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A METHOD FOR THE DETERMINATION OF HEPARIN IN BLOOD

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Best (1946) has pointed out that an accurate method for the determination of small amounts of heparin in blood is very desirable for many studies in the field of blood clotting and thrombosis. The methods available for this purpose up to the present have been the correlation of heparin concentration with the clotting time (cf. Jaques, 1939), the titration of heparin with protamine (cf. Jaques & Waters, 1941), and the isolation of the heparin by the procedure of Charles & Scott (1933). The first method is applicable only to a very limited range of concentrations and requires that there be no change in factors affecting the clotting system other than heparin. The protamine titration also requires a clotting system and is of little value with small concentrations of blood heparin. The Charles & Scott procedure can be applied only to large volumes of blood containing high concentrations of heparin. For these and other reasons, experiments have been undertaken in this laboratory in an attempt to develop a direct method for blood-heparin estimations.

The method for the recovery of heparin from blood described in this paper is an application of the fact that the strongly acid heparin will form salts with proteins or complex bases in stoichiometric proportions (Fischer & Astrup, 1935; Fischer, 1935; Jaques, 1943). In a private communication, Dr A. F. Charles of the Connaught Medical Research Laboratories, University of Toronto, informed us that heparin combines with the straight-chain amine, *N*-octylamine. On the basis of his suggestion, tests were made of this reagent and we found that *N*-octylamine hydrochloride reacted with heparin at the pH of the blood, and also precipitated heparin directly from plasma. Furthermore, a critical concentration of octylamine precipitated over 90% of heparin added to plasma with a minimal precipitation of protein. The stable octylamine salt of heparin could then be hydrolysed by heating to 70° C. in relatively strong (0·1 N)-NaOH, and the opaque, colloidal solution obtained could be purified by precipitating the heparin with brucine at pH 5·5–6·0.

METHODS

A. *The extraction of heparin from blood*

Reagents

Sodium citrate: 3·8% solution.

Octylamine: 7% (v/v) solution. To 7 ml. of *N*-octylamine add 7 ml. of distilled water and 3·4 ml. of concentrated hydrochloric acid. After the octylamine has gone into solution add enough distilled water to make up to a volume of 100 ml. The pH is usually between 7·0 and 8·5. If above or below this range, the pH must be adjusted by the cautious addition of concentrated hydrochloric acid or NaOH. A fresh octylamine solution must be made weekly.

0·1 N-NaOH *and* 0·05 N-NaOH.

Ether.

Ethyl alcohol 95%.

Brucine phosphate solution: 10 g. of brucine alkaloid are dissolved in 50 ml. of warm 95% ethyl alcohol. Concentrated phosphoric acid (Merck, sp.gr. 1·750) is diluted 1 : 10 with distilled water and 14 ml. of the dilute acid are added to the warm brucine solution. The solution is set aside to evaporate to dryness. The crystalline brucine phosphate is obtained as white needles; 5 g. of brucine phosphate are dissolved in 100 ml. of water. The pH of this solution must be 5·5.

Procedure

Nine ml. of blood are drawn and added to 1·0 ml. of sodium citrate solution and centrifuged. To the plasma, 1·0 ml. of the octylamine solution is added, and after standing 2 min., the mixture is centrifuged at 2000 r.p.m. for 10 min. The supernatant is decanted and the tube allowed to drain several minutes. The precipitate is broken up thoroughly with a stirring rod and 2·0 ml. of 0·1 N-NaOH are then added and the tube placed in a water-bath at 70° C. for 15 min. The tube is cooled to 30–40° C., 4·0 ml. of ether are added and the octylamine extracted from the aqueous layer by inverting the tube gently three or four times. The ether layer is removed with a pipette and the remainder of the ether removed by placing the tube in a water-bath at 40° C., the temperature of which is gradually raised to 70° C.

The tube is then cooled and 4 ml. of the brucine phosphate solution are added. After standing 5 min., the precipitate of brucine-heparin is collected by centrifuging at 2000 r.p.m. for 5–10 min. The supernatant is then decanted off and the tube allowed to drain. The precipitate is washed by mixing with 4·0 ml. of 95% ethyl alcohol, using a stirring rod. After centrifuging, the precipitate is similarly washed with 4·0 ml. of ether and centrifuged, the ether is decanted off, and the remaining ether is removed by drying the precipitate in a vacuum desiccator for at least 1 hr.

To the precipitate are added 2·0 ml. of 0·05 N-NaOH solution and the tube is placed in the water-bath at 70° C. for 15 min. It is then cooled to room temperature.

The heparin present in this extract can now be estimated by one of the known methods, after adjusting the pH by the addition of buffer. The method we have used routinely for the determination of heparin is the estimation of its metachromatic activity with Azure A as described by Jaques, Mitford & Ricker (1947). We have also used the thrombin method described by Jaques & Charles (1941), particularly for low concentrations of heparin in blood.

Remarks on the procedure

Citrate must be used as the anticoagulant, since octylamine precipitates with oxalate and in fact will clot oxalated blood. The amount of octylamine used appears to be sufficient to precipitate the heparin quantitatively and also to precipitate a small portion of the plasma proteins. A larger quantity of octylamine precipitates increasing amounts of protein. The small quantity of protein precipitated with this quantity of octylamine appears necessary to

ensure satisfactory flocculation of the octylamine-heparin precipitate. With an occasional sample, it is necessary to add slightly more octylamine to obtain satisfactory separation of the precipitate. This is observed particularly in fatty human plasmas. On standing 30 min., the octylamine may cause a gelling of the plasma and hence it is necessary to centrifuge immediately. For hydrolysis of the octylamine-heparin compound, a temperature of 70° C. is required and it is advisable to use a constant temperature water-bath. After hydrolysis, an opaque, colloidal solution is obtained. The opacity clears on warming after treatment with ether but reappears when all the ether has been driven off.

Under the conditions described (a brucine phosphate concentration of 3·3% and a pH of 5·5–6·0), brucine precipitates heparin quantitatively. Washing with alcohol and ether removes not only excess brucine but also lipids and colour which interfere with the determination of heparin. If much colour is present as a result of haemolysis this can be removed by longer contact with the alcohol.

After hydrolysis of the brucine-heparin compound, a clear solution is usually obtained. Rarely, a slight protein precipitate appears and may give a weak gel. It is then necessary to add the buffer, used in the assay procedure presently to be described, to the tube in order to provide sufficient volume of solution that the protein can be removed by centrifuging.

B. *The determination of heparin in blood extracts*

The method used routinely by us is the method of Jaques *et al.* (1947) based on the measurement of the metachromatic activity of heparin with the dye, Azure A. Certain minor modifications to increase the sensitivity of this method have been made and are included in the following description. For a discussion of the principles involved in this method and sources of error (interfering substances, etc.), reference should be made to the original paper.

Reagents and equipment

Azure A: 100 mg. % of Azure A (certified biological stain of dye content 82%). This is diluted 1 : 8·5 before use.

Phosphate buffer: a potassium phosphate buffer, pH 7·3 and 0·15 M-phosphate is prepared. This is in place of the borate buffer used by Jaques *et al.* The latter is not suitable for use when the heparin is to be determined at the same time by its antithrombin activity. If other methods are used for the final determination of the heparin, the buffer may need corresponding modification.

Lovibond tintometer: model no. 1. The slide carrying the neutral tint glasses is replaced with a slide carrying each of 10 and 20 red, 10 and 20 blue glasses. To increase the length of light path without a marked increase in volume of the solution, a specially constructed cell (inside dimensions 9 mm. wide, 27 mm. long, and 38·5 mm. high) is used. The light apertures of the tintometer box are partially masked to reduce the width to less than that of the glass cell (9 mm.).

Procedure

Unknown heparin solutions are dissolved in saline or distilled water as desired. No measurable metachromatic difference has been observed whether the heparin is dissolved in saline or in distilled water. Saline is used if an assay for anticoagulant activity is to be conducted on the same solution.

To a given volume of the heparin solution is added saline to 2·0 ml. Two ml. of buffer are added

and then 1·0 ml. of Azure A solution. The solution is mixed and transferred to the glass cell. The colour of the cell solution is matched immediately in a Lovibond tintometer using the red and blue glasses. From the red reading obtained, the heparin content of the sample is determined on the standard curve (Fig. 1). For maximum accuracy, a dilution which will give a red reading between 5 and 10 is found for the final assay. With unknown heparin solutions of high concentrations, the dilution required for this may be determined approximately by diluting the first assay with an equivalent dilution of the dye in buffer and saline until the red reading falls into the desired range. As recommended by Jaques *et al.*, instead of a duplicate determination, a second reading is made with a quantity of the unknown less or greater than that used in the first determination.

The calibration curve for the Lovibond tintometer is established using a series of dilutions of the standard heparin solution. Some difference in the values for the calibration curve are found with different observers but the values are reproducible for any given observer.

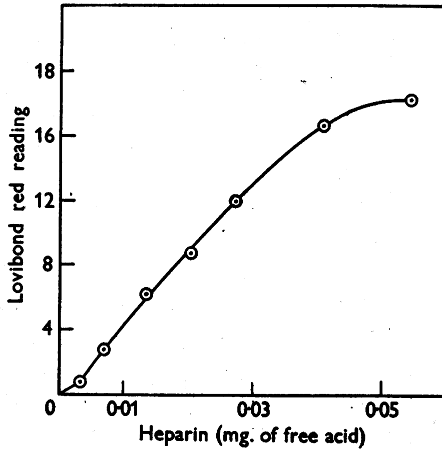

Fig. 1. Calibration curve for the estimation of heparin metachromatically.

Expression of heparin concentration

The concentration of heparin in blood and tissues, in agreement with general biochemical usage, should be expressed in terms of the weight of heparin in a given volume or weight of the material. Up to the present there have been relatively few investigations on heparin where this was a problem. While heparin is issued commercially on the basis of anticoagulant units, it would appear advisable to reserve this for expressions of the anticoagulant activity, *per se*. Heparin concentration might be expressed in milligrams of equivalent barium salt, sodium salt, or of the free heparin acid. The use of the barium salt of Charles & Scott as the reference expression has the advantage of ease of convertibility from weight to anticoagulant units (1 mg. = 100 units). The sodium salt will be the actual comparison standard used in most cases. Both these salts are produced *in vitro* and do not represent the actual heparin present, probably in the form of a protein salt. In agreement with the custom used for many biologically active substances we express the concentration of heparin as mg. of free acid heparin. The value reported by Jaques & Charles (1941) for the

anticoagulant potency of beef heparin is 156 units/mg. of free acid heparin present.

In all cases, we determine the concentration of heparin in terms of equivalent weight of the Provisional International Standard of heparin, issued by the National Institute for Medical Research, London, for the Health Organization of the League of Nations. In the absence of figures for the salt content of the International Standard, it is not possible to determine directly the amount of free acid heparin present. However, from sulphur content is obtained a conversion factor of 0·884 for converting the weight of International Standard to weight of free acid heparin. It is to be understood then that all values will be reported as $0{\cdot}88 \times$ equivalent weight of International Standard. While the factor used may be in error by as much as 3% and is subject to future correction, this error is not significant for the purposes of the method. This represents an assigned value of 148 units per mg. for the anticoagulant activity of the free acid heparin in the International Standard.

Heparin standard

As standard, a sample was used of the Provisional International Standard of heparin (130 i.u./mg.) supplied through the courtesy of Dr F. C. MacIntosh. A solution of $76{\cdot}9\,\mu$g. (10·0 i.u./ml.) was prepared, and with this the standard curve (Fig. 1) has been constructed. For routine standard solutions, a secondary standard prepared from Connaught heparin and assayed against the International Standard has been used.

For the experiments on the recovery of added heparin, Connaught heparin was used. The lot used was found to have 90% of the metachromatic activity of the International Standard. The difference represents the water content of the sample.

RESULTS

In Table 1 is shown the recovery obtained by the method of commercial heparin added to canine blood. From 0·02 to 0·36 mg. of heparin was added to a total volume of 10 ml. The average recoveries in two series were 87 and 90%. The recovery was as high with the lowest concentration of heparin (0·002 mg./ml.) as with the highest (0·036 mg./ml.). As shown on comparing series B with series A, incubation of heparin with blood at 37° C. for $3\frac{1}{2}$ hr. had no effect on its recovery. Recovery of the heparin on addition to the plasma of the same blood was complete (98%). This suggests that the 10% not recovered on the addition of heparin to blood is due to loss of heparin in the cells. This is partly due to failure completely to remove the plasma from the centrifuged cells. It may also be partly due to adsorption of heparin on the white cells as reported by Fischer (1936). While routine recoveries are not always as high as in this series, recoveries are always better than 80% of the added heparin, indicating

that losses due to the manipulative procedures are less than 10%. On the basis of these results, a correction factor of 12% is added to the found value to give the amount of heparin present in samples of dog blood.

TABLE 1. Recovery of heparin after addition to dog blood

Heparin added (free acid, mg.*)	Heparin recovered A mg.	A %	B mg.	B %	C mg.	C %
0·000	0·000	—	0·000	—	0·000	—
0·022	0·020	92	0·019	87	0·022	100
0·045	0·038	85	0·041	91	0·039	87
0·090	0·072	80	0·081	90	0·098	109
0·181	0·156	86	0·172	95	0·172	95
0·362	0·326	90	0·312	87	—	—
Average	—	87	—	90	—	98

A, 1·0 ml. of heparin solution added to 9·0 ml. of citrated blood. B, Same but blood plus heparin incubated for 3½ hr. before centrifuging. C, 1·0 ml. of heparin solution added to the plasma from 9·0 ml. of citrated blood.

* To convert to International anticoagulant units, for beef heparin multiply by 148. For other species of heparin, this factor must be determined (cf. Jaques & Charles, 1941).

TABLE 2. Recovery of heparin after addition to human blood

Heparin added (free acid, mg.)	Heparin recovered Blood A mg.	Blood A %	Blood A mg.*	Blood A %	Blood B mg.	Blood B %	Blood C mg.	Blood C %
0·000	0·000	—	0·000	—	0·005	—	0·000	—
0·022	0·011	50	0·011	50	—	—	0·015	68
0·045	0·029	65	0·030	67	0·027	60	0·025	56
0·045	0·030	67	0·028	62	—	—	0·033	73
0·090	0·068	76	—	—	—	—	0·055	61
0·181	0·123	68	—	—	0·167	92	0·117	65
0·362	0·292	81	—	—	0·296	82	0·232	64
Average	—	68	—	—	—	78	—	65

1 ml. of heparin solution added to 9·0 ml. of blood.

* From serum: for this the citrate was omitted, the blood allowed to clot, and the serum removed next morning.

Table 2 shows recoveries obtained with human blood. The average recovery was 18% lower than obtained with canine blood, i.e. ran from 60 to 80% of the amount of heparin added. This means that a larger correction factor (+31%) must be added to the values obtained for human blood. As shown in Table 2, no difference between plasma and serum has been found for the recovery of heparin.

As shown in Table 2, blood sample B, without addition of heparin, yielded a material by this method which showed metachromatic activity. The amount of this material in 10 c.c. of normal blood is usually below the sensitivity of the metachromatic test. Treatment of 40 ml. of human blood provided sufficient

material for tests of metachromasia, in which the material showed qualitatively the same metachromatic powers as heparin. The material also showed good antithrombin activity. These results have indicated that the normal concentration of 'heparin' in human blood is of the order of 0·0001 mg./ml. Studies of this material are now in progress. Canine blood yielded a precipitate by this method but only occasionally did this material show metachromasia.

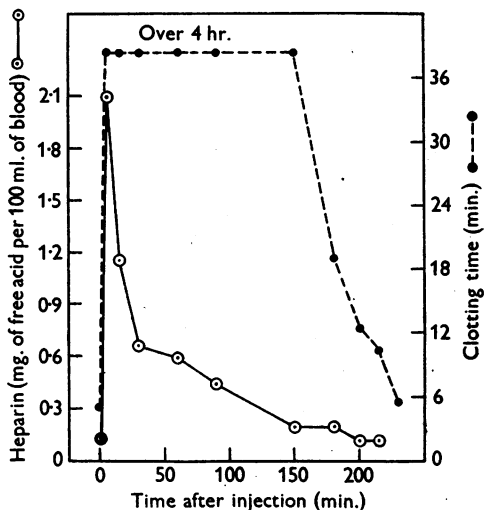

Fig. 2. Clotting times and blood-heparin concentrations after intravenous injection of heparin in the dog. Dog of 22·5 kg. given 45 mg. of sodium heparin at zero time. Concentration of heparin expressed in mg. of free acid per 100 ml. of blood.

In Fig. 2 is shown the clotting time and blood-heparin concentration for a dog weighing 22·5 kg., following the intravenous injection of 45 mg. of sodium heparin. Immediately after the injection the blood-heparin concentration was 0·021 mg./ml. expressed as free acid heparin. Calculated from the quantity of heparin injected and the blood volume (assumed to be one-eleventh of the body weight) the blood-heparin concentration should have been 0·0175 mg./ml. This indicates that the method is satisfactory for the determination of heparin added to blood *in vivo*. Also of interest as a new observation is the very rapid fall in the blood-heparin concentration until a level of 0·005 mg./ml. (expressed as free acid heparin) is reached. Thereafter the disappearance of heparin from the blood is more gradual. The clotting time only returned to measurable values 3 hr. after the injection, when the blood-heparin concentration was 0·0017 mg./ml. The clotting time then rapidly returned to normal with the further decrease in blood-heparin concentration.

SUMMARY

1. A method for the separation of heparin from blood is described.

2. Blood is collected in citrate and the heparin precipitated from the plasma with *N*-octylamine. The heparin is reprecipitated with brucine.

3. Recoveries of 80–90% are obtained for heparin added to blood, while complete recovery of added heparin is obtained from plasma.

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