Standardization in the laboratory control of oral anticoagulants

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SUPPLEMENTUM XXXX AD THROMBOSIS ET DIATHESIS HAEMORRHAGICA

35. Standardization in the Laboratory Control of Oral Anticoagulants

E. A. Loeliger and H. C. Hemker

I believe that the final goal of our Subcommittee must be to propose lines along which it would be possible to achieve comparability of the results obtained by the various assay procedures used in the different countries for the control of oral anticoagulant treatment. Our motivation, I think, has been to define the so-called therapeutic range of hypocoagulability, which is the range of hypocoagulability resulting in effective thrombosis prophylaxis and an acceptable bleeding tendency. Such a definition is badly needed for the design and evaluation of clinical trials and to improve the treatment of individual patients.

Our personal efforts in this field have been facilitated by the extensive material offered by the Leiden Thrombosis Service, which now comprises more than 10,000 patients, a large proportion of whom have been and are still treated on long-term schedule. In such a large material the rate of thrombosis and bleeding complications, if carefully watched, can serve as a reliable parameter to determine the therapeutic range.

Over the years, three different thromboplastins have been used by our Service; in their terms the therapeutic range is as shown in Table 1.

Table 1. Therapeutic range as defined for different thromboplastin preparations by the Leiden Thrombosis Service.

Thromboplastin	Therapeutic range percentage acc. to Quick and Owren
Human brain (Owren) (1954—1958)	15—30
Rabbit lung (Roche) (1959—1962)	15—30
Thrombotest (Nyegaard) (1963—1969)	5—10

With all three of these thromboplastin preparations, similar clinical results were obtained as judged from the rate of complications. Therefore, the conclusion to be drawn from our practical experience is that different procedures and thromboplastins can be equally useful.

Haematology Section of the Department of Medicine, Leiden University Hospital, and the Leiden Thrombosis Service, Leiden, The Netherlands.

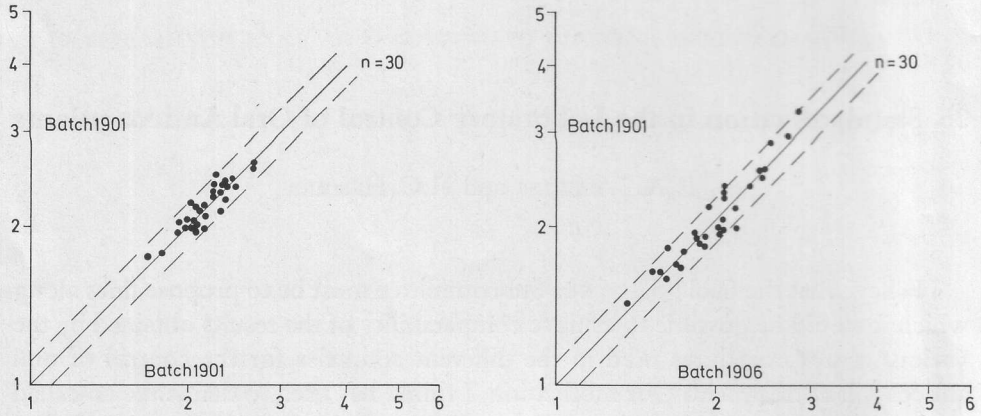


Fig. 1. Correlation diagrams as obtained by comparison of prothrombin time ratios found in a series of individual patients with two identical samples of one batch (batch 1901, left) and two samples of two different batches (batches 1901 and 1906, right) of human brain thromboplastin.

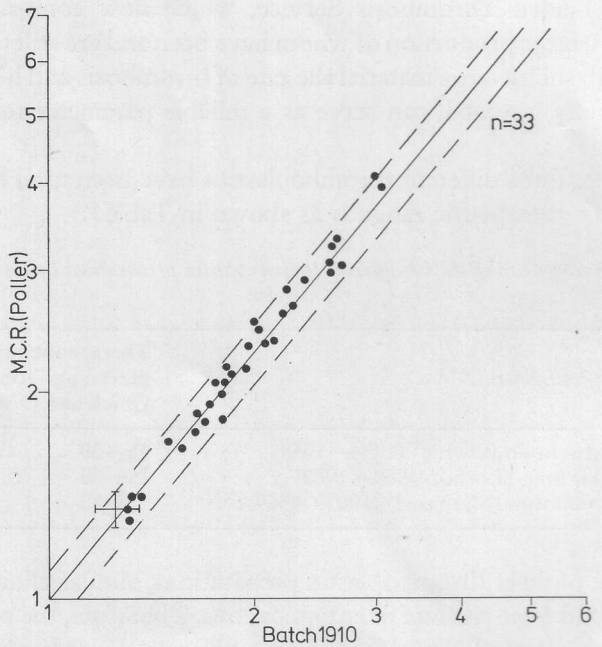


Fig. 2. Correlation diagram as obtained by comparison of prothrombin time ratios found in 33 patients with two different preparations of human brain thromboplastins, one prepared according to Owren (batch 1910), the other being Poller's Manchester Comparative Reagent. Normal ranges are indicated by a cross.

This is not surprising if we suppose that the three procedures and thromboplastins used measure and indicate the defect induced by coumarin congeners similarly, and hence in comparable terms. This should be true at least in stabilized patients, independent of whether they are treated with short- or long-acting coumarin congeners.

In the first part of my presentation I hope to substantiate this hypothesis once again by evidence.

To begin with, we may consider the experimental error. The error in coagulation times found by a skilled operator is about 5% in terms of coefficient of variation of that time. The error amounts to about 7% when ratios are considered which are calculated by dividing the patient's time by the normal time. This is illustrated by Figs. 1 and 2.

Fig. 1 shows on the left the results obtained in individual patients when two apparently different human brain thromboplastins are compared. As can be expected for identical preparations (two identical samples of one batch prepared in our laboratory were compared), the correlation line is rectilinear, the steepness of the line is 45°, and the deviations from the line are such as to be expected for operators skilled in the performance of coagulation tests. Note that the magnitude of deviation is independent of the size of the ratio. This is consistent with the experience that the error is a constant percentage of the values found. The data presented on the right in Fig. 1 show that a very similar picture is arrived at when results obtained with two different batches of human brain thromboplastin are compared. A very similar picture is found also if the results obtained with human brain thromboplastin prepared in two different laboratories with two slightly different methods are compared (Fig. 2).

If we consider the results obtained with two different procedures and thromboplastins, i.e., Quick's procedure with the use of human brain thromboplastin and Thrombotest, the correlation is still good, though with more scattering. This is shown in Fig. 3.

The greater scatter is consistent with an additional variation of about 8% (coefficient of variation). This is due not to a larger experimental error, but to the fact that in Quicks's procedure more than just the vitamin K-dependent coagulation factors are rate-limiting and in Owren's procedure no hematocrit correction has been applied; in addition, a different sensitivity of the two thromboplastins for the coagulation defect induced by coumarin congeners contributes to the greater variation.

From the data so far presented and our experience with many other commercial preparations it follows that the coagulation defect expressed in coagulation times or ratios is indeed indicated in comparable terms by different methods and thromboplastins. No outliers were observed (Fig. 4).

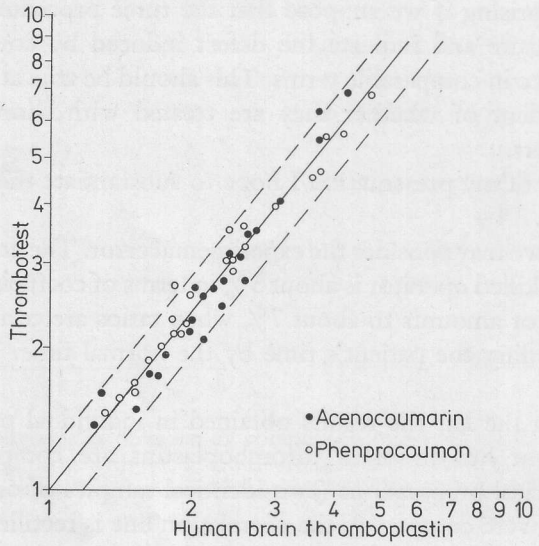


Fig. 3. Correlation diagram as obtained by comparison of coagulation time ratios found in 47 patients (23 treated with acenocoumarin and 24 with phenprocoumon) with two different assay procedures. Thrombotest according to Owren and the prothrombin time assay procedures according to Quick with the use of human brain thromboplastin were compared.

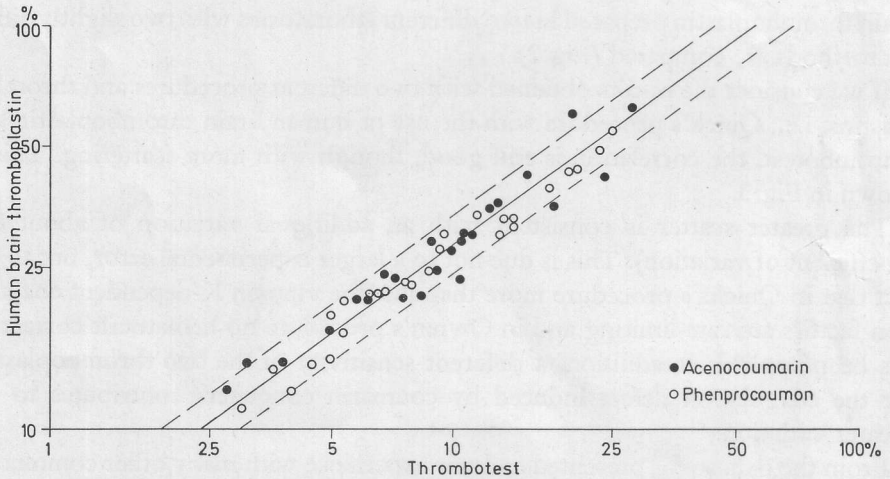


Fig. 4. Correlation diagram as obtained when conventional percentages of coagulation activity found with two different assay procedures are compared in patients treated with acenocoumarin (dots) and phenprocoumon (circles).

Further, if we consider the correlation curves as obtained when conventional percentages* found with all the different thromboplastins are compared, it becomes quite clear that the percentages defined for the different procedures have a widely different biological significance and are hardly ever comparable. Table 2 shows the therapeutic range aimed at by our Thrombosis Service in terms of these conventional percentages belonging to different procedures and thromboplastins commonly used on the Continent.

Table 2. Therapeutic range adopted by the Leiden Thrombosis Service for different thromboplastins and assay procedures.

	Equivalent to levels of 10 to 20% coagulation factor activity	Manufacturer's proposal
Geigy's thromboplastin	20—34	15—25
Human brain thromboplastin	16—30	15—30 (Poller)
Roche thromboplastin	16—30	15—25
Simplastin	18—35	11—25
Thrombotest	5.4—10.5	10—20

Equivalents to 10—20% coagulation factor activity (mean activity of factors II and X) are given in terms of conventional percentages as defined by the different authors and manufacturers.

For patients without disease states providing contraindications and stabilized on long-term treatment, the optimal range of the coagulation factors II, VII, IX, and X has empirically proven to be 10—20% of normal. The data given in Table 2 show that the percentages defined by Quick are distinctly higher than those found for the coagulation factor activity, whereas for Thrombotest the opposite is the case. The main reason for the fact that percentages as defined by Quick are all distinctly higher than those found for coagulation factors is that for Quick's method the percentages are calculated by reference to coagulation times obtained with normal plasma diluted in saline, which means that dilution of factor V plays a role. However, mutual differences between percentages according to Quick as obtained with the various thromboplastins are also present. These, and particularly the low percentages found with Thrombotest, are the result of a second biological action of coumarin congeners, known since 1963 (4), namely the appearance in the circulation of a more or less constant amount of metabolic precursor of prothrombin, called PIVKA (*P*rotein *I*nduced by *V*itamin *K A*bsence or *A*ntagonists). PIVKA competitively inhibits thrombin formation; the degree to which PIVKA is monitored by the different thromboplastins varies

* Percentages defined by Quick (prothrombin time assay procedure) and Owren (Thrombotest).

widely: for instance, rabbit brain preparations have proven to have little sensitivity to PIVKA, whereas ox brain and human brain preparations are clearly sensitive. These differences in sensitivity appear to be of no principal importance, given the good correlation between results obtained with insensitive (e.g., Simplastin) and sensitive (e.g., Thrombotest) thromboplastins in patients on long-term anticoagulant treatment (Fig. 5).

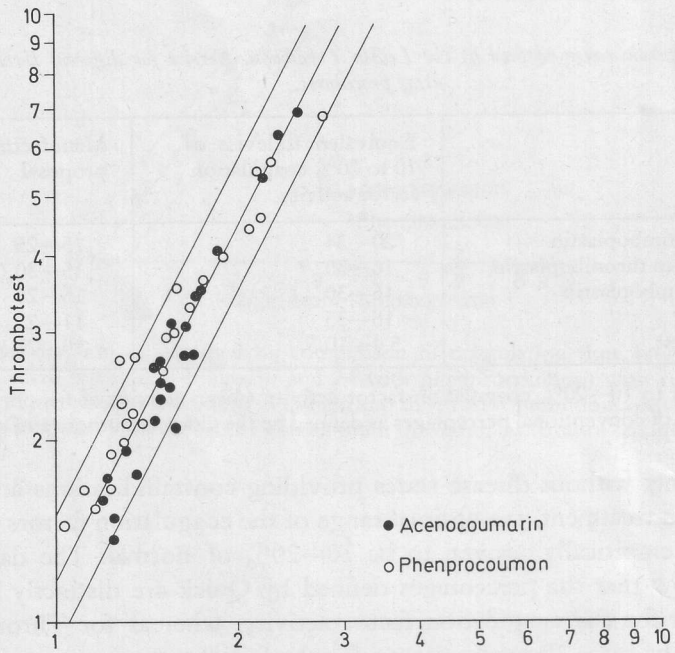


Fig. 5. Correlation diagram as obtained when coagulation time ratios found with Thrombotest are compared with those found with Quick's one-stage assay procedure performed with the use of Simplastin.

The correlation does not differ from that shown in Fig. 3 for results obtained with two PIVKA-sensitive thromboplastins. This means that interindividual differences in PIVKA activity may not be as large as has been supposed.

On the basis of the data obtained in our comparative study on the various thromboplastin preparations and assay procedures in individual patients, we can only conclude that these preparations and procedures do not differ principally in their monitoring of the degree of individual coumarin-induced hypocoagulability. Differences between the preparations pertain mainly to the over-all sensitivity. This conclusion is not new; in fact, it is only a confirmation of results reported by Biggs as early as 1964 (1, 2). It is reassuring, however, with respect to the stand-

ardization problem, because it means that any thromboplastin preparation can be used as a reference. This brings us back to our major concern, the problem of standardization.

It is clear that once we have proven that all thromboplastin preparations give us similar information in different terms — whether they be times, ratios, or percentages — we must next look for a common denominator. The most sensible definition of the defect induced by coumarin congeners would be to express it in biochemical terms, as Eilers so rightly pointed out during the Princeton meeting in 1968 (3). Our own approach has indeed been in this direction (5, 6). We have attempted to define the coagulation defect in terms of shortage of the vitamin K-dependent coagulation factors and the appearance in circulation of PIVKA. The shortage was chosen as a parameter for the magnitude of the defect, the level of PIVKA being non-informative in this respect, since it is independent of the intensity of treatment.

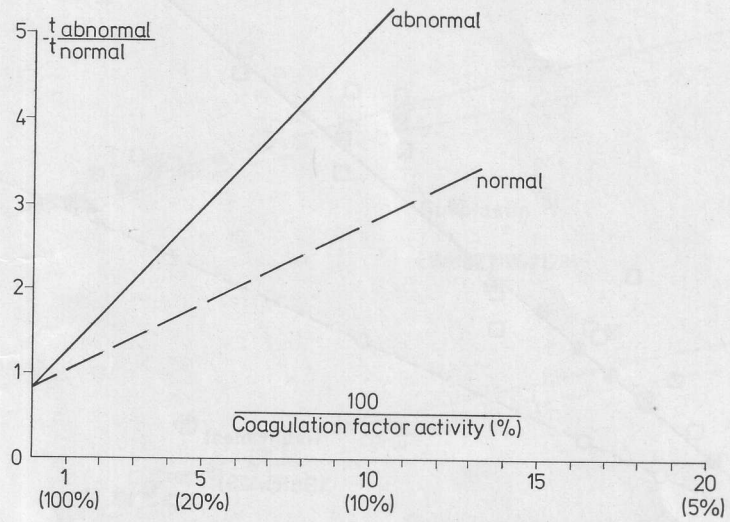
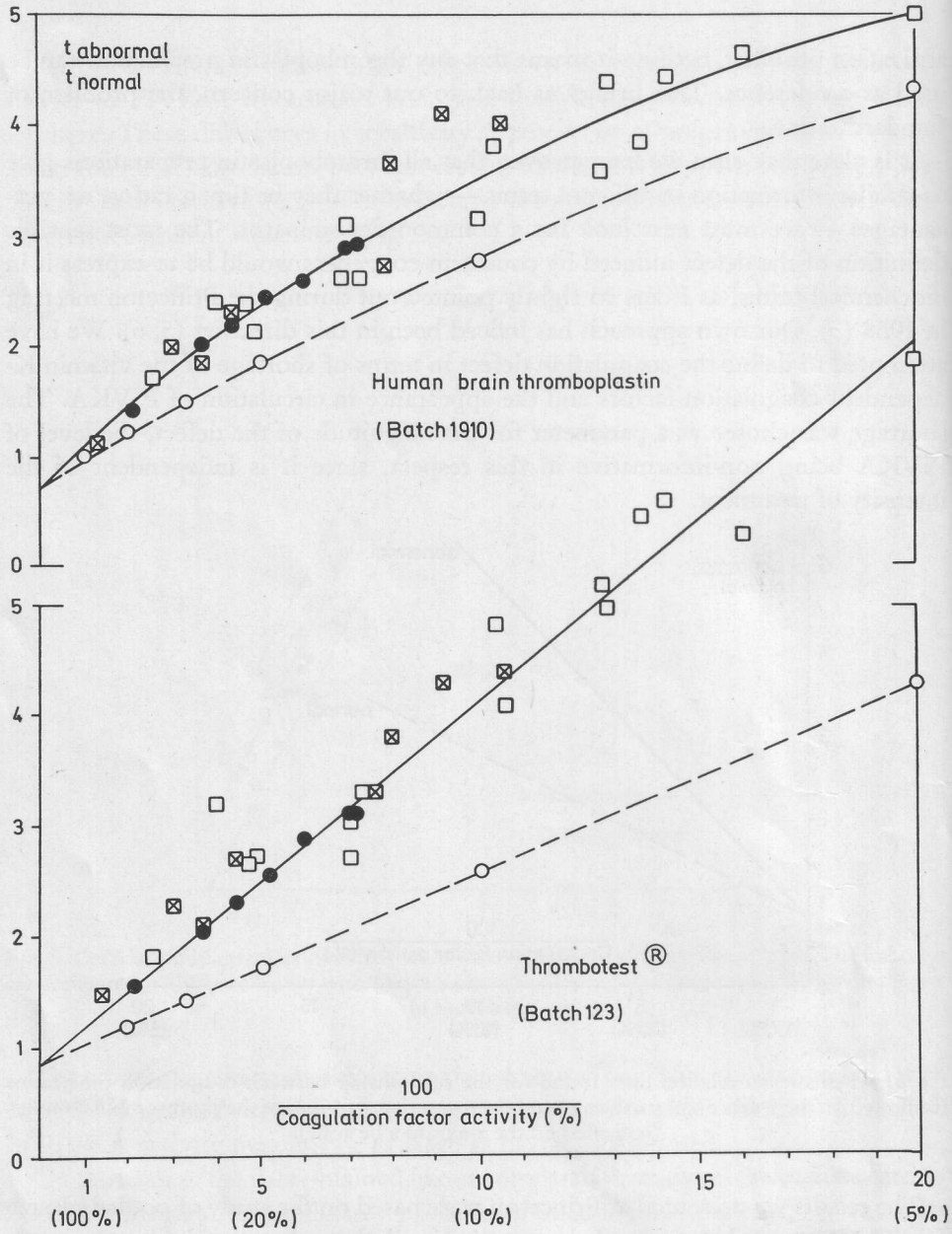
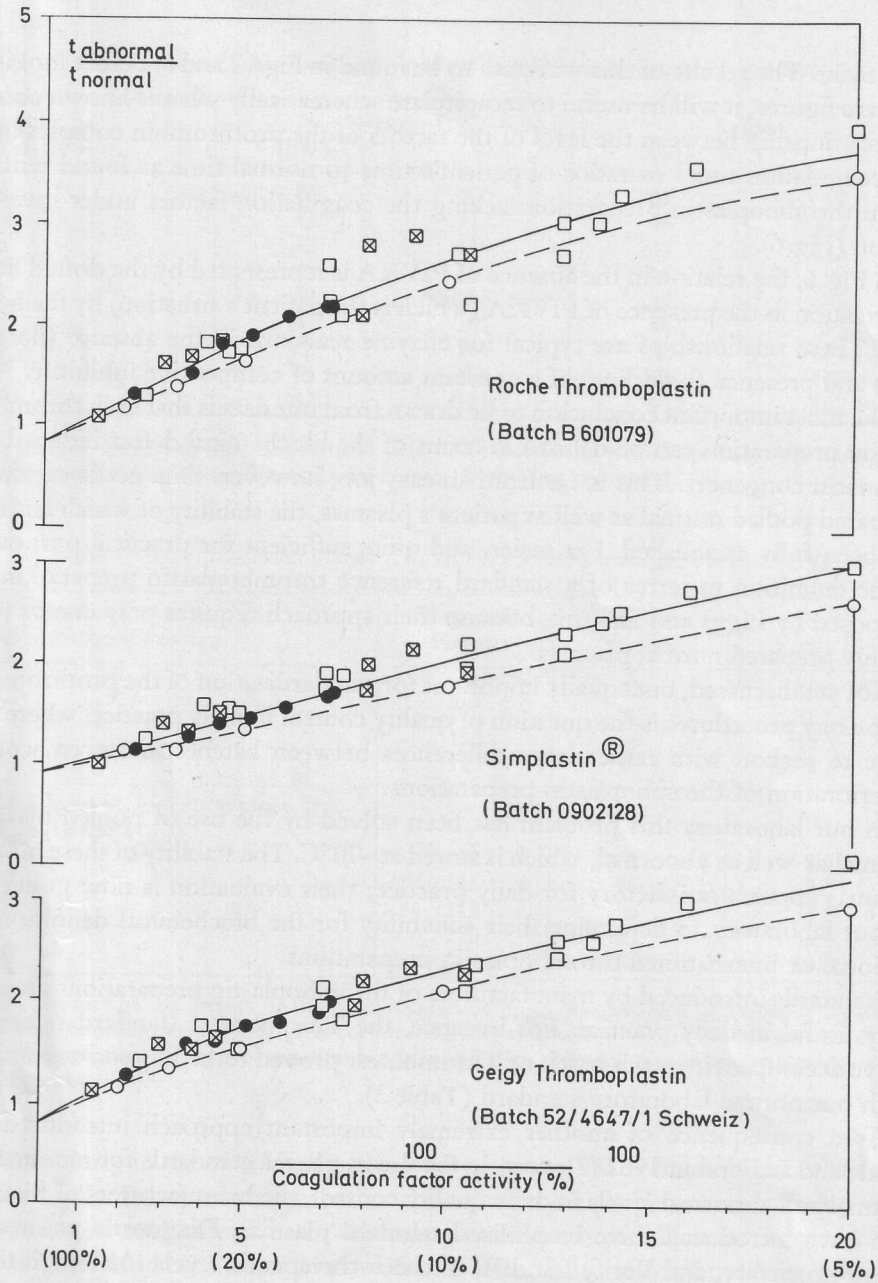


Fig. 6. Schematic correlation lines found for the relationship between coagulation time ratios (indicated on the y-axis on normal scale) and the respective activities of the prothrombin complex (indicated on the x-axis in a l/c scale).

The results we presented at Princeton were based on the study of pooled plasma (6). This year, we have extended our activities in three directions: first, the study of seven different pooled plasmas, each containing equal amounts from 30 individual patients treated at a certain level of stabilized anticoagulation; second, the investigation of 15 individual patient plasmas; and third, the investigation of plasmas obtained from one individual taken on eight different occasions during long-term



Figs. 7 and 8. Correlation diagrams showing the relationship referred to in Fig. 6 as obtained for different assay procedures and thromboplastins. The solid lines show the correlation as estimated by eye for results of tests of abnormal plasma; the dotted line was obtained from normal plasma (batch 1502) and its dilutions with Al $(OH)_3$-adsorbed normal plasma. Solid dots represent the results obtained with the seven pooled plasmas, open squares refer to the 15 randomly chosen patients, and crossed squares to one volunteer checked on eight different experimental anti-



coagulations at different levels of intensity. Note that only results obtained with Thrombotest are consistent with the hypothesis of a rectilinear correlation. Deviations from rectilinearity are particularly obvious for human brain, Roche, and Geigy thromboplastin. However, in the therapeutic range (between 5 and 10 on the x-axis, which is 20—10% in terms of coagulation factor activity) all lines may be considered rectilinear.

treatment. The results of this work are to be found in Figs. 7 and 8. Before looking at these figures, it will be useful to recapitulate schematically what is known about the relationship between the level of the factors of the prothrombin complex and the coagulation times or ratios of patient's time to normal time as found with a given thromboplastin preparation lacking the coagulation factors under investigation (Fig. 6).

In Fig. 6, the relation in the absence of PIVKA is represented by the dotted line, the relation in the presence of PIVKA (which is the patient's situation) by the bold line. These relationships are typical for enzyme reactions in the absence (dotted line) and presence (bold line) of a constant amount of competitive inhibitor.

The most important conclusion to be drawn from our data is that each thromboplastin preparation can be defined in terms of the biochemical defect induced by coumarin congeners. This is far from an easy job, however. One needs carefully prepared pooled normal as well as patient's plasmas, the stability of which has not yet been fully established. Far easier, and quite sufficient for practical purposes, is the definition in terms of a standard reference thromboplastin preparation as proposed by Biggs and Denson, because their approach requires only one or two freshly prepared normal plasmas.

Not yet discussed, but equally important for standardization of the prothrombin time assay procedures is the question of quality control in daily practice, where we have to reckon with rather large differences between batches, or, even worse, deterioration of thromboplastin preparations.

In our laboratory this problem has been solved by the use of pooled plasma, normal as well as abnormal, which is stored at –70 °C. The stability of these pooled plasmas appears satisfactory for daily practice; their evaluation is now going on in our laboratory to determine their suitability for the biochemical definition of national or international thromboplastin preparations.

Standards introduced by manufacturers of thromboplastin preparations are also very useful in daily practice. For instance, the Thrombotest standard reference curve accompanying each batch of Thrombotest proved to be in good agreement with our normal laboratory standard (Table 3).

As a consequence of another extremely important approach introduced by Miale and LaFond in 1962 (7), namely the use of plasma standards for monitoring normal and abnormal levels in daily quality control, the manufacturers of Simplastin have introduced three lyophilized standard plasmas, Diagnostic plasma, to check normality, and Verify I and II, to check therapeutic levels. Although these preparations have been very helpful for users of Simplastin, they appear to be unsuitable as standardized quality control preparations for other thromboplastin preparations because of large differences in the activity of factors VII and X, contained in these preparations (Table 4).

Table 3. Normal values found with a series of seven different batches of Thrombotest.

Thrombotest (batch no.)	Nyegaard		Leiden	
	100%	10%	100%	10%
796	40	100	41	99
860	44	112	44	115
915	43	110	44	115
916	43	105	41	99
917	39	98	39	96
918	43	101	44	98
919	42	104	42	101
937	43	106	42	100
mean	42.1	104.5	42.1	102.9

Values as defined by the manufacturer (left) and those found with deep-frozen normal pooled plasma used by the Leiden Thrombosis Service (right) are compared.

Table 4. Results found with Warner Chilcott's Diagnostic plasma and Verify I and II.

Read from specific assays	Diagnostic Plasma	Verify I	Verify II
II	95	22	10.5
V	105	90	75
VII	150	55	10.5
X	120	10	4.5
Read from prolongation ratios (Figs. 1a and 1b)			
G	80	9	3.5
HB	115	35	8.5
R	115	23	6.5
S	120	23	6.5
TT	200	57.5	10.5

The activity of the factors of the prothrombin complex was assessed individually by means of specific one-stage assay procedures (upper columns) and collectively (lower columns) obtained by transformation of the prolongation ratios found with different thromboplastin preparations (see Figs. 7 and 8).

Particularly in the two Verifys the level of factor VII is much higher than that of factor X. This probably explains the relatively high percentages found with Thrombotest and human brain thromboplastin, both of which are known to be factor VII sensitive, whereas values found with Geigy thromboplastin, which is factor VII insensitive, closely parallel the factor X activity.

Summary

1. Quick's one-stage assay procedure with the use of human brain or rabbit lung thromboplastin and Owren's Thrombotest assay procedure with the use of ox brain thromboplastin have proved equally useful in practice.

2. Thromboplastin standardization according to Biggs and Denson has again proven to be satisfactory for all practical purposes; different PIVKA sensitivity of the different thromboplastins has no primary importance in this respect.

3. The various assay procedures and thromboplastin preparations do not differ principally in their monitoring of the degree of the individual coumarin-induced hypocoagulability.

4. For the definition of the coumarin action in biochemical terms, and, equally important, for daily quality control, plasma standards are needed and hence must be developed.

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