THE ASSAY OF HEPARIN

BY J. PRITCHARD

From The Evans Biological Institute, Runcorn

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FOR many years the potency of heparin has been assayed in these laboratories by a method which originated as a modification of that of Kuizenga, Nelson and Cartland[1] but which has since been so greatly modified that it is now similar to the method suggested by Foster[2,3] and adopted in the U.S.P. XIV.

As in the U.S.P. XIV, the assay consists of comparing by eye the clots produced when sheep plasma is recalcified in the presence of varying amounts of the standard heparin and the heparin under test. There are however, many technical differences between the two methods of assay.

In 1953 Blombäck, Blombäck, Corneliusson and Jorpes[4] published a paper on the reliability of the methods used in the assay of heparin and gave the anticoagulant activity of 20 samples of heparin sodium which had been assayed by four different methods—a fresh whole blood method, a thrombin method on plasma, and the methods of the U.S.P. XIV and the B.P. 1953. They found the U.S.P. XIV method to give 10 to 15 per cent. lower figures than the thrombin method for samples with 25 to 110 I.U. per mg., and sometimes lower figures for commercial samples.

In 1954 Jorpes, Blombäck and Blombäck[5], published the results of further research on the assay of heparin and included an *in vivo* method using sheep. When comparing the new and old Swedish standard heparins against the International Standard heparin, the U.S.P. XIV and the B.P. 1953 methods of assay again gave lower readings than the *in vivo* sheep, the fresh ox blood and the thrombin methods. They considered the U.S.P. XIV method the most difficult to handle and the least reliable.

In view of these statements it was thought necessary to check the reliability of our method and to show that on identical samples it did not give rise to similar discrepant results. Through the courtesy of Professor J. E. Jorpes of the Karolinska Institutet, Stockholm, samples of the two Swedish standard heparins were obtained and assayed against the International Standard heparin and the U.S.P. Heparin Sodium Reference Standard. Table I shows that the results obtained compared with those of Jorpes and others.

TABLE I

STRENGTH OF NEW SWEDISH STANDARD HEPARIN (I) AND THE OLD SWEDISH STANDARD HEPARIN (II) USING DIFFERENT METHODS OF ASSAY

International units per milligram of water-free substance

	In vivo sheep (Jorpes and others)	Fresh ox blood (Jorpes and others)	Thrombin method (Jorpes and others)	U.S.P. XIV (Jorpes and others)	B.P. 1953 (Jorpes and others)	Sheep plasma (Pritchard)
(I)	110	108	107	96	103	111
(II)	80	81	82	67	73	79

These results are in such close agreement with those given by the Swedish workers with their *in vivo* sheep, whole ox blood and thrombin methods that it was thought that a full description of our method of assay would be of value to others.

EXPERIMENTAL

Reagents

Sheep Plasma. Sheep are bled with aseptic precautions by cannula from the jugular vein, 225 ml. blood being taken into 25 ml. normal saline solution containing 3·8 per cent. sodium citrate B.P. The blood is stored at 4° C. for 5 days before the cells are separated by centrifuging for 45 minutes at 2800 r.p.m. and the plasma drawn off aseptically into a sterile bottle. Several sheep have been reserved for this purpose only and three or more are bled at a time as required and the plasma from these bulked. In order to maintain health and the quality of the plasma, the sheep are not bled more often than once every three weeks.

The bulked plasma is distributed into sterile 60 ml. bottles (filled only to the shoulder), capped with overstyle rubber caps held by rubber bands and stored in a cold room at — 10° C. until required. Stored in this manner, it may be used up to 4 or 5 months after the bleeding.

In the test, the bottle containing the solid frozen plasma is placed in a water bath at 37° C. and occasionally shaken until the plasma is completely liquid. This is then filtered through a small piece of cotton wool in a funnel to remove the small amount of coagulum which occasionally is deposited in some samples; those containing heavy deposits are not used. The thawed plasma may be used on the next day if kept overnight at 4° C. and may, if necessary, be diluted with normal saline to obtain a suitable clotting range.

Calcium Chloride Solution. A 2·5 per cent. solution of $CaCl_26H_2O$ (= 1·3 per cent. $CaCl_2$) in distilled water is used. The quantity per tube is kept constant at 0·25 ml. for all plasma.

Heparin Standard

The standard used, whether the International Standard, the U.S. Reference Standard or a subsidiary standard, is weighed rapidly and dissolved in sufficient sterilised normal saline solution (containing 0·13 per cent. chlorocresol B.P. as a preservative) to produce a solution containing exactly 10 International Units per ml. Sufficient may be made to last 4–8 weeks provided it is stored at 4° C. and not allowed to become contaminated by micro-organisms. The solution is not filtered or otherwise sterilised and it has been found useful to keep it in the graduated measure in which it is made, replacing the ground glass stopper with an overstyle rubber cap through which the solution is withdrawn by syringe.

Heparin Solutions for Assay

All solutions are diluted with normal saline, or powders dissolved in normal saline and further diluted, if necessary, to produce a solution

which may be expected to contain 10 units per ml. Preliminary tests may be made to determine the approximate potency but the final tests are made on a dilution which should be within 2–3 per cent. of the expected potency.

For samples of good quality a preliminary test using the Toluidine Blue method of MacIntosh[6] will give a reasonable estimate of the potency. Some samples of crude heparin, or heparin which has been degraded give less accurate but useful information by this test.

Apparatus

(1) Racks, wood or metal to hold 10 or more rows of 7 tubes.

(2) Test tubes, 10 mm. internal diameter, 65 mm. long, as uniform as possible.

After use, the tubes are rinsed and kept in a 1·5 per cent. Lissapol N solution overnight. They are then cleaned with a brush and soap, followed by thorough rinsing in tap water and distilled water and are dried in a hot-air dryer.

(3) Rubber stoppers for test tubes. Thoroughly washed in tap water and dried after use.

(4) Good quality accurate 1 ml. tuberculin syringes graduated in 0·01 ml. These are used instead of pipettes or burettes to measure the various solutions. There is no chance of "overshooting" the graduation marks as with a pipette or burette and errors due to variations in "draining time" when measuring such a viscous material as plasma are eliminated. It is essential, however, to test these syringes to ensure that the accuracy is within suitable limits (having an error of not more than 1·0 per cent. when tested at both 0·5 ml. and 1·0 ml.) and that there is no leak past the piston and between the nozzle and needle mount.

(5) Glass stoppered test tubes holding 10 ml. "Exelo" test tubes 100 mm. × 16 mm. with size C14 interchangeable ground glass stoppers are satisfactory.

Performance of Test

A heparin-plasma mixture is prepared for the standard and each of the unknown samples by placing 0·7 ml. of the standard heparin (10 units per ml.) and 0·7 ml. of each batch of heparin to be tested (diluted to contain an estimated 10 units per ml.) into a series of suitably labelled glass stoppered tubes. 6·3 ml. of plasma is added to each tube and the contents mixed thoroughly by inversion. This amount is sufficient for two identical rows of the standard and of each batch to be tested. If only preliminary tests are needed 0·4 ml. heparin and 3·6 ml. plasma is sufficient for one row. The following amounts of plasma are delivered into each row of the tubes in the rack: 0·76, 0·71, 0·65, 0·58, 0·50, 0·40 and 0·28 ml. The following amounts of the standard heparin-plasma mixture are placed in the first two rows of tubes: 0·24, 0·29, 0·35, 0·42, 0·50, 0·60 and 0·72 ml. The subsequent pairs of rows are filled in the same manner with similar doses of the heparin-plasma mixtures of each batch to be tested or in the case of preliminary tests a single row is used.

Not earlier than 20 minutes after the original heparin-plasma mixture is made 0·25 ml. calcium chloride solution is added to each tube which is then stoppered with a rubber stopper and the contents mixed by inverting 7–8 times making sure that the whole of the inside surface of the tube is wetted by the solution. The tubes are then allowed to stand overnight at room temperature.

It has been found that the most convenient order of adding the reagents to the tubes is to work along the columns from front to back with the plasma, along the rows from right to left with the heparin-plasma mixtures, and again from front to back with the calcium chloride solution.

Reading the results

The amount of clotting is estimated by eye using an arbitrary scale in terms of symbols which are later converted to numerical values and plotted as a curve. It has been found in practice (*cf.* Foster[2]) that it is easier to read the degree of clotting as trace, slight, ±, + or + + than to visualise it in figures such as 2, 3, 4, 6, and 10, or as a percentage. The examination of the tubes is best carried out by looking through the tubes which are held above eye level against a north light window. Tubes having clots of a value of "trace" (numerical value 2) or less can be shaken to detach the clot from the tube to make the comparison of the amount of clot easier.

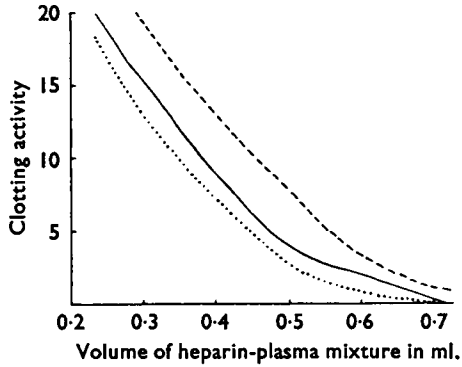

FIG. 1 Curves correlating clotting activity with heparin dosage.
—— International standard.
..... P6842. – – – P6853.

The first to be read are the standard tubes and having estimated the degree of clotting in each tube this is converted to its numerical value. The sum of the values of each pair of tubes containing the same dose of heparin-plasma mixture is used for the plotting of the graph, correlating the dose of heparin-plasma mixture and the clotting value, which is usually a smooth curve.

TABLE II

CLOTTING VALUES

Symbol	Numerical value	Appearance of clot
+ +	10	Clear, transparent, yellowish solid clot.
+ (+)	8	Transparent, yellowish solid clot with slightly granular appearance.
+	6	Opaque, granular, whitish solid clot.
±	4	Pale translucent clot filling whole tube. Slightly more opaque in upper portion.
S (slight)	3	Pale translucent clot extending to ¾ or over of tube. Nearly clear below.
tr (trace)	2	Thin but opaque clot extending ¼ to ½ of tube. Clear below.
?	1	Transparent ring of clot at surface of fluid in top quarter of tube.
—	0	No clot.

Having finally determined the degree of clotting in the standard tubes, the two rows of tubes for each product to be tested are examined and the degree of clotting in each tube directly compared with the clots in the standard tubes. In most instances the two rows of tubes for any one batch, standard or test, will be identical or very nearly so. Where tests are done on one row only, the numerical value of each tube is doubled.

Table II is intended as a guide to evaluate the degrees of clotting with the symbols and numerical values allotted to them. With practice it is easy to read intermediate values between those given in this Table.

Calculation of Results

From the plotted curve of the standard (see Fig. 1), the amount of heparin required to give clotting values of 12½, 10, 7½, 5 and 3 are obtained.

TABLE III

READINGS AND CALCULATIONS FOR A COMPLETE TEST

Row No.	Batch	Primary dilution in normal saline	Dilution in plasma	0·24 ml.	0·29 ml.	0·35 ml.	0·42 ml.	0·50 ml.	0·60 ml.	0·72 ml.
8	P6853	1/20	0·4/4	++	++	+(+)	+	±	?/tr.	−/?
7	P6842	1/21	0·4/4	+(+)/++	+/+(+)	±/+	S	?/tr.	−/?	−
6	P6019	1/11	0·7/7	++	+ (+)	+	±	tr.	?	−
5	P6019	1/11	0·7/7	++	+ (+)	+	±	?/tr.	?	−
4	P6018	1/8	0·7/7	++	+ (+)	+	±	>tr.	?	−
3	P6018	1/8	0·7/7	++	+ (+)	+	±	>tr.	?	−
2	I.S.	10 u./ml.	0·7/7	++	+ (+)	+	±	tr.	?	−
1	I.S.	10 u./ml.	0·7/7	++	+ (+)	+	±	tr.	?	−
	Numerical values		P6853	20	20	16	12	8	3	1
			P6842	18	14	10	6	3	1	0
			P6019	20	16	12	8	3½	2	0
			P6018	20	16	12	8	4½	2½	0
			I.S.	20	16	12	8	4	2	0

Volumes of heparin-plasma mixtures required to produce similar clots at different clotting levels

Clotting level	International Standard	Batch P6018	Batch P6019	Batch P6842	Batch P6853
12·5	0·34	0·34	0·34	0·31	0·41
10·0	0·385	0·385	0·385	0·35	0·46
7·5	0·43	0·43	0·43	0·39	0·51
5·0	0·475	0·485	0·47	0·44	0·555
3·0	0·54	0·565	0·52	0·50	0·60

Potency as percentage activity of International Standard at different clotting levels

Clotting level	International Standard	Batch P6018	Batch P6019	Batch P6842	Batch P6853
12·5	100	100	100	109·5	83
10·0	100	100	100	110	83·5
7·5	100	100	100	110	84·5
5·0	100	98	101	108	85·5
3·0	100	95·5	104	108	90
Average ..		98·7	101	109·1	85·3

P6018. A 1 mg./ml. solution of the old Swedish Standard heparin
A 1/8 dilution contains 98·7 per cent. of 10 u. = 9·87 units.
P6018 contains 79 units/ml.

P6019. A 1 mg./ml. solution of the new Swedish Standard heparin
A 1/11 dilution contains 101 per cent. of 10 u. = 10·10 units.
P6019 contains 111 units/ml.

P6842. Production control sample
A 1/21 dilution contains 109·1 per cent. of 10 u. = 10·91 units
P6482 contains 229 units/ml.

P6853. Production control sample
A 1/20 dilution contains 85·3 per cent. of 10 u. = 8·53 units
P6853 contains 170 units/ml.

From the curves of each test sample the amount required to give clotting values over the same range are obtained and a percentage activity of the sample obtained for each value. The average of the five readings is taken as the percentage potency of the diluted sample compared to 10 units per ml., and the unitage of the undiluted sample calculated from this figure. The readings and calculations for a complete test are given in Table III.

Selection and Adjustment of Plasma

For most satisfactory results a clot with the value of $\pm$ (4), should be produced in the tube containing 0·42 ml. of the standard heparin-plasma mixture. This can be ensured by adjusting the amount of heparin contained in the 7 ml. heparin-plasma mixture, using proportionately more or less heparin according to the results given in a preliminary test. An alternative method, where the clots are very opaque and for ease in calculation, is to maintain the heparin-plasma mixture at 0·7 ml./7·0 ml. and to adjust the plasma by diluting with normal saline.

Discussion

It will be seen that the method of assay described above differs in many respects from that of the U.S.P. XIV. Whereas the latter requires the test to be read exactly one hour after recalcification of the plasma, in the above test, clotting is allowed to proceed for approximately 16 hours, after which time the amount of clot produced has nearly reached a maximum and thus does not change even if left standing for several hours longer. The degree of clotting in the tubes increases greatly between the 1 hour period and a period of approximately 16 hours, and it has been found that tests read at these two times do not give concordant results.

By allowing the reaction to proceed to near completion it is not necessary to select only those plasma which when recalcified form a solid clot within 5 minutes. In practice very few batches of plasma are found to be entirely unsuitable for use, and these are rejected not on the speed with which they clot but because the various grades of clot are not easily differentiated.

The use of plasma obtained by careful bleeding from the jugular vein minimises its contamination by tissue juices. Storage of the blood at 4° C. for 5 days before separating the plasma in the centrifuge has resulted in a very satisfactory plasma for this test, requiring slightly more heparin per unit volume to inhibit clotting than plasma separated immediately after bleeding.

Variations in the amount of calcium chloride used in the recalcification do tend to affect the degree of clotting produced in the tubes but complete tests carried out using amounts of calcium chloride 25 per cent. above and below the suggested amount gave results showing that the accuracy of the test is not dependent on the use of a critical amount of calcium chloride and that preliminary recalcification tests on the plasma are not necessary. Other differences in technique have been made with the object of reducing the time and labour involved in putting up the test without reducing its accuracy.

It is satisfactory to know that Professor Jorpes[7] is prepared to accept the results of this method of assay although he has on several occasions criticised the U.S.P. method.

The advantages of the method are that—

(1) It is reliable and accurate. With samples of commercial grade heparin the fiducial limits of error (P = 0·95) of a single assay are ± 1·7 per cent. and for the mean of a duplicate assay ± 1·2 per cent.

(2) It is easy to perform. After a very short period of training most technicians can carry out the assay and obtain results very close to those of more highly experienced workers.

(3) It allows a direct comparison between standard and sample at various clotting levels—essential for accuracy and an advantage in the detection of abnormal heparins.

(4) As the clotting is allowed to proceed to near completion there is no urgency in reading the results which can be done by several observers and their findings correlated.

(5) The plasma can be stored for several months and is thus immediately available when a test is required.

(6) The amount of calcium chloride solution necessary for recalcification of the plasma is not critical and need not be adjusted for individual batches of plasma.

Summary

1. A method is described for the assay of heparin consisting essentially in the comparison by eye of the clots produced in approximately 16 hours when sheep plasma is recalcified in the presence of varying amounts of Standard Heparin and the heparin under test.

2. The results obtained are believed to be accurate and reliable and the technique of the assay reasonably simple.

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