 0.940 (0.0060) and the interlaboratory coefficient of variation 4.7%.

Introduction

International Reference Preparations (IRP) are required to determine the International Sensitivity Index (ISI) of thromboplastins used in the prothrombin time (PT) test for the laboratory control of oral anticoagulant treatment (1). The first IRP, coded 67/40, was a human brain extract to which adsorbed bovine plasma was added (combined reagent). In 1984, 67/40 was replaced by BCT/253, a human brain extract (plain reagent). Presently, there are two additional IRPs from different species available from the World Health Organization (WHO): OBT/79 (combined, bovine brain) and RBT/90 (plain, rabbit brain). Each IRP is characterized by an ISI value, that is the slope of the relationship with the primary IRP 67/40. The stocks of the IRP BCT/253 are now practically exhausted. This report deals with the results of an international collaborative study organized under the auspices of the Scientific and Standardization Committee (SSC) of the International Society on Thrombosis and Haemostasis (ISTH) for the calibration of the replacement candidate.

Materials and Methods

Material Provided

1. A 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 and C); IRPs BCT/253, RBT/90 and OBT/79; candidate replacement thromboplastins (X/95 and Y/95); placental thromboplastin (PL).
3. Vacuum tubes for blood collection containing 0.105 M Na-citrate.
4. Appropriate diluents to reconstitute X/95, Y/95, BCT/253 and OBT/79.
5. Sterile redistilled water to reconstitute RBT/90, PL and lyophilized plasmas.

Design of the Study

All thromboplastins and plasmas have been tested over 10 different working days (not necessarily consecutive). Fresh plasmas from 2 normal subjects and 6 patients stabilized on oral anticoagulant treatment have been used on each working day along with 3 lyophilized plasmas. The order of testing plasmas was as follows: lyophilized plasmas, normal plasma 1, patient plasmas 1 through 6 and normal plasma 2. Each plasma was tested as a single determination with all thromboplastins before proceeding to the next. The order of testing with different thromboplastins was changed each day. On odd days the order of testing was BCT/253, RBT/90, OBT/79, X/95 and Y/95 and PL, whereas on even days it was reversed. Participants were instructed to select healthy subjects among ambulant adults (females taking oral contraceptives not excluded) and to take a different pair each day. Patients had to be different on each day and chosen among those who were in good health and had been stabilized for at least 6 weeks in the range of treatment between 1.5 and 4.5 INR, according to the routine reagent of the laboratory. At the end of the study clotting times (seconds) for patients and normals were sent to the coordinating Center (Milano) for statistical analysis.

Statistical Analysis

The statistical methods were those employed for the calibration of previous IRPs (2-4). In particular, orthogonal regression was used to estimate the slope of the relationship between the log-transformed PTs obtained with the candidates and the IRPs (vertical axis). Values exceeding the interval

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1.5-4.5 INR as measured with the IRPs 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 IRP. ISI values within each route of calibration were calculated as the mean of the separate regression lines calculated for the different laboratories. The final ISI value assigned to the candidates as well as their standard errors of the mean (SE) were calculated from the individual ISI values obtained with the three different routes of calibration after exclusion of outliers. These were detected within each route of calibration by the algorithm described previously (5) and used for the calibration of RBT/90 (2). Coefficients of correlation of log-PT for the combined data of patients plus normals obtained with the candidates vs. the IRPs were assessed after outlier exclusion. The assumption that the mean log-PT of normals lies on the orthogonal regression line drawn through patients data points only was tested according to Tomenson (6) and the level of significance was set at 0.01. Conversion of patient PT into INR was performed for each laboratory by dividing the geometric mean PT for patients by the geometric mean PT for normals and then raising this ratio to a power equal to the ISI value. For BCT/253, RBT/90 and OBT/79 the ISI values were 1.085, 1.000 and 1.011 (2-4), whereas for X/95 and Y/95 the values were 0.940 and 1.004 as determined in this study.

**Results**

**ISI Assignment**

Nineteen out of 20 laboratories provided results. Overall, the mean percentage (range) of PT values excluded from the calculation of the ISI values for being outside 1.5-4.5 INR was 5.5% (0-17.5%) for both candidates, whereas those excluded for being outliers (i.e., more than 3 SD from the regression line) were 0.5% (0-2.5%) and 0.3% (0-2.5%) for X/95 and Y/95. The geometric mean values of the PTs from fresh normal plasmas are shown in Table 1. The values obtained with the IRPs are in close agreement with those obtained at the time when they were calibrated against their predecessors (2-4). Slopes and their coefficients of variation (CV) for calibration of X/95 and Y/95 vs. different IRPs are shown in Fig. 1. The CV values, which estimate the within-laboratory precision of the calibration, were within acceptable limits (≤ 3%) (7) in the majority of laboratories. Laboratory 13 scored high CV values with both candidates (and PL, not shown) in the calibration vs. OBT/79. Laboratory 13 also scored high CV values for the lyophilized control plasmas included in the study (not shown), suggesting that the operator might have had problems in dealing with OBT/79. It was, therefore, decided to exclude the ISI value obtained in laboratory 13 with OBT/79 from the final calculation. The ISI values obtained with the calibration of X/95 and Y/95 vs. the three IRPs are shown in Figs. 2 and 3 and in Tables 2 and 3. There were two outliers in the calibration of X/95 (Table 2 and Fig. 2): one in the calibration vs. BCT/253 (laboratory 14) and one in the calibration vs. OBT/79 (laboratory 1). There were no outliers in the calibration of Y/95 (Table 3 and Fig. 3). The between-laboratory CV values for X/95 ranged from 5.0% to 6.3% (including the outliers) or from 3.8% to 5.0% (excluding the outliers) (Table 2). The between-laboratory CV values for Y/95 ranged from 7.8% to 8.1% (Table 3). The final mean ISI (SE) value for X/95, i.e.,
the average value of the 54 calibrations generated in this study and retained for calculation after exclusion of the outliers, was 0.940 (0.0060) (Table 2). The correspondent value for Y/95 was 1.004 (0.0106) (56 calibrations) (Table 3). Results on calibration of PL and results on lyophilized control plasmas will be reported separately.

**INR Conversion for Fresh Patient Plasmas**

Table 4 reports mean INR values and ranges calculated for the fresh plasmas at each laboratory with IRPs and candidates. As expected there were considerable between-laboratory differences in the INR values due to the different intensity of anticoagulation used by different laboratories. However, the mean values for all laboratories were similar for the three IRPs and X/95, but were slightly lower for Y/95.

**Comparison of the Candidates**

The criteria used to judge the calibration of the two candidates were as follows (in the order of importance):
1. Within-laboratory precision of the calibration, expressed as the CV of the slope.
2. Between-laboratory precision of the calibration, expressed as the CV of the ISI.
3. Conformity of the calibration model i.e., (i) linearity of the relationship between log-PTs obtained with the candidates vs. existing IRPs and (ii) testing the assumption that the mean log-PT of normals lies on the orthogonal regression line drawn through patient data points only.

**Within-laboratory precision.** Table 5 summarizes the percentage of calibrations which scored a CV value equal or below the recommended 3% (7). Overall, these were 88% for X/95 and 65% for Y/95.

**Between-laboratory precision.** Overall, CV values (after exclusion of outliers) were 4.7% for X/95 (Table 2) and 7.9% for Y/95 (Table 3).

**Conformity of the calibration model.** Table 6 summarizes the results of correlation of log-PTs for the combined data of patients plus normals obtained with candidates vs. different IRPs. Correlation coefficients (r) were acceptable for both candidates. Overall, the percentage of calibrations which scored r values greater than 0.98 were 61% for X/95 and 20% for Y/95. Figs. 4 and 5 show examples of calibration plots for both candidates with and without significant deviation from the assumption that the mean log-PT of normals lies on the orthogonal regression line drawn through patient data points only. Significant deviation was observed in 7% of the cases for X/95 and in 83% for Y/95 (Table 7).

**Discussion**

The study included all three existing IRPs. Following a previous deliberation of the SSC of the ISTH it was decided that the ISI of the candidate replacement be the average value of three calibrations against the three existing IRPs (8). This was deemed necessary in order to minimize differences that have been observed between the three routes of calibration (2). This strategy, that had already been implemented for the
calibration of RBT/90 (2), proved to be fruitful because the ISI values for X/95 calculated in the present study vs. BCT/253 and RBT/90 are very close each other (i.e., 1.7% difference), whereas the ISI values calculated vs. BCT/253 and OBT/79 are 3% apart. This difference, which is however negligible, might be reduced when OBT/79 will be replaced following the proposed calibration procedure. It is, therefore, reasonable to assume that the proposed scheme of calibration of new IRPs will considerably reduce the bias between the three routes of calibration. On the other hand, the proposed scheme does not substantially change the original scheme established by WHO. The recommendation of like-vs.-like calibration will still remain in place for commercial reagents, thus reducing the variability in the assignment of the ISI value, particularly when small number of laboratories perform the calibration. On the other hand, this rule can be broken for calibration of IRPs because the considerable number of laboratories participating in the calibration minimizes the risk of increasing the standard deviation of the ISI (2, 9).

Potential candidates to replace BCT/253 were human brain, human placenta and human recombinant relipidated tissue factor. To avoid any risk of viral transmitted diseases the SSC of the ISTH, after consultation with WHO, decided to restrict the choice to available human recombinant preparations that previous studies have shown to be equivalent to the extractive preparations (9, 10). Additional requirements were that the material should have to be provided in glass sealed ampoules and a suitable number of them (at least 10,000) 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 coded as X/95 and Y/95 (courtesy of Dr. A. R. Hubbard, NIBSC, Potters Bar, UK) by people neither involved in the calibration exercise nor in the statistical analysis and calibrated in an international collaborative study. Twenty laboratories from Europe, Australia, Canada and Argentina were invited to participate in the study. The vast majority of them had already participated in the collaborative exercise to calibrate RBT/90 (2) and had experience with the manual (tilt tube) technique for PT testing. All except one laboratory eventually provided results.

The criteria to judge the calibration of the two candidates were (in the order of importance), the within- and between-laboratory precision of the slope. Percentage of calibrations with CV of the slope equal or lower than 3%.

<table>
<thead>
<tr>
<th>IRPs vs.</th>
<th>Candidates</th>
<th>X/95</th>
<th>Y/95</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCT/253</td>
<td>100</td>
<td>74</td>
<td></td>
</tr>
<tr>
<td>RBT/90</td>
<td>68</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td>OBT/79</td>
<td>95</td>
<td>79</td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>88</td>
<td>65</td>
<td></td>
</tr>
</tbody>
</table>

The ISI values for X/95 calculated in the present study vs. BCT/253 and RBT/90 are very close each other (i.e., 1.7% difference), whereas the ISI values calculated vs. BCT/253 and OBT/79 are 3% apart. This difference, which is however negligible, might be reduced when OBT/79 will be replaced following the proposed calibration procedure. It is, therefore, reasonable to assume that the proposed scheme of calibration of new IRPs will considerably reduce the bias between the three routes of calibration. On the other hand, the proposed scheme does not substantially change the original scheme established by WHO. The recommendation of like-vs.-like calibration will still remain in place for commercial reagents, thus reducing the variability in the assignment of the ISI value, particularly when small number of laboratories perform the calibration. On the other hand, this rule can be broken for calibration of IRPs because the considerable number of laboratories participating in the calibration minimizes the risk of increasing the standard deviation of the ISI (2, 9).

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The criteria to judge the calibration of the two candidates were (in the order of importance), the within- and between-laboratory precision of the slope. Percentage of calibrations with CV of the slope equal or lower than 3%.

<table>
<thead>
<tr>
<th>IRPs vs.</th>
<th>X/95</th>
<th>Y/95</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCT/253</td>
<td>1(19) 5.3%</td>
<td>16(19) 84.2%</td>
</tr>
<tr>
<td>RBT/90</td>
<td>0(19) 0%</td>
<td>13(19) 68.4%</td>
</tr>
<tr>
<td>OBT/79</td>
<td>3(19) 15.8%</td>
<td>18(19) 94.7%</td>
</tr>
<tr>
<td>Overall</td>
<td>4(57) 7.0%</td>
<td>47(57) 82.5%</td>
</tr>
</tbody>
</table>

Fig. 4 Examples of calibration plots for X/95 in which the line drawn through patients (broken) either does pass (left) or does not (right) through the log mean of normals. Solid line, overall regression line.

Fig. 5 Examples of calibration plots for Y/95. See also Fig. 4.

Table 5 Summary of within-laboratory precision of the slope. Percentage of calibrations with CV of the slope equal or lower than 3%.

Table 6 Summary of the correlation of log-PTs for the combined data of patients plus normals obtained with the candidates vs. IRPs. Percentage of calibrations with coefficients of correlation (r) within the specified range of values.

Table 7 Adequacy of the WHO model. Number (out of total) and percentage of laboratories with significant deviations from the assumption that the log-PT of normals lies on the orthogonal regression line drawn through patients data points.
mended 3% (7) were 88% vs. 65%. Also the between-laboratory precision was better for X/95 than for Y/95, with CV values of 4.7% vs. 7.9%. Finally, X/95 was more adequate to fulfill the requirements of the calibration model. First, it is required that in a given calibration there is a linear relationship between log-PT values measured with the candidates and the IRPs. Overall, the correlation of log-PTs determined with the candidates vs. the IRPs was satisfactory for both candidates, but better for X/95. Second, it is required that the overall regression line describes patient and normal data points adequately. Visual inspection of the calibration plots to assess whether the patient-only regression line passed through the scatter of normals revealed a greater percentage of deviations for Y/95 than X/95. However, according to Tomenson (10) this can be achieved more objectively 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 X/95 deviated from the assumption in 7% of the cases, whereas Y/95 did deviate in 83% of the cases.

On the basis of these results the SSC of the ISTH agreed unanimously that X/95 was the preferred candidate IRP to be submitted to WHO (11).

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Appendix

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References