Laboratory Control of Oral Anticoagulants

Definition of Therapeutic Range in Terms of Different Thromboplastin Preparations

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Introduction

The so-called therapeutic range in oral anticoagulant treatment is the intensity of hypocoagulability to be attained and maintained in patients in order to prevent the occurrence or progression of thromboembolic disease. The hypocoagulability is assessed by biological methods, i.e. coagulation time assay procedures; the range to be aimed at is usually defined by a lower and an upper limit, expressed as percentages of normal coagulability.

The assay procedures currently used for the assessment of the therapeutic range are all based on the original procedure first described by Quick in 1935 (1), which still enjoys an excellent reputation as the so-called Quick one-stage prothrombin time estimation, usually abbreviated to Quick test or prothrombin time. In this test, oxalated plasma is recalcified at 37°C after the addition of a suspension of acetone-dried rabbit brain powder, called thromboplastin. However, the great majority of the patients are no longer checked by Quick’s original procedure, because many modifications include the use of capillary blood instead of venous blood and the substitution of citrate for oxalate in decalcification. Of much greater impact have been the modifications in the preparation of thromboplastin and, even more important, the replacement of rabbit brain thromboplastin by various tissue preparations deriving from other species. The latter change was made for economic as well as biological reasons. Human brain material, for instance, was not only readily available in some countries, but also appeared to excel over rabbit brain material in sensitivity to changes in coagulability.

Expression of Results as “Percentage of Normality”

Results obtained with the coagulation time assay procedure are often expressed as a “percentage of normal”. However, it is not sufficiently known that the percentages obtained with the various methods differ widely in biological significance. Percentages obtained with different methods are hardly ever comparable. One of the main reasons for this divergence is that for the Quick method the percentages are calculated by reference to coagulation times obtained with normal plasma diluted in saline, so that the dilution of Factor V plays a role, whereas for Thrombotest as well as the P & P method the reference curves are obtained with normal plasma diluted in a milieu in which only the vitamin K-dependent factors are rate limiting. However, with both procedures introduced by Owren (the P & P method and Thrombotest) the results found in patients are also mutually divergent, the Thrombotest “percentages” being
about one half those found with the P & P method. The reason for this divergence is
that Thrombotest is particularly sensitive for PIVKA, a protein anticoagulant
appearing in the circulation during coumarin treatment (PIVKA being the abbrevia-
tion of Protein Induced by Vitamin K Absence or Antagonists (4)).

Unawareness of the wide divergence in the significance of these “percentages” has
led to ambiguities and hence to dangerous confusion with regard to optimal therapeu-
tic levels. This situation explains many failures in anticoagulant treatment during
the past twenty years, and makes standardization imperative. These difficulties may
be illustrated by experience in The Leiden Thrombosis Service.

In the Leiden Thrombosis Service over more than 30,000 patient years of coumarin
treatment, it has been shown that the case with which adequate anticoagulant therapy
is maintained depends, in addition to tight supervision of the patient and the choice of
the anticoagulant, to a great extent on the reliability of the method used to evaluate
couagulation. At the Leiden Thrombosis Service, three different methods and thrombo-
plastin preparations have been tried during the past fifteen years: in the beginning,
Quick’s one-stage prothrombin time assay procedure with human brain thromboplastin
was used; later, because of a shortage of human brain material, a commercial rabbit
lung thromboplastin preparation (Roche) was applied, and finally, since 1963,
Thrombotest (Nyegaard) has been used. Thrombotest was chosen upon the recommenda-
tion of the Scientific Committee for the Thrombosis Services of the Dutch Red
Cross, which considered Thrombotest to offer the most reliable results, particularly
because it provides a reproducible value for normal plasma (16).

The search for the so-called therapeutic range, i.e. the range between the limits of
which hypocoagulability induced by coumarin congeners should be maintained, has,
for any given assay procedure, been a matter of trial and error. In the extensive
material of the Leiden Thrombosis Service, a therapeutic range of 15 to 30 “per cent”
according to Quick has proven to be adequate when use was made of human brain and
Roche thromboplastin. With the introduction of Thrombotest after an extensive
comparative study (16), a range of 5 to 10 “per cent” was adopted instead of the
recommended 10 to 25 “per cent”. For cases of active venous thrombosis, even lower
values (4 to 8 “per cent”) were aimed at, and in patients with relative contra-indica-
tions, values lower than 7 “per cent” were avoided as much as possible. It is interesting to
note that Poller, who covers a population of 15 million with his Manchester Compar-
ative Reagent (a human brain material), proposes a therapeutic range of 15 to 30 “per
cent”, a range he showed as early as 1962 to equal 5 to 10 “per cent” Thrombotest (17).
Following these guidelines, we have obtained highly satisfactory prophylaxis of venous,
intracardial, and arterial thrombosis as well as restriction to an acceptable number of
bleeding complications (18, 19, 20). Similar experience has been reported by other
investigators (21, 22). However, for the P & P method, a therapeutic range of 10 to
25 “per cent” was originally proposed (2), but 10 to 20 “per cent” may even be more
adequate (23). Unfortunately, manufacturers of thromboplastin preparations recom-
mend therapeutic ranges that may result in insufficient anticoagulation (24). The
reverse – institution of excessive anticoagulation – may also be the case when using
the therapeutic ranges recommended by manufacturers (25, 26).

From the foregoing it is clear that “percentages” found with one thromboplastin
preparation cannot be equated with the percentage found with another. These dif-
fferences are explained by what is known about the mode of action of coumarin congeners,
the assay procedures, and the interaction of coagulation factors. For these reasons the
percentage activity derived from (saline) dilution curves is indicated by the use of
inverted commas in this communication.
The Sensitivity of Thromboplastin Preparations to the Defect Caused by Coumarin Drugs

The sensitivity of a thromboplastin preparation may be assessed by the extent of prolongation of clotting time in a test sample compared to normal. Since the various methods have different normal clotting times this sensitivity may be recorded using the clotting time ratio (the clotting time of the test sample divided by the clotting time of the normal sample).

If the "therapeutic range" adopted by the Leiden Thrombosis Service is used for purposes of comparing different preparations, it is found that the most sensitive assay procedure is that introduced by Owren in 1951, the so-called P & P method (2). For the range adopted as optimal by the Leiden Thrombosis Service, the difference in these ratios with this procedure is approximately 3.3 (e.g. a range of 3.7-7). Quick's assay procedure with the use of human brain thromboplastin displays a distinctly lower sensitivity, from 1.0 to 1.5 (e.g. a range of 2.3-3.4), dependent on the mode of preparation of the thromboplastin. For commercially available thromboplastin preparations the sensitivity is still lower, rabbit brain preparations displaying a difference in ratios as small as 0.50 (a range of 1.5-2.0). Thrombotest, a reagent introduced by Owren in 1959 (3) contains bovine brain thromboplastin and an excess amount of fibrinogen and factor V, by which specifically the vitamin K-dependent coagulation factors become rate limiting in the coagulation reaction. This reagent is unequivocally more sensitive than any other commercially available thromboplastin preparation, the difference between the prolongation ratios limiting the therapeutic range being 1.6 (a range of 2.5-4.1).

Standardization

From this discussion the need for a reliable standardization procedure by which the results of one centre may be correlated with those of another is obvious. In 1963, Miale proposed the use, in addition to meticulously standardized assay techniques, of standard plasmas containing a normal amount of factor V and fibrinogen and known amounts of factors II, VII, and X at different levels (5); coagulation times found with these plasmas could then be used as monitors of normality and of the limits of the therapeutic range. Commercially prepared standards have already been used in daily quality control (6, 7). Biggs and Denson (1967) approached the problem from a different angle. Using the clotting time-ratio for comparison they found that the results with one thromboplastin were well correlated with those using another when a number of samples from patients receiving coumarin drugs were tested (8). They thought that a normal reference plasma would not be needed. Loeliger and co-workers used pooled normal and pooled coumarin plasma stored at $-70^\circ$ C, which permit standardization in "absolute" terms, i.e. in terms of the activity of two coagulation factors, the factors II and X, circulating in the patient’s blood (9). This last approach has been extended, giving the results reported in this paper. The results reported here also suggest that the most profitable approach may result from a combination of the methods of Miale (1963 Plasma Standards) and Biggs and Denson (1967 Thromboplastin Standards).

Material and Methods

Normal plasma

Normal plasma consisted of a pool of equal parts of platelet-poor plasma prepared from 30 healthy individuals with an average of approximately 30 years and a sex ratio of 1:1. The material was stored at $-70^\circ$ C in small portions for daily use (batch 1502). Batch 1504 was prepared similarly, but was stored at $-25^\circ$ C.
Plasma to be tested

Plasma to be tested was obtained from patients and one volunteer displaying stable anticoagulation. The patients had been treated for coronary or peripheral atherothrombosis on a long-term basis under the supervision of the Thrombosis Service of Leiden. Individual plasmas were tested without delay, whereas pooled plasma and plasma from the volunteer were checked only after storage for 1-4 months at -70°C. Seven pools were prepared, each of them from 30 patients; to obtain pools with mutually differing levels of hypoocoagulability, the patients were carefully selected according to intensity of anticoagulation. It proved difficult to collect on one day from 30 patients displaying insufficient anticoagulation the material required for a pool with a relatively high activity of coagulation factors. Most of the patients whose plasma was included in the pools had been treated with the long-acting coumarin preparation phenprocoumon, but several of the patients whose plasma was investigated individually had been treated with the short-acting coumarin preparation acenocoumarin.

Preparation of plasma

In both patients and normals, venapuncture was performed with a disposable needle and blood was collected, without the use of a syringe, directly in plastic tubes containing 0.55 M sodium citrate, so as to obtain a final concentration of 0.011 M sodium citrate. Blood for immediate investigation was centrifuged once at room temperature for 10 min at 3,000 rpm. For plasma to be stored at -70°C, a second centrifugation, at 4°C for 30 min at 20,000 g, was applied. The duration of the interval between venapuncture and storage of the plasma at -70°C amounted to up to 8 hrs (patient pools); plasma was kept at room temperature for not more than 8 hrs. Except for the venapuncture needle, blood and plasma were handled with plastic or siliconized materials.

Lyophilized 'standard' plasma

Diagnostic plasma (batch 0479078), Verify I (batch 0906128), and Verify II (batch 0907128), all 3 plasmas produced by Warner & Chilcott, Morris Plains, New Jersey, USA.

Standard I, II, and III: obtained through the courtesy of Dr. J.B. Miale, University of Miami, Coral Gables, Florida.

Al(OH)$_3$ adsorbed plasma

1.0 ml normal pooled plasma was adsorbed with 0.1 ml 20% Al(OH)$_3$ suspension for 3 min at 37°C. To get rid of the Al(OH)$_3$, the plasma was carefully centrifuged three times, first for 10 min at 3,000 g and then twice for 20 min each at 20,000 g.

Prothrombin-deficient plasma

Prepared according to Koller et al. (10). In a sterile bloodbank bottle containing 1 ml human brain thromboplastin diluted 1:100 with barbital buffer, and under continuous shaking of the bottle, 500 ml blood from an arbitrarily-chosen donor is collected and allowed to coagulate for 24 hrs at 37°C. The bottle is then centrifuged for 60 min (750 g), after which the supernatant serum is pipetted off, centrifuged for 30 min (750 g), and mixed with a 0.1 M sodium oxalate solution in a ratio of 5:1. The oxalated serum is held for 48 hrs at 4°C and then mixed in a ratio of 1:1 with freshly prepared oxalated bovine plasma deficient in prothrombin and factors VII, IX, and X, through adsorption onto BaSO$_4$, 100 mg/ml. The mixture, which generally has a prothrombin content of <1%, is stored in 1 ml aliquots in rubber-stoppered glass test-tubes at -25°C.

Thromboplastin

5 different thromboplastin preparations were used:

1. Human brain thromboplastin prepared according to Owren and Aas (11), batch No. 1910.
3. Thrombokinase Geigy, batch No. 52/44971/Schweiz; produced by Geigy Ltd., Basel, Switzerland. (Dutch repr.: Propharma, Haarlem).
4. Thromboplastin Lösung 'Roche', batch No. B 60 1079, produced by Roche Ltd., Basel, Switzerland. (Dutch repr.: La Roche N.V., Mijdrecht).
5. Thrombotest, batch No. 123, produced by Nyegaard, Oslo, Norway (Dutch repr.: Pharmachemie, Haarlem).
Calcium Chloride
Solution of \( \frac{1}{100} \) M, except for Thrombotest, for which a 3.2 mM solution is used.

Buffer
Barbital sodium acetate buffer (pH 7.4), according to Michaelis.

Test Tubes
Blood and plasma were stored in polystyrol crystal tubes with polythene cap. Determinations of clotting times were performed in disposable glass test-tubes (10-11 x 55 ml) held at 37°C in a waterbath.

Thromboplastin Time Assay Procedure
The thromboplastin time assay procedure was meticulously standardized and performed according to Quick's and the manufacturer's directions. Patient and normal plasmas were tested in parallel to exclude time influences as much as possible. All determinations were performed in duplicate, except for those performed on normals tested in parallel with the pooled patient plasma, which were tested in quadruplicate. For the calculations, the mean of the 2 (or 4) values was used. The coefficient of variations for Geigy thromboplastin, Simplastin, and Thrombotest times are distinctly lower than those for human brain and Roche thromboplastin: in skilled hands, this value is about 3% for the first 2 of these preparations and 4-8% for the other 3. For Geigy thromboplastin, however, the normal value depends on the scrupulousness with which the suspension is prepared. The normal value obtained with Roche thromboplastin depends to an important degree on how the reading is done; values lying between 15.5 and 16.5 sec were obtained.

Factor II Assay Procedure
The activity of prothrombin was determined according to the one-stage principle. Routinely, 0.1 ml artificially factor II deficient plasma, 0.1 ml diluted test plasma (normal or abnormal), and 0.1 ml human brain thromboplastin are successively brought into a test tube at 37°C and thoroughly mixed. Recalcification is performed without delay with 0.1 CaCl₂ \( \frac{1}{100} \) M. The plasma-thromboplastin-calcium mixture is vigorously mixed in an automatic mixer. Abnormal plasma is diluted 1:10 and 1:20, whereas the normal reference plasma (here batch 1504) is diluted 1:20, 1:40, 1:80, and 1:160. The determination of normal and abnormal plasma is done in parallel, to rule out changes of the activity of thromboplastin with the duration of incubation. Results are read from a straight line drawn on double log paper through the 4 points obtained from the values obtained for the dilutions of normal plasma. The coefficient of variation of the resulting percentage approximates 10%.

Factor X Assay Procedure
From normal pooled plasma and test plasma with a Thrombotest time shorter than 100 sec, dilutions with Michaelis buffer are prepared in siliconized or plastic test tubes (1:2, 1:3, 1:4, and 1:5 for undiluted patient's plasma and 1:3, 1:5, 1:7, and 1:10 for normal plasma). For samples with Thrombotest times of more than 100 sec, normal pooled plasma is added to the test plasma 1:10 before the dilutions are prepared. The reason for the addition of this normal plasma is to obtain shorter coagulation times, since Thrombotest times longer than 250 sec are difficult to read. The plasmas and each of their 4 dilutions are tested in duplicate; 0.05 ml is added to 0.4 ml Thrombotest reagent. The results are plotted on a t/D plot, and the best-fitting straight line is drawn through the points. The steepness of the curve obtained with the test plasma in relation to that obtained with normal plasma gives the factor X activity in per cent as has been shown by Hemker et al. (12). From the percentage obtained for a test plasma to which normal plasma has been added, 10% must be subtracted and the resulting percentage multiplied by \( \frac{10}{9} \). The coefficient of variation of the result obtained approximates 10%.

Factor V Assay Procedure
Done according to Borchgrevink (13).

Factor VII Assay Procedure
This procedure is very similar to the factor II assay except for the use of factor VII-poor plasma, which was prepared according to Lechner and Deutsch (14).
Results

Most of the results obtained from the comparative study of thromboplastin preparations are shown in Figs. 1a, 1b, and 2. In these graphs, the clotting time ratios (patient's time / normal time) determined with different thromboplastin preparations, are plotted on the y-axis on normal scale; on the x-axis, the corresponding coagulation factor activities (mean of the factor II and X-activity) are plotted on a 1/e-scale (where e is the concentration). The samples tested were 7 samples of plasma pooled from different patients under treatment at different levels of anticoagulation (solid circles) and 22 individual coumarin samples of which 8 belonged to a volunteer studied over a period of time.

With Thrombotest, a straight line was obtained for the normal plasma and its dilutions, and the results obtained with Thrombotest in patients and the volunteer also strongly suggest rectilinearity; this rectilinearity is particularly obvious for the results obtained with pooled coumarin plasma samples. The curves found with the other 4 thromboplastin preparations become flatter towards the right. The curve ascends most sharply in the therapeutic range for Geigy's preparation, which contains large amounts of factor VII-like material (15). Human brain thromboplastin and Roche thromboplastin display almost straight lines in the therapeutic range, the flattening becoming evident between 5 and 10% coagulation factor activity. Finally,
Fig. 1 a and 1 b. A t/D plot showing the relation between prolongation ratios obtained with various thromboplastin preparations and the level of factors II and X. The solid line represents the correlation line as estimated by eye for coumarin plasmas; the dotted line was obtained from normal plasma (batch 1502) and its dilution in Al(OH)₃-adsorbed normal plasma. Solid dots represent results found with pooled coumarin plasma, open squares refer to randomly chosen patients, and crossed squares to one volunteer checked on 8 different occasions during experimental anti-coagulation studies at different levels of intensity.

For Simplastin, the deviation from rectilinearity becomes apparent only at very low coagulation factor activities (2.5-5%), which are not encountered in patients under therapeutic conditions. The correlation lines given for patients were fitted by eye. Particularly when the results obtained from pooled patients plasma are considered, it is obvious without statistical estimations that the patient lines run above the lines found for normal plasma and its dilutions (open circles), the distance between them increasing progressively with decreasing coagulation factor activity. This is again particularly obvious for Thrombotest, the 'patient line' of which certainly does not pass through point 1:1 of the diagram. As shown in Fig. 2, the correlation lines in the therapeutic range can for all practical purposes safely be considered as rectilinear.

If we consider the lines resulting from the comparison of coagulation times c.q. ratios obtained from normal plasma and its dilutions in Al(OH)₃-adsorbed plasma (Fig. 2), rectilinearity is obvious only with respect to Thrombotest, which is appa-
Fig. 2. Synopsis of the correlation lines found experimentally; for each thromboplastin, both the lines found for patients and those based on normal plasma and its dilutions in Al(OH)₃-adsorbed normal plasma obtained with dilutions of up to 2.5% are presented. The vertical dotted and solid lines are the limits of the therapeutic range empirically taken at (25 to) 20–10 (to 8)% The segments of the patient’s lines in the 10–20% range are accentuated. It is clear that for all practical purposes these parts of the lines may be considered rectilinear.

rently free of coagulation factors of the prothrombin complex. No statistical estimations are needed to show that the curves obtained for all other thromboplastins are not rectilinear but flatten towards the right. For Geigy’s preparation the main and rather abrupt change takes place between 1/c = 1 and 1/c = 5 (100–20%), whereas at higher dilutions the shape of the curves is similar to those obtained with Roche and human brain thromboplastin, displaying distinct flattening in the region >1/c = 10 (<10%).

The early change in the direction of the curve belonging to Geigy’s preparation is caused by the high amounts of factor VII or factor VII-like activity present in this preparation (9, 15); the flattening of the rest of the curve, as in the curves found with the other preparations, indicates contamination with factor VII and/or factor X activity. Such contamination is to be expected for human brain thromboplastin, since the brains are not freed of blood remaining in the intracerebral regions. Roche and Warner Chilcott’s preparations are probably also not completely free of coagulation factors.

The patients’ curves seem to justify two main conclusions: 1. the general pattern follows that of the lines obtained with normal plasma, and 2. a shift to the left in comparison with normal plasma dilutions is not to be denied, being particularly obvious for Thrombotest, human brain and Roche thromboplastin, and very probable for Geigy’s preparation and Simplastin. The first conclusion suggests similarity between normal plasma and patient’s plasma, whereas the second indicates the presence of an additional factor. Indeed, plasma obtained from patients under stable anti-coagulation may be considered as diluted plasma as far as the activity of factors II, VII, IX, and X is concerned; these 4 factors, like the dilutions of normal plasma,
displaying similar activities (29, 30). Regarding the second conclusion, we would like
to stress that, for Thrombotest, the patient’s line, in all probability intercepts the
y-axis at the same point as does the normal line, a finding to be expected in the pres-
ence of a competitive inhibitor, the amount of which is independent of the level of
anticoagulation, such as is the case for PIVKA (4). Similar reasoning holds for the
results obtained with the other thromboplastin preparations, although a much larger
series will be needed for statistical confirmation.
The scatter around the curves is distinctly smaller for the pooled coumarin plasma
samples than that for individual plasmas. This is due to the fact that (a) pools were
tested by only one operator; but, more important, (b) individual plasmas show a
rather large variation in the activity of the factors depressed by coumarin drugs; ex-
perience has shown that this variation amounts to about 20% in terms of the coef-
ficient of variation (29, 30). The scatter is largest for human brain thromboplastin and
Roche thromboplastin, due to the rather inconsistent normal values found with these
preparations. The coagulation time end-point with Roche thromboplastin is less
clearly defined than that found with the other preparations, and human brain material
is slightly activated during incubation at 37°C.

![Manufacturer’s proposal](image)

Table 1. Therapeutic Range in Currently Used Terms.

<table>
<thead>
<tr>
<th>Thromboplastin</th>
<th>Equivalent to levels of (8) 10-20% (25) coag. factor activity</th>
<th>Manufacturer’s proposal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Geigy’s Thromboplastin</td>
<td>(16) 20-34</td>
<td>(42) 15-25</td>
</tr>
<tr>
<td>Human Brain Thromboplastin</td>
<td>(14) 16-30</td>
<td>(35) 15-30 (Poller)</td>
</tr>
<tr>
<td>Roche Thromboplastin</td>
<td>(13) 18-30</td>
<td>(35) 15-25</td>
</tr>
<tr>
<td>Simplastin</td>
<td>(10) 18-35</td>
<td>(45) 11-25</td>
</tr>
<tr>
<td>Thrombotest</td>
<td>(4.2) 5.4–10.5</td>
<td>(13.5) 10-20</td>
</tr>
</tbody>
</table>

Limits of the therapeutic range accepted on the basis of experience at the Leiden Thrombosis
Service, expressed in percentages defined by the calculators furnished by the manufacturers of
thromboplastin preparations. For human brain thromboplastin, a saline dilution curve obtained
from normal plasma has been taken as reference. The values in the righthand column refer to the
therapeutic ranges recommended by the manufacturers.

The values given in Table 1 are derived from the data presented in Figs. 1 and 2. If it
is accepted that the coagulation factor activity (plotted on the x-axis) is the ultimate
criterion for comparison, then the different thromboplastin preparations may be
assessed by determining the clotting time range with each thromboplastin which
corresponds to a particular range of factor activity. The therapeutic range adopted at
the Leiden Thrombosis Service corresponds to a factor activity range of (8–10 to
20–25) per cent. For the various thromboplastin preparations this absolute criterion
may be converted to “percentages” as determined by the manufacturers from dilution
curves. It will be seen once again that the “percentages” determined by the manufac-
turers bear little relation to the percentage coagulation factors. The therapeutic ranges
suggested by manufacturers (last column) also differ very markedly from the range
adopted by the Leiden Thrombosis Service with the exception of the Manchester
reagent and Roche thromboplastin.

Table 2 shows results obtained with 2 samples of lyophilized normal plasma (Dia-
gnostic plasma and Standard 1) and 4 abnormal dried samples. It will be seen that
Table 2. Coagulation Factor Activities (%).

<table>
<thead>
<tr>
<th></th>
<th>Read from specific assays</th>
<th>Read from prolongation ratios (Figs. 1+2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>II</td>
<td>V</td>
</tr>
<tr>
<td>Diagnostic plasma</td>
<td>95</td>
<td>105</td>
</tr>
<tr>
<td>Verify I</td>
<td>22</td>
<td>90</td>
</tr>
<tr>
<td>Verify II</td>
<td>10.5</td>
<td>70</td>
</tr>
<tr>
<td>Standard I¹</td>
<td>110</td>
<td>70</td>
</tr>
<tr>
<td>Standard II²</td>
<td>14</td>
<td>60</td>
</tr>
<tr>
<td>Standard III³</td>
<td>6</td>
<td>45</td>
</tr>
</tbody>
</table>

¹ courtesy Dr. J.B. Miale, Miami, USA.

Coagulation factor activities found in 'standard' plasmas, Warner Chilcott's Verify I and II, and 3 experimental standards obtained through the courtesy of Dr. Miale. The activity of the factors of the prothrombin complex was assessed individually by means of specific one-stage assay procedures (left-hand columns) and collectively (right-hand columns) by transformation of the prolongation ratios found with different thromboplastin preparations, using the correlation given in Fig. 2.

these samples differ markedly one from another in the relative amounts of factor II, V, VII, and X that they contain. It will be seen also that the various thromboplastin preparations assess these dried plasma samples differently depending on the particular sensitivity of the Thromboplastin. With factor X sensitive preparations (such as Geigy's thromboplastin) the results approximate the factor X assay figures. On the other hand with Thrombotest, which is sensitive also to factor VII, the figures parallel those for the factor VII assay.

Table 3 gives the results of testing various batches of Thrombotest reagent with a pool of normal plasma samples. The results show the great uniformity of this reagent from one batch to another.

Table 3. Comparison of Thrombotest Batches.

<table>
<thead>
<tr>
<th>Thrombotest (batch no.)</th>
<th>Nyegaard 100%</th>
<th>Nyegaard 10%</th>
<th>Leiden 100%</th>
<th>Leiden 10%</th>
</tr>
</thead>
<tbody>
<tr>
<td>796</td>
<td>40</td>
<td>100</td>
<td>41</td>
<td>99</td>
</tr>
<tr>
<td>860</td>
<td>44</td>
<td>112</td>
<td>44</td>
<td>115</td>
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<td>915</td>
<td>43</td>
<td>110</td>
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<td>916</td>
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<td>919</td>
<td>42</td>
<td>104</td>
<td>42</td>
<td>104</td>
</tr>
<tr>
<td>937</td>
<td>43</td>
<td>106</td>
<td>42</td>
<td>100</td>
</tr>
<tr>
<td>mean</td>
<td>42.1</td>
<td>104.5</td>
<td>42.1</td>
<td>102.9</td>
</tr>
</tbody>
</table>

Comparison of values found with a series of different Thrombotest batches used to test pooled normal plasma (batch 1504) with the values taken from the reference curve furnished by the manufacturer.

Discussion

With respect to the mode of action of coumarin congeners, three points may be made on the basis of personal experience (27). First, factors II, VII, IX, and X show a
common pattern of response to an initial loading dose of any of several coumarin congeners. This pattern is determined by the biological half-times of the 4 coagulation factors (28). Second, after the initial phase of unequal decrease, the 4 factors - under the influence of a constant maintenance dose - reach a constant and mutually similar level of lowered activity as assessed by one-stage assay techniques (29, 30). Third, a metabolic precursor of (one of the factors of the) prothrombin (complex) appears in the circulation. This precursor, which competitively inhibits prothrombin conversion and the amount of which is independent of the intensity of treatment, was initially called preprothrombin (31) and later, less specifically, PIVKA (4). Recent investigations have shown that at least part of this precursor may really be preprothrombin, since an appreciable and constant amount of slowly reacting prothrombin has been detected in patients suffering from vitamin K deficiency (32).

Concerning the assay procedures used in the present study and the interaction of coagulation factors, several important remarks must also be made. As to the stability of coagulation factors: there is no measurable difference between prothrombin times as assessed with a series of different thromboplastin preparations before and after storage at -70° C, particularly because factor V appears to be rather stable in platelet-free citrated plasma stored at this temperature; activity of the vitamin K dependent factors remains unaltered for years even at -25° C (34). These observations show that pooled samples from normal persons and patients stored at -70° C may be used for standardization.

Concerning the specificity of the assay procedures: the one-stage prothrombin time test appears to be insensitive to rather large differences in the relative amount of prothrombin (factor II) as demonstrated by a thorough study of carriers of severe congenital prothrombin deficiency; all these carriers had normal prothrombin times in spite of a 50% shortage of prothrombin (33). Nevertheless, prothrombin can be measured with great accuracy by a one-stage assay procedure (34). The fact that the result is independent of the dilution shows that PIVKA has no inhibitory effect within the test-system, and a falsely high activity due to PIVKA-thrombin is equally unlikely, as may be concluded from the very low factor II activities found in cases of severe long-lasting coumarin intoxications (34).

Factor VII, the second factor to be discussed in this context, can be safely monitored with human brain thromboplastin and Thrombotest (15, 28); but Geigy's porcine lung preparation, on the contrary, is highly insensitive to factor VII. The specific assay procedures give highly satisfactory results (29, 30). However, because factor VII is easily activated by contact and other not yet avoidable influences (35), we think that it should not be considered as an equally reliable monitor in cases of stabilized treatment.

With regard to factor X, studies on the reaction kinetics of the extrinsic coagulation system have disclosed that this factor is rate-limiting in the Thrombotest reaction. This implies that Thrombotest can be used as a specific factor X assay procedure if applied to a series of dilutions of the plasma to be tested (12). The accuracy of the assay closely approaches that known for the prothrombin estimation, and is clearly higher than the accuracy of the results found with the currently available one-stage factor X assay procedures (34).

We may now return to the central difficulty in the use of the one-stage prothrombin time, the problem of the large differences in the "percentages" currently used. It has been proven that the presence of PIVKA is responsible for the fact that the "percentages" obtained with Thrombotest are considerably lower than those obtained with less sensitive or PIVKA-insensitive assay procedures such as the P & P method (4). On the
other hand, “percentages” calculated according to Quick are higher than would be expected from the depressed coagulation-factor level in patients’ plasma. This difference is the result of the steepness of the standard reference curve constructed on the basis of Quick’s original procedure from the results obtained with saline dilutions of normal plasma; we know that dilution of factor V contributes considerably to the relatively slow thrombin formation in saline dilutions. For such reasons the normal plasma dilution curves cannot be used for standardization.

Using the coagulation factor dilution curves (Figs. 1 and 2), it is found that in the therapeutic range, all curves may be considered rectilinear (Fig. 2). This means that the results obtained with different thromboplastins can easily be converted to a single scale taking any one of the preparations taken as a reference thromboplastin (method of equivalent ratios of Biggs and Denson). We have already emphasised that the therapeutic ranges indicated by various manufacturers when assessed in terms of coagulation factor activity are very different (Table 2). In our opinion these divergences may be dangerous. For example, the Geigy recommendation of a range of 15–25 “per cent” according to the Geigy calculator corresponds to 8–13% of coagulation factor activity a level which, in our opinion, should only be instituted in acute thrombosis with no contra-indications. Similarly, the recommended Simplastin range of 11–25 “per cent” corresponds to a coagulation factor range of 6–14%, which is in our opinion, a dangerous degree of anticoagulation for most patients. This last conclusion is in agreement with the findings of Rabiner, who proposes that we aim at a prolongation ratio of approximately 1.7, which corresponds to 25 “per cent” in terms of Simplastin “percentages”.

Another conclusion to be drawn from our results is that Thrombotest and human brain thromboplastin display the highest sensitivity, Geigy’s thromboplastin and Simplastin the lowest. This is important, as the accuracy of results obtained, expressed as percentages, depends not only on the experimental error in the reading of coagulation times, but also on the steepness of the correlation curve. The more sensitive a thromboplastin preparation, the more accurate the result. Thrombotest, e.g., which displays the same low experimental error as Simplastin, surpasses its reliability because of a much higher sensitivity. The most important conclusion to be drawn from our data, which has already been alluded to, is that the almost rectilinear correlation between thromboplastin time and coagulation factor concentration can be used as a sound basis for standardisation. In fact, Biggs and Denson based their standardization proposal on this correlation in 1967, although they used a reference thromboplastin rather than the coagulation factor activity as a standard. The conclusion of Biggs and Denson that a rectilinear relationship exists between the ratios found with two different thromboplastin preparations in a series of randomly chosen coumarin plasmas, has been proven statistically and is consonant with our findings, at least as long as “therapeutic” values are considered. Not considered by Biggs and Denson, but equally important for standardization, is the question of a constant normal comparison.

Owren in 1959 introduced, together with Thrombotest (3) a painstakingly defined standard reference curve which, as experience has shown, gives a constant normal value at 100 “per cent”. However, the dilution curve does not reflect the true coagulation defect induced by coumarin congeners, as explained earlier.

Miale and La Fond have suggested that in addition to meticulous standardization of technique, carefully checked plasma standards for monitoring normal and abnormal levels should be used as part of the daily quality control in the producers’ and users’ laboratories. Their approach, although still under investigation, has led to the pre-
paration of commercially available diagnostic plasmas. Table 2 shows the results we obtained in an investigation of both experimental and commercially available plasma standards. It appears that particularly two of the latter (Verify I and Verify II) display rather large mutual differences in the activity of the factors II, VII, and X. The activity of factor VII is clearly higher than that of factor X. This explains the relatively high percentages found when the overall activity is tested with Thrombotest and human brain thromboplastin, both of which are known to be factor VII sensitive, whereas the values found with Geigy’s thromboplastin, which is factor VII insensitive, closely parallel the factor X activity found in Verify I and II. Hence, these standards hardly seem suitable for the standardization of different thromboplastins. Moreover, they lack PIVKA, so that in this respect too they do not reflect the true coagulation defect induced by coumarin congeners. On the other hand, Verify I and Verify II appear highly suitable for the daily laboratory evaluation of Simplastin (6, 7). The deep frozen (−70°C) samples derived from patients treated with coumarin drugs have not shown these variations from one thromboplastin preparation to another (Fig. 1). This is presumably because all were from patients treated with long-term anticoagulation. In these samples the concentrations of factors II, VII, and X were equally reduced.

In this paper we hope to have demonstrated that the preparation of reference plasmas to be used for standardization of different assay procedures monitoring the coumarin-induced hypocoagulability is feasible. As long as no internationally accepted plasma standard and/or reference thromboplastin(s) is (are) available, painstakingly defined deepfrozen pooled normal and pooled patients’ plasma (the factor II and factor X activities of which are easily assessed) may facilitate understanding. We hope that the data on many aspects of the problem presented in this paper may assist in the ultimate choice of a method of standardization which in our opinion should involve the use of a reference thromboplastin reagent and well defined plasma samples from normal persons and from patients treated with coumarin congeners.

Summary

The therapeutic range in oral anticoagulation, which is defined as the range of hypocoagulability resulting in optimal thrombosis prophylaxis and an acceptable bleeding tendency, is monitored by assay procedures of which the majority are modifications of Quick’s original prothrombin time test. With respect to individual patients on stabilized anticoagulation, the different modifications commonly used give the same information, the reliability depending more on the accuracy than on the sensitivity of the tests. The results do not appear to be comparable in terms of conventional reference curves or prolongation ratios of the coagulation times found, but on the basis of painstakingly defined normal and patient plasma, the mean of the factor II and factor X activity present in patients’ plasma proved to be a useful common denominator. In terms of this common denominator, the therapeutic range to be aimed at lies between (8 to)10 and 20(to 25)percent.

Résumé

La zona thérapeutique au cours de l’anticoagulation orale est définie par la zone d’hypocoagulabilité entre une protection optimale contre la thrombose et une tendance aux hémorragie encore acceptable. Le contrôle est fait par des méthodes de dosage en grande partie dérivées du temps de prothrombine d’après Quick. En ce qui concerne
l'anticoagulation stabilisée, cinq modifications paraissent donner la même information. La qualité dépend davantage de l'exac- titude que de la sensibilité des systèmes. Les résultats ne sont pas comparables par usage simple des courbes de référence conventionnelles ni par le calcul des rapports du temps de coagulation. Ils le sont seulement sur la base de plasmas normaux et de malades très soigneusement définis. La moyenne d'activité des facteurs II et X s'est révélée être un utile dénominateur commun. Calculée sur la base de ce dénominateur commun, la zone thérapeutique recommandée va de 0 à 10 à 20 (25) pour cent.

Zusammenfassung


Addendum

Since this paper was written, a series of results similar to those obtained in the volunteer described here, have been obtained in 12 patients on long-term anticoagulant treatment, each of whom was tested longitudinally on 7 different occasions (half of these patients were treated with the short-acting acenocoumarin, the other half with the long-acting phenprocoumon). Preliminary analysis of the findings confirms the tentative conclusion that the results obtained with the 3 different thromboplastins, i. e. human brain, rabbit brain, and Thrombotest-thromboplastin, after transformation into a common denominator, do not differ significantly.

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References


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