HEPARIN ASSAY USING THROMBIN INHIBITION

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ABSTRACT

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by Mary Lynne Schwab

One of the disadvantages so far associated with heparin therapy has been the lack of a convenient and satisfactory control procedure.

The relation of thrombin clotting times and the ability of heparin to inhibit thrombin activity was studied.

In the first series a relationship was found between the time it took to clot fibrinogen in the plasma and the amount of thrombin present. The second series showed that the units of thrombin inactivated depended upon the concentration of heparin. From this information 2 factors were calculated (A = 3.45 and B = 34.13). These 2 factors plus the clotting time of 0.4 ml. plasma to which 0.1 ml. thrombin (10 units/ml.) has been added can be used to calculate the concentration of heparin in the plasma. This is expressed in the equation:

$$\text{Heparin conc. } (\mu g./ml.) = 3.45 - \frac{34.13}{\text{clotting time (seconds)}}$$

The proposed method is a one-step thrombin clotting time procedure that can be done in a relatively short period of time. The standard deviation of the heparin concentration from the mean is .007 µg./ml. Therefore, the accuracy of the method is quite good in that 95% of the time the calculated concentration will be ±0.014 µg./ml. from the actual concentration.

HEPARIN ASSAY USING THROMBIN INHIBITION

By

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For

My Parents and Dinesh

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INTRODUCTION

With the increasing use of heparin therapy the need for a reliable heparin assay test has become important. Because of heparin's fast anti-coagulant activity and its rapid removal from the circulating blood, the test employed in following its effect should be relatively quick and easy.

At the present time the Lee-White Clotting Time, which measures the time for a known quantity of whole blood to clot, is widely used for following the anticoagulant effect of heparin *in vivo*. This test is tedious with many variables which, if not closely controlled, can lead to erroneous results.

The purpose of this project was to develop a method for the assay of heparin in blood that could easily be adopted in any clinical laboratory to provide a useful guide for the management of heparin therapy.

REVIEW OF LITERATURE

Blood Clotting Mechanism

Even though the fact that blood coagulates is probably the best known property of blood, the precise mechanism of clotting is still an object of controversy. The physical and chemical changes responsible for turning a solution of blood to a gel have been studied for centuries. Since 1666, when Malpighi first considered the problem, a large mass of experimental data has been collected giving rise to numerous theories on the mechanism of blood coagulation. Howell in 1935 recognized that most of these theories crossed and overlapped each other.

Finally, after years of confusion, a pattern does seem to be emerging. Macfarlane (1964) proposed the idea that all the reactions involved in blood coagulation are enzymatic. Clotting is first initiated by contact of the blood with a foreign material. Then a cascade of proenzyme-enzyme transformations takes place with each enzyme activating the next until the final substrate, fibrinogen, is reached. At present, the most acceptable schemes for the coagulation of blood are based on this enzyme cascade hypothesis.

Although the proposed scheme of Macfarlane simplifies the overall reaction of blood clotting considerably, each step taken individually still presents a set of intricate problems. The visible or last step of the clotting reaction alone involves thrombin, fibrinogen and fibrin along with the factors in the plasma which

affect the reactions of these molecules.

Thrombin

Howell reviewing the literature in 1935 found many accepted theories in which thrombin played no part in the conversion of fibrinogen to fibrin. Howell reported that thrombin was present but he was not sure of its exact mode of action.

To answer the numerous questions involving the chemistry of coagulation, it was of fundamental importance to isolate and purify the various proteins involved without altering their inherent properties. The first in a series of experiments on the purification of prothrombin was published in 1938 by Seegers, Brinkhous, Smith and Warner. In 1942 Seegers and McGinty described a purified thrombin prepared from their prothrombin preparations by the addition of calcium and thromboplastin.

Using ultracentrifugation and electrophoresis, Lamy and Waugh (1954) concluded that the formation of thrombin from prothrombin was complicated to the extent that no definite scheme could be proposed.

Seegers (1954) stated that a combination forms between thromboplastin, calcium and prothrombin conversion factors which in turn has the ability to convert prothrombin to thrombin. This occurs any time thromboplastin is liberated into the plasma.

Several methods for the dissociation of prothrombin into fragments and for their reassociation have yielded a whole series of thrombin compounds. Some of these compounds have only minimum properties of thrombin. For research involving clotting, Lamy and Waugh (1954) suggested that only thrombin, which has been obtained

under near normal physiological conditions of pH and ion strength through the use of thromboplastin, calcium, accelerator globulin and other trace materials, be employed.

In their work Therriault, Gray and Jensen (1957) found that in the presence of traces of thrombin the conversion of prothrombin became greatly accelerated. It appears that thrombin speeds up its own formation from prothrombin but the specific manner of this action of thrombin is not completely resolved.

The action of thrombin as an enzyme in coagulating figrinogen is well recognized (Laki, 1953; Sherry, Troll and Glueck, 1954).

The role of thrombin is viewed as a splitting off of peptides from the fibrinogen molecule to make the fibrin clot (Laki, 1953). In 1964 Laki and Gladner showed that this protein enzyme with esterase activity had a specificity for certain peptide bonds formed between arginine and glycine residues. They estimated the molecular weight of thrombin to be 8000.

Besides acting on fibrinogen thrombin has another important function. It is needed for release of a contractile protein from platelets for clot retraction to occur (Therriault et al., 1957).

Fibrinogen

Fibrinogen is a protein with a molecular weight of 330,000 and is present in the plasma in quantities of 100 to 800 mg./100 ml. (Laki and Gladner, 1964). Its purification is simplified because of its high concentration and unusual solubility. However, these advantages are somewhat outweighed because fibrinogen is extremely labile and is easily denatured (Jaques, 1943; Ware and Lanchantin,1954).

Fibrinogen is the primary substrate for thrombin. Lorand in 1954 viewed the fibrinogen-thrombin reaction as a striking phenomena with the net result being the formation of a gel from a solution of fibrinogen merely by the addition of trace amounts of thrombin. Laki (1953) stated that almost any amount of thrombin no matter how minute could clot any amount of fibrinogen.

Ferry in 1954 reported that the first step in the conversion of fibrinogen appeared to be a proteolytic one with the removal of peptide fragments from the fibrinogen. The activated fibrinogen then underwent a spontaneous but reversible polymerization resulting in the formation of fibrin. The previous works of Lorand and Middlebrook (1952) and Bettleheim and Bailey (1952) were in accord with this view.

The current concept of the conversion of fibrinogen to fibrin allows for three steps. In the first or enzymatic phase thrombin splits 4 peptide bonds from the fibrin molecule yielding fibrin peptides and fibrin monomer. The second phase deals with the formation of intermediate polymers from the fibrin monomers. These intermediate polymers may grow to a length of 10,000 Å before they aggregate to form the fibrin clot which is the third and last phase of clotting (Ellias and Iyer, 1967).

In 6 species of fibrinogen examined, Laki and Gladner (1964) found that the 4 peptide bonds, hydrolyzed by thrombin in its action on fibrinogen, were between arginine and glycine residues. Because of these bonds thrombin is highly specialized for fibrinogen. The peptides are by-products, since once they are liberated, they are

not needed for the polymerization that follows (Lorand, 1954). However, these peptides may sensitize smooth muscles of the capillaries to chemical or other stimulation. Certain cases of hypertension have shown a drop in blood pressure when clotting was inhibited by the infusion of heparin (Laki and Gladner, 1964).

Synthesis of fibrinogen occurs in the liver, and the rate of formation is adjusted to the fibrinogen level in the plasma. If the level becomes too low the rate of formation may increase 6 to 8 fold (Guyton, 1961). Ordinarily, the concentration of fibrinogen in the plasma remains fairly constant. It may drop, however, during pregnancy, after extensive muscular exercise, exposure to sunshine, or in cases of severe liver damage (Hodgkinson, 1958); but only rarely, even in pathological conditions, does it fall low enough to interfere with normal coagulation (Guyton, 1961).

Heparin

Guyton (1961) reports that over 30 different substances affecting coagulation have been found in the blood and related tissues. Those that promote coagulation are called procoagulants; those inhibiting clotting are called anticoagulants. Three anticoagulants of considerable importance are antithromboplastin, antithrombin and heparin.

In 1916 McLean, trying to purify cephalin, discovered a powerful anticoagulant which he called heparphosphatid. In 1918 Holt and Howell changed the name to heparin after extracting this anticoagulant from a dog's liver. Heparin probably can be produced

by many cells of the human body but especially large quantities are present in the granules of mast cells (Wintrobe, 1967). Eiber and Daneshefsky (1957) reported that the highest concentrations of heparin were found in the liver and lungs with only minute amounts in the blood. They estimated the concentration of heparin in the circulating blood to be 0.5 mg./100 ml.

Lenahan, Frye and Phillips (1966) described heparin as a naturally occurring mucopolysaccharide with the power to decrease rapidly the activity of the coagulation system.

Since McLean first discovered heparin, controversy over its mode of action has been lively. Because heparin does not inhibit the reaction between purified thrombin and purified fibrinogen, Howell (1935) believed heparin to be an antiprothrombin. Quick in 1937 showed that heparin did not influence the conversion of prothrombin to thrombin. He called heparin an antithrombogen; by this he meant an agent which reacts with a constituent in the plasma to form a true antithrombin. In the experiments of Monkhouse and Clarke (1957) heparin did not significantly affect the thrombin clotting time unless a plasma component, heparin cofactor, was present.

Henstell and Kligerman(1967) demonstrated that antithrombin in the plasma was bound to an inhibitor and found very little free in the plasma. Since heparin increased antithrombin activity, they proposed that heparin disrupts a bond between antithrombin and inhibitor of antithrombin. This free antithrombin may then react with any thrombin present.

Using antithrombin prepared from ox plasma, Astrup and Darling (1942) obtained a straight line when they plotted the amount of antithrombin added against the percent of thrombin inactivated. However, applying this same principle Astrup and Darling (1943) could not satisfactorily duplicate their results with the antithrombin formed from heparin. They then plotted units of thrombin inhibited against mg./ml. of heparin added to blood in vitro and obtained a curve for a dissociable compound in which dissociation was forced in one direction by the addition of one of the components. They concluded that the normal antithrombin in the plasma is not identical with the thrombin inhibitor which is released after addition of heparin.

Seegers in 1954 recognized 4 categories of antithrombin: Antithrombin I-concerned with the adsorption of thrombin on fibrin; Antithrombin II-involved heparin and heparin cofactor; Antithrombin III-acted directly with thrombin to inhibit its activity; Antithrombin IV-inhibited activation of prothrombin.

Heparin Assay

Murray in 1937 was the first to use heparin as an in vivo anticoagulant. Because of its relative non-toxicity and rapidity of action, heparin is now widely used clinically in the control of thromboembolic disorders. Recommended dosage for intravenous injections of heparin range from 200 mg. every 12 hours (Miale, 1962) to 40,000 units per day (Stamm, 1963). Both these authors strongly pointed out the need for periodic determinations of clotting time in heparin therapy.

So far the Lee-White clotting time has been universally used for control of heparin therapy (Rezansoff and Jaques, 1967). Schatz and Hathaway in 1963 suggested the thrombin time as an adequate criterion for controlling the administration of heparin. They recommended the use of platelet-rich plasma which is less sensitive to small amount of heparin and thus a better indicator of therapeutic prolongation of coagulation.

Recently the partial thromboplastin time has been proposed by Lenahan et al. (1966) for monitoring heparin therapy. They mentioned, however, that if the partial thromboplastin time was going to be adopted for routine clinical use the technologist must take extra precautions to control the various reagents involved. They thought the lack of sensitivity of the partial thromboplastin time reported previously for heparin was due to the oxalated blood and could be corrected by using citrated specimens.

Jaques and Charles in 1941 described several methods for the assay of heparin using chemical, biochemical or biological tests. They classified the various tests into 2 categories, clotting time methods and titration methods. The clotting time methods recorded the time for a given amount of unknown to clot. The titration methods fixed the clotting time and found the amount of unknown necessary for this time.

Blombäck, Blombäck, Cornelius and Jorpes (1953) investigated the reliability of the various methods used in heparin assays. They found that the thrombin clotting time method gave the best results with very little deviation from day to day or with different standard preparations. No such agreements were obtained with the other methods.

Rezansoff and Jaques (1967) pointed out that in vitro tests may fail to reflect the effect on coagulation in vivo. Jaques and Bell (1959) thought that methods in which heparin was added to freshly drawn blood were preferable as being closer to the parameter measured by the clinician using heparin. Jaques and Richer (1948) found that the clotting times of samples incubated with heparin for 30 minutes were much longer than the times from freshly prepared samples. They discovered that the anticoagulant activity of heparin was at a minimum at 25 C. and a maximum at 37 C. and that coating the glassware with silicone gave a surface which was almost inert to the clotting system.

Jaques and Richer (1948) studied the relationship of clotting time to heparin dosage in the dog. They obtained a straight line using heparin dosage plotted against the logarithm of the clotting time.

MATERIALS AND METHODS

Source of Plasma

Venipunctures were performed on humans with a 1.5 inch 20-gauge needle. Sodium oxalate (M/10)* was used as the anticoagulant (0.5 ml./ml. of blood). To obtain platelet-rich plasma the whole blood was then spun in a centrifuge for 20 minutes at 60 G. Freshly drawn plasma was used in all the experiments.

Chemicals Used in the Tests

Thrombin. Thrombin, Topical (bovine origin)** was used in all the tests. Each bottle contained 1000 N.I.H. units† of dried thrombin produced through a conversion reaction with prothrombin and thrombokinase in the presence of calcium chloride.

A stock thrombin solution, according to the methods of Rapport and Ames (1957), was prepared as follows: 5 ml. of oxalated saline (1 part 0.1 M oxalate and 5 parts 0.85% sodium chloride) were added to the contents of one bottle of Thrombin, Topical. The solution

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*Sodium oxalate - 1.34 gm. of sodium oxalate (F.W. 134.01) dissolved in 100 ml. of distilled water.

**Thrombin, Topical (Bovine Origin) - Parke Davis and Co., Detroit, Michigan

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

was then adsorbed with 100 mg. of barium sulfate for 20 minutes at 37 C. After centrifuging, the supernatant was transferred to a 10-ml. volumetric flask. To this 2.5 ml. of glycerol were added and the volume diluted to the mark with physiological saline. The solution was stable for about 2 weeks at 4 C.

Heparin. A stock heparin solution containing 1 mg./ml. (100 units) was prepared from a heparin (ammonium salt)* solution of 1000 mg./ml. and stored at 4 C.

Thrombin Times

From the stock thrombin solution (100 units/ml.) serial dilutions of 20, 15, 10, 8, 5, 4 and 2 units/ml. were prepared with ice-cold (4 C.) physiological saline. These solutions were prepared fresh for each experiment. A 0.4 ml. sample of platelet-rich plasma was placed in a tube (10x75 mm.) and incubated for 3 minutes in a 37 C. water bath. Then 0.1 ml. of diluted thrombin was added to the preincubated plasma and the clotting time in seconds noted. Each dilution was run in duplicate and the average taken.

Heparin Assays

The stock solution of heparin (1 mg./ml.) was further diluted with distilled water to make solutions of 0.01, 0.02, 0.03 and 0.04 mg./ml. To 5 siliconized iced tubes, 2 ml. of platelet-rich plasma were added. Then 0.1 ml. of the diluted heparin was added to make final concentrations of 0.5, 1.0, 1.5 and 2.0 µg./ml. of plasma.

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

A 0.4 ml. aliquot of the plasma containing heparin was placed in a 10 x 75 mm. tube and incubated for 3 minutes at 37 C. in a water bath. Next 0.1 ml. of thrombin (10 units/ml.) was added and the clotting time recorded. Duplicate clotting times were run on each sample of plasma containing the various concentrations of heparin and the average taken.

RESULTS

Thrombin Dilution Curves

In the first series of determinations the time in seconds for 0.4 ml. of platelet-rich oxalated plasma to clot was plotted against the reciprocal of thrombin units added. Equation of the line was obtained by the method of least squares. A summary of the results is represented in Figure 1.

The equation used for the thrombin curves is $y = Kx$ in which y is the reciprocal of the number of thrombin units added and x the observed clotting time. Using the data from all 16 experiments (7 dilutions of thrombin/experiment) K was found to be 0.0101. K represents a factor by which the clotting time can be multiplied to obtain the reciprocal of the number of thrombin units present.

Heparin Assays

For the second series 0.1 ml. of thrombin (10 units/ml.) was added to 0.4 ml. of platelet-rich oxalated plasma containing the various concentrations of heparin and the clotting time recorded.

Using the factor (K) from the first series the clotting times were multiplied by 0.0101. This gave the reciprocal of the number of thrombin units not inhibited by heparin. Dividing the reciprocal into 1 the value for the active thrombin units was obtained. By subtracting this number from 10 (thrombin units added) the thrombin

units inactivated or inhibited by heparin were found.

The units of inactivated thrombin were plotted against the heparin concentrations. Using the data from 16 separate experiments (4 concentrations of heparin/experiment), a straight line was obtained with the formula $y = k\,x$ in which y = inactivated thrombin units and x the concentration of heparin in μg./ml. (Figure 2).

Including all the data from the 16 experiments k was found to be 2.897.

Combined Information from Both Series

The results of the thrombin dilution curves and the heparin assay curves were combined into the equation $10 - \frac{1}{Kt} = kx$. In the preceding equation t = the clotting time and x = the heparin concentration. The whole formula can be rearranged to $x = \frac{10}{k} - \frac{1}{(kK)t}$ or rewritten as $x = A - \frac{B}{t}$.

Taking K = 0.0101 and k = 2.897 and substituting these in the equation gives A = 3.45 and B = 34.13.

Hypothesis

The last set of calculations test the hypothesis that when 0.1 ml. of thrombin (10 units/ml.) is added to 0.4 ml. oxalated plasma the heparin concentration can be calculated. The equation is

$$\text{Heparin concentration } (\mu\text{g./ml.}) = 3.45 - \frac{34.13}{t}$$

(clotting time in seconds)

Table 1 compares the calculated values for the concentration of heparin in the plasma with the actual amounts of heparin added (μg./ml.). The standard deviation of the calculated values from the actual amounts was .007 μg./ml.

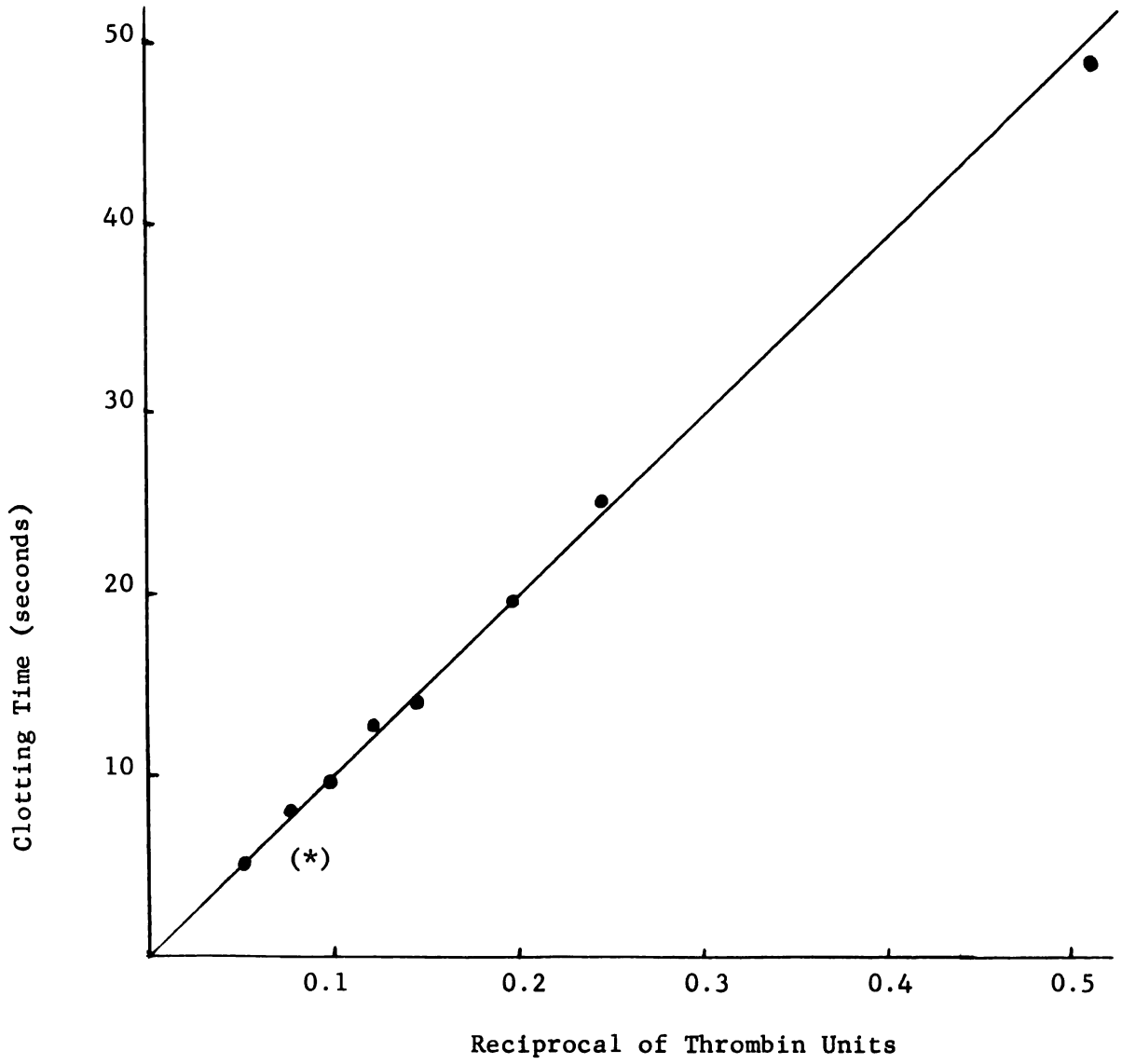

Figure 1. Thrombin Dilution Curve (summary of data)

* Each point represents the average of 16 different determinations

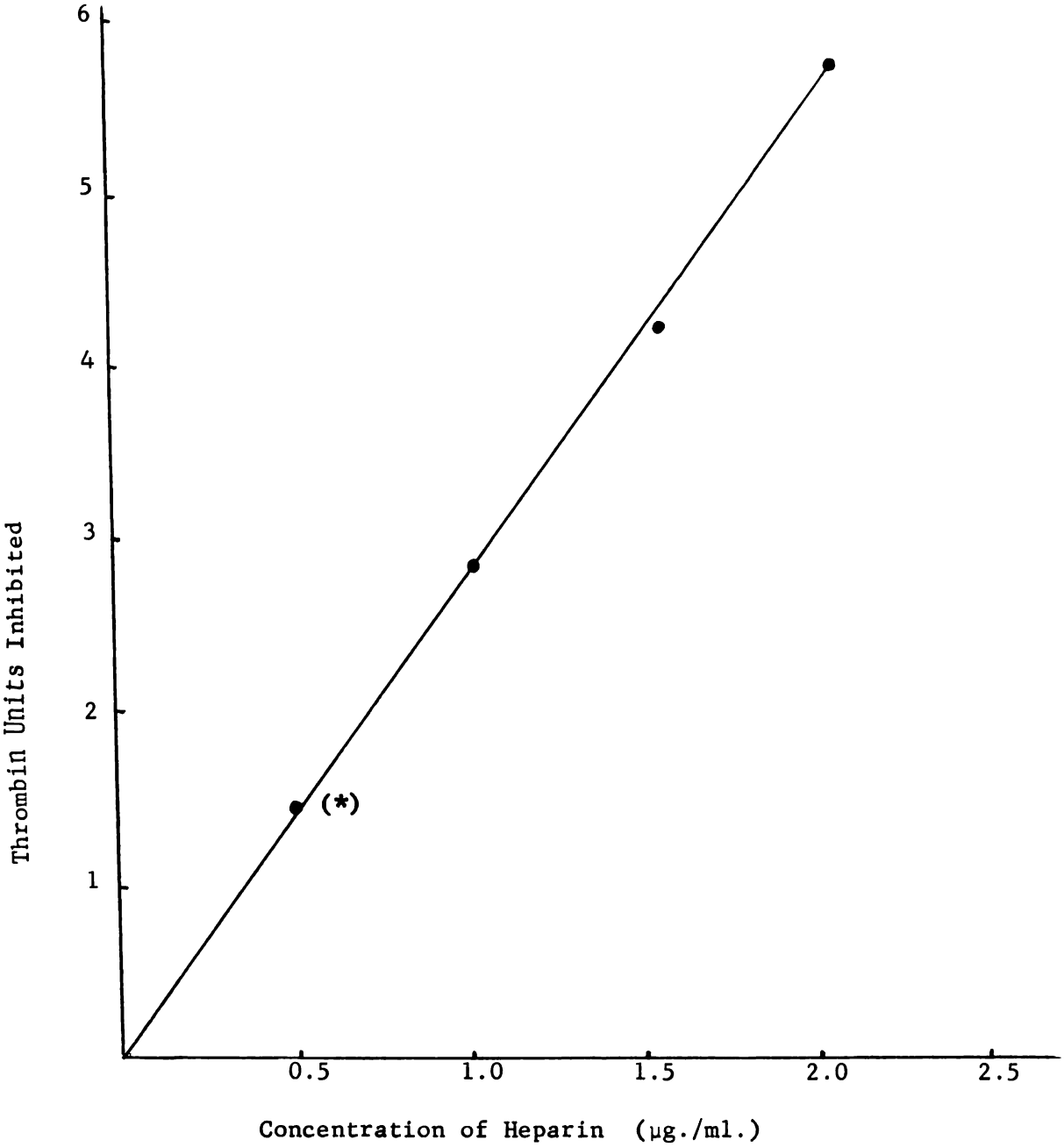

Figure 2. Units of Thrombin Inactivated by Various Concentrations of Heparin

* Each point represents the average of 16 different determinations

Table 1. Comparison of the calculated value for the amount of heparin present with the actual amount of heparin added

	(x (a))*	(x (c))**	(x (a))	(x (c))	(x (a))	(x (c))	(x (a))	(x (c))
1.	0.5	0.43	1.0	1.01	1.5	1.55	2.0	1.98
2.	0.5	0.41	1.0	1.01	1.5	1.65	2.0	2.37
3.	0.5	0.46	1.0	0.98	1.5	1.48	2.0	1.84
4.	0.5	.29	1.0	0.96	1.5	1.45	2.0	1.84
5.	0.5	.48	1.0	0.92	1.5	1.61	2.0	1.98
6.	0.5	.58	1.0	.89	1.5	1.36	2.0	1.86
7.	0.5	.46	1.0	1.05	1.5	1.55	2.0	2.02
8.	0.5	.48	1.0	.99	1.5	1.50	2.0	1.98
9.	0.5	.46	1.0	.997	1.5	1.62	2.0	2.00
10.	0.5	.48	1.0	.997	1.5	1.48	2.0	1.96
11.	0.5	.46	1.0	1.01	1.5	1.46	2.0	2.01
12.	0.5	.54	1.0	.91	1.5	1.41	2.0	1.97
13.	0.5	.54	1.0	1.01	1.5	1.58	2.0	1.99
14.	0.5	.48	1.0	1.08	1.5	1.56	2.0	2.02
15.	0.5	.38	1.0	.997	1.5	1.50	2.0	2.01
16.	0.5	.54	1.0	1.01	1.5	1.55	2.0	1.97

S.D. = 0.007 µg./ml.

* x (a) - µg.ml. of heparin added

** x (c) - µg./ml. of heparin calculated from the equation $x = A - \frac{B}{t}$

DISCUSSION

With the increasing use of heparin the need for an accurate and rapid heparin assay method has gained in importance. At the present time the Lee-White, which is a whole blood coagulation time test, is the standard procedure for monitoring heparin therapy. Due to its disadvantages and inadequacies a substitute procedure was sought.

Heparin acting as an anticoagulant blocks the formation of fibrinogen by inhibiting the enzyme action of thrombin. With this in mind, thrombin curves were run to see if there was a correlation between the amount of thrombin present and the clotting time of fibrinogen. The results indicate that there is a definite relationship. Figure 1 shows that a factor obtained from the curve may be multiplied by the observed clotting time to give the amount of active thrombin present.

Figure 2 shows that there is also a relation between the amount of inactivated thrombin and the concentration of heparin. In higher dilutions than 2 μg./ml. the relationship no longer holds true. This may account for the results of Astrup and Darling (1943) in which they obtained a curve by graphing inactivated thrombin units against concentration of heparin. If a concentration of over 2.5 μg./ml. is found a dilution of the original sample of plasma should be made and run again.

Combining the thrombin dilution curve and the heparin assay curve 2 constants were found. Using these constants and the thrombin

clotting time the concentration of heparin in the plasma can be calculated. The validity of this last statement is confirmed in Table 1. Platelet-rich plasma was used to more nearly simulate the effect of heparin *in vivo*. This also assured that sufficient amounts of heparin cofactor and fibrinogen were present. Heparin as stated earlier will not inhibit coagulation unless a cofactor is present. Also, as previously mentioned, the fibrinogen level rarely falls low enough to interfere with coagulation.

The platelet-rich plasma appears to be less sensitive to the action of heparin. In several trial runs, using platelet-rich and platelet-poor plasma, the platelet-poor plasma failed to coagulate even with the lower concentrations of heparin.

In addition, to more closely resemble conditions *in vivo*, only freshly drawn samples were used. Since Jaques and Richer (1948) found that clotting times lengthened after incubation of the sample with heparin, all samples were prepared immediately before use and incubated for only 3 minutes before the thrombin was added.

As pointed out by Monkhouse and Clarke (1957), thrombin in the presence of heparin and cofactor will often cause a tiny precipitate of fibrin to form which floats to the top of the solution. This precipitate was noticed in some of the experiments and seemed to bear little relation to the concentration of heparin. For this reason all solutions were considered to be clotted only when a firm gel had formed.

Because of the extreme rapidity of the action of heparin after its injection and its relatively quick removal from the circulation,

the clinical test used for checking heparin therapy should be simple, rapid and efficient. The proposed method for determining heparin concentration and thus its anticoagulant activity fulfills these basic requirements. It takes only a few minutes to set up and run. The only materials necessary are purified thrombin, pipettes, centrifuge, stopwatch and a constant-temperature water bath. The degree of accuracy is quite high with a standard deviation from the mean of 0.007 μg./ml. This means that 95% of the time the calculated heparin concentration will be ±0.014 μg./ml. from the actual concentration of heparin in the plasma.

In 1953 Blombäck, Blombäck, Corneliusson and Jorpes assayed the anticoagulant activity of heparin by 4 different methods: a fresh whole blood method, a thrombin method on plasma and the methods of the U.S.P XIV and B.P.1953. They found that the thrombin clotting times 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.

SUMMARY AND CONCLUSIONS

One of the disadvantages associated with heparin therapy has been the lack of a convenient and satisfactory control procedure.

The purpose of this project was to develop a method employing a one-step thrombin clotting time for assaying accurately the concentration of heparin in blood.

A relationship was found between the time it took to clot fibrinogen in the plasma and the amount of thrombin present. It was also found that the units of thrombin inactivated depended upon the concentration of heparin. From this information 2 factors were calculated (A - 3.45 and B - 34.13). These 2 factors plus the clotting time of 0.4 ml. of platelet-rich oxalated plasma with thrombin (10 units/ml.) added can be used to calculate the concentration of heparin in the plasma. The equation is

$$\text{Concentration of heparin } (\mu g./ml.) = 3.45 - \frac{34.13}{\text{clotting time (seconds)}}$$

Thus the proposed method is a one-step thrombin clotting time procedure that can be done in a relatively short time. The degree of accuracy for determining heparin concentration is quite high with a standard deviation from the mean of 0.007 μg./ml.

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