82. A METHOD FOR ESTIMATING THE POTENCY OF HEPARIN PREPARATIONS

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(*Received* 6 *June 1941*)

THE earliest, crude preparations of heparin, made by the method of Howell & Holt [1918], were labelled in terms of a purely biological unit, defined as the minimum quantity of the material necessary to keep 1 ml. of cat's blood fluid for 24 hr. at 0°. The disadvantages of units so defined, in terms of some potentially variable animal reaction, are now generally recognized. Activity ought rather to be stated in terms of a standard preparation, a unit being then the specific activity contained in a fixed weight of a standard preparation, and measurable by any comparative biological test which is quantitative and specific. The crystalline barium salt of heparin [Charles & Scott, 1936] would appear to furnish a suitable standard of this kind, and Murray & Best [1938] have already proposed the use of a heparin unit, defined as the activity present in 0·01 mg. of this material. Before such a standard and unit can be recommended, however, for general adoption, there are several points concerning this barium salt on which further information is required.

(1) It is important to know whether the crystalline barium salt can be regarded as a pure and accurately reproducible chemical entity, so that all samples of it, complying with certain physical and chemical criteria of purity, can be assumed to be identical in the specific heparin activity. Even if the evidence were to show that different samples of the salt, indistinguishable by ordinary physical and chemical tests, nevertheless vary in activity, this would not, by itself, condemn the barium salt as an unsuitable material for a standard. It would, however, necessitate the definition of the standard, not simply as any pure sample of the barium salt complying with certain criteria, but as a particular quantity of the barium salt, kept in a recognized control laboratory under conditions satisfactorily guaranteeing the complete stability of its activity. Similarly, the unit would then have to be defined as the specific activity contained in a certain weight, not of the crystalline barium salt, wherever prepared, but of this particular standard sample of it.

(2) In any case, if the barium salt is to be regarded as suitable for adoption as a standard material, on any basis, it is important to know whether conditions can be found which will ensure the permanent stability of its specific heparin-activity; and, if so, whether these conditions are compatible with the convenient issue of the samples of the standard, and with its use by those requiring it as a basis of practical biological assay.

(3) A material standard should, ideally, furnish a basis for the definition of the unit applicable in any comparative biological test, and therefore independent of the details of the test so used, provided only that this measures the specific activity in question with sufficient accuracy.

My purpose in the present paper is to describe a method which gives results of good reproducibility, is very economical in time and animal material and

requires no special skill or judgement, since its end-point is sharp and objective. On the other hand, it reveals differences in activity between different samples of heparin, which are definitely greater than those determined for the same samples by methods using whole mammalian blood. The contrast between the results obtained by the two different types of method is sufficient to suggest that even the crystalline barium heparin contains more than one active material; that the activities of different components impede different factors concerned in the complex process of the clotting of shed blood; and that the component which is measured in the test using the clotting of recalcified plasma, as here described, is less stable than another component or components, the measurement of which also enters into a test using the clotting of whole blood.

Principle

Oxalated horse plasma, to which heparin has been added, is recalcified in the presence of an excess of thrombokinase. The clotting time depends on the concentration of heparin. A simple apparatus facilitates the simultaneous measurement of the clotting time in several vessels, containing serial concentrations of the unknown and of the standard heparin respectively.

Reagents

(1) *Oxalated horse plasma.* 9 vol. of blood are run from the jugular vein into a chilled paraffined vessel containing 1 vol. of 0·1 *M* potassium oxalate. Prompt and thorough mixing is essential. The oxalated blood is kept on ice until the plasma can be removed by centrifuging for 20 min. at 2000 r.p.m. The separated plasma is stored at 2°. Its clotting time when recalcified increases with storage, and plasma more than a week old is unsatisfactory for the assay: at room temperature it deteriorates still more rapidly. If, however, the oxalated horse plasma is completely dried in the frozen state while it is still fresh, and then stored under dry nitrogen in the cold, it retains its clotting properties unchanged for an indefinite period. The use of such a dried preparation greatly increases the convenience of the method. The bulked plasma is best distributed into 1 oz. bottles, each containing 10 ml., and kept frozen until drying can be commenced: the contents of one bottle suffice for a day's assays. The reconstituted plasma deteriorates at the same rate as fresh plasma: 4 hr. at 20° cause a measurable loss of clotting activity, which is, however, too small to affect the accuracy of the assay; any longer exposure at room temperature should be avoided.

Human, rabbit or cat plasma gives less satisfactory results, the end-point being less sharp in any of these cases than that obtainable with horse plasma.

(2) *Heparin* is dissolved in 0·9% NaCl. The concentration should be that which produces a clotting time of between 70 and 500 sec. in the recalcified system. With the use of fresh oxalated plasma this will be approximately 2 to 4 Toronto units per ml. It is convenient to use 3 concentrations each of standard and unknown: these may be in the ratio 80 : 100 : 125.

(3) *0·025*M $CaCl_2$.

(4) *Thrombokinase solution.* A rabbit is anaesthetized with nembutal or dial and bled out from the abdominal aorta under artificial ventilation. The right ventricle is incised, and the fore parts are perfused from the thoracic aorta with Locke's solution until the fluid returning by the jugular vein is colourless. The brain is then removed, freed from dura and ground in a mortar to a smooth paste. This is spread in a thin layer on a glass plate and dried, first in air at 37°,

and then *in vacuo* over P_2O_5. The powdered material can be kept for several months in the refrigerator without appreciable loss of potency. On the day of the experiment, a 3 % suspension is made in 0·9 % NaCl. This is warmed to 55° for 10 min. in a water bath, with thorough stirring; solid particles are then removed by centrifuging for 10 min. at 2000 r.p.m., and the milky supernatant is used. More prolonged centrifuging gives a clearer but less active fluid.

Glass-distilled water is used for all solutions, and all glassware must be thoroughly clean.

Procedure

Double-bulbed pyrex clotting tubes of the form shown in Fig. 1 are used. The design is of some importance: the bulbs should be spherical rather than tubular in shape, and the angle between them should be great enough to permit the contents of one bulb to drain completely into the other when the tube is inclined through 70° from the vertical. Six such tubes are conveniently held by spring clips on a horizontal wooden rack affixed to a rod which can be tilted by hand about its long axis. The whole apparatus is so arranged that it can be placed in a thermostat at 37°.

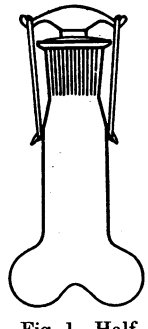

Fig. 1. Half actual size.

0·1 ml. of thrombokinase solution and 0·1 ml. of 0·025 *M* $CaCl_2$ are measured into one bulb of each tube, and 0·1 ml. of heparin solution and 0·1 ml. of oxalated plasma into the other bulb. A 0·5 ml. micrometer syringe of the type devised by Trevan [1926] is useful for measuring the reagents. Since heparin, added to plasma, does not immediately become fully effective [Quick, 1936], the plasma and heparin in each tube should be mixed at as nearly the same moment as possible. The rack carrying the tubes is placed in the thermostat, and left for 20 min., to enable the effect of the heparin on the plasma prothrombin to reach a practically uniform degree in all tubes. The reagents are then mixed simultaneously in all tubes by tilting the rack several times. The end-point is the formation of a solid clot, which is not displaced when the tube is tilted, and the time required is noted for each tube. It is important not to tilt the rack too often or too far, since the incipient clot may thereby be dislodged or broken, and the apparent clotting time prolonged. A horizontal mark, on each bulb of the series in which the clotting mixtures are kept for observation, facilitates the determination of the end-point.

The clotting time can, with a little practice, be measured with an accuracy of 5–10 %. Since an increase of 25 % in the heparin concentration may, over the optimal range, more than double the clotting time, the precision of the method is potentially high, and, indeed, is limited in practice by other errors than those involved in judging the end-point.

Calculation

If clotting occurs at the same time in two tubes, these are judged to contain the same number of anticoagulant units. Intermediate values are obtained by plotting the clotting time against concentration of the standard heparin, and interpolating for the clotting time of the unknown. No simple formula expresses the relationship between clotting time and heparin concentration: this varies somewhat from experiment to experiment, unless elaborate precautions are taken to maintain constant activity of the reagents.

A specimen protocol shows the type of result obtained.

Heparin	Concentration (μg./ml.)	Clotting time (sec.)
Standard	20	110
,,	25	230
,,	31·2	500
Unknown	64	100
,,	80	190
,,	100	420

Whence 80 μg. of unknown = 23·2 μg. of standard; 100 μg. of unknown = 29 μg. of standard; and potency of unknown = 29 % potency of standard.

Error of the method

Five dilutions of a standard heparin solution, the strengths of which were unknown to the experimenter, were assayed in triplicate, 3 tubes of standard and 3 tubes of unknown being used in each experiment. The standard deviation from theory of the 15 single assays was 2·9 %, the largest error being 5·6 %. The standard deviation of the 5 triplicate assays was 1·1 %, the largest error being 1·5 %.

The result given by the method is unchanged by the addition, to one part by weight of the sample to be assayed, of two parts of any of the following substances: dried rabbit's brain, Ba (as $BaCl_2$), NaOH, HCl, tricresol, mucoitin sulphuric acid, heparin inactivated by prolonged boiling with acid alcohol. The method can therefore be used for the assay of preparations of the barium salt of heparin, or of solutions for clinical use containing tricresol as preservative, and, presumably, for following the activity of crude material during its purification.

Comparison with other methods

While this method was being developed, it was observed, unexpectedly, that certain highly purified preparations of heparin showed differences in activity of as much as 35 %, which could not be explained by differences in ash or water content. Such variations in potency were found among samples of the crystalline barium salt, as well as among samples of the sodium salt prepared from it, and were perfectly reproducible from one assay to another. It was therefore important to know whether such qualitative differences would lead to the assignment of different relative potencies for these preparations, when they were assayed by other methods. I was fortunate in obtaining the co-operation of Dr F. J. Dyer and Dr R. Wien of the Department of Pharmacology of the College of the Pharmaceutical Society, and of Mr W. A. Broom and Mr E. M. Bavin of the Pharmacological Department of Boots Ltd., who had been using for the assay of heparin, in their respective laboratories, methods based on the use of fresh rabbit's blood. They will themselves report in more detail some of their results, but they permit me to summarize them here. Briefly, where unexplained differences of potency were found by the present method, they were also found by the rabbit's blood assay, but were smaller in the latter case: the difference between the results of the two methods was, in two cases out of five, statistically significant. One of these was a crystalline barium salt preparation which, compared with the temporary British Standard (110 units/mg.) assayed 73 units/mg. by the plasma-kinase method, and 92 units/mg. by the rabbit's blood method. The other was a specimen of the same barium salt, whose activity had been deliberately reduced by treatment with acid methyl alcohol: it assayed 19 units/mg. by the plasma-kinase method and 40 units/mg. by the rabbit's blood

method. From these last results it is clear that the partial inactivation caused by acid treatment appears greater by the plasma-kinase test than by the whole-blood test. It seems probable that most of the differences of potency found among samples of 'pure' heparin, and the divergent results obtained by different assay methods, may be due to the presence of varying amounts of material whose activity has thus been reduced by acid treatment. In one experiment, in fact, recrystallization of the barium salt in the presence of warm acetic acid, according to the usual technique, was found to reduce the potency by some 35% as found by the plasma-kinase test; on the other hand, recrystallization at nearly neutral reaction, in the presence of excess barium acetate, left the potency unchanged. If it is true that any submaximally active heparin preparation has been partly denatured by acid during the processes leading to its isolation, it follows that the plasma-kinase test gives a more delicate indication of the extent of the denaturation than does the whole-blood test. It does not follow, however, that the plasma-kinase test supplies more reliable data on the therapeutic efficiency of the material, for nothing is known about the relative activity of partially denatured heparin in the human circulation.

The present method is believed to offer several advantages over other biological methods on ground of convenience. By its use one worker can complete within a working day 8 or 10 comparisons, the results of which are reproducible within limits of $\pm 5\%$; no particular skill or judgement is required, and the cost in animal material is low, one rabbit's brain supplying enough thrombokinase for some 40 determinations. The question of the suitability of this method, as a measure of that action of heparin required in practical use, is considered further in the following paper.

The choice of a standard heparin

It has already been noted that Murray & Best [1938] have suggested that the crystalline barium salt would be a suitable material. It is clear, however, that samples of the barium salt, in spite of their crystalline nature and their apparently identical behaviour in physico-chemical tests, may vary considerably in anticoagulant potency, even when prepared from the same animal species. Recent work by Jaques & Waters [1940] and Jaques [1940] shows that still larger potency differences may be expected for crystalline preparations of barium-heparin from different animal species, again with no corresponding differences which can be detected by the physical and chemical methods so far applied. If the standard were to be made of the crystalline barium salt of heparin, it would, therefore, be necessary to choose a particular sample of the salt as the standard, and to define the unit of anticoagulant activity in terms of this sample. It must be remembered, however, that the chief advantage expected from using the barium salt as a standard depended on its supposed purity and invariability. If it is not, in fact, invariable in activity, we have to consider whether it is otherwise suitable.

On such other grounds it would seem preferable to use a sample of the sodium salt rather than the barium salt as a standard. The barium salt as crystallized contains some 12% of water and its aqueous solutions are strongly acid [Charles & Todd, 1940]. In view of the well-known susceptibility of heparin to acid inactivation, its stability over a long period might be doubted. I have, in fact, found that a sample of crystalline barium salt, which had been kept at laboratory temperature for about a year, had retained only about one-third of the potency expected for it. The possibility of a slow inactivation in the refrigerator, though a longer period might be required for its certain detection,

cannot be ruled out. The sodium salt, on the other hand, can be prepared in the fully neutralized condition, from material purified by crystallization as the barium salt. I have found it to be extremely stable, even in dilute solution and at high temperatures: unlike the barium salt, it can be sterilized by autoclaving without loss of potency. The standard sodium salt might be stored in the completely dried state in the central control laboratory, and (to avoid the difficulties of weighing small quantities of hygroscopic material) issued in the form of a sterile solution having a defined period of currency.

Summary

1. A method for the standardization of heparin preparations is described. It involves the use of oxalated horse plasma, which is recalcified in the presence of heparin and an excess of thrombokinase. It gives results reproducible to $\pm 5\%$.

2. Oxalated horse plasma, though inconveniently unstable in the fluid condition, appears to keep indefinitely when completely dried in the frozen state, and retains its clotting properties unchanged when redissolved.

3. The sodium salt of heparin, prepared from the crystalline barium salt, is probably more stable than the barium salt itself, and is therefore a more suitable material for use as a permanent reference standard.

4. The limitations of methods for the assay of heparin are discussed.

I wish to thank Sir Henry Dale for his interest and advice, and Prof. A. R. Todd for several samples of heparin. I am much indebted to Dr R. I. N. Greaves of Cambridge University for the preparation of a large stock of stable dried plasma.

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Addendum

Since this paper was written, Jaques & Charles [*Quart. J. Pharm. Pharmacol.* 1941, **14**, 1] have described, among other methods for the standardization of heparin, one which depends on its anticoagulant activity in a system consisting of thrombin and oxalated blood. They note two drawbacks of the method: (*a*) barium interferes by precipitating oxalate, so that samples of barium heparin cannot be accurately assayed; (*b*) the clotting properties of oxalated blood (and still more so those of oxalated plasma) change with keeping, so that reproducible results are secured with difficulty. The method which I have described in this paper, although it uses oxalated plasma, avoids these objections. The quantity of barium added in the form of heparin barium salt alters the Ca concentration in the clotting mixture by less than 2%, a change insufficient appreciably to affect the clotting time, as shown by my control experiments in which barium was deliberately added in the corresponding proportion; and the use of plasma stored in the stable dry form removes any uncertainties due to variations in the composition of this reagent.