Reappraisal of Thromboplastin

F. D. ZIEGLER, Ph.D.,* S. A. RICH, Ph.D.,* M. J. FASCO,* CAMERON RUSSELL, F.I.M.L.S.,† and J. H. KELLY, M.D.‡

*Division of Laboratories and Research, New York State Department of Health, Albany, NY 12201
and
†Bender Hygienic Laboratory, Albany, NY 12208

ABSTRACT

Proficiency testing surveys in the state of New York indicate that despite increased sophistication in instrumentation, there has been no real improvement in interlaboratory reproducibility in prothrombin-time determinations over the last 10 years. This lack of improvement is most pronounced in the therapeutic range of 20 to 30 sec. One reason may be that thromboplastins are variable from one manufacturer to another and even between types produced by the same manufacturer. While it has been possible in several laboratories to synthesize experimentally a thromboplastin with known content of lipid and active protein, no efforts have been made to make such a product commercially available. Blood levels of warfarin were measured but cannot be reliably used to monitor anticoagulation. In a preliminary study, factor Xa activity was measured using chromogenic substrate S2222. Factor Xa activity gave a positive correlation with prothrombin times of patients receiving warfarin therapy. Chromogenic substrate factor assays may represent a future method of choice for controlling anticoagulant therapy.

Introduction

Thromboplastins are tissue extracts which, in the presence of calcium, initiate the clotting activity of plasma. In the laboratory, thromboplastin is important for its use in the prothrombin-time test to monitor or control anticoagulant therapy and as a tool to investigate bleeding disorders. Extrapolating from a European report,9 it is estimated that, in the United States alone, more than 45 million tests are performed each year to control anticoagulant therapy, at an estimated cost of over $100 million. This estimate is based solely on the population and frequency of testing; taking account of routine work-ups for liver disease or bleeding disorders would vastly increase the total.

Warfarin has been widely used for over three decades to treat thromboembolism, myocardial infarction, and atherosclerosis. Although doubts have been cast on its efficacy,11,26 statistical evidence still supports its use.6,12,32 Intensive research dur-
The prothrombin-time determination, devised in its present form by Quick in the mid-1930s, is a technically easy procedure. Thromboplastin (an aqueous suspension of acetone- or saline-extracted tissue, usually brain and/or lung) is added to plasma together with calcium. The end point is a solid fibrin clot. The prothrombin-time determination is dependent on the extrinsic coagulation system and involves three of the four vitamin K-dependent clotting factors. Factor VII is present in the least amount, and its turnover is the fastest. It is, therefore, rate-limiting when therapy is initiated. Thromboplastin activates factor VII to factor VIIa, which then activates factor X to factor Xa. Factor Xa joins with factor V and Ca\(^{2+}\) to convert prothrombin to thrombin. Thrombin hydrolyzes four small peptides from fibrinogen to form fibrin, which aggregates to form the clot. Sufficient fibrinogen and factor V must be present to produce a detectable clot, and no interfering substances may be present. When these conditions are met, the prothrombin time reflects the activity level of three vitamin K-dependent factors: VII, X, and II.

Under a New York State-mandated proficiency testing program, the performance of prothrombin-time tests by most clinical laboratories in New York State was surveyed by us. In these surveys, commercial plasma samples with normal and abnormal prothrombin-time target values are supplied to participants. An early survey (1969) showed that the results were quite variable, with coefficients of variation of 10 percent for normal-range target values of 10 to 12 sec and 11 percent for a therapeutic-range target value of about 30 sec. In the survey of 1979 reported here, the coefficient of variation had decreased to 8 percent for the normal range but had increased to 14 percent for the therapeutic range.

Apart from poor technique, this problem in achieving uniformity of prothrombin time may be due either to instrumentation and methods or to the source of thromboplastin.

### Instrumentation

Advances in the technique for measuring prothrombin time have been primarily in the development of automated devices to detect fibrin strands. The number of automated instruments in common use has tripled over the last 10 years. There are now 10,—six mechanical and four photo-optical. The most recent instruments are photo-optical.

A survey in 1978 by the College of American Pathologists (CAP) reported analyses with six automated instruments and nine thromboplastins. Of the 54 possible instrument-thromboplastin combinations, only 24 were used frequently enough to be treated statistically. Over 3000 laboratories, about 50 percent of the total in the CAP survey, used combinations common to 18 or fewer other laboratories. Similarly, a survey of laboratories in New York State showed that only a few method-thromboplastin combinations were used in sufficient numbers to be treated individually (table I). Only the data for major categories, such as the manual method with brain-lung thromboplastins, could be analyzed statistically with such an approach.

The effects of thromboplastin source and method on prothrombin time in a recent (1979) New York State survey are shown in figure 1. Standard plasma samples with a target value of 30 sec, all from the same lot (purchased from General Diagnostic Division, Warner Lambert &
When various thromboplastins were compared regardless of method, one (Hyland, dried brain),* which had relatively few users, seemed to be least variable. This thromboplastin gave a mean value of 25 seconds, lower than the other products tested. Several thromboplastins, which were individually less popular (Thromboplastin 1 in figure 1A), showed the greatest variability. The most popular product (Ortho Pure Brain) f also showed a wide range.

When prothrombin time was graphed by method, regardless of thromboplastin source, all methods appeared variable (figure 1B). The manual method was actually slightly less variable than the mechanical or optical detection methods. Similar results have led the British to recommend a return to manual methods.24

### Thromboplastin

The thromboplastin used in the prothrombin-time test is an acetone or saline extract of animal or human tissues; the highest yields are from brain, placenta, or lung. The extract is a mixture of phospholipid and protein associated with the microsomal membrane fraction. The preferred thromboplastin composition and source, whether animal or human, varies from one country to another and from one commercial supplier to the next. One manufacturer† in the United States supplies three rabbit brain-lung thromboplastins; the brain components are extracted with acetone, the lung components with saline. Another§ supplies several pure-brain acetone-extracted preparations. These products have been compounded to meet various needs, most recently the demands of photo-optical instruments.30

Rabbit brain and brain-lung combinations are used in the United States and Canada, but there is no consensus either nationally or internationally on thromboplastin source. Reference thromboplastins are used by the Netherlands, United Kingdom, and Australia to

---

* Hyland Diagnostics Division, Travenol Laboratories
† General Diagnostics Division, Warner-Lambert
§ Dade Diagnostics
**#BioData
†† Medical Laboratory Automation
§§Mechrolab
†† Baltimore Biological Laboratory

---

Co.), were distributed to 312 laboratories. When various thromboplastins were compared regardless of method, one (Hyland, dried brain),* which had relatively few users, seemed to be least variable. This thromboplastin gave a mean value of 25 seconds, lower than the other products tested. Several thromboplastins, which were individually less popular (Thromboplastin 1 in figure 1A), showed the greatest variability. The most popular product (Ortho Pure Brain) f also showed a wide range.

When prothrombin time was graphed by method, regardless of thromboplastin source, all methods appeared variable (figure 1B). The manual method was actually slightly less variable than the mechanical or optical detection methods. Similar results have led the British to recommend a return to manual methods.24

### Thromboplastin

The thromboplastin used in the prothrombin-time test is an acetone or saline extract of animal or human tissues; the highest yields are from brain, placenta, or lung. The extract is a mixture of phospholipid and protein associated with the microsomal membrane fraction. The preferred thromboplastin composition and source, whether animal or human, varies from one country to another and from one commercial supplier to the next. One manufacturer† in the United States supplies three rabbit brain-lung thromboplastins; the brain components are extracted with acetone, the lung components with saline. Another§ supplies several pure-brain acetone-extracted preparations. These products have been compounded to meet various needs, most recently the demands of photo-optical instruments.30

Rabbit brain and brain-lung combinations are used in the United States and Canada, but there is no consensus either nationally or internationally on thromboplastin source. Reference thromboplastins are used by the Netherlands, United Kingdom, and Australia to

---

* Hyland Diagnostics Division, Travenol Laboratories, Deerfield, IL 60015.
† Ortho Diagnostics, Raritan, NJ 08869.
equalize interlaboratory differences. The Netherlands Thrombosis Service uses predominantly Thrombotest, with whole blood rather than plasma as a standard. The British correctly maintain that thromboplastin is species-specific. They feel that thromboplastins derived from rabbit or bovine tissue lack human specificity, and strongly support a human brain product as a standard, using ratios of normal to abnormal plasma prothrombin times and activity coefficients to compare results between laboratories. Australia has followed the example of Britain and provides a phenol-stabilized saline suspension of human brain as the Australian Reference Thromboplastin. In the United States, a standard thromboplastin has been rejected as too complex for agreement by commercial sources, and a standard plasma is used instead. Canada follows the practices of the United States, while Scandinavian countries use a system based on Thrombotest, Normotest, or the F and P test. These reagents contain bovine or rabbit brain thromboplastin supplemented with bovine factor V.

Our observations of widely variable results in prothrombin-time determinations in 1969 prompted us to fractionate several commercial thromboplastins. At that time great emphasis was placed on the phospholipid composition. It was shown that the phospholipid classes of the most popular commercial thromboplastins were the same and that those with the highest percentage of phosphotidyl ethanolamine were the most active. Phosphotidyl ethanolamine then was considered the most important phospholipid component of thromboplastin. Recently, however, Wijngaards et al demonstrated that the charge on the phospholipid must be about -7 mV. These workers found that several phospholipid combinations, each with a net charge of about -7 mV, were all highly active. It may be concluded that contamination of supposedly pure phosphotidyl ethanolamine fractions with other phospholipids was responsible for the activity reported by us for this fraction.

The assumption that a protein fraction is necessary for thromboplastin activity has recently been further investigated and refined, largely by Nemerson's group. Using differential centrifugation, it was demonstrated that tissue factor is con-
tained in the microsomal membrane fraction of many tissues, including brain and lung. It is also present in blood vessel


intima, both in endothelial cells and in the atherosclerotic plaque.

Isolation of the protein fraction of rabbit brain and brain-lung thromboplastins was achieved by us following the procedure shown in figure 2. The protein was solubilized in sodium dodecyl sulfate and electrophoresed on poly-acrylamide gel according to published procedures.28 The electrophoreogram (figure 3) shows that a major band and one minor band are common to both types of thromboplastin. Other minor bands are different between thromboplastins. The two major bands may correspond to those in bovine thromboplastin which have molecular weights of 220,000 and 330,000.16,20 This protein is specific and necessary for thromboplastin activity.16,20 The corresponding human brain apoprotein is of lower solubility and molecular weight (55,000).5 The protein components vary from species to species,3 and often display differing activity. This is in part the rationale of the British group, who elect to use human brain as a source for thromboplastin.

It can now be stated with certainty that active thromboplastin is a lipoprotein from the microsomal fraction of many tissues, including vessel walls. Both phospholipid and protein are necessary for activity; neither purified component is active alone. It has been suggested7,14 that hydrophobic lipid-protein interactions are the basis for formation of the active complex. In this scheme (figure 4), the phospholipid forms a vesicle, with the charged portion oriented toward the aqueous phase (plasma). This vesicle without protein is inactive. However, the protein has considerable hydrophobicity,3 and the hydrophobic portion can be inserted into the nonpolar portion of the lipid vesicle. This arrangement provides a specific surface for binding factor VII in the proper conformation to cleave an Arg-Ile bond, releasing a peptide and leaving behind the active factor. It may also help to align factor Xa, factor V, and prothrombin in the correct spatial conformation to

FIGURE 3. Electrophoreogram of protein fraction from commercial thromboplastin. BL, brain-lung combination (General Diagnostics, Simplastin).—PB, pure brain (Ortho, Pure Brain Thromboplastin).
**Figure 4.** Schematic diagram of combination of lipid and protein components of thromboplastin to form a surface for the interaction of coagulation factors. Diagram based on data from Baugh, R. F. and Houghie, C. Clinics Hemat. 81:3–30, 1979.

**Figure 5.** High-performance liquid chromatograms of (A) normal serum spiked with a warfarin standard and (B) serum from a patient on warfarin therapy. Peaks are detected by absorbance at 313 nm. Retention time in seconds is given beside the peak(s) of interest. Warfarin is the peak with retention time of 666 sec.
release active thrombin. Factors VII, X, and II are bound to the lipoprotein surfaced by Ca\(^{++}\) bridges between carboxyglutamic acid residues of the protein and phospholipid.\(^{34}\)

These studies and other work\(^{16,20,22,31}\) demonstrate that it is possible to isolate and recombine the specific protein and the active phospholipids of thromboplastin. It should, therefore, be possible to produce a standard, specific, sensitive, thromboplastin. So far this has not been attempted on a large scale.

**Plasma Warfarin**

The diversity of methods and thromboplastins for prothrombin-time tests has led to a search for alternatives to thromboplastin for monitoring anticoagulant therapy. On the rationale that one can measure therapeutic blood levels of a drug—as is done with digoxin, theophylline, and Dilantin—high performance liquid chromatography was used to measure warfarin levels in sera from normal subjects and from patients receiving warfarin. Normal serum spiked with a warfarin standard and serum from a patient receiving warfarin therapy are compared in figure 5. While it is possible to quantitate the warfarin in the patient sample, a number of other peaks, possibly due to other drugs or caffeine, are also present.

Even though warfarin in serum was measurable, there was no apparent correlation between warfarin level and anticoagulation effect (figure 6). This might be expected because of variations in the human response to and rate of metabolism of warfarin. Also, warfarin binds strongly to albumin and only the small unbound portion is active. Drugs which displace warfarin from albumin enhance its anticoagulant effect while drugs which stimulate hepatic enzymes enhance degradation of warfarin and, hence, reduce its anticoagulant effect. A wide variety of xenobiotic compounds affect warfarin pharmacokinetics and pharmacodynamics,\(^{10}\) so it appears that measurements of warfarin in the blood can make no contribution to management of warfarin therapy. This position is also held by Duffield et al.\(^{9}\)

**Chromogenic Substrates**

A more hopeful alternative may be the use of synthetic substrates for coagulation
factors. Each has an amino acid sequence which makes it highly specific for one of the active coagulation factors.

The feasibility was investigated by using one of these commercially available substrates, S 2222,* which is specific for factor Xa. Plasma was collected from 15 patients receiving warfarin therapy. A pool of plasma from 10 normal subjects was prepared and used as a control. All plasmas were collected in citrate and frozen at -20° until tested. The normal plasma pool suffered no loss of activity after freezing and thawing.

For testing, factor X in buffered plasma was first activated by incubation with Russell viper venom (1:10,000) and CaCl₂ at 37° for five min. Substrate S 2222 was added, and the 37° incubation was continued for an additional 5 min. The reaction was arrested with excess acetic acid, and the 405 nm absorbance was read in a spectrophotometer.† Activity varied linearly with serial dilutions of the pooled normal plasma (figure 7) and was thus apparently Xa-dependent in agreement with the findings of Lammle et al. Patients receiving warfarin had much less factor Xa activity than the normal plasma pool (figure 8). Interestingly, the patients with the longest prothrombin time had the least factor X activity. The correlation coefficient of the regression line was 0.84, equivalent to that observed by Lammle et al. While the number of patients was small, enough correlation was observed to warrant further investigation. It must be remembered, however, that the method is sensitive only to factor Xa while warfarin reduces the activity of factors II, VII, and IX as well as factor X.

The prothrombin time test, in use in its various forms for over 40 years, is sensitive to three of the four factors suppressed by warfarin and had the advantages of reasonable cost and ease of performance.

---

* Manufactured by AB-Kabi, Stockholm, Sweden and distributed in the United States by Ortho Diagnostics, Raritan, NJ 08869.
† Beckman Model 25. Beckman Instruments Co., Fullerton, CA 92634.

Although it has been used for years, interlaboratory standardization, both nationally and internationally, has proved an intractable problem. Chromogenic substrates are now, and for the foreseeable future will be, more costly and their use more technically demanding than thromboplastins and prothrombin time determinations. Still, the fact that they are based on a specific enzyme reaction may gain them acceptance in the search for standard, reliable, and reproducible laboratory methods for the control of anticoagulant therapy.

References

6. Chandler, A. B., Chapman, I., Erhardt, L. R., Roberts, W. C., Schwartz, C. J., Smapius,


