



PROCESS FOR PREPARING MUCOPOLYSACCHARIDE COMPOSITIONS
HAVING HIGH ANTITHROMBOTIC ACTIVITY.

5 BACKGROUND OF THE INVENTION

The invention relates to a process for preparing mucopolysaccharide compositions endowed with high anti-thrombotic activity.

10 It is known that compositions of this type have been obtained from heparin by alcoholic extraction, or again, by depolymerisation by chemical or enzymatical route.

In general, these processes enable the production of compositions or fractions consisting of mucopolysaccharides or MPS whose chains contain about 25 to 30 units at the most and possess an anti-Xa activity measured by the Yin Wessler titer (YW) higher than that of heparin and an overall anti-coagulant activity, expressed by the USP titer, less than that of heparin, this titer can even have a value
20 which is very low to practically nil.

These products have the interest of exerting a more specific activity than heparin on certain steps brought into play in the coagulation process.

In a given treatment, the product is chosen with regard to its YW titer and YW/USP ratio. In that respect the inventors have studied means enabling the easy production of compositions corresponding to a given profile as regards, in particular, their YW titer and their ratio of YW to USP titers. Applicants have observed that by operating under particular conditions, it is possible to isolate
30 such products from other MPS compositions.

It is therefore an object of the invention to provide a novel process for preparing compositions of MPS with high antithrombotic activity based on the treatment
35 of another MPS composition containing them, whose simple

employment enables products of great usefulness to be made easily available.

5 The process of the invention is characterized in that MPS compositions formed from a majority of MPS chains of molecular weight (MW) of about 1800 to 8000 daltons, having a YW titer and a ratio of YW/ USP titers higher than that of heparin are subjected to at least one fractionation step, in order to separate
10 selectively from said compositions at least the major part of chains having MW lower than about 2000 and those having a higher MW but a sulfate content lower than the sulfate average content of the mixture chain and that the fractions freed from said chains are recovered. These fractions
15 show a ratio of YW to USP titer less than the one of the starting compositions but higher than the one of heparin, with a USP titer higher than that of the starting compositions.

 Preferably, the fractionation step is carried out
20 by means of a mixture a) of water containing an inorganic salt and b) of an organic solvent, this solvent being selected from among those in which at least a majority of the products sought is selectively insoluble and precipitates.

 The relative proportions of inorganic salt and of
25 solvent are adjusted and this according to the pH of the medium to obtain the desired precipitation of the MPS chains.

 According to a preferred feature of the invention the organic solvent is advantageously an alcoholic solvent, more especially ethanol.

30 According to another preferred feature, the organic salt used is constituted by a salt miscible in the organic solvent used, such as sodium or potassium chloride.

 According to another preferred feature, the pH of
35 the reaction mixture is adjusted to a value corresponding

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to an acid pH, more especially to a pH less than 4.

According to a preferred embodiment of the invention, the MPS compositions employed for the fractionation contain a majority of chains of (1) MW in the range of about 1800 to 8000, (2) possessing a ratio of the YW to USP titers of at least about 10 and (3) a YW titer of about 200 to 250 u/mg.

The YIN-WESSLER titer is used for measuring the anti-Xa activity according to YIN E.T., WESSLER S., BUTLER J.V. in J.LAB.CLIN.MED. 1973, 81, p.298-310.

These compositions are advantageously obtained by a partial depolymerisation process of heparin under the action of chemical agents such as nitrous acid. Recourse is had particularly to the process described by Applicants in the second application for a certificate of addition n° 80 06282 of 20.03.1980 to patent FR 78 31357 of 6.11.1978. Advantageously, the depolymerisation process which is performed is based on self-regulation of the depolymerisation reaction such as described in patent application FR 81 07283 of 10.04.1981 in the name of Applicants.

According to the most general aspect of this auto-regulated depolymerisation, heparin is reacted with nitrous acid in an aqueous medium at a pH of about 2 to 3, advantageously of 2.5, in amounts such that heparin concentration is of at least about 8% in weight and nitrous acid molarity of about 0.02M to 0.1M, and when the depolymerisation reaction stops by itself, after having consumed all the nitrous acid, the MPS mixture of the kind of those which can be precipitated by an organic solvent is recovered.

Nitrous acid is generated in situ by adding an acid having advantageously biologically acceptable anions, such as HCl or CH_3COOH , to a nitrous acid derivative, particularly a salt, an ether-salt and more especially an alkaline or an alkaline-earth salt. NaNO_2 is more particularly used at a molarity of 0.03 to 0.05M.

The process according to the present invention is preferably performed with a NaNO_2 molarity of 0.040M to 0.046M more particularly of about 0.043M.



Due to the process used for their preparation, the MPS compositions possess at the reducing end, a unit of 2,5 anhydromanno structure, preferably selected from among 2,5-anhydromannonic acid groups.

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The starting MPS compositions are placed _____ in solution in a proportion of 5% w/v in water containing 10g/l of NaCl.

After adjustment of the pH to 3.8, the fractionation
10 of these MPS is carried out by means of an organic solvent enabling the selective precipitation of the MPS chains possessing the highest molecular weights and/or the most sulfated of the mixture .

Preferably the organic solvent is an alcoholic
15 solvent, more especially ethanol.

The elimination of low MW chains and of the low sulfate content chains results in fractions of a higher USP titer, the YW titer being practically maintained.

The MPS compositions recovered on precipitation
20 are characterised in that they are essentially formed of chains (1) of an average molecular weight of 4000 to 5000 daltons, particularly about 4500 (2) possessing a YW titer of about 180 to 200 u/mg and a ratio of the YW/USP titers, less than 10, particularly of about 6 to 3 more especially
25 of about 4. Furthermore, said compositions are formed of a majority of chains which are terminated by units of 2.5-anhydromanno structure when the starting compositions are obtained by a nitrous heparin depolymerisation process.

The compositions obtained according to the process
30 of the invention are endowed with biological activities enabling them in particular to control specifically certain steps of blood clotting.

These compositions are hence particularly valuable for developing medicaments which can be used, particularly,
35 for the prevention of thrombosis and aging of the tissues.

Said medicaments are used under the usual administration forms and doses for a given indication.

- MPS compositions obtained according to the invention
- 5 are useful for the constitution of biological reagents useful in the laboratory, particularly as a comparison reference for the study of other products of which the anticoagulant activity is tested, particularly at the level of inhibition of the Xa factor.
- 10 Regarding the products soluble in the water organic solvent mixture used in the fractionation step, they are devoid or practically devoid of antithrombotic activity.

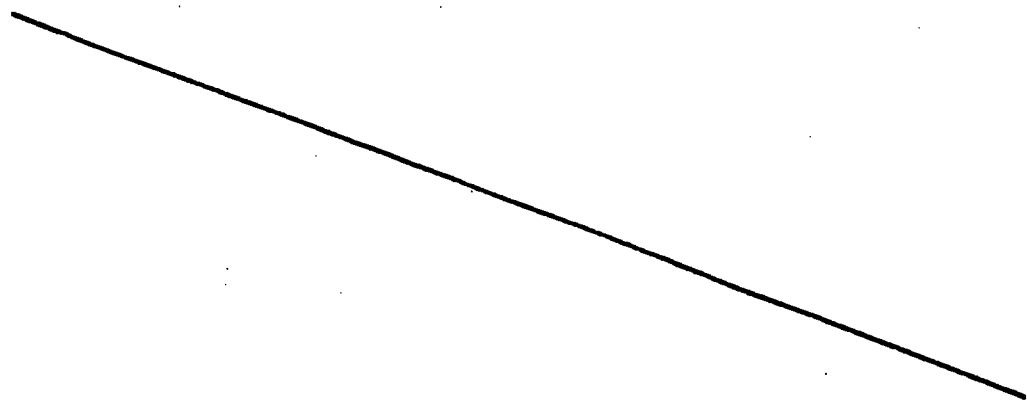
Said products can be recovered by gel filtrating the soluble fraction.

- 15 By using an exchange ion chromatography, different kinds of oligosaccharides can be separated, particularly hexosaccharidic chains which have a high activity on inhibition of neoangiogenesis. The recovery of the oligosaccharides from said fractions are advantageously performed by alcoholic precipitation for example and lyophilisation.
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- Other characteristics and advantages of the invention will appear in the examples which follow. Examples 1 and 2 relate to the preparation of MPS compositions possessing a ratio of the YW to USP titers of the order of 4,
- 25 examples 3 and 4 to the activity of the products respectively obtained according to examples 1 and 2 and example 5 to the obtention from the soluble fractions of products.

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practically devoid of antithrombotic activity.

EXAMPLE 1:

Process for obtaining MPS compositions with YW/
USP ratio of about 4.

5 500 g of injectable heparin, in the form of sodium salt, are placed in solution in 4500 ml of demineralized water, at a temperature of 18°C.

 The YW/USP ratio of the heparin used is in the vicinity of 1, these titers having a value of the order
10 of 160-170.

 The solution obtained is subjected to vigorous stirring, and its pH is lowered to 2.5 by the addition of concentrated hydrochloric acid. Then 15 g of sodium nitrite dissolved in 300 ml of water are added. The pH of the reaction is adjusted to 2.5 by concentrated hydrochloric acid,
15 and the total volume of the solution is brought to 5000 ml. The reaction is left to take place for 45 minutes and then the absence of residual nitrous ions in the reaction solution is checked, by means of indicator paper impregnated
20 with starch potassium iodide (development of a blue-violet colour in the presence of NO_2^- ions).

 The reaction is allowed to continue up to the total disappearance of nitrous ions and the absence of reaction with iodo-starch paper, by carrying out checks
25 every 3 or 4 minutes.

 When these checks become negative, the reaction is considered as having been completed.

 The pH of the solution is then raised to 10 by means of concentrated soda, and 5 g of sodium-tetrahydride-
30 borate is added.

 The solution is kept under stirring for 15 hours.

 The unreacted sodium tetrahydrideborate is destroyed by lowering the pH to 3.0 by means of concentrated hydrochloric acid. The solution is subjected to stirring
35 for 15 minutes, then the pH is readjusted to 7.0 by means

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of concentrated soda.

The reaction products are recovered by the addition of 10 l of ethanol. After 48 hours standing, the product is decanted and the supernatant liquor removed.

5 The precipitate is redissolved in 9 litres of demineralized water. 100 g of sodium chloride are added, and the pH of the solution is lowered to 3.8 by means of concentrated hydrochloric acid. The volume is adjusted exactly to 10 litres by means of demineralized water, and
10 with vigorous stirring 10 litres of ethanol are added. This is left to stand 48 hours. The supernatant liquor is siphoned off and put aside. The precipitate is recovered, washed with ethanol, ground, dried under vacuum.

230 grams of product having the following characteristics are obtained :

USP titer = 52 uI/mg

Yin and Wessler titer = 225 uI/mg

Average molecular weight = 4000 to 5000 daltons

EXAMPLE 2: PROCESS FOR OBTAINING MPS FRACTIONS WITH YW/USP RATIO OF ABOUT 4.

20 5000 g of injectable heparin are dissolved in 45 litres of demineralized water, at 18°C.

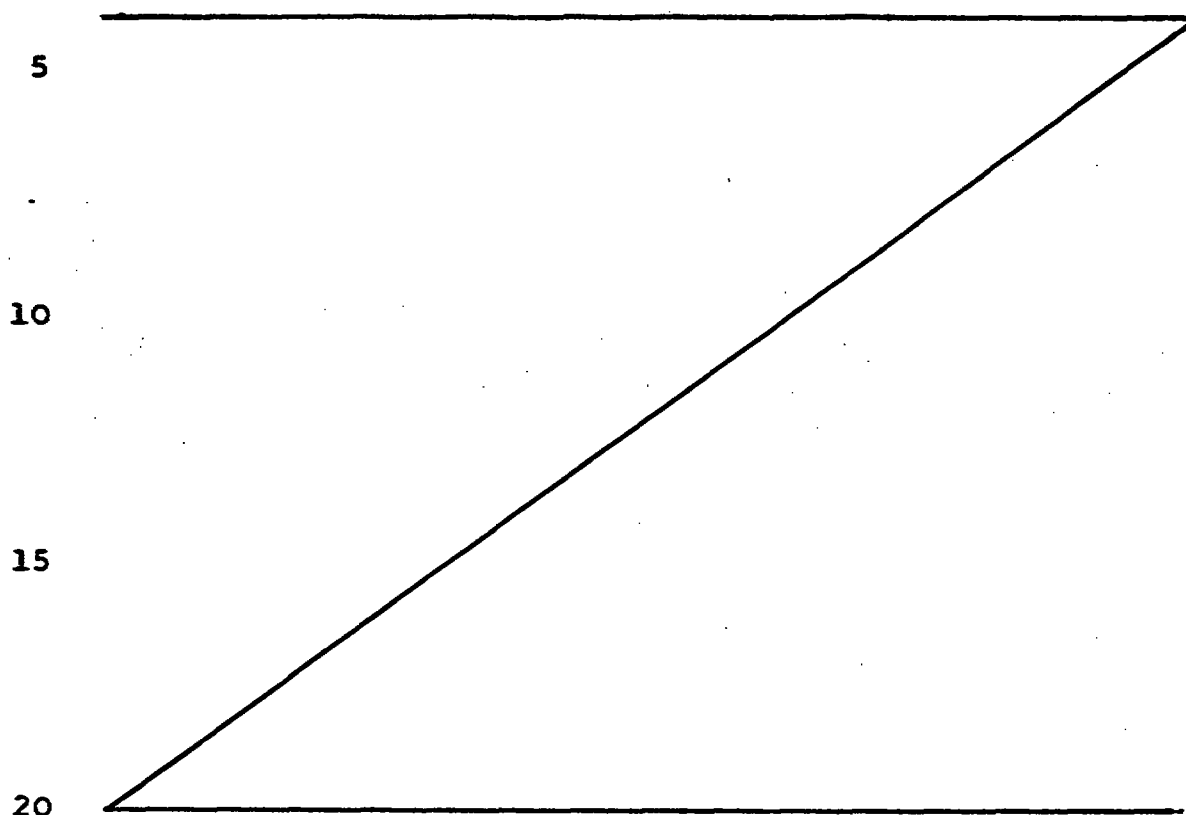
In similar manner to example 1 the procedure is followed multiplying the amounts of reagents by 10, that is to say:

- 25 - by adding 150 g of sodium nitrite in solution in three litres of water.
- by adjusting the volume of the reaction to 50 litres.
- by adding 50 g of tetrahydrideborate.
- by precipitating by means of 100 litres of ethanol.
- by redissolving 90 l of water.
- 30 - by adding 1000 g of sodium chloride.
- by adjusting the final volume to 100 litres.
- by finally adding 100 litres of ethanol.

In this way 2230 g of product are obtained having the following characteristics :

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USP titer = 54 uI/mg
Yin and Wessler titer = 232 uI/mg
Average molecular weight = 4000 to 5000 daltons



In Example 3 given below, the results of pharmacological tests carried out with the product of example 1, are reported.

EXAMPLE 3:

25 In vitro activity of the composition of example 1:

On figures 1 to 4 are reported the curves obtained by studying, in vitro, the variation of the clotting times induced in human blood plasmas by increasing doses of commercial heparin and the product of example 1.

30 In the tests corresponding to the results given in figures 3 and 4, plasmas free of platelets were used, and impoverished in factor XIA.

These figures show the variations in seconds of thrombin time (figure 1), of cephalin-kaolin (figure 2),
35 of coagulation in presence of concentrated thromboplastin

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(figure 3) and of dilute thromboplastin (figure 4), induced by the tested products, namely, the product of example 1 (curves a1, a2, a3 and a4) and of heparin (curves b1, b2, b3, b4) according to the doses respectively used.

5 The thrombin times and the cephalin-kaolin times both constitute types of measurement reflecting rather the action of the preparation studied respectively on the inhibition of the activated factor II and the overall coagulation. Study of the curves of figures 1 and 2 shows
10 that the MPS according to the invention exert a distinctly smaller effect than that of heparin on the inhibition of activation of prothrombin and at the level of the overall coagulation. Figures 3 and 4 which are representative of
15 enzymatic reactions, characteristics of extrinsic coagulation (particularly in the relative absence of factor XIA show the advantageous effect of the MPS of the invention with respect to heparin.

 The example 4 below concerns the results of tests
20 in vivo in the rabbit with the MPS of example 2.

EXAMPLE 4: Study of the antithrombotic activity of the product of example 2 in the rabbit.

 The administration of 500 u YW to the rabbit causes a considerable anti-Xa activity whilst the overall
25 anticoagulant activity remains relatively low.

 Two hours after the administration, the YW activity is of the order of 0.88 u/ml whilst the cephalin kaolin time is 0.09 ui.ml.

 The in vitro and in vivo tests show therefore
30 the distinctly more selective action of the MPS of the invention, particularly at the level of inhibition of the Xa factor, than that of heparin.

EXAMPLE 5: Process for obtaining MPS fractions practically devoid of antithrombotic activity.

35 The soluble fraction of example 1 is recovered.



Said fraction contains chains with 2 to 14 units.

Additional alcoholic fractionation step of said fraction results in a precipitate, which is separated, and a soluble fraction from which is recovered composition y.

- 5 Said composition comprises chains with 6 to 8 units at most and has a YW titer lower than 70 and a USP titer lower than 5.

By gel filtrating composition y, according to the usual techniques, hexasaccharidic compositions are recovered.

- 10 Said compositions are practically devoid of whole anticoagulant activity and of YW activity.

Example 6:

Activity on the neoangiogenesis of compositions y and z.

- 15 The trials in vitro concerning anti angiogenesis were carried out on a chicken embryo chorioallantoic membrane 6 days old in culture in petri dishes.

- Pellets are formed by mixing 5 µg of hydrocortisone with 10 µl of methylcellulose, compositions y and z are
20 added in amounts varying from 5µg to 100 µg. The pellets obtained are dried and placed on the chorioallantoic membrane to which they adhere. For each test 4 eggs are used.

- After 48 hours of incubation, avascular zones are found and an important drop in the vascular zones can be
25 seen. These results are reached in a remarkable way using only 6 µg of the tested products.

Example 7:

Action of a hexasaccharidic z composition on a Bl6 mouse melanoma.

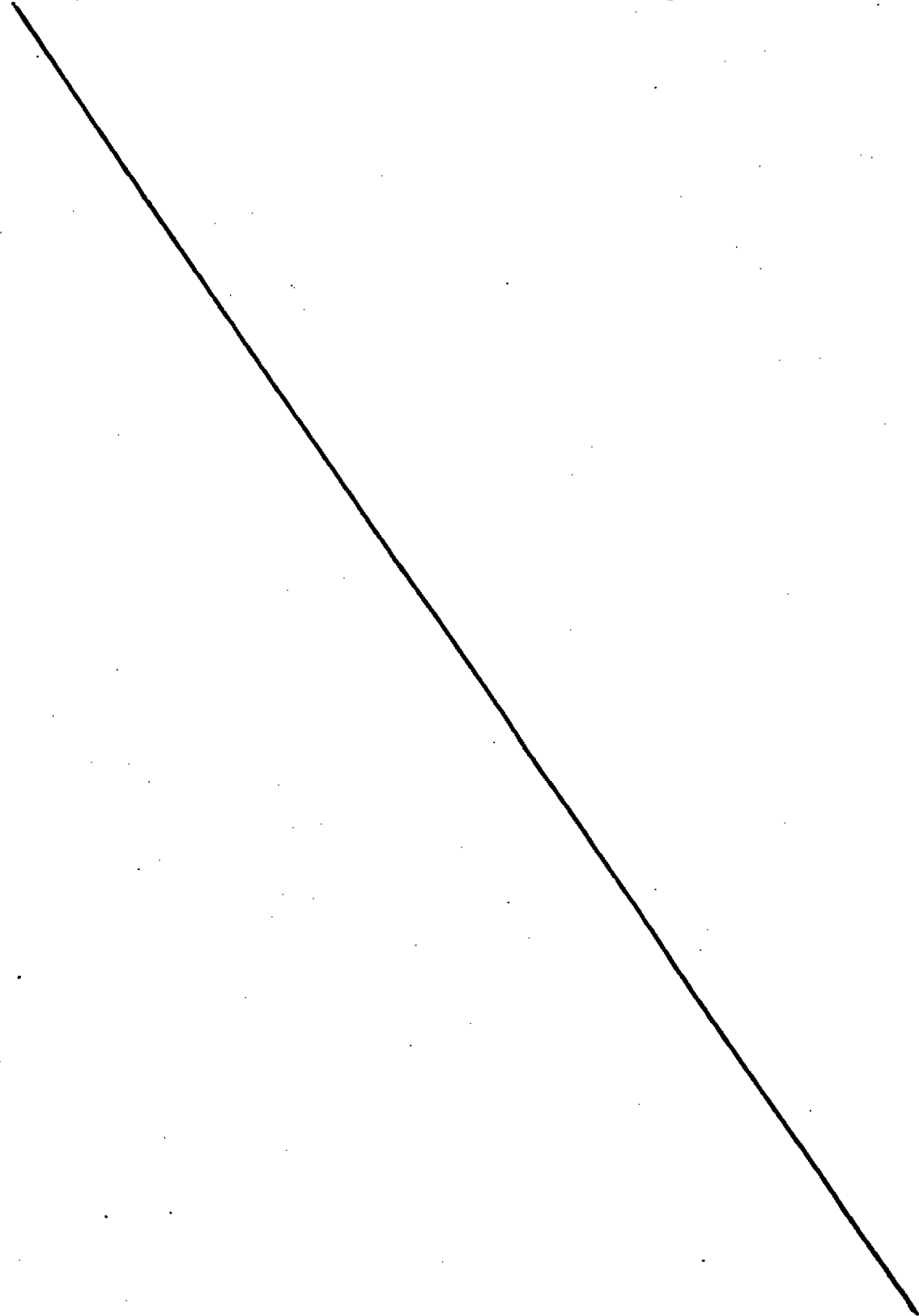
- 30 Tests carried out on C57 BL/6 mice carrying Bl6 melanomas.

- The treatment carried out during a period of 15 days, administered by sub-cutaneous injection twice a day to each mouse of 0.15 mg of the hexasaccharide composition of
35 acetate of cortisone in varying doses reduced during the

(34)

treatment from 250 mg/kg/j to 40-50 mg/kg/j. At the end of the 15 day period the growth of the tumours has ceased.

A regression of the tumours is advantageously observed.





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CLAIMS

1. Process for preparing mucopolysaccharides or MPS possessing high antithrombotic activity, characterized by the fact that compositions formed form a majority of MPS chains having a molecular weight of about 1800 to 8000 daltons, a YW titer and a ratio of the YW/USP titers higher than those of heparin to at least one fractionation step, in order to selectively separate from said compositions at least the major part of chains having MW lower than about 2000 and those having a higher MW but a sulfate content lower than the sulfate average contents of the mixture chain and that the fractions devoid from said chains are recovered, said fractions having a lower ratio of YW/USP titers than the starting compositions but higher than the one of heparin with USP titer higher than the one of the starting compositions.

2. A process according to claim 1, wherein the fractionation is carried out by means of a mixture of water containing an inorganic salt and of an organic solvent in which at least the majority of the desired products is selectively insoluble.

3. A process according to claim 2, wherein the fractionation is carried out by means of an organic solvent enabling the selective precipitation of MPS formed of chains having an average MW of 4000 to 5000 daltons, particularly of about 4500, having a ratio of the YW to USP titers less than about 10, with a YW titer of about 180 to 200 u/mg.

4. A process according to claim 3, wherein the ratio of the YW to USP titers of the precipitated MPS is of about 6 to 3, particularly in the vicinity of 4.



(36)

5. A process according to anyone of claims 2 to 4, wherein the organic solvent is an alcoholic solvent.

5 6. A process according to claim 5, wherein the alcoholic solvent is ethanol.

7. A process according to anyone of claims 2 to 6 wherein the inorganic salt is constituted by a salt miscible in the organic solvent used, such as sodium or potassium chloride.

10 8. A process according to anyone of claims 2 to 7, wherein the pH of the reaction mixture is adjusted to an acid value.

9. A process according to claim 8, wherein the pH is below 4.

15 10. A process according to claim 1, comprising placing in solution in water 5% w/v of MPS containing 10 g/l of NaCl and after adjustment of the pH to 3.8, effecting the fractionation by the addition of one volume of ethanol and recovering the precipitate formed.

20 11. A process according to anyone of claims 1 to 10, wherein the MPS compositions employed for the fractionation possess a ratio of the YW to USP titers of at least about 10 and a YW titer of about 200 to 250 u/mg.

25 12. A process according to anyone of the preceding claims, wherein the MPS compositions employed possess at the reducing end a unit of 2,5-anhydromanno structure selected preferably from among the groups
30 2,5-anhydromannose, 2,5-anhydromannitol or 2,5-anhydromannonic acid.