RESEARCH PAPERS

AN *IN VIVO* METHOD FOR THE ASSAY OF HEPARIN

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WITH the increasing use of heparin, the question of its assay has gained in importance. The matter has recently become topical, owing to the difficulties encountered in determining the anticoagulant activity of heparin. It has been found that the results obtained are greatly dependent on the methods applied, which is a serious drawback in a biological assay.

In a recent study[1], we pointed out that commercial samples of heparin assayed by the U.S.P. XIV method tended to give lower figures for the anticoagulant activity than when assayed with fresh whole blood or by a thrombin method. The discrepancy was, in fact, remarkable. Commercial samples of the Swedish heparin (Vitrum), when assayed by the U.S.P. method, frequently gave 20 per cent. lower figures than with the fresh whole blood method. A sample of the Danish heparin (Novo) analysed in 1953 showed an activity of 3335 I.U./mg. as assayed against the International Heparin Standard, instead of the declared strength of 5000 I.U./mg. The discrepancy was certainly not due to a deficiency in the strength of the heparin samples. Since the U.S.P. XIV method will be automatically accepted in most countries, the question of its reliability is of considerable importance.

In our previous communication[1], we reported on the analyses of 20 samples of heparin sodium, ranging in strength from 25 to 130 I.U./mg., performed by 4 different methods. They were: a fresh whole blood method, a thrombin method on plasma, the U.S.P. XIV method and the B.P. 1953 method. In samples with a strength of 25 to 110 I.U./m.g., the U.S.P. XIV method yielded 10 to 15 per cent. lower figures than the thrombin method. A striking deviation was noted in assays of the old Swedish standard heparin (see Table I).

TABLE I

ASSAY OF THE OLD SWEDISH STANDARD HEPARIN AGAINST THE INTERNATIONAL STANDARD HEPARIN

Whole blood method of Jalling, Jorpes and Lindén[2]	Thrombin method of Studer and Winterstein[3]	Plasma method of the U.S.P. XIV, 1950
I.U./mg.	I.U./mg.	I.U./mg.
80	83	65
80	79	68
85	86	70
82	83	70
80* (25.11.51)	83	65
81** (23.7.52)	78	65
76** (6.5.52)	—	68
81	82	67

* Checked against Liquemin (Roche). ** Checked against Heparin (Novo).

It is seen from Table I that the Swedish standard heparin had a potency of 81 I.U./mg. when assayed by the whole blood method, whereas it was 82 I.U./mg. by the thrombin method of Studer and Winterstein. The U.S.P. method gave, however, only 67 I.U./mg. All the figures recorded in the Table I were obtained on different days.

In view of the difficulties of obtaining uniform results by the use of one or other of the *in vitro* methods, we concentrated our efforts on devising an *in vivo* method. There is every reason to attempt to dispense with the artificial systems hitherto in use, since they imply a lack of one or more of the normal coagulation factors, or the introduction of seriously disturbing components, such as an excess of thrombokinase or an unbalanced quantity of calcium.

Howell[4] preferred to use fresh cat blood, to which heparin was added *in vitro*. His method was improved and described in detail by Scott and Charles[5] and by Jaques and Charles[6]. One of us (E. J.) found ox blood to be preferable[7]. For the sake of convenience, however, artificial systems have been considered more advantageous. Among them are those with citrated plasma (U.S.P. XIV), with oxalated or citrated plasma and thrombin (Studer and Winterstein), and with salted whole blood (B.P. 1953).

As already pointed out, it seems to be a hopeless task to ascertain which of the aforementioned systems is to be regarded as the method of choice. Consequently, we have attempted to determine the anticoagulant activity in the only physiological way, *i.e.*, by injecting it intravenously into living animals, and determining the coagulation time a few minutes later. This technique ensures the presence of all the factors entering into the coagulation system, and no disturbing elements are introduced. Since we found that unanæsthetised sheep could be used, the influence of narcosis could also be eliminated.

Our experience with the *in vivo* technique in animals has hitherto been favourable. Moreover, we have been able to confirm that the methods using fresh, unaltered whole blood are fully reliable.

Experimental

Our first experiments were made on cats and dogs under nembutal anæsthesia. Heparin of known strength was injected intravenously in small doses, increasing in size by 10 to 20 per cent., at intervals of 3 to 4 hours. The coagulation time of the blood, taken from the femoral vein, was determined 4 minutes after each injection. The values were plotted on a curve, from which the strength of an unknown sample could be read off after injection of a suitable dose. It soon became evident that application of this principle was feasible. Still better results were, however, obtained in sheep, since general anæsthesia was unnecessary and capillary bleeding could be avoided.

Technique

The wool is cut, and the area over a jugular vein is anæsthetised by injecting 5 to 10 ml. of a 0·3 per cent. solution of lidocaine. In order to support the polyethylene tubing to be inserted into the jugular vein, a

specially constructed coupler—a cannulated plate as used by Hallgren and Björck[8] (see Fig. 1)—is fixed to the skin by means of two safety-pins. The plate (A. B. Jacoby, Stockholm) is provided with an elongated nozzle at one end and a Record fitting at the other; in the centre it has a stopcock.

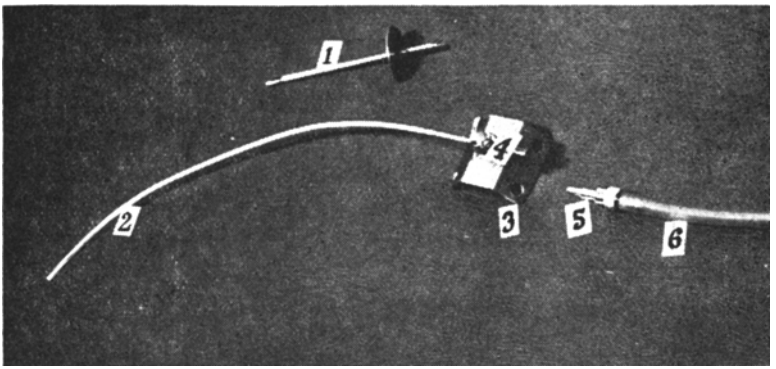

FIG. 1. The polyethylene tubing (2) fixed to the supporting plate (3).

The polyethylene tubing is drawn over the nozzle, and the rubber tubing attached to a drip flask containing physiological saline solution is connected to the Record fitting.

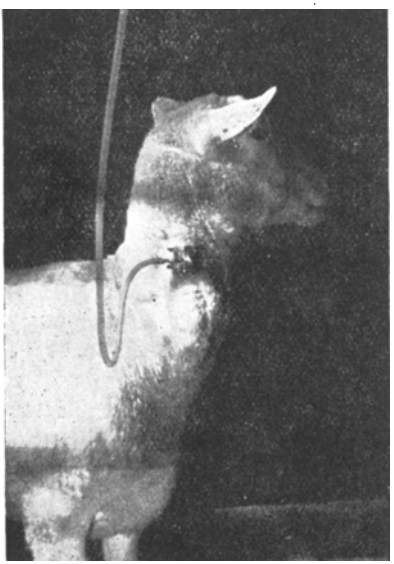

FIG. 2. The polyethylene tubing *in situ*.

A metal cannula with an inner diameter of slightly over 2 mm. is inserted into the jugular vein, and through it is passed a plastic tube (Polyethylene Medical Tubing, inner diameter 1·57 mm., outer diameter 2·08 mm., Pe 205, Clay Adams Co., Inc., New York). The tube is 20 cm. long and 10 cm. of its length is inserted. A single suture is passed through the skin and around the tube; the free end of the tube is then drawn over the nozzle of the plate. The polyethylene tubing is firmly attached in this way and cannot be displaced by the movements of the animal. The rubber tubing (inner diameter 5 mm.) which connects the plate with the flask containing physiological saline solution is sufficiently long to permit freedom of movement; the animal is able to eat, drink and lie down unhindered throughout the experiment, which lasts for up to 20 hours. (Fig. 2.)

During the whole experiment, a continuous flow of sterile, physiological saline solution runs into the vein at a rate of 15 to 20 drops per minute. This is to prevent the formation of clots in the polyethylene tubing. The heparin is injected through the rubber tubing, close to the plate, and is flushed down with a little saline solution. The blood samples are withdrawn with a 10-ml. Record syringe. For this purpose, the saline drip is disconnected, the syringe is connected to the fitting on the plate and is filled rapidly, the time being recorded with a stopwatch. The saline drip is then reconnected without delay.

Only small doses of heparin are given. The maximal effect is reached 4 minutes after the injection (Fig. 3); consequently, all the samples for determination of the coagulation time should be taken exactly 4 minutes after injection as recorded with a stopwatch.

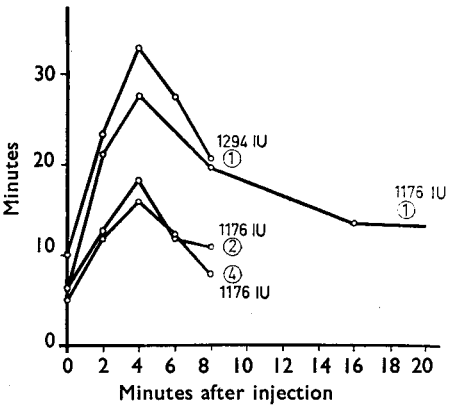

FIG. 3. The effect of small doses of heparin on the coagulation time at different times after injection.

We found a modified Bergquist chamber[9] to be convenient for the determinations of the coagulation time. The chamber, which is 17 by 6 by 4·6 cm., is made of transparent plastic and has a removable lid (Fig. 4). Moistened wads of cotton wool prevent the blood from drying. The floor of the chamber is elevated and has 3 holes, each of which contains a removable plastic cup. The upper surface of the cup is spherically concave, and on it is placed a watch-glass of standard size. A surface of plastic material is unsuitable. The cups, together with the watch-glasses, are rotated by means of a rod provided with cogs, which engage the cogs on the outer surface of the cups. 2 drops of blood are placed on each watch-glass with the Record syringe. When reading off, the chamber should be tilted at an angle 45° against a white background. Owing to the movement of the cups, the readings are fairly distinct.

2 chambers with 3 watch-glasses in each are used for each determination, thus giving 6 single determinations for each sample. In the individual sample, the normal values—i.e., 5 (4 to 6) minutes—usually deviate from each other by 15 to 20 seconds. With longer coagulation times, approaching 20 minutes, the readings are less distinct, and there is difficulty in fixing the end-value. If air bubbles or other impurities are present, the sample must be discarded. A single determination showed in the 10 to 15 minutes group (210 analyses) a spreading (σ) of 1·01 $\pm$ 0·05 minutes, in the 15 to 20 minutes group (157 analyses) 1·20 $\pm$ 0·07 minutes and in the 20 to 30 minutes group (74 analyses) 1·50 $\pm$ 0·12 minutes.

After injecting 800 to 1000 I.U. of heparin, an interval of about 4 hours must elapse before a new dose is given. The excretion apparently proceeded

more slowly at the end of an experiment, when the animal had been given, e.g., 4 injections. A similar delayed excretion has been reported by Merz[10] to occur in man, following the accumulation of repeated doses of heparin. In addition, the coagulation time must have returned to normal

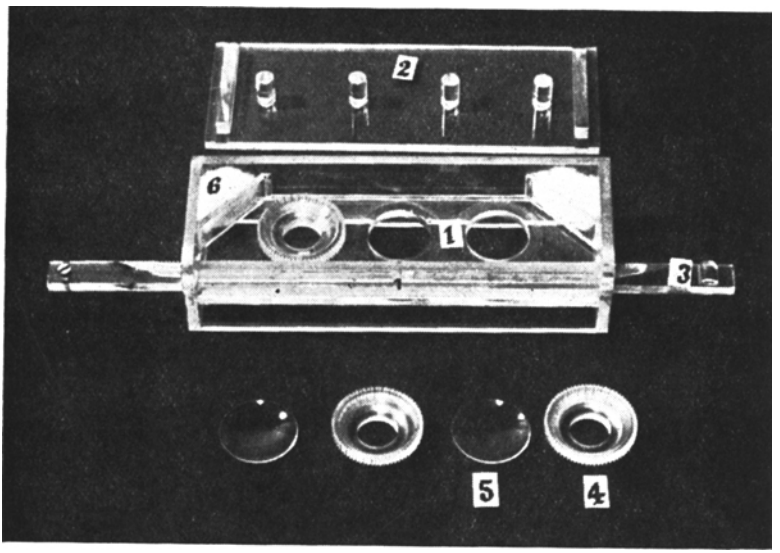

FIG. 4. The modified Bergqvist chamber for the determination of the coagulation time. The perforated floor (1), the lid (2) lying upside down, the rod keeping the cups rotating (3), a plastic cup (4), supporting the watch-glass (5), moistened cotton (6).

before administering a further dose. An elevation of only 1 minute necessitates a new test after ¾ to 1 hour. It is desirable to use 4 or 5 animals concurrently.

In our series of experiments, none of the animals suffered any ill effects. The only precaution taken was to cleanse the operation field with ethanol at the beginning of the procedure, as well as after removal of the polyethylene tubing, the suture around it and the metal plate. Finally, in order to lessen the risk of thrombosis, 50 mg. of heparin were injected at the end of the experiment.

RESULTS

As seen in Figure 5, a difference of 10 per cent. in the quantity of heparin injected resulted in a distinct difference in the coagulation time. Consequently, it should be possible to locate the strength of an unknown sample of heparin between two points differing in strength by 10 per cent. In fact, this proved to be the case.

The first sample assayed was a new Swedish national standard heparin. Its strength had been thoroughly analysed with the fresh whole blood method[1,2] and with the thrombin method of Studer and Winterstein. The former method gave 108·6 I.U./mg. as assayed on 2 days against the old

Swedish standard heparin, and 108·1 I.U./mg. as assayed likewise on 2 days against the international standard heparin. On each of the 4 days, 15 or 18 stands were used. With the thrombin method, and assaying against the international standard heparin, the results on 3 different days were 104, 110 and 105 I.U./mg., respectively (mean: 107 I.U./mg.).

Of the sample assumed to contain 108 I.U./mg. doses of 600 to 1100 units were injected into 4 sheep and curves plotted. Then doses of 1000 and 660 I.U., respectively, of the international standard heparin were given to 2 of the animals. Found: 990 and 614 I.U. 2 doses of 1000 and 1100 I.U. respectively were given to a third animal. Found: 990 and 1025 I.U. Thus, the international standard heparin was 1·0, 7·0, 1·0 and 7·5 per cent., respectively (mean 4·1 per cent.) weaker than the sample used in plotting the curves.

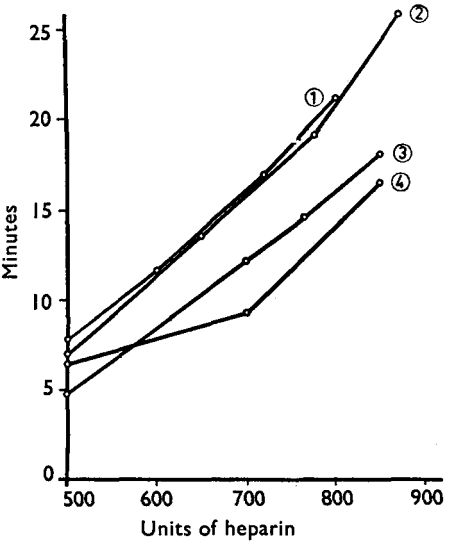

FIG. 5. The effect upon the coagulation time of injecting successively increasing small doses of heparin intravenously in sheep as measured 4 minutes after injection. 1, 2, 3, 4, different animals.

On another day, the international standard heparin was found to contain 123 and 139 I.U./mg. (mean 131 I.U./mg.) when assayed in the same way in 2 animals.

Consequently, the new Swedish national standard heparin contains 108 I.U./mg. of water-free substance as assayed with the fresh whole-blood method, 107 I.U./mg. when assayed with thrombin and citrated plasma, and approximately 110 I.U./mg. when assayed in living animals (sheep).

The next sample analysed was the old Swedish standard heparin, which had earlier been found to contain 81 I.U./mg. (Table I) with the fresh whole-blood method. 4 animals were given injections of the new national standard heparin (108 I.U./mg. of water-free substance) and curves were plotted. The figures found for the old standard heparin in 3 of the animals were 78·3, 80·5 and 82·1 I.U./mg. respectively (mean: 80·3 I.U./mg.). In the fourth animal, the previous dose of heparin had evidently not been completely eliminated when the unknown was given. It is remarkable how closely this figure is in agreement with the previous one, 81 I.U./mg., obtained earlier with the fresh whole-blood method (Table I). The figure found with the U.S.P. XIV method was 67 I.U./mg.

For the sake of comparison the new Swedish standard heparin was also assayed by the U.S.P. XIV method and by the B.P. 1953 method. The first gave on 2 different days with different samples of sheep plasma and 2

dilutions of each of the international standard heparin, and of the new Swedish standard heparin 94·4 and 98·0 (mean: 96·2) I.U./mg. of water-free substance. The B.P. 1953 method gave 106 I.U./mg. on one day and 100 I.U./mg. on another day.

With the B.P. 1953 method the old Swedish heparin standard was found to contain 70·7 I.U./mg. In a series of earlier experiments, performed on 7 different occasions during the course of one year, the mean figure 73 I.U./mg. had been found.

Discussion

As pointed out in previous papers[1,2], fresh whole blood seems to be the most adequate menstruum for measuring the anticoagulant activity of heparin. In all the artificial systems, some factor is lacking or foreign influences are introduced, thus making it impossible to compare the respective methods. In fresh whole blood, both the heparin co-factor and other labile factors are present, and the salt and thromboplastin content is normal. The only unphysiological factor is the mixing of blood with the anticoagulant *in vitro*. Logically, the next step would be to inject heparin directly into the animal and to follow the changes in the coagulation time. The living animal does, in fact, react to increasing doses of heparin in a stoichiometric way. Consequently, the anticoagulant heparin can be assayed in the same way as the other hormones.

The artificial coagulation systems used in the assay of heparin are far from reliable[2]. Some of them, e.g. the recalcified sheep plasma used in the *U.S.P. XIV method*, may give 20 per cent. lower figures than the whole blood method and the *in vivo* technique. The peculiarities of this method are discussed in our earlier paper[2] (page 1039). Of all the methods suggested, this is the most difficult to handle and the least reliable.

The *thrombin method of Studer and Winterstein* has many advantages. It is easy to work with and gives usually a correct level for the strength. Nevertheless, it has its pitfalls. At one time, we consistently obtained values too high by 20 per cent. on analysing 16 different samples of heparin, until we found that a new batch of plasma gave the correct figures. The method can, in fact, give the most peculiar results. Thus, the β-heparin of Marbet and Winterstein[11,12] was found to contain about 50 (sheep) and 25 (ox) I.U./mg. With the whole blood method, the figures were 3 to 4 and 8 I.U./mg., respectively, and with the *in vivo* method 3 and 8 I.U./mg., respectively, as assayed in both the cat and the dog. The U.S.P. XIV method showed no anticoagulant activity whatsoever, or less than 2 to 3 I.U./mg. (Yamashina[13]).

The B.P. 1953 method worked out by Adams and Smith[14] is rapid and simple in performance. As pointed out by Smith the data can be submitted to analysis of variance and the fiducial limits can easily be obtained. Unfortunately, however, this tells very little about the anticoagulant activity, the heparin sample would exert under physiological conditions. In several instances we also found the level of strength indicated by this method to be erroneous. Thus a sample of Liquemin Roche, recently analysed by us, gave only 4480 I.U./ml. on one occasion and 4130 I.U./ml.

on another day instead of 5000 I.U./ml. It is evident that difficulties will arise, if this method alone is applied in assaying commercial samples of heparin.

Since the different methods for the assay of heparin, which utilise artificial coagulation systems, tend to give deviating and sometimes evidently erroneous results (see Table II), there is a need for a reliable technique. For this purpose it seems to be possible to use fresh whole blood *in vitro*. Judging by our experience the fresh whole-blood method, using ox blood, gives figures equivalent to those obtained in injecting heparin *in vivo* in the sheep.

TABLE II

THE STRENGTH OF THE NEW SWEDISH STANDARD HEPARIN (I) AND THE OLD SWEDISH STANDARD HEPARIN (II) AS FOUND IN USING DIFFERENT METHODS OF ASSAY. I.U./MG. WATER-FREE SUBSTANCE

In vivo (sheep)	Fresh ox-blood (Jalling *et al.*[2])	Thrombin method (Studer and Winterstein.)	U.S.P., 1950	B.P., 1953
I 110	108	107	96	103
II 80	81	82	67	73

SUMMARY

1. The anticoagulant activity of heparin has been assayed in sheep, by giving small consecutive doses intravenously at intervals of 4 hours, and determining the coagulation time 4 minutes after injection. A curve is plotted, using doses of a standard heparin varying in strength by 10 to 20 per cent. This is followed by 1 or 2 injections of the unknown heparin in suitable doses. The figures found agree, with a margin of a few per cent., with the theoretical ones.

2. The fresh whole-blood method, using ox blood *in vitro*, is found to give figures identical with those obtained with the *in vivo* method.

3. The unreliability of the methods using artificial systems with citrated plasma or salted whole blood, including the U.S.P. XIV method, is discussed. An *in vivo* method is devised as a reference technique in assaying heparin.

REFERENCES

1. Blombäck, Blombäck, Corneliusson and Jorpes, *J. Pharm. Pharmacol.*, 1953, **5**, 1031.
2. Jalling, Jorpes and Lindén, *Quart. J. Pharm. Pharmacol.*, 1946, **19**, 96.
3. Studer and Winterstein, *Helv. Physiol. Pharmacol. Acta*, 1950, **9**, 6.
4. Howell, *Amer. J. Physiol.*, 1924, **71**, 553.
5. Charles and Scott, *J. biol. Chem.*, 1933, **102**, 425, 437.
6. Jacques and Charles, *Quart. J. Pharm. Pharmacol.*, 1941, **14**, 1.
7. Jorpes, *Biochem. J.*, 1935, **29**, 1817.
8. Hallgren and Björck, *Nord. Vet. Med.*, 1953, **5**, 1.
9. Bergqvist, *Acta chir. scand.*, 1945, **92**, Suppl. 101.
10. Merz, *Schweiz. med. Wschr.*, 1954, **84**, 315.
11. Marbet and Winterstein, *Helv. chim. Acta*, 1951, **34**, 2311.
12. Marbet and Winterstein, *Experientia*, 1952, **8**, 41.
13. Yamashina, *Acta chem. scand.*, 1954. In print.
14. Adams and Smith, *J. Pharm. Pharmacol.*, 1950, **2**, 836.