INTRODUCTION

It is 15 years ago since the International WHO Reference Preparation of Thromboplastin, Bovine, Combined (NIBSC 68/434) was prepared. Although its stocks would probably suffice for a further decade and although recalibration in 1979 by the group of experts working with the European Community Bureau of Reference (BCR) confirmed its sensitivity value established in 1979, replacement seems appropriate in order to facilitate the calibration procedure for bovine thromboplastins. After replacement, national control laboratories and manufacturers of thromboplastins dispose of the same material, which obviously enhances accuracy of calibration, as is the case for the second WHO reference thromboplastin Rabbit, Plain, established in 1982.

Therefore, after consultation at WHO, biologicals unit, and in accordance with representatives of the International Committee on Standardization in Haematology (ICSH) and the International Committee on Thrombosis and Haemostasis (ICTH), a proposed secondary WHO reference preparation thromboplastin, bovine, combined, has been prepared and calibrated against the WHO primary International Reference Preparations of Thromboplastin, Human, Combined (NIBSC 67/40) and Bovine, Combined (NIBSC 68/434).

The same statistical treatment was applied as for the Second International Reference Preparation of Thromboplastin, Rabbit, Plain.

The calibration occurred in the framework of the activities of the European Commission (EC) in Brussels.

MATERIAL

Fourteen thousand ampoules, sealed by the fusion of the glass containing aliquots of freeze-dried bovine brain thromboplastin were prepared in November 1978 by Nyegaard & Co., Oslo, Norway. The samples were received summer 1979 and have since then been stored at the National Institute of Public Health in Bilthoven, The Netherlands, at -70°C.

From this stock, 6000 ampoules were earmarked for use by the World Health Organization, 6000 for the Bureau Communautaire de Référence, and 800 for CDC, Atlanta (the latter to relate the American system for standardization of the prothrombin time with the WHO system) and for a long-term stability study.

The material has the code OBT/79.
Characteristics of OBT/79 as supplied by the manufacturer:

- **Batch number**: Nyegaard & Co
- **Filling weight of liquid material and its SD**: 2.211 ± 0.005 g
- **Vacuum**: double-checked
- **Water mass fraction after lyophilization (kg/kg)**: 2.2 ml sterile CaCl₂ 3.2 mmol/l
- **To be reconstituted with**: satisfactory
- **Solubility**: 7.73
- **pH**: below detection limit
- **Haemoglobin**: 140 mmol/l
- **Sodium concentration**: 1.2 mmol/l
- **Calcium concentration**: good
- **Clot formation**: CV of the mean coagulation times obtained from investigation of 20 ampoules on two control plasmas (displaying mean values of 85.9 sec. and 54.4 sec.) approximated 0.9 and 0.8%, respectively.
- **Uniformity tests**: twenty ampoules stored at 37°C for three weeks were compared with two ampoules stored at 4°C. The testing showed that the mean coagulation time for the heated ampoules increased by 1.2 sec., compared to the times obtained with unheated ampoules. Testing occurred with "Control Plasma 25".
- **Stability data**: Stability study (Leiden):

(a) **Accelerated degradation of lyophilized OBT/79**

Accelerated degradation tests to detect thermal instability of OBT/79 were completed in 1980. The material, stored for 75 days at -70°C, 29°C, 38°C, and 43°C, was tested on plasmas which are kept deep frozen (-196°C) or lyophilized in capped vials (-20°C). Normal plasma was a pooled normal obtained from 24 volunteers. Abnormal plasmas were pooled plasmas prepared from more than 100 patients per pool, treated long-term with phenprocoumon. Seven different levels of anticoagulation were used.

The results of testing with deep-frozen plasmas are presented in Fig. 1. Those obtained with lyophilized plasmas are not given because of the even more accentuated scatter of the observation points due to the relatively large inter-vial variability of the prothrombin times when lyophilized plasmas are tested.
It is clear from Fig. 1 that the prothrombin times show an increase when the temperatures to which OBT/79 is exposed are raised. The increase is relatively small, however, at 29°C. Under the latter condition the prothrombin time ratios (patient plasma time divided by a single normal plasma (-196°C) time determined in the same test) display no significant trend although the scatter is still considerable (Fig. 2).

(b) Long-term stability of OBT/79

Beginning in the spring of 1981, the stability of OBT/79 was tested according to an agreed protocol for long-term stability of the material stored at -70°C. Results of 12 checkings (until October 1982) indicate no difference with the 1980 data and no changes in the prothrombin times of OBT/79 during 1981/82.

(c) Stability on the reconstituted OBT/79

No substantial change of the prothrombin times was found within six hours after reconstitution, using reconstituted lyophilized plasma.

COLLABORATIVE CALIBRATION STUDY

The collaborative calibration study took place under the directives of the BCR, in December 1979. The candidate WHO reference preparation of thromboplastin, bovine, combined (OBT/79) was calibrated, together with the International WHO Reference Preparations of Thromboplastin, Bovine, Combined (NIBSC 68/434), against the WHO primary International Reference Preparation of Thromboplastin, Human, Combined (NIBSC 67/40). Ten expert laboratories took part in the study, seven of which were European, and three American.\(^\text{a}\)

On an agreed protocol, the OBT/79 and the three International WHO Reference Preparations of Thromboplastins were tested on freshly prepared patient plasmas and freshly prepared normal plasmas. The clinical practice was followed as far as possible. The laboratories were urged to select patient plasmas with prothrombin times 1.5-5 times that of normal prothrombin times in terms of the WHO primary International Reference Preparation of Thromboplastin, Human, Combined (NIBSC 67/40). It was argued that the patient plasmas with values outside this interval would reflect unstable anticoagulation and, therefore, would be unsuitable for the calibration of thromboplastins.

As many as 10 different laboratories were considered to be sufficient to show the effect of inter-laboratory variation. To include the effect of within-laboratory variation, prothrombin time determinations were performed in each laboratory on at least six, and preferably 10 different days. On each day, each laboratory included plasmas from two normal subjects and six patients. The order in which the 6 determinations were made was balanced and prescribed in the agreed protocol of tests.

In all, prothrombin times of about 160 individually different fresh normal plasmas and about 400 individually different patient plasmas were assessed.

RESULTS OF THE CALIBRATION STUDY

After extensive statistical consultation\(^\text{b}\) it was decided not to follow the WHO\(^1/\)ICSH\(^6\) calibration procedure, but to relate the log seconds, as obtained with the thromboplastins to be calibrated, to the log seconds as obtained with the primary International WHO Reference

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\(^{a}\) Professor Beeser, Bonn; Dr Evatt, Atlanta; Professor Flute, London; Dr Gralnick, Bethesda; Professor Loeliger, Leiden; Dr Nielsen, Herlev; Professor Samama, Paris; Professor Tentori, Rome; Dr Triplett, Muncie; Professor Verstraete and Professor Vermeylen, Leuven.

\(^{b}\) Dr Kirkwood, NIBSC, London; Dr Hermans and Dr Van der Velde, Leiden; Dr Weis Bentzon, Copenhagen, Dr Yee, Manchester.
Preparation of Thromboplastin, Human, Combined (NIBSC 67/40). Two parameters, the intercept \( a \) and the slope \( b \), define the calibration line \( y = a + bx \), where \( y \) is the log prothrombin time determined with the primary International WHO Reference Preparation and \( x \) is the log prothrombin time determined with the thromboplastin to be calibrated. To account for the variability in \( y \) and \( x \), the parameters \( a \) and \( b \) are assessed by means of the orthogonal regression equation.\(^7-9\)

Fig. 3 shows the orthogonal regression lines \( y = a + bx \) for each of the 10 laboratories separately, with \( y = \) prothrombin time/second in a logarithmic scale using the WHO primary International Reference Preparation of Thromboplastin, Human, Combined (NIBSC 67/40), and similarly, the value \( x \) is that of the respective International Thromboplastin.

Table I shows the parameters of the calibration lines for the proposed secondary WHO reference preparation of thromboplastin, bovine, combined (OBT/79) and the established International Thromboplastin Bovine, Combined 68/434 (NIBSC 68/434).

**TABLE I. UNWEIGHTED MEANS OF THE 10 ORTHOGONAL REGRESSION LINES**

\( y = a + bx \) FOR THE TWO THROMBOPLASTINS OBT/79, AND 68/434

\( (x\text{-AXIS) VERSUS 67/40 (y\text{-AXIS})} \)

\( x \) and \( y \) are in log (time/seconds)

<table>
<thead>
<tr>
<th></th>
<th>Intercept</th>
<th>Slope</th>
<th>Standard deviation(s) between laboratories</th>
<th>Intercept I=a+bx in therapeutic range</th>
<th>Standard deviation(s) between laboratories</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( a )</td>
<td>( b )</td>
<td>( s_a ) ( s_b )</td>
<td>( x )</td>
<td>( I )</td>
</tr>
<tr>
<td>OBT/79</td>
<td>-0.297</td>
<td>0.996</td>
<td>0.073 0.046</td>
<td>2.0</td>
<td>1.696</td>
</tr>
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<td>68/434</td>
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<td>1.009</td>
<td>0.088 0.048</td>
<td>2.0</td>
<td>1.644</td>
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**CONCLUSIONS**

In view of the above-mentioned properties of the thromboplastin OBT/79 the International Committee on Standardization in Haematology requests the Expert Committee on Biological Standardization of the World Health Organization to accept the thromboplastin, bovine, combined (preparation OBT/79) and establish it as the second International Reference Preparation of Thromboplastin, Bovine, Combined which, when compared with the International Reference Preparation, Human, Combined (NIBSC 67/40) has an orthogonal regression line with an intercept \( a \) of -0.297 and a slope \( b \) of 0.996.

**INSTRUCTION FOR USE OF THE MATERIAL**

The second WHO reference preparation of thromboplastin, bovine, combined (OBT/79), will be used by national reference laboratories for the calibration of national reference thromboplastins bovine, along the lines recently recommended by the World Health Organization.

The ultimate goal remains to define the optimal therapeutic ranges to be aimed at in the clinical practice for any given batch of bovine thromboplastin in terms of the primary International WHO Reference Preparation of Thromboplastin, Human, Combined (NIBSC 67/40).
REFERENCES


Fig. 1: Prothrombin times as a function of the number of days of storage of thromboplastins OBT/79. Storage was at four different temperatures. Results are depicted for four plasmas: deep-frozen normal: upper left; lyophilized normal: upper right; deep-frozen abnormal IV: lower left; deep-frozen abnormal V: lower right. Curves are fitted by eye.
Fig. 2: Prothrombin time ratios (abnormal time/normal time) as a function of the number of days of OBT/79 storage at 4 different temperatures.

Fig. 2a depicts ratios as calculated from the prothrombin times shown for abnormal IV in Fig. 1.

Fig. 2b gives the means of the ratios as calculated from the prothrombin times of the 7 abnormal (patient) plasmas.
Fig. 3 Calibration lines for OBT/79 and 68/434 for the 10 laboratories separately.