THE USE OF SULPHATED WHOLE BLOOD IN THE ASSAY OF HEPARIN

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With an Interpretation of the Data by K. L. Smith

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Numerous *in vitro* methods have been described for the assay of heparin. The more popular ones have been reviewed by Jorpes[1], and it is not proposed to consider them in detail here. They include both physicochemical and biological methods, but only the latter, which determine the inhibitory effects of heparin on the clotting of fresh blood or stored blood systems, in comparison with a standard preparation, give results which are related to the activity of the anticoagulant in the animal organism[1,2].

On theoretical grounds, assays using fresh whole blood are to be preferred to those using stored blood or plasma systems as in them the heparin activity is more closely related to that which occurs *in vivo*. The practical disadvantages of the fresh blood methods are considerable[3], and stored blood systems, since they are easier to manipulate, are therefore favoured. These systems consist of blood, or more usually plasma, which is preserved by oxalate or citrate, and in which clotting is induced by the addition of calcium, calcium and thrombokinase, or thrombin. Possible sources of error are, however, introduced, due to the effect of the salts on the blood proteins, and the addition of unsuitable amounts of clotting promoters.

When blood is collected into an equal volume of half-saturated sodium sulphate solution it will remain fluid, but simple dilution with water will promote coagulation[4]. If aqueous heparin solutions are used to dilute this " salted " blood, and thus induce coagulation, they cause prolongation of the clotting times over the water controls. The application of this system to the assay of heparin was considered; its use for this purpose has not hitherto been recorded. It was hoped many of the objections raised against other artificial systems would be avoided since aqueous dilution would produce a reconstituted though diluted whole blood, in which the clotting components would be in normal physiological proportions. Unfortunately the recorded coagulation times were too long for a rapid assay, and the addition of an unsuitable excess of thrombokinase extract was necessary to reduce them to a favourable range. Data presented later will show that this addition did not lead to erroneous results.

The design of heparin assays can be divided into two classes as described by Jaques and Charles[3].

(a) The clotting time method, in which clotting times are determined

for one or a given number of heparin solutions and which entails continuous examination.

(b) The titration method, in which the clotting mixture and a series of heparin dilutions are examined once only, after a certain fixed incubation time varying from 2 to 24 hours.

In the design of this assay, the clotting time method was chosen, so that data amenable to standard methods of analysis could be obtained and the lengthy incubation periods which many titration methods require could be avoided.

Reagents Used

Sulphated Whole Blood. Ox blood is collected from a freshly slaughtered beast into a 250-ml. wide neck glass stoppered bottle, containing 50 ml. of a 7 per cent. w/v solution of exsiccated sodium sulphate B.P., and stored below 4°C. until required.

Thrombokinase Extract. 1·5 g. of acetone-dried ox brain is extracted with 60 ml. of distilled water for 10 to 15 minutes at 50°C., centrifuged for 2 minutes at 1,500 r.p.m., and the suspension filtered through a Whatman No. 1 filter paper. This extract will retain its activity for several days when kept in the refrigerator, but rapidly undergoes bacterial decomposition at room temperature. The addition of tricresol (0·3 per cent.) added as a bacteriostatic does not affect the accuracy of the method.

Acetone-Dried Ox Brain. A fresh ox brain, freed from vascular and connective tissue, is cut into pea-size pieces and placed in acetone for preliminary dehydration. 30 g. of this material is pounded in a mortar with successive 75 ml. volumes of acetone until dehydration is complete. This is evident when a dry whitish buff powder remains, after Buchner funnel filtration. This preparation is finally dried at 37°C. for 2 hours to remove all traces of acetone.

Procedure

The clotting times produced by 3 dilutions of standard heparin and 3 equivalent dilutions of the unknown heparin are determined simultaneously, and this is repeated 4 times for a complete assay. It has been found convenient to make the highest concentration of heparin 2 units/ml., and to use an 80 per cent. interval between successive levels. The method of determining the clotting times is as follows.

1 ml. of a heparin dilution in distilled water is pipetted into a 6″ × ½″ soda glass test tube followed by 0·1 to 0·2 ml. of thrombokinase extract. 1 ml. of sulphated ox blood is then added, and the whole mixed by gentle inversion to prevent the formation of air bubbles. The time from this addition to the formation of a firm clot which remains in the bottom of the tube when it is completely inverted, is recorded.

The change in the fluidity of the blood observed by gentle tilting indicates the onset of coagulation, and by shortening the interval between examinations the operator can determine the end-point to within 15 seconds and with practice avoid the breaking of the clot by premature

inversion. If a tube is inverted before complete coagulation, the formation of the clot is hindered, and the whole run of 6 tubes is abandoned. This, however, seldom happens except in the initial training of an operator, as the end-point is extremely good. The amount of thrombokinase extract to be added may be varied slightly according to conditions, but is generally 0·2 ml. if correctly prepared. The most accurate assays are obtained when the longest clotting times range from 9 to 12 minutes, and longer times than these are not recommended. This can be arranged either by altering the range of heparin concentrations or the concentration or volume of the thrombokinase extract. It is perhaps easier to keep the heparin concentrations fixed and to dilute the thrombokinase extract until favourable clotting times are obtained with the 2 units/ml. standard solution.

Table I shows the clotting times in minutes recorded during a normal assay. The interpretation of such data, using the linear relationship which exists between the log. coagulation time and the log. concentration

TABLE I

CLOTTING TIMES IN MINUTES

Runs	Standard Heparin			Unknown Heparin—Assumed Strength		
	1·28 μ/ml.	1·6 μ/ml.	2 μ/ml.	1·28 μ/ml.	1·6 μ/ml.	2 μ/ml.
1	3¼	5¼	10¼	2¾	4¾	9¾
2	2½	4	6¼	2¾	3½	5¼
3	3½	5½	10¾	3¼	5	8¾
4	3¼	5	8½	3	4½	7½

of heparin gives potency estimates with a high order of accuracy, as is shown later in this paper. More rapid assays with a slight loss of accuracy may be carried out using 2 doses only of standard and single doses of the unknown. The potency estimates for such assays may be made graphically or arithmetically.

Sulphated ox blood is a more stable system than many citrated or oxalated bloods or plasmas. It is stable for a period of at least 3 to 4 weeks if stored below 4°C., and small granular clots which do sometimes form do not affect the assay and can be filtered off through muslin.

FACTORS INFLUENCING THE ACCURACY OF THE ASSAY

Tube Diameter. The choice of a test tube of a definite size, in which to conduct the assay, is of great importance, since tubes of varying diameters give clotting times which differ appreciably. Tubes of ½-inch diameter give excellent results, smaller ones than this causing errors in the end-point determinations, due to surface tension effects.

The Clotting End-Point. The clotting end-point is a very definite one, easily determined, and of rapid onset, such that it can be assessed to within 15 seconds of its formation. This enables different operators to obtain readily reproducible results after a very brief training.

Temperature at which the Assay is Conducted. Nearly all types of heparin assay are conducted at a constant temperature, usually 37°C. With sulphated blood however this is not necessary, and all operations are performed at room temperature. Experiments in which the assay was conducted at 37°C. showed no increase in accuracy. Furthermore it is not essential to keep the system in a refrigerator immediately prior to use, as is the case with some clotting time methods involving oxalated or citrated plasmas[5,6].

The Effect of Thrombokinase on the Accuracy of the Assay. Jalling, Jorpes and Linden deprecate the use of thrombokinase in heparin assays, because of the possibility of it removing some of the heparin, especially if the barium salt is used[2]. They quote results obtained by MacIntosh[7], in substantiation of this, in which he obtained lower values with recalcified plasma, and thrombokinase, than with fresh whole blood.

In order to determine if similar results were to be expected using the sulphated blood assay, four samples of sodium heparin were tested, and compared with results obtained using fresh whole blood. Table II shows that there is no significant difference between the two sets of figures.

TABLE II

Heparin Sample	Sulphated Blood Assay		Fresh Blood Assay
	Potency μ/mg.	Limits P = ·95	Potency μ/mg.
Crude Heparin	25	23·7—26·3	25·3
H 217—226	59·6	57·1—62·4	56·3
HP 50	100·7	92·7–108·6	101·2
HP 50 Hydrolysed	50	46·3—53·5	51·7

The Influence of Electrolytes on Clotting Times. The presence of electrolytes in heparin solutions was found to delay considerably the clotting times of sulphated blood, and this is in accord with the findings of other workers using other coagulation systems[3,5]. For this reason all solutions and extracts are made in distilled water. The maximal concentration of sodium chloride in the heparin solutions which causes no added anticoagulant action varies from 0·5 to 0·15 per cent. w/v. This factor has however no effect in the assay of injection of heparin B.P., as the chloride concentrations in the final dilutions are much less than those which show an apparent anticoagulant action.

Interpretation of the Data and Accuracy of the Method

The use of sulphated whole blood with added thrombokinase for the assay of heparin yields data in a form amenable to standard methods of examination, since, over the practical range, the log. of the time for coagulation is linear to log. concentration; this is a relationship which we have used also for the treatment of heparin assays by the method of MacIntosh[7] following a suggestion made to us by Mr. E. C. Fieller. The data are so readily obtained that the various hypotheses needed to calculate potency and its fiducial limits may be confirmed separately for each assay by conducting four runs in each of which both the standard and the unknown are represented at three concentrations. If the concentrations are in geometric progression the arithmetical procedures described by Bliss and Marks[8], and the British Standards Institution[9] may be applied. In practice we use parts of each method and have retained the symbols used respectively by them to denote the equivalent numerical values. The example Table I represents the data obtained in one of two assays carried out on a sample of heparin assumed to obtain 5,000 units per ml. and will be used to illustrate the analysis.

Arithmetical Procedures. The logarithms of the coagulation time are recorded to the nearest second decimal place, and these together with the appropriate summations are shown in Table III.

TABLE III

Run	1·28 units S_1	1·6 units S_2	2·0 units S_3	1·28 units U_1	1·6 units U_2	2·0 units U_3	Sum
1	0·51	0·72	1·01	0·44	0·68	0·99	4·35
2	0·40	0·60	0·80	0·44	0·54	0·72	3·50
3	0·54	0·74	1·01	0·51	0·70	0·94	4·44
4	0·51	0·70	0·93	0·48	0·65	0·88	4·15
Sum	1·96	2·76	3·75	1·87	2·57	3·53	16·44

An analysis of variance is made by the recognised method in which errors between runs and concentrations are removed (Table IV).

In practice we do not isolate the variance due to the separate effects but calculate the Residual Sum of Squares as Total Sum of Squares + Correction Term − Sum of Squares for Runs − Sum of Squares for Concentrations.

The further essential numerical values are then obtained using factorial coefficients after the method described by Bliss and Marks[8] with the exception that we prefer to examine the linearity of separate log. concentration response lines. The extraction of these values are shown in Table V.

Since the values A_1, A_2, A_3, in turn do not exceed $s^2 \times 4{\cdot}54$ the linearity of the respective lines and their parallelism is accepted. It may happen that one or more of the criteria will exceed $s^2 \times 4{\cdot}54$ but in this

TABLE IV

ANALYSIS OF VARIANCE ON DATA IN TABLE I

Source	Sum of squares	Correction term	Reduced sum of squares	df	Variance
Total	$0{\cdot}51^2 + \text{———} + 0{\cdot}88^2$	$16{\cdot}44^2/24$	0·8722	23	
Between runs ...	$\left[4{\cdot}35^2 + \text{———} + 4{\cdot}15^2\right]/6$	$16{\cdot}44^2/24$	0·0900	3	0·0300
Between doses ...	$\left[1{\cdot}96^2 + \text{———} + 3{\cdot}53^2\right]/4$	$16{\cdot}44^2/24$	0·7597	5	0·1519
Residual ...			0·0225	15	$0{\cdot}0015 = s^2$

Reduced sum of squares = Sum of squares − Correction term.

TABLE V

Treatment effect	Coefficients for x S_1	S_2	S_3	U_1	U_2	U_3	Nx^2	$SN(xYp)$	$\frac{S^2N \times Yp}{Nx^2}$
Samples	−1	−1	−1	+1	+1	+1	24	$-0{\cdot}5 = T$	
Slope	−1	0	+1	−1	0	+1	16 = P	3·45 = Q	
Linearity of curve for standard	+1	−2	+1				24	0·19	$0{\cdot}0015 = A_1$
Linearity of curve for unknown				+1	−2	+1	24	0·26	$0{\cdot}0029 = A_2$
Parallelism of lines	+1	0	−1	−1	0	+1	16	−0·13	$0{\cdot}0011 = A_3$
Yp	1·96	2·76	3·75	1·87	2·57	3·53			$0{\cdot}0018 = A = \frac{A_1 + A_2 + A_3}{3}$

case we still proceed with the analysis providing the value A does not exceed $s^2 \times 3{\cdot}29$. The values 4·54 and 3·29 being the respective 5 per cent. variance ratios for $n_1 = 1$ and 3 when $n_2 = 15$ (Fisher and Yates[10]) which are the conditions that apply in the given example.

Calculation of Potency and its Fiducial Limits. In general the log. activity ratio of the unknown to the standard may be calculated from tests with three concentrations of standard and three of unknown

as $M = 1{\cdot}33\ TI/Q$

where I is the log. of the ratio of successive concentrations.

Its fiducial limits at a prescribed probability level may be obtained by using the appropriate Student value t and calculating

$$C = Q^2/[Q^2 - t^2 s^2 P]$$

and applying this in the expression

$$CM \pm \sqrt{(C-1)\,(2{\cdot}667\ I^2 + CM^2)}$$

which is the appropriate modification of that given by Fieller[11].

In designs similar to that discussed, and working at P = 0·95 these expressions reduce to

$$M = 0{\cdot}1292\ T/Q$$

$$C = Q^2/Q^2 - 72{\cdot}659\ s^2$$

$$CM \pm \sqrt{(C-1)\,(0{\cdot}02507 + CM^2)}$$

and when applied to the specific example yield the following estimates.

$$M = -0{\cdot}01872 = \bar{1}{\cdot}98128 = \log.\ 0{\cdot}9579$$

$$C = 1{\cdot}00924$$

$$CM \pm \sqrt{(C-1)\,(0{\cdot}0257 + CM^2)} = -0{\cdot}00356 \text{ and } -0{\cdot}03421$$

$$= \bar{1}{\cdot}99644 \text{ and } \bar{1}{\cdot}96578 = \log.\ 0{\cdot}9917 \text{ and } \log.\ 0{\cdot}9243$$

Thus we estimate the potency of the unknown to be 95·79 per cent. of that assumed with fiducial limits P = 0·95 of 99·17 per cent. and 92·43 per cent.

In a second assay conducted on this sample the following values were obtained.

T = −0·16	Q = 2·94	s^2	= 0·0003
A1 = 0·0028		$s^2 \times 4{\cdot}54$	= 0·0013
A2 = 0·0021	A = 0·0017	$s^2 \times 3{\cdot}29$	= 0·0010
A3 = 0·0002			

Since in this instance both A_1 and A_2 exceed $s^2 \times 4{\cdot}54$ and A exceeds $s^2 \times 3{\cdot}29$, it would not be permissible to use s^2 in the analysis of this assay but we may proceed using A in place of s^2 taking due note that A has been established with 3 df.

The same formulae are used to calculate M and its fiducial limits at P = 0·95 except that the value of C is now taken as

$$C = Q^2/[Q^2 - t^2 AP]$$

which in examples similar to that described reduces to

$$C = Q^2/[Q^2 - 162A].$$

Applying the formulæ to the above example we obtain the following estimates

$$M = -0{\cdot}00703 = \bar{1}{\cdot}99297 = \log.\ 0{\cdot}9840$$

$$C = 1{\cdot}03291$$

$$CM \pm \sqrt{(C-1)\,(0{\cdot}02507 + CM^2)} = +0{\cdot}02146 \text{ and } \bar{1}{\cdot}96402$$

$$= \log.\ 1{\cdot}051 \text{ and } \log.\ 0{\cdot}9204$$

Concordancy and Combination of Results. It is our practice to weight each estimate inversely as its variance and to use this to establish the concordancy of the estimates and to calculate the mean potency.

In general terms the variance is calculated as

$$s_m^2 = \frac{s^2}{b^2}\left(\frac{1}{N_{St}} + \frac{1}{N_u} + \frac{M^2}{PI^2}\right) = \frac{1}{w}$$

where b = Q/PI, N_{st} and N_u = the number of responses to standard and unknown s^2 being replaced by A on the appropriate occasions.

If X represents the estimated per cent. potency the concordancy is checked by comparing the value

$$Sw\,(X-\bar{X})^2 \text{ calculated as } SwX^2 - S^2wX/Sw$$

with the appropriate value of χ^2 with n corresponding to the number of estimate less 1.

The mean log. estimated percentage potency is then calculated as

$$\bar{X} = SwX/Sw$$

and its limits of error as $\bar{X} + \sqrt{6^2/Sw}$. Where t is the appropriate Student value corresponding to the summed degrees of freedom with which s^2 (or A) have been established. In a design similar to that described the weight may be calculated as

$$w = \frac{6b^2}{s^2} \Big/ [1 + 40M^2]$$

In applying this treatment to the examples given we have used the value (X − 2) in place of X to reduce the number of digits to be handled and have extracted the values shown in Table VI.

TABLE VI

Test	Estimated potency	X	w	w(X−2)	w(X−2)²
	per cent.				
1	95·79	1·9813	19136	−357·8432	6·6917
2	98·4	1·9930	12628	−88·3960	·6188
			31764	−446·2392	7·3105
			Less correction term $S^2w(X-2)/Sw$		6·2690
				$Sw(X-\bar{X})^2$	1·0415

The value $Sw(X-X)^2$ of 1·0415 for two estimates indicates satisfactory agreement between them at P=0·95. We therefore calculate the mean log. estimated potency as $2 - \frac{446{\cdot}2392}{31764} = 2 - 0{\cdot}0140 = 1{\cdot}9860 = \log.$ 96·83 per cent. and using in this instance the value t=2·1 the limits of error of this estimate at P=·95 as 1·9860±0·0118=1·9978 and 1·9742=log. 99·49 per cent. and 94·23 per cent.

Characteristics of the log. concentration response lines. During 115 tests of the linearity of the l.c.r.l. for standard and for the unknown and their parallelism were acceptable without question 83 times. In 15 of the remaining tests one or more of the criteria were in doubt, but since A did not exceed $s^2 \times 3{\cdot}29$ the linearity and parallelism of the lines were still accepted, but in the other 17 tests A had to be used in place of s^2 for the subsequent calculations. In all, the linearity of the l.c.r.l. for standard was questioned on 8 occasions, that of the unknown on 19 occasions, and the parallelism on 14 occasions.

Theoretical Accuracy of the Assay. In the 83 tests acceptable without question the value of b^2/s^2 ranged from 92·5 to 9586·0 and had a mean value of 2,764·0. (In the 17 tests in which A was used in place of s^2 the mean value for b^2/A was 755·3.)

Thus on the average when M is zero the weight of the test described should be 16,584, which at P=0·95 would indicate an error of the order ±3·5 per cent.

The accuracy of the method in practice. The data shown in Table VII illustrate the accuracy of the method in practice. The results are those obtained during the examination of dilutions of the standard treated as samples of unknown strength. The error of these estimates are within that expected from the internal evidence of the tests.

TABLE VII

Standard prepared to be	Potency found	P=0·95 limits
76·9 per cent.	80·16 per cent.	75·89—84·8
91·6 ,,	89·12 ,,	82·4 —97·12
110 ,,	108·2 ,,	103 –114
110 ,,	109·2 ,,	104·5 –114·5

The application of the method to a rough assay. In tests carried out at intermediate stages in the production of heparin B.P. the linearity of the line relating log. coagulation time to log. concentration is accepted and assays are conducted using two levels of standard (2 U/ml., 1·28 U/ml.) and one only of the sample under test. This is prepared so that it would be equivalent to the high concentration of standard if the potency assumption has been made correctly. On these occasions four samples are examined at one time and the assay is completed by carrying out 6 runs. The estimation of potency could be made graphically, but it is perhaps easier to calculate it arithmetically.

If S_2, S_1 and U_2 represent the sums of the log. coagulation times for high and low concentratilons of standard and for the unknown respectively.

Then $$Q=S_2-S_1 \text{ and } T=U_2-S_2$$
$$\text{and } M=TI/Q$$

where I=log. ratio of the Standard concentrations.

On theoretical grounds the accuracy of such an estimate when $b^2/s^2=2700$ would be of the order ± 5 per cent at P=0·95. There is some objection to calculating the fiducial limits from such an assay since occasions do occur when the linearity or parallelism of the respective lines are in question. When fiducial limits are desired it is preferable to carry out the full assay which has been described.

SUMMARY

1. A new method of heparin assay using " sulphated " ox blood, and thrombokinase is described.

2. The method has the following advantages:—

(a) The method is rapid, accurate, and agrees well with whole blood methods.

(b) Equipment and technique are simple.

(c) The blood system, if stored below 4°C. is stable for at least 3 weeks.

(d) Reproducibility of results between different operations is good.

(e) The assay is conducted at room temperature.

(f) The data may be interpreted by standard methods of analysis and a treatment is described.

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DISCUSSION

The paper was presented in abstract by Mr. S. S. Adams.

THE CHAIRMAN asked if any members had compared the existing methods with that proposed in the paper.

MR. R. MAXWELL SAVAGE (Barnet) reported some observations on clotting times. They occasionally used a method for determining clotting times which was almost identical with that described in the paper, and he could confirm that it worked very well. It gave a sharp end-point. They did not do it at room temperature, because it was so easy to control the temperature with the water-bath. The method had the drawback that it could not be used for systems which were clotting very rapidly. There was an alternative method which consisted of putting very small drops of plasma and of the solution of heparin side by side on a microscope slide and mixing them with a capillary stirrer. The time was observed with a stop-watch and clotting times as low as 15 seconds were recorded with quite small errors. His next point concerned the preparation of a clotting system. Dried plasma, if stored in a screw-capped bottle in a small desiccator in a refrigerator, retained its clotting properties for periods of a year or more. Thrombin was a stable reagent, so that, by the use of dried plasma and thrombin solution, with a little attention to storage, a clotting system could be set up very easily without previous notice and in a few minutes. Previous reports had suggested 25°C., i.e., the temperature of the body at the normal site of clotting,

to be a more suitable temperature than 37°C. At about that temperature many clotting processes changed very little and control of temperature might therefore not be necassary. In the author's method, the standard obviated the need for controlling the temperature, as both the standard and test solution were equally affected, but in the method he had described there were difficulties in operating without a thermostat. The clotting process was not completely indifferent to temperature, and if one did the determination of the standard and of the test solution at too great an interval of time, the disturbing effects of, say, a shaft of sunlight on a neighbouring bench might put up the temperature sufficiently to upset the experiment.

DR. G. E. FOSTER (Dartford) asked if the author had tried to make heparin possessing a retarded action, and whether he had tried hirudin which was used a great deal in physiological work.

MR. S. S. ADAMS, in reply, said he was aware that the system which they had used was not very suitable for the more rapid clotting times, but they were not very interested in that, because with clotting times which ranged from 3 to 10 minutes the operator had plenty of time in which to put down the clotting times accurately. When the clotting times varied only between about 30 and 90 seconds, it was difficult to record the times for 3 or 4 test tubes accurately. He had not tried a method using dried plasma, but he had tried several methods, and their modifications, using thrombin. The major difficulty was that both the plasma and thrombin had to be kept at 0°C. immediately prior to use, because otherwise the clotting times differed quite appreciably from minute to minute. He had done some work with retarded heparins, and this method would give a result for absolute potency. He was referring to preparations in which the heparin was in a gelatin base. The measurement of retarded effect *in vivo* was made by injecting a standard solution of normal heparin into rabbits and determining the clotting times of the blood at half-hourly or hourly intervals, and then injecting the retarded heparin into another batch of rabbits and determining the clotting times for those. It was not a quantitative method. They had no experience of hirudin.