A CLINICAL EVALUATION OF THE THROMBIN INHIBITION TEST

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ABSTRACT

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by Darlene Elaine Clingenpeel

A major disadvantage in the use of heparin therapy is the lack of a convenient and accurate system of controlling dosage.

The thrombin inhibition test, a method for monitoring heparin therapy, was modified and clinically tested.

A linear relationship was found to exist between the clotting time of a fibrinogen solution and the reciprocal of the thrombin concentration. There is also a linear relationship existing between the number of micrograms of heparin present and the number of inactivated thrombin units. From these results the following formula was derived to calculate the concentration of heparin:

$$\text{Heparin concentration } (\mu g./ml.) = 2.11 - \frac{19.7}{t}$$

(clotting time in seconds)

This method has a variance of 0.0159, thus; 95% of the time the calculated heparin concentration will be $\pm$ 0.256 µg./ml. from the actual concentration in the plasma.

In the clinical evaluation of this assay method, the calculated heparin concentration was compared to the Lee-White clotting time. A linear relationship exists between these 2 methods. The precision and accuracy of the thrombin inhibition test is much greater than that of the Lee-White clotting time.

A CLINICAL EVALUATION OF THE THROMBIN INHIBITION TEST

By

Darlene Elaine Clingenpeel

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For

My Parents and Family

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TABLE OF CONTENTS

	Page
INTRODUCTION	1
REVIEW OF LITERATURE	2
Blood Clotting Mechanism	2
Thrombin	3
Fibrinogen	5
Heparin	6
Heparin Assay	8
MATERIALS AND METHODS	11
Source of Plasma	11
Reagents Used in This Experiment	11
Thrombin Activity	12
Heparin Assays	12
Plasma Stability	13
Clinical Experiment	13
RESULTS	15
Thrombin Activity	15
Heparin Assays	15
Plasma Stability	16
Clinical Experiment	17
DISCUSSION	23
SUMMARY AND CONCLUSION	26
REFERENCES	28
VITA	31

LIST OF FIGURES

Figure		Page
1	Thrombin dilution curve (summary of data)	18
2	Thrombin activity curve	19
3	The number of active thrombin units that remained after the addition of various concentrations of heparin	20
4	Comparison of calculated heparin concentration to the Lee-White clotting time	21
5	Comparison of Lee-White clotting time to calculated heparin concentration	22

INTRODUCTION

Heparin is used when a rapid anticoagulant effect is desired. One great disadvantage in using heparin therapy is the lack of a convenient and satisfactory control system. A relatively quick and simple test is needed to determine reliably the heparin concentration in the patient's plasma.

The common test for monitoring heparin therapy is the Lee-White clotting time, which measures the time it takes for a known quantity of whole blood to clot. This time-consuming and inconvenient method has many variables which, if not closely controlled, can lead to erroneous results.

Schwab (1969) developed a thrombin inhibition test to determine the concentration of heparin in blood. The purpose of this project was to continue the development of this method, in order to provide a useful guide for the management of heparin therapy.

REVIEW OF LITERATURE

Blood Clotting Mechanism

The phenomenon that blood coagulates was beyond human comprehension and was not the subject of investigation before the late 1600's. In 1666, Malpighi discovered that pale fibers remained, when blood clots were washed in water. This substance which formed these fibers was called "fibrin". He was the first of many researchers trying to discover the physical and chemical changes that cause a fluid substance, blood, to turn to gel. The experimental data, collected by these researchers, gave rise to numerous theories on the mechanism of blood coagulation. The exact mechanism, however, is still the subject of controversy.

At present, there are 2 schools of thought about the coagulation of blood. Macfarlane and his associates in Britain (1964) and Davie and Ratnoff in the U.S.A. (1964) believe that all the reactions in blood coagulation are enzymatic. Coagulation is first initiated by contact with foreign material. Then a cascade of proenzyme-enzyme transformations take place until the final substrate, fibrinogen, is reached. This hypothesis is widely accepted today.

However, Seegers (1964) strongly opposes this theory. According to him, some of the clotting factor precursors thought to be converted to enzymes may not exist. He believes that prothrombin contains 2 enzymes in precursor form, thrombin and autoprothrombin C (Factor X). The proteolytic degradation of prothrombin activates this molecule. Seegers

concludes that the activation of prothrombin occurs by an autocatalytic process rather than an enzymatic cascade.

If each step in coagulation of blood is taken individually, it presents a set of intricate problems. The last step in coagulation, alone, involves thrombin, fibrinogen, fibrin, and certain other factors in the plasma. When any one of these factors is inhibited, normal clotting does not occur.

Thrombin

Many theories have been proposed to explain or summarize the studies on coagulation; most of these were listed in an extensive review of the literature up to 1935 by Howell. It has generally been accepted that thrombin is formed from some chemical union of prothrombin, calcium, and tissue factors. Thrombin, in turn, initiates the final conversion of fibrinogen to fibrin by the hydrolytic effect.

Because of the numerous questions involving the chemistry of coagulation, it was important to isolate and purify the various proteins involved without altering their properties. Seegers, Brinkhous, Smith, and Warner (1938) purified prothrombin from oxalated beef plasma, then by the addition of thromboplastin, thrombin was obtained. They defined a unit of thrombin as the amount that will clot 1 cc. of a standardized fibrinogen solution in 15 seconds. If dried or stored at -30 C., thrombin will retain its activity indefinitely. Thrombin was further purified by Seegers and McGinty in 1942.

Certain impurities have been found in bovine thrombin commercially prepared by Parke, Davis & Company. The experiments of Yin and Wessler (1968) show that this commercial thrombin is contaminated with a large amount of Factor X. These researchers reported that the results obtained

in the studies of coagulation or enzyme kinetics may be misleading when this product is used.

Many substances that have the properties of thrombin can be formed from prothrombin by several methods. When these substances were tested, they exhibited chemical and physical differences which were important to understanding the activation and inactivation of prothrombin and thrombin (Lamy and Waugh, 1954).

Lamy and Waugh (1954), using ultracentrifugation and electrophoresis, tried to explain the transformation of prothrombin into thrombin. They concluded that this transformation was so complicated that no definite scheme could be proposed.

It was found by Therriault, Gray and Jensen (1957) that the rate of conversion of prothrombin was accelerated in the presence of trace amounts of thrombin. Apparently thrombin speeds up its own formation by activating a plasma accelerator-globulin (Factor V). However, the specific mechanism of this action is not completely resolved.

The action of thrombin as an enzyme in the conversion of fibrinogen to fibrin is widely accepted (Laki, 1953; Sherry, Troll and Gluek, 1954). Thrombin hydrolyzes 4 peptide bonds from the fibrinogen molecule (Laki, 1953). This proteolytic enzyme was shown by Laki and Gladner (1964) to have a specificity for certain peptide bonds formed between arginine and glycine residues. The molecular weight of thrombin was estimated to be 8000 by their experiments.

Besides acting as an enzyme, thrombin has another important function. It induces platelets to aggregate, contract, and to release serotonin, nucleotides, potassium, and other substances (Ganguly, 1969).

Fibrinogen

Fibrinogen is a protein present in plasma in the concentration of 1 to 8 mg./ml. with a molecular weight of 330,000 (Laki and Gladner, 1964). The isolation of this protein is simplified considerably, because of its unusual solubility characteristics. Since fibrinogen is easily denatured and is extremely labile, purification is difficult (Jacques, 1943; Ware and Lanchantin, 1954).

Lorand in 1954 considered the fibrinogen-thrombin reaction to be the most striking phenomenon in protein chemistry. The addition of trace amounts of thrombin to a solution of fibrinogen results in a gel meshwork. It has been reported that only minute amounts of thrombin are necessary to clot a very large quantity of fibrinogen (Eagle, 1935; Laki, 1953). Because of the results of several researchers, fibrinogen is considered to be the primary substrate for thrombin.

The chemical difference between fibrinogen and fibrin indicates that peptide bonds are split from the fibrinogen molecule by thrombin, resulting in the formation of fibrin (Laki, 1953). By 1954, it was reported that the proteolytic removal of fibrino-peptide from the fibrinogen molecule was necessary to activate it. The activated fibrinogen then spontaneously but reversibly polymerizes and fibrin is formed (Ferry, 1954; Lorand, 1954). The previous results of Lorand and Middlebrook (1952) were in agreement with this view.

Laki and Gladner (1964) examined fibrinogen from 6 species. They suggested that the 4 peptide bonds of fibrinogen hydrolyzed by thrombin are between arginine and glycine residues. This is in accordance with the earlier experiments of Sherry, Troll and Gluech (1954). The fibrinopeptides are by-products once they are removed and are not needed for the polymerization that follows (Laki and Gladner, 1964).

The currently accepted concept of the conversion of fibrinogen to fibrin suggests that this conversion occurs in 3 steps. The first step, as described above, is the enzymatic action of thrombin on fibrinogen, resulting in the formation of a fibrin monomer plus fibrino-peptides. In the next step, fibrin monomers combine to form intermediate fibrin polymers. Finally, there is a secondary aggregation of the intermediate polymers to form the clot network (Ellias and Iyer, 1967). The formation of the fibrin clot occurs by 3-dimensional polymerization. Electrostatic forces and hydrogen bonding are believed to be responsible for this type of polymerization (Ferry, 1954; Laki and Gladner, 1964).

The liver has long been regarded as the source of fibrinogen. The precise cellular localization of synthesis remained uncertain. It was shown by an immunologic technique that fibrinogen was contained in the liver. Barnhart and Anderson (1962) reported that fibrinogen is synthesized by the microsomes and stored in the soluble part of the parenchymal cell. This was consistent with the experiments of Forman and Barnhart (1964). The rate of formation of this protein is adjusted to its plasma level.

Heparin

In the early 1900's, McLean discovered the first physiologic anticoagulant while trying to extract cephalin from livers. The term, heparin, and the description of the material were reported later by Howell and Holt (Vigran, 1965). Heparin, a polysulfated mucopolysaccharide, is a naturally occurring anticoagulant found in most tissues of the body. The highest concentrations were found in the liver and lung, with only minute amounts in the blood (Eiber and Danishefshy, 1957). The large metachromatic granules of mast cells are loaded with heparin. The term heparinocyte is applied to these cells (Jorpes, 1962).

Heparin is one of the most potent inhibitors of blood coagulation and, because of this property, it has been used extensively in the clinical treatment of thrombosis (Eiber and Danishefsky, 1957). This anticoagulant, together with a cofactor in the plasma, acts as an antithrombin and will inhibit the action of thrombin on fibrinogen. It prevents platelet stickiness and agglutination (Vigran, 1965). Heparin also interferes with thromboplastin generation and the conversion of prothrombin to thrombin (Lenahan, Frye, and Phillips, 1966).

The clotting time is rapidly increased after an intravenous injection of heparin (Jaques and Ricker, 1948). The experiments of Eiber and Danishefsky (1957) demonstrated that the clotting time would return to normal within 5 hours. According to their work, the quick return of the normal clotting time was due to rapid elimination of heparin rather than deactivation.

Heparin also has a lipolytic effect. Triglycerides are cleared from the serum of animals and men when heparin is administered by any injectable route. This clearing activity is the result of the release of an enzyme, *lipoprotein lipase* (Vigran, 1965).

Since the discovery of heparin, there has been active controversy about the mode of action of this anticoagulant. Howell, in 1935, believed heparin to be an antiprothrombin, because heparin did not inhibit purified thrombin's action on purified fibrinogen. Quick (1936) reported that heparin is not an antiprothrombin. His experiment demonstrated that heparin, itself, was not an antithrombin, but will react with an unknown substance in the plasma to form a strong antithrombin. Hence, he called heparin an antithrombogen. By 1957, Monhouse and Clarke noted that heparin did not significantly increase the thrombin clotting time, unless there was a heparin cofactor present in the plasma.

Astrup and Darling (1942) used antithrombin, prepared from ox plasma, in their experiments. When they plotted the amount of antithrombin added against the percent of thrombin inactivated, a straight line was obtained. Since they were able to obtain a quantitative measurement of naturally occurring antithrombin, they applied the same principle to antithrombin formed from heparin (1943). The results were not too satisfactory, because the curves obtained were not always straight lines. From their data they concluded that the thrombin inhibitor which is released by the addition of heparin is not identical with naturally occurring antithrombin.

Seegers (1954) reported 4 different categories of antithrombin: Antithrombin I is concerned with thrombin adsorption on fibrin; Antithrombin II is associated with heparin and heparin cofactors; Antithrombin III directly inhibits thrombin activity; Antithrombin IV inhibits prothrombin's activity.

It was demonstrated by Henstell and Kligerman (1967) that antithrombin in the plasma is bound to an inhibitor, leaving little or no free antithrombic activity. Since heparin increases antithrombic activity, it was proposed that heparin splits the bond between antithrombin and its inhibitor. A new bond is formed between heparin and antithrombin, which may react with any thrombin present.

Abildgaard (1968) suggested that the increase in clotting time is caused by the formation of thrombin-heparin-fibrinogen complexes. At present, his results confirm that heparin inhibits the enzymatic action of thrombin on purified human fibrinogen by the formation of a complex.

Heparin Assay

Heparin was first used as an *in vivo* anticoagulant by Best in 1935. Because of its rapid action and its relatively nontoxic properties, it

has been used widely in the control of thromboembolic disorders (Vigran, 1965). The recommended intravenous dosage of heparin ranges from 200-300 mg. every 12 hours (Miale, 1967) to 30,000 units per day (Penner, 1967). It is necessary to determine periodically whether the patient has an adequate level of anticoagulant in his blood.

The method used universally in controlling heparin therapy is the Lee-White clotting time. This method has many variables such as the diameter of the tube used, the frequency of tilting the tube, environmental temperature, and the venipuncture technique. These variables cause the end results of the Lee-White clotting time to be neither reproducible nor reliable (Vigran, 1965). Sufficient amounts of heparin will have a prolonged effect on several of the coagulation systems. Many researchers have suggested that the Lee-White clotting time could be replaced by one of the following tests: whole blood recalcification time, activated whole blood, the partial thromboplastin time, or the thrombin clotting time (Spector and Corn, 1967).

The relationship of the clotting time to heparin dosage was studied in 1948 by Jaques and Ricker. When they plotted the heparin dosage against the logarithm of the clotting time, a straight line was obtained.

Blombäck, Blombäck, Corneliusson and Jorpes (1953) investigated the reliability of the following methods used in heparin assays: a whole blood method, a thrombin method on plasma, the method of U.S.P. XIV, 1950, and the sulphated whole blood method. They found that the thrombin clotting time method gave the best results, varying only 2-5% from the standard heparin concentration. The other methods gave concentrations 5-10% lower than the thrombin method.

The data obtained by Rappaport and Ames (1957) indicates that a properly standardized plasma thrombin time can be used effectively to

follow heparin therapy. They suggested the use of platelet-poor plasma to rule out residual heparinemia and platelet-rich plasma to check the therapeutic heparin level. Since platelet-rich plasma is less sensitive to heparin, Schatz and Hathaway (1963) recommended it as an indicator of therapeutic prolongation of coagulation.

Penner (1967) measured heparin by its antithrombic activity in his laboratory. The assay required the addition of a measured amount of thrombin to the patient's blood. He found the normal thrombin clotting time to range from 18 to 24 seconds. In the presence of heparin, this clotting time is lengthened. His results were correlated with values obtained using the Lee-White clotting time.

Lenahan *et al.* (1966) recently proposed that the activated partial thromboplastin time could monitor heparin therapy. Because sufficiently standardized and sensitive reagents are not available, this test cannot be adopted for routine clinical use (Harmeling and Cordray, 1967).

Schwab (1969) proposed a one-step thrombin clotting time method for assaying accurately heparin concentration in platelet-rich plasma. A relationship was found between the time it took the fibrinogen in the plasma to clot and the amount of thrombin added. This procedure could easily be adapted for a clinical laboratory to provide a useful guide for the management of heparin therapy. However, Rezansoff and Jaques (1967) point out that *in vitro* tests may fail to reflect the effect of heparin on coagulation *in vivo*.

MATERIALS AND METHODS

Source of Plasma

A 1.5 inch, 21-gauge needle was used to draw blood from human subjects. Four and one-half milliliters of blood were added to a tube containing 0.5 ml. of 0.1 M sodium oxalate. Platelet-rich plasma was obtained by centrifuging the whole blood for 20 minutes at 60 G.

Reagents Used in This Experiment

Thrombin. Topical Thrombin (bovine origin)* was used in all tests. Each vial contained 1000 N.I.H. units** of dried thrombin. A stock solution of thrombin was prepared using the method of Rappaport and Ames (1957): 5 ml. of oxalated saline (1 part 0.1 M sodium oxalate and 5 parts 0.85% sodium chloride) was added to the dried contents of one vial. The solution was then absorbed with 100 mg. of barium sulfate for 20 minutes at 37 C. After centrifuging at 750 G. for 10 minutes, the supernatant was transferred to a 10 ml. siliconized volumetric flask. To this solution, 2.5 ml. of glycerol was added. Physiologic saline was then used to dilute the mixture to 10 ml.

The stock thrombin solution (100 units/ml.) was stored at 4 C. for 1 day before it was used. This solution was stable for 1 week when stored

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*Topical Thrombin (Bovine Origin). - Parke, Davis & Company, Detroit, Michigan.

**National Institute of Health Units - the amount required to clot 1 ml. of standardized fibrinogen solution in 15 seconds.

at 4 C. and for 3 months if frozen in polyethylene tubes (12 x 75 mm.).

Heparin. A stock heparin solution containing 100 units (1 mg./ml.) was prepared from a heparin ammonium salt* solution of 1000 units by diluting with deionized water. This stock solution was stable for 1 week if stored at 4 C.

Thrombin Activity

Using a stock thrombin solution (100 units/ml.) a series of dilutions were prepared with cold physiologic saline (4 C.) to give the following concentrations: 20, 15, 10, 8, 6, 5, 4, and 2 units/ml.

Approximately 2 ml. of pooled platelet-rich plasma was incubated in polyethylene tubes (12 x 75 mm.) for 3 minutes in a 37 C. water bath. Next, 0.4 ml. of incubated plasma was placed in each of 4 prewarmed plastic reaction cups.** To each reaction cup, 0.1 ml. of the diluted thrombin was added and the clotting time*** in seconds was recorded. Each dilution was repeated 16 times and the average clotting time was calculated. A thrombin activity curve was constructed by the method of least squares.

Heparin Assays

The stock heparin solution (1 mg./ml.) was diluted with deionized water to make solutions of 0.01, 0.02, 0.03, 0.04, 0.05 mg./ml. To iced polyethylene tubes (12 x 75 mm.) 1.9 ml. of pooled platelet-rich plasma and 0.1 ml. of diluted heparin were added. The final concentration of heparin was then 0.5, 1.0, 1.5, 2.0, and 2.5 µg./ml. of plasma. Platelet-rich plasma (1.9 ml.) was placed in an iced polyethylene tube and used

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*Heparin (Ammonium Salt) - Biological Research, Inc., St. Louis, Mo.

**Fibrotube; BD, Division of BioQuest, Cockeysville, Md.

***Fibrometer System; BD, Division of BioQuest, Cockeysville, Md.

as a control. Each tube was kept in crushed ice until thrombin times were performed. Next, each tube was incubated 3 minutes in a 37 C. water bath. To 0.4 ml. of incubated control plasma or heparin-containing plasma, 0.1 ml. of thrombin (8 units) was added and the thrombin times recorded in seconds. This was repeated 16 times for each heparin dilution and the control.

Plasma Stability

Pooled platelet-rich plasma was stored at 4 C. in 12 air-tight polyethylene tubes (12 x 75 mm.). One tube was removed from storage every hour and 4 thrombin times were performed. This procedure was repeated 12 times and the average clotting time was calculated for each hour the plasma was stored at 4 C. The thrombin concentration was 8 units for this experiment.

Clinical Experiment

Blood of patients* receiving heparin therapy was used for this experiment. An oxalated sample of blood was obtained when a Lee-White clotting time was performed on these patients. Four thrombin times were determined for each recorded Lee-White clotting time. The average thrombin time was determined for each sample. The heparin concentration was calculated by the following formula:

$$\text{Heparin concentration } (\mu g./ml.) = 2.11 - \frac{19.7}{t}$$

(clotting time in seconds)

*Patients were from St. Luke's Hospital, Saginaw, Mich., St. Lawrence Hospital, Lansing, Mich., and Ingham Medical Hospital, Lansing, Mich.

This formula was determined from the results of the heparin assay experiment (page 12).

RESULTS

Thrombin Activity

The reciprocal of the thrombin units added was plotted against the time in seconds it took 0.4 ml. of platelet-rich plasma to clot, and a straight line was obtained. The equation of the line was determined by the method of least squares. The results of the first series of determinations are summarized in Figure 1.

Figure 2 is a thrombin activity curve constructed from the above results. The equation for this curve is $y = Kx$ in which y is the reciprocal of the thrombin units that was added and x is the observed clotting time. Using the data from the 16 experiments (8 dilutions of thrombin/experiment) K was calculated to be 0.0092. K represents the factor the clotting time can be multiplied by to obtain the reciprocal of the concentration of thrombin present. This was the procedure used for determining the residual activity of thrombin alone or in the presence of heparin.

Heparin Assays

For the next set of determinations 0.1 ml. of varying concentrations of heparin were added to 1.9 ml. of iced platelet-rich plasma and the thrombin times determined as before. A control was performed for each series of heparin dilutions and the concentration of thrombin (8 units) added, remained constant for these experiments. From the first series of determinations, one concludes that the clotting time multiplied by the thrombin concentration equals a constant ($t \times T = k$) where t is the clotting

time in seconds, T is the number of active thrombin units, and k is a constant). The k was calculated from the control experiments, and the number of active units of thrombin that remained after the addition of known amounts of heparin was determined ($T = \frac{k}{t}$).

The number of active units of thrombin was plotted against the concentration of the heparin added. Using the data from 16 separate experiments (3 concentrations of heparin/experiment) a straight line was obtained by the method of least squares. A summary of the results is represented by Figure 3.

If the number of active units of thrombin equals 8 (number of units of thrombin added) minus Kh (some constant times the heparin concentration added) then a formula for determining heparin concentration can be written as $\frac{8}{K} - \frac{k}{Kt} = h$. This formula can be rewritten as $y = B - Ax$ (where y is the heparin concentration and x is the reciprocal of the clotting time). The values for A and B were calculated by the method of least squares (A = 19.7, B = 2.11).

When 0.1 ml. of thrombin (8 units) is added to 0.4 ml. oxalated platelet-rich plasma, the heparin concentration can be calculated by the following equation: Heparin concentration = $2.11 - \frac{19.7}{t}$

(μg./ml.) (clotting time in seconds)

This method has a variance of 0.0159 with 95% confidence limits of $\pm$ 0.256 μg./ml.

Plasma Stability

The effect of storage of platelet-rich plasma was studied. Thrombin times were not prolonged when stored 12 hours at 4 C. in air-tight polyethylene tubes. The slight variation that occurred was the same as

exhibited by fresh platelet-rich plasma. For 48 determinations performed, the average thrombin clotting time was 10.5 seconds with a standard deviation of 0.77.

Clinical Experiment

The purpose of the following studies was to evaluate the clinical application of this assay method. The calculated heparin concentration in 18 patients was compared to their Lee-White clotting times. Their heparin concentrations were plotted against their Lee-White clotting times. A straight line was obtained by the method of least squares. The linear relationship that exists between the calculated concentration and the Lee-White clotting times is summarized in Figure 4.

If the Lee-White clotting times are compared to the calculated heparin concentration, there is also a linear relationship. This is shown in Figure 5.

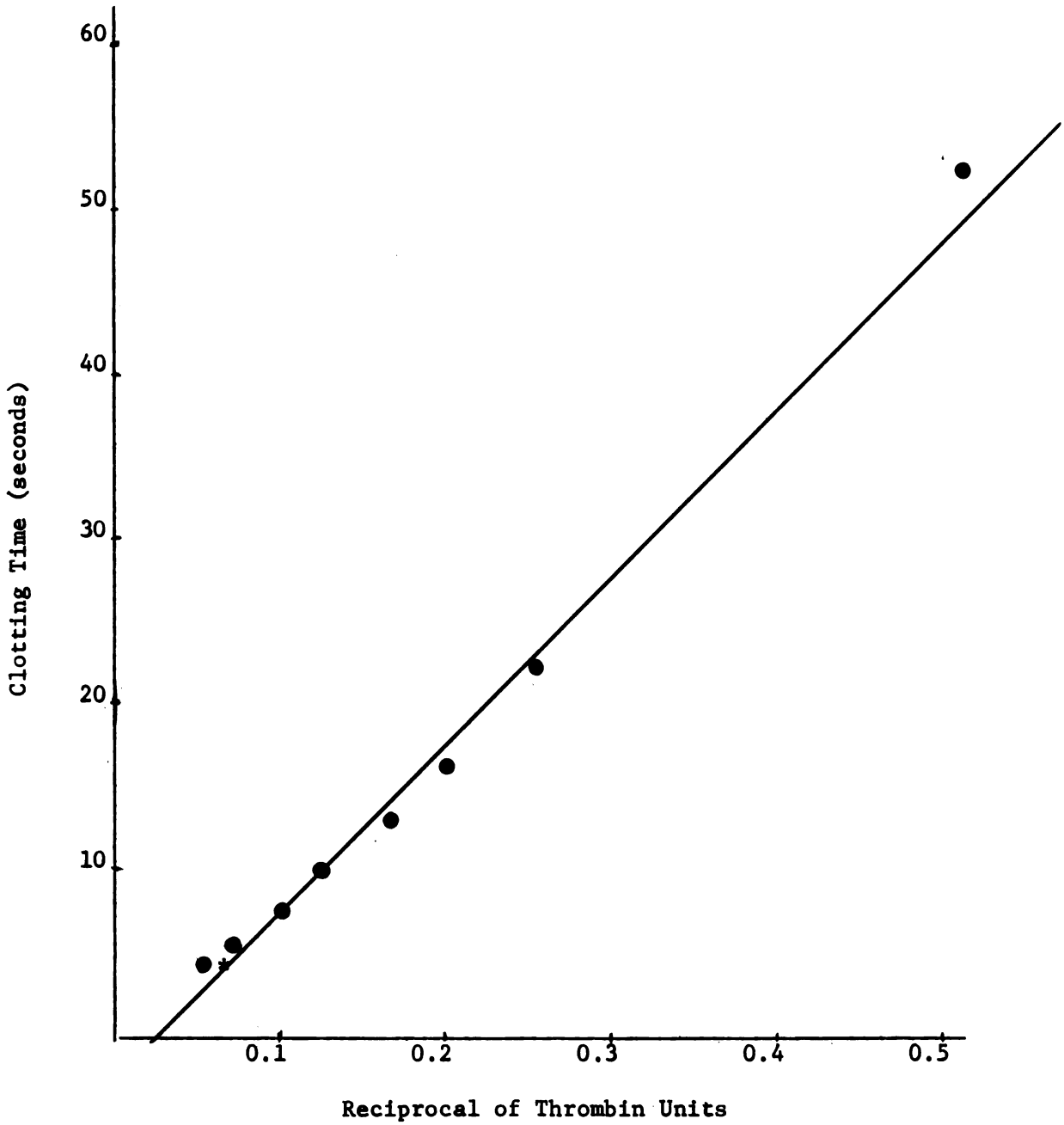

Figure 1. Thrombin dilution curve (summary of data).

*Each point represents the average of 16 different determinations. Ninety-five percent confidence limits ± 5.5 seconds.

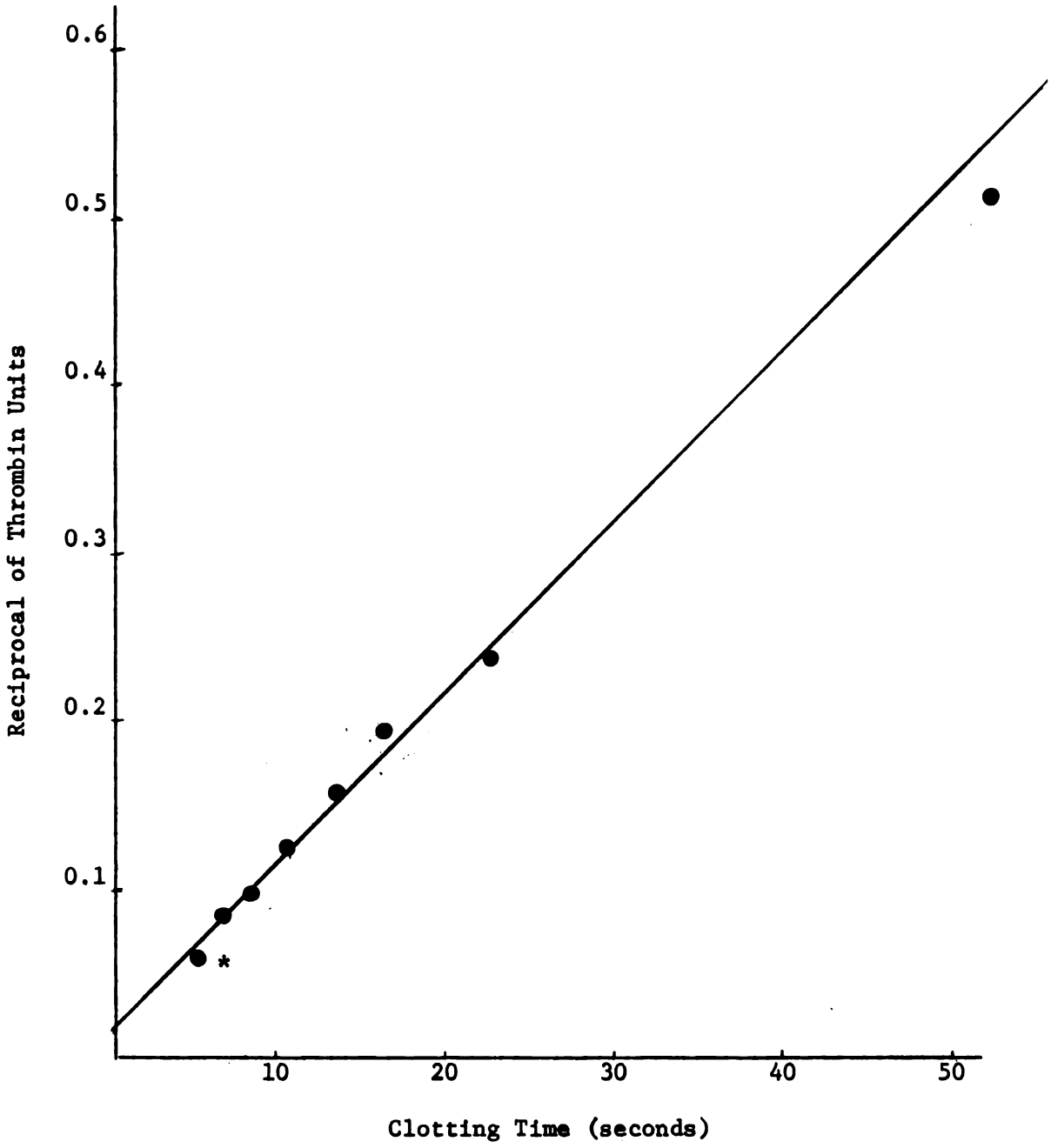

Figure 2. Thrombin activity curve.

*Each point represents the average of 16 different determinations. Ninety-five percent confidence limits ± 0.054 reciprocal units of thrombin.

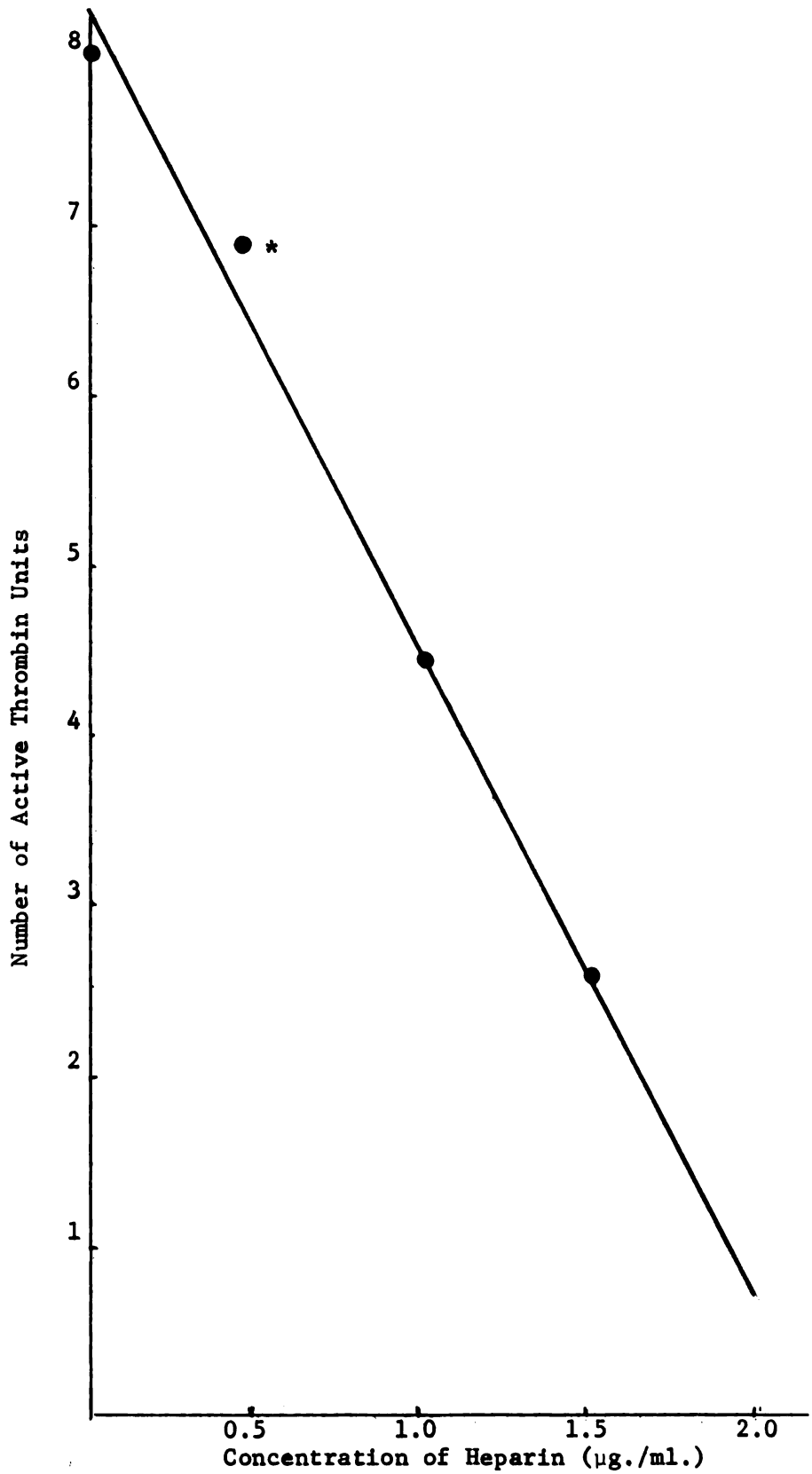

Figure 3. The number of active thrombin units that remained after the addition of various concentrations of heparin.

*Each point represents the average of 16 different determinations. Ninety-five percent confidence limits ± 1.4 thrombin units.

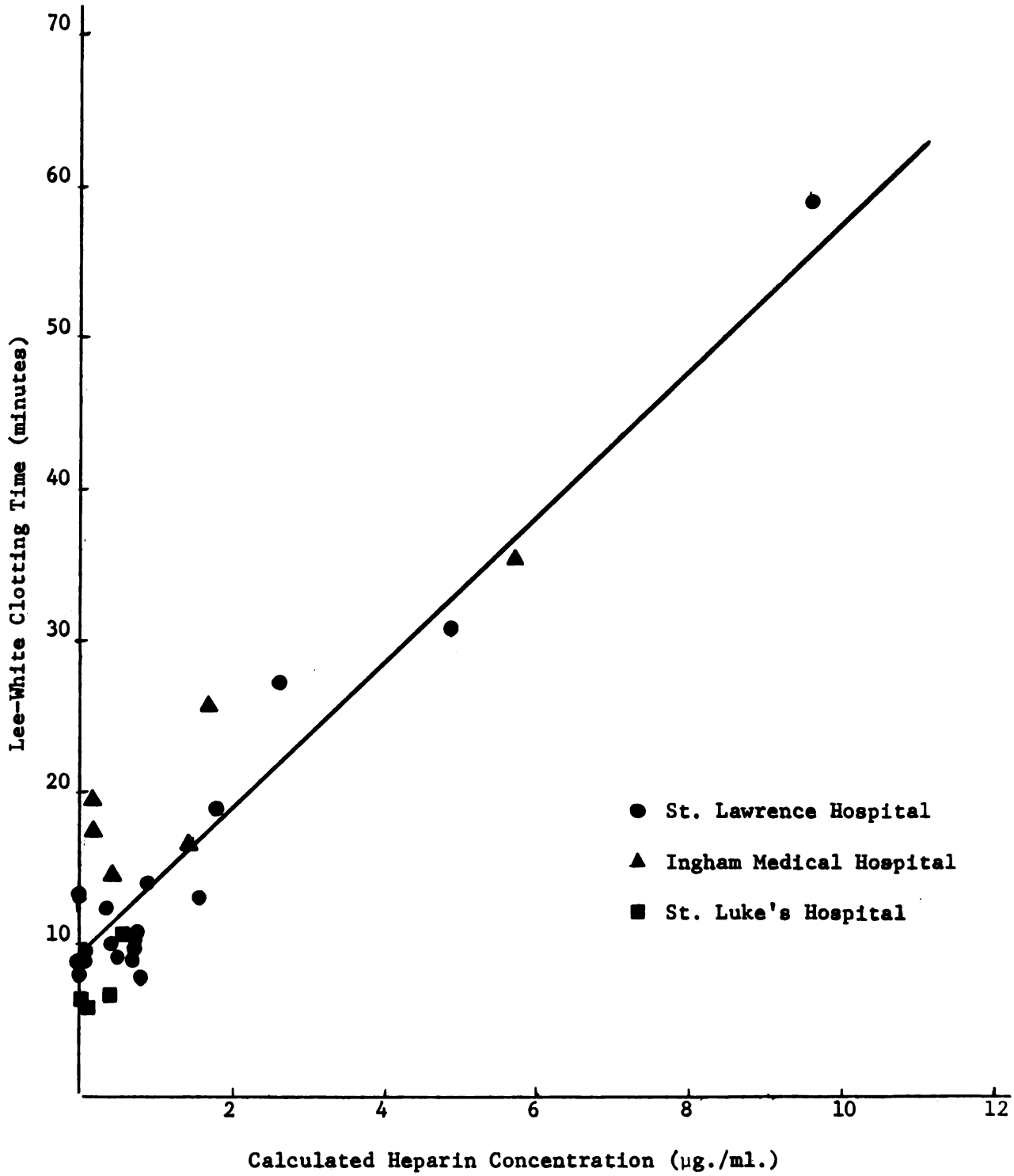

Figure 4. Comparison of calculated heparin concentration to the Lee-White clotting time.

Ninety-five percent confidence limits ± 8.4 minutes.

Rank correlation coefficient 0.703.

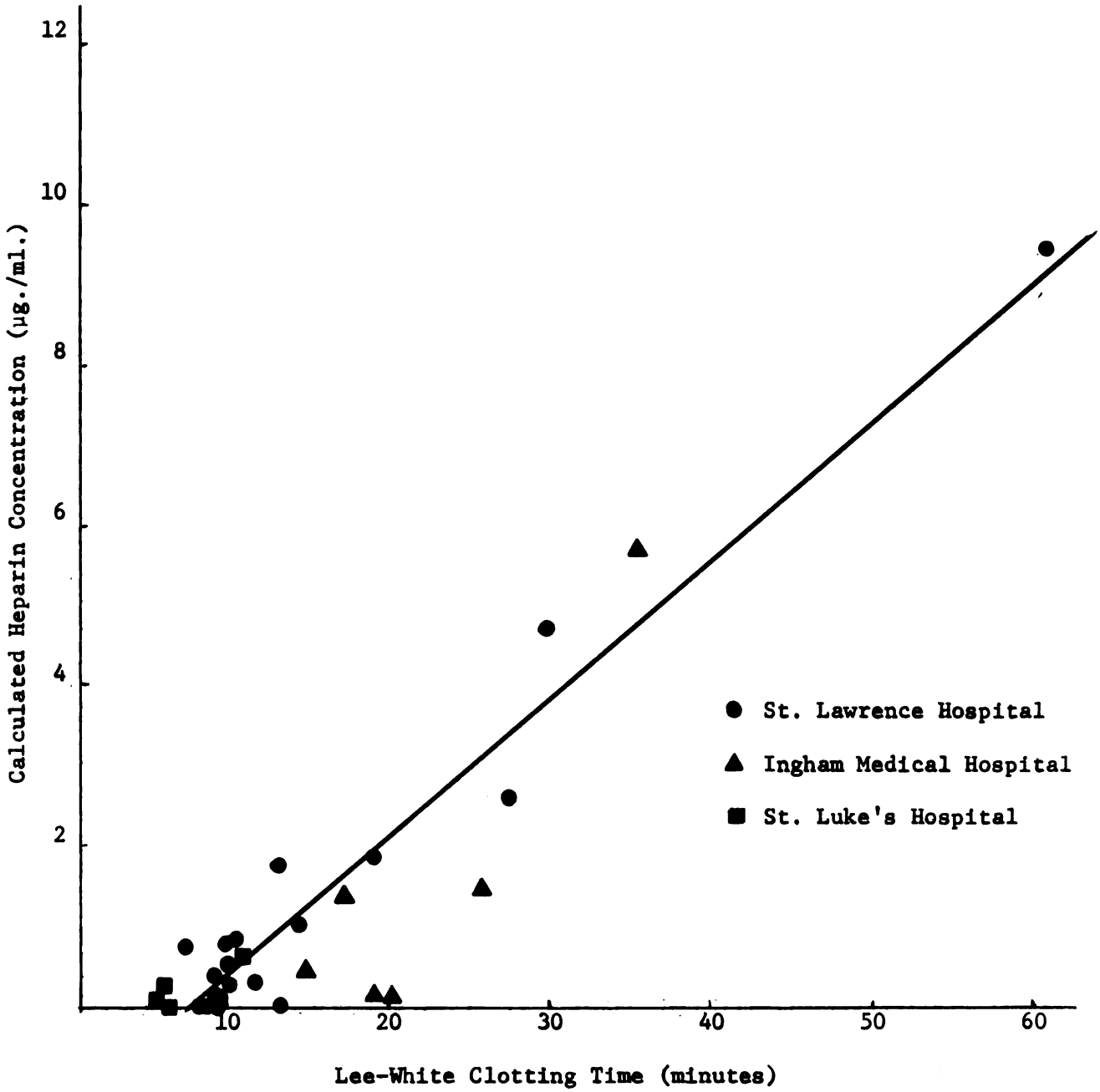

Figure 5. Comparison of Lee-White clotting time to calculated heparin concentration.

Ninety-five percent confidence limits ± 1.59 µg./ml.

DISCUSSION

The replacement of the Lee-White clotting time by some other method has been reported by many researchers. Due to the disadvantages and inadequacies of the whole blood clotting time, a substitute procedure that rapidly and accurately assays heparin in plasma was sought.

Coagulation is a complicated process that depends on the presence of many substrates, enzymes, and cofactors. The visible or last step requires fibrinogen, thrombin, and plasma factors to result in the formation of a fibrin clot. The *in vivo* formation of thrombin requires many steps. If thrombin is added to plasma, all these steps can be bypassed.

Heparin acts at several stages of the coagulation system, but one of the more effective sites is its inhibition of the action of thrombin on fibrinogen. By relating the time required for clot formation to the reciprocal of thrombin concentration, it became possible to measure thrombin activity. Then by adding a known amount of thrombin to an unknown amount of heparin, it should be possible to estimate the concentration of heparin. Fortunately, simple linear relationships exist between: (1) the clotting time of fibrinogen and the reciprocal of the thrombin concentration, and (2) the number of micrograms of heparin present and the number of units of thrombin inactivated. These relationships are summarized in Figures 1, 2, and 3. In concentrations of heparin above 1.5 µg./ml. the relationship no longer holds true. When the concentration is over this value, the original sample should be diluted with normal plasma, and a thrombin time repeated.

After determining the activity of thrombin, the effect of heparin on this enzyme can be summarized by the equation $y = B - Ax$. By using the value of the 2 constants and the thrombin clotting time, the heparin concentration can be calculated. This method has a variance of 0.0159; therefore, 95% of the time the calculated heparin concentration will be $\pm$ 0.256 µg./ml. from the actual concentration in the plasma.

Schwab in 1969 reported that platelet-rich plasma was needed for the thrombin inhibition test. This assured that a sufficient amount of fibrinogen and heparin cofactor were present. It was stated early that heparin is not a strong antithrombin unless a cofactor is present. According to the literature, the fibrinogen level rarely decreases in concentrations low enough to interfere with coagulation. In several trial tests, a platelet factor* was added to platelet-poor plasma in an attempt to reproduce the results obtained by platelet-rich plasma. The platelet substance failed to reproduce the same effect as platelet-rich plasma in assaying thrombin activity.

The data in Figure 4 suggests a linear relationship between the calculated heparin concentration and the Lee-White clotting time. The precision and accuracy of the heparin assay, using a thrombin inhibition test, is greater. This test is relatively simple and requires only a few minutes to perform.

A similar linear relationship exists between the Lee-White clotting time and the calculated heparin concentration. Figure 5 summarizes this relationship. This would provide one with a method of estimating heparin concentration from published Lee-White clotting times. If the Lee-White clotting method is used to determine heparin concentration, there is a variance of 0.222.

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*Platelin; Warner-Chilcott Company, Morris Plains, N.J.

The time-saving element of this method makes it an improvement over the time-consuming Lee-White test. The potential sources of error lie in the preparation of the plasma samples and the dilution of the thrombin. A centrifuge with predetermined settings for platelet-rich plasma will eliminate plasma inaccuracy. Stability of the dilute thrombin can be insured by making the dilution in polyethylene tubes and using the resulting mixture within 20 minutes.

With a method available that assays the concentration of heparin in blood, a dosage schedule may be determined. This could be predicted on the bases of kinetic studies of the rate of heparin clearance from the circulatory system for each individual patient. Research is needed in this area to prove the validity of this hypothesis.

SUMMARY AND CONCLUSION

A major disadvantage in the use of heparin therapy is the lack of a convenient and accurate system of controlling dosage.

The purpose of this project was to continue the development of a method for the assay of heparin in blood that would provide a useful guide for the management of heparin therapy.

A linear relationship was found to exist between the clotting time of a fibrinogen solution and the reciprocal of thrombin concentration. There is also a linear relationship that exists between the number of micrograms of heparin present and the number of inactivated thrombin units. From these results, the following formula was derived to calculate the concentration of heparin in plasma:

$$\underset{(\mu g./ml.)}{\text{Heparin concentration}} = 2.11 - \frac{19.7}{\underset{\text{in seconds)}}{\underset{\text{(clotting time}}{t}}}$$

To evaluate the clinical application of this assay method, the measured heparin concentration was compared to the Lee-White clotting time. A linear relationship exists between these 2 methods.

There is a variance of 0.0159 if the heparin concentration is determined by using the thrombin inhibition test. However, if the Lee-White clotting time is used to predict the heparin concentration there is a variance of 0.222.

The thrombin inhibition test is a one-step procedure requiring a relatively small amount of time to perform. The precision and accuracy appears to be higher than the Lee-White clotting test for monitoring heparin therapy.

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VITA

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