A SIMPLE METHOD FOR THE PURIFICATION OF HEPARIN

by

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INTRODUCTION

A very useful method for the extraction of heparin from animal material has been described by CHARLES AND SCOTT[1, 2]. From the literature it appears that this extraction method is generally adopted.

On the other hand a great number of methods have been described for the further purification of the crude product thus obtained. Often impurities have been precipitated by variation of the acidity, either with or without the addition of alcohol or acetone[2, 3, 4]. In addition adsorption methods[1, 3, 4, 5, 6] have been used for the removal of impurities. LLOYD'S reagent and charcoal have been mentioned as adsorbents. With somewhat purified heparin, precipitation of further impurities by salts of heavy metals (Cd, Pb, Ba) is frequently employed[3, 6, 7].

A rather important problem in the purification process of crude heparin is the removal of proteins. FISCHER[8] observed an association between heparin and proteins at the acid side of the isoelectric point of the latter. This linkage is reversibly dissolved at the alkaline side of the isoelectric point, a property of great importance when choosing the conditions for the purification of crude preparations. For instance, it is at once obvious that extraction of the animal tissues with alkaline solutions as proposed by CHARLES AND SCOTT[1, 2] will be the method of choice.

By acidification of this extract proteins are precipitated from the solution together with practically all the heparin.

These proteins in the crude product may give rise to serious losses of heparin in those purification methods where an acid reaction is imperative. In particular the adsorption methods are not without objections. If the heparin is purified with heavy metals an acid reaction is again required, involving losses by co-precipitation with proteins.

In this paper a method will be described which avoids these difficulties by introducing a partition of the crude heparin between water and phenol. Many proteins can be dissolved in phenol and, on the other hand, heparin remains in the aqueous phase. This partition can be effected at neutral or even weakly alkaline reaction. The losses of heparin seldom amount to 10% and, apart from the proteins, many of the coloured impurities are removed with the phenol phase.

The purification with phenol has been successfully employed with crude heparin obtained from cattle lungs and livers and from hog livers.

A previous digestion of proteins with trypsin is to be recommended, as it cuts down the number of necessary partitions.

If in the purification of the heparin from lungs the tryptic digestion is omitted and

References p. 337.

two preceding purifications, as described later, are inserted, it is necessary to shake out twice with phenol instead of once in order to remove all of the considerable amount of proteins then present. In this case the loss of heparin is correspondingly larger and may amount to 15%.

From the heparin solutions, obtained after the phenol purification, the non-protein and coloured impurities may be succesfully precipitated from an acid solution by lead acetate. The heparin-lead salt is precipitated by the addition of alcohol. The lead salt is converted into the neutral sodium salt. In this way colourless preparations, completely soluble in water, are obtained.

The activity of the dried product, containing on the average 10% of moisture, is invariably around 100 International Units per mg when cattle lungs or livers have been used as the starting material. From hog liver, heparin with an activity around 60 International Units per mg was obtained. By far the best yields were obtained from cattle lungs. The cattle heparin was non-toxic to rabbits on intravenous administration of doses of 1 g.

Anaphylactogenic reactions in guinea pigs were never observed with the many batches investigated. Neither was there any effect on the blood pressure.

EXPERIMENTAL

1. *Extraction and preliminary purification*

In the first stages of our process the method of CHARLES AND SCOTT is adopted in principle. In accordance with KUIZENGA AND SPAULDING[7] it has been found that the method of autolysis of cattle lung greatly influences the yield. The crude precipitate, obtained by acidification to p_H 2.5 of the extract by means of sulphuric acid is washed twice with water adjusted to p_H 2.5 with the same acid. This removes ammonium sulphate, originating from the crude extract, which would otherwise interfere later on in the lead-acetate purification. After washing the residual precipitate with alcohol it is subjected to tryptic digestion according to CHARLES AND SCOTT. The digestion is continued until no further change in p_H is apparent.

The solution thus obtained is strongly coloured and still contains proteinous material, in particular if the acid precipitate has been abundant. The amount of this precipitate may vary considerably and it is therefore recommended to use the following purification method, before fractionating with phenol.

2. *Purifications with carbonate and by heat*

All data refer to quantities obtained from 10 kg of original material. To the tryptic digest, with a volume of 1–1.5 l, 200 ml N-ammonia and 300 ml of 20 W/V% ammonium carbonate are added. The solution is brought to 60° C and slowly cooled to room temperature. The inactive precipitate, which, amongst others, contains all the insoluble inorganic carbonates, is removed by centrifuging. The solution is again brought to 60° C and the p_H adjusted to 7.5 with acetic acid. At this p_H the solution is boiled gently until a distinct coagulation occurs. This operation takes less than an hour. After cooling slowly to room temperature, the dark inactive precipitate is removed by centrifuging. The crude heparin is precipitated by the addition of two volumes of alcohol to the heated solution. The quantity and purity of this heparin may vary. From cattle lungs and livers the activity is from 10 to 20 International Units per mg. The yield in this stage for

References p. 337.

cattle lung will be some 200000 Units. Often the phenol purification can be used successfully without applying the last-mentioned coagulation method, but since many dark-coloured pigments are removed by boiling the solution, it is recommended.

The heparin, thus obtained is suspended in 200 ml 20 V/V% alcohol, to which 4 ml 20 W/V% ammonium carbonate solution have been added. The suspension is heated to 60° C and the p_H adjusted to 7.5 if necessary. The inactive precipitate formed after cooling to room temperature is removed, and the supernatant liquid subjected to the phenol purification.

The losses in these preliminary purifications do not surpass 10%. If the p_H of the 20% alcohol is too low, considerable losses may occur by adsorption of heparin on the precipitate. For instance if the above-mentioned supernatant liquid of p_H 7.5 is adjusted to p_H 5, the loss after 3 minutes at 60° C will amount to 30%; after one hour at 90° C all heparin is lost in the precipitate.

3. *Phenol purification*

The above-mentioned heparin solution is diluted with water to 400 ml and subsequently shaken with 200 ml of phenol liquified with 20% water. The p_H is adjusted to 7–7.5. The mixture is left overnight to settle. Usually there is a good separation of the two layers; if not, it can be promoted by centrifugation. Usually the aqueous phase is completely free from proteins after having only once been shaken out, and does not give a reaction with sulphosalicylic acid after dilution and acidification.

On the average the loss of activity amounts to less than 8%. If the tryptic digestion is omitted, a second treatment with phenol becomes necessary with a correspondingly greater loss.

From the phenol phase dark-coloured inactive proteinous material can be precipitated by acetone and ether.

The heparin is isolated from the aqueous solution by the addition of 0.7% NaCl, followed by two volumes of alcohol. The heparin is centrifuged down and washed with alcohol and ether for the complete removal of phenol. In the air-dried state this preparation contains 30–50 Units per mg.

4. *Final purification*

The heparin powder is dissolved in 200 ml 0.2–0.3% ammonium acetate solution. The p_H is brought to 5 with acetic acid and the solution heated to 70° C. A 10% solution of lead acetate, adjusted to p_H 5 with acetic acid, is now added in such amounts that the impurities give a coarse precipitate. The supernatant liquid is almost colourless. The inactive precipitate, containing lead, is removed at room temperature in the centrifuge. From the solution the heparin is precipitated with 1.5 volume of alcohol at 60° C. The dry precipitate is transferred to 80 ml 2.5% ammonium acetate solution, the p_H adjusted to 5 and the temperature is raised to 60° C. If on cooling again to room temperature a precipitate occurs, it is removed by centrifuging. If this precipitate contains much lead sulphate serious lossed of heparin will be found, owing to adsorption. For this reason the sulphate ions were removed earlier in the process (see 1).

In the filtrate 0.7% NaCl is dissolved and the active lead salt is isolated by precipitation at 60° C after the addition of 1.5 volume of alcohol.

The active lead salt is dried with alcohol and ether and dissolved in 60 ml water at ordinary temperature; while stirring the p_H is brought to 5 and any impurity is removed

References p. 337.

by centrifuging. At 60° C 12 ml of 20% sodium carbonate solution are added. The resulting lead carbonate is removed after the solution has cooled again. The acid sodium salt of heparin[9] is precipitated from the solution by 9 volumes of acetic acid, and is washed once with acetic acid and twice with ether. This acid salt is converted into the neutral salt in the following way: The material is suspended in a mixture of equal volumes of alcohol and ether and a freshly prepared 1% solution of NaOH in alcohol/ether 1 : 1 is added dropwise until a drop of the suspension gives a bluishgreen colour with a small amount of bromothymol blue solution.

After centrifuging the neutral sodium salt it is washed twice with alcohol/ether 1 : 1 and finally with ether. It is first dried in air and subsequently for some days in vacuo over P_2O_5, until the moisture content does not exceed 10%. The total losses involved in the lead purifications are from 2 to 5%. The losses in the conversion to the sodium salt are equally small.

The residue on ignition, calculated for the dry substance, is 38–41%. The preparations contain small amounts of inorganic salts (a.o. sodium-sulphate). Batches from cattle liver or lung usually showed an activity of 100 provisional International Units per mg. In a few cases a slightly lower activity (90 Units per mg) was observed.

Using the method of CHARLES AND SCOTT[3] the acid barium salt[9] is smoothly obtained in the typical shape of micro rosettes. From this barium salt a sodium salt with an activity of 120–130 Units per mg can easily be prepared.

As mentioned above the best yields, varying from 0.1 to 0.2 g per kg of starting material, were registered for cattle lung.

5. *Assay*

All preparations were assayed against a substandard, which had been compared with the provisional International standard of 130 Units per mg.

In the majority of the standardisations use was made of the increase of the clotting time by heparin on the system: citrate-plasma, thrombine. Citrate-plasma from both cattle and sheep was used though sheep plasma has distinct advantages as it gives a very sharp transition point from the liquid to the clotted state[7].

These assays were frequently checked, in particular with the end products, with the assay method employing recalcified citrate plasma. The "clotting time method"[11] was always used. The results of both methods of assay agreed within the experimental error.

SUMMARY

Description of a purification method for heparin. The solution of the crude product is shaken with phenol to remove the proteins.

RÉSUMÉ

Description d'une méthode pour la préparation de l'héparine. La solution du produit brut est agitée avec du phénol pour la séparation des protéines.

ZUSAMMENFASSUNG

Beschreibung eines Verfahrens zur Herstellung von Heparin. Die Lösung des Rohproduktes wird mit Phenol ausgeschüttelt um Eiweisskörper zu beseitigen.

References p. 337.

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