

HEPARIN-DERIVED OLIGOSACCHARIDE MIXTURES, PREPARATION
THEREOF AND PHARMACEUTICAL COMPOSITIONS CONTAINING SAID
MIXTURES

5 The present invention relates to oligosaccharide mixtures derived from heparin, with an average molecular weight of from 1800 to 2400 daltons, characterized by high anti-Xa (aXa) activity and an absence of anti-IIa (aIIa) activity, to a process for
10 preparing them and to pharmaceutical compositions containing them.

Heparin is a mixture of sulfated mucopolysaccharides of animal origin, used especially for its anticoagulant
15 and antithrombotic properties.

However, heparin has drawbacks that limit its conditions of use. In particular, its substantial anticoagulant activity (aIIa) can cause hemorrhaging
20 (Seminars in Thrombosis and Hemostasis, vol 5 sup. 3 (1999)).

Low molecular weight heparins, obtained especially by basic depolymerization of heparin esters, which are
25 currently marketed, such as Enoxaparin, also show substantial aIIa activity.

More recently, very low molecular weight heparins have been described in the prior art. For example, in patent
30 US 6 384 021, the products show anti-Xa activity of between 100 and 120 IU/mg and anti-IIa activity of between 2 and 8 IU/mg. In the international applications WO 02/08295 and WO 2004/033503, the products have anti-Xa activities which are especially
35 between 100 and 190 IU/mg for anti-IIa activities of less than 5 IU/mg. However, none of these very low molecular weight heparins effectively has anti-Xa activity of greater than 190 IU/mg while at the same

time having no, or virtually no, anti-IIa activity.
(IU = International Unit)

The expression "virtually no anti-IIa activity" (in
5 other words, showing virtually no anti-IIa activity)
means an activity of less than 0.2 IU/mg.

Disclosed herein is oligosaccharide
mixtures with very selective activity toward activated
10 factor X (factor Xa) while at the same time showing no,
or virtually no, anti-IIa activity.

A first aspect of the present invention is thus an
oligosaccharide mixture having the general structure
15 of the constituent polysaccharides of heparin and
having the following characteristics:

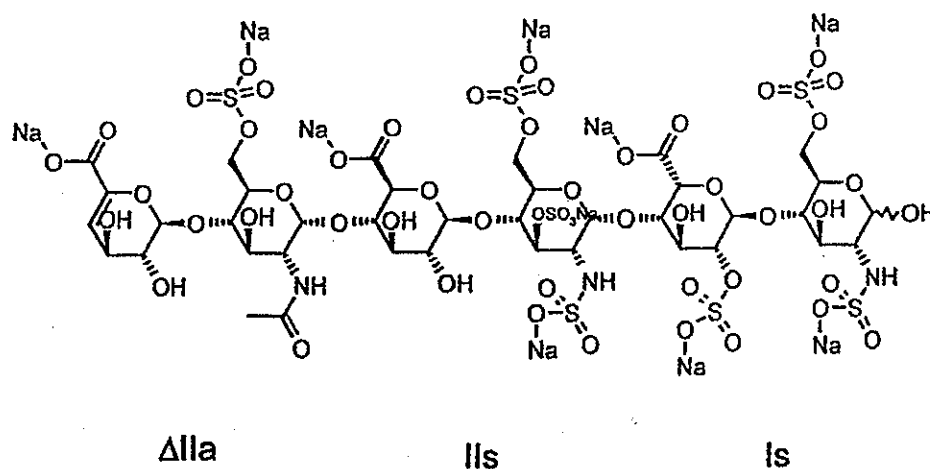
- an average molecular weight of from 1800 to
2400 daltons, anti-Xa activity of between 190 IU/mg and
20 450 IU/mg and no, or virtually no, anti-II activity,

- it being understood that the constituent
oligosaccharides of the mixtures

25 - contain from 2 to 16 saccharide units,

- have a 4,5-unsaturated uronic acid 2-O-sulfate
unit at one of their ends,

30 - and contain the hexasaccharide of the following
formula:



in the form of an alkali metal or alkaline-earth metal salt.

According to a second aspect of the invention, there is provided a process for preparing the oligosaccharide mixture as defined above, wherein a very low molecular weight heparin with aXa activity of greater than 140 IU/mg, aIIa activity of greater than 5 IU/mg and an average molecular mass of between 2000 and 3000 daltons is subjected to the following chemical reactions:

- a) transsalification by the action of benzethonium chloride to obtain benzethonium heparinate,
- b) esterification of the benzethonium heparinate obtained by the action of benzyl chloride, and treatment to obtain the sodium salt of the benzyl ester of the very low molecular weight heparin,
- c) transsalification of the benzyl ester obtained and production of the quaternary ammonium salt,
- d) depolymerization by means of a strong organic base with a pKa value preferably of greater than 20, so as to obtain a depolymerized very low molecular weight heparin,
- e) conversion of the quaternary ammonium salt of the depolymerized very low molecular weight heparin into the sodium salt,
- f) saponification of the residual esters and optional purification.

According to a third aspect of the invention, there is provided a oligosaccharide mixture as defined above, which may be obtained by the process as defined above.

According to a fourth aspect of the invention, there is provided a medical product, the oligosaccharide mixture as defined above.

According to a fifth aspect of the invention, there is provided a medicinal product with antithrombotic activity, the oligosaccharide mixture as defined above.

According to a sixth aspect of the invention, there is provided a medicinal product as defined above, for treating or preventing venous and arterial thrombosis, deep vein thrombosis, pulmonary embolism, unstable angina, myocardial infarction, cardiac ischemia, occlusive diseases of the peripheral arteries and atrial fibrillation, smooth muscle cell proliferation, atherosclerosis and arteriosclerosis, cancer by modulating angiogenesis and growth factors, and also diabetic disorders such as diabetic retinopathy and diabetic nephropathy.

According to a seventh aspect of the invention, there is provided a pharmaceutical composition containing at least one medicinal product as defined in the fourth aspect and one or more pharmaceutically inert excipients or vehicles or additives.

According to an eighth aspect of the invention, there is provided a method for determining the anti-Xa activity of the oligosaccharide mixture as defined above, wherein an amidolytic method is used on a chromogenic substrate in which the reconstitution buffer is Polyethylene Glycol 6000 (PEG 6000).

The hexasaccharide Δ IIa-II_s-Is contained in the oligosaccharide mixture described in the present invention can be a sequence with high affinity for ATIII and can be characterized by aXa activity of greater than 740 IU/mg.

Alkali metal or alkaline-earth metal salts that are preferred are the sodium, potassium, calcium and magnesium salts.

The average molecular weight is determined by high-pressure liquid chromatography using two columns in series, for example those sold under the name TSK G3000 XL and TSK G2000 XL. The detection is performed by refractometry. The eluent used is lithium nitrate and the flow rate is 0.6 ml/minute. The system is calibrated with standards prepared by fractionation of Enoxaparin by chromatography on agarose-polyacrylamide gel (IBF). This preparation is performed according to the technique described by Barrowcliffe et al, Thromb. Res., 12, 27-36 (1977-78) or D.A. Lane et al, Thromb. Res., 12, 257-271 (1977-78). The results are calculated using the GPC6 software (Perkin Elmer).

The anti-Xa activity is measured via the amidolytic method on a chromogenic substrate according to the principle described by Teien et al, Thromb. Res., 10, 399-410 (1977). The assays are performed according to the method described in the monograph on low molecular weight heparins of the European pharmacopea in force, except for the reconstitution buffer : the alubumin in the tris-NaCl buffer, pH 7.4, is replaced aby polyethylene glycol 6000(PEG 6000). The anti Xa activity is measured relative to a standard Very Low Molecular Weight Heparin (VLMWH) which measures from 140 to 180 U/mg (dry weight)

The activity of the standard VLMWH is measured relative to the international low molecular weight heparin standard. This standard VLMWH was prepared according to the teaching of patent applications WO WO 02/08 295 and in particular of WO 2004/033503. The activity of the standard VLMWH is measured relative to the international low molecular weight heparin standard.

The anti-IIa activity is measured via the amidolytic method on a chromogenic substrate according to the method described in the monograph on low molecular weight heparins of the European pharmacopea in force. The aIIa activities are measured relative to a standard Very Low Molecular Weight Heparin (VLMWH) with a measured activity of 2.1 IU/mg. The activity of the standard VLMWH is measured relative to the international low molecular weight heparin standard.

According to one preferred embodiment, the oligosaccharide mixture according to the invention contains from 20% to 100% of a hexasaccharide fraction. In particular, this mixture contains from 30% to 60% of hexasaccharide fraction.

Moreover, the mixtures according to the invention contain from 20% to 70% of the hexasaccharide Δ IIa-II $\underline{s}$ -Is in the hexasaccharide fraction of the oligosaccharide mixture. In particular, this fraction
5 Δ IIa-II $\underline{s}$ -Is is present in the hexasaccharide fraction to a proportion of 25% to 50%.

The percentage of the hexasaccharide fraction may be determined analytically by high-pressure liquid
10 chromatography on TSK G3000 XL and TSK G2000 XL columns or by preparative separation of the hexasaccharide fraction.

In this case, the mixture is chromatographed on columns
15 filled with gel of polyacrylamide agarose type. The mixture is eluted with a sodium hydrogen carbonate solution. Preferably, the sodium hydrogen carbonate solution is a solution of from 0.1 mol/l to 1 mol/l. Even more preferably, the separation is performed at a
20 concentration of 1 mol/l. The detection is performed by UV spectrometry (254 nm). After fractionation, the hexasaccharide fraction in solution in the sodium hydrogen carbonate is neutralized with glacial acetic acid. The solution is then concentrated under reduced
25 pressure so as to obtain a sodium acetate concentration of greater than 30% by weight. The hexasaccharide fraction is precipitated by addition of 3 to 5 volumes of methanol. The hexasaccharide fraction is recovered by filtration through a No. 3 sinter funnel. The
30 hexasaccharide mixture obtained may be analyzed by high performance liquid chromatography (HPLC) to determine its content of hexasaccharide Δ IIa-II $\underline{s}$ -Is. The hexasaccharide Δ IIa-II $\underline{s}$ -Is may be isolated by preparative HPLC or by affinity chromatography on an
35 antithrombin III sepharose column according to the techniques used by those skilled in the art (M. Hook, I. Bjork, J. Hopwood and U. Lindahl, *F.E.B.S letters*, vol 656(1) (1976)).

Preferably, the mixtures according to the invention have an average molecular weight of between 1900 and 2200 daltons and in particular from 1950 to 2150 daltons.

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According to one preferred embodiment, the oligosaccharide mixture according to the invention is characterized in that it has anti-Xa activity of between 190 IU/mg and 410 IU/mg and no, or virtually no, anti-IIa activity. Most particularly, the anti-Xa activity is between 200 and 300 IU.

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Disclosed herein are thus, most particularly, mixtures having the following characteristics:

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- an average molecular weight of between 1950 and 2150 daltons,

20

- anti-Xa activity of between 190 IU/mg and 410 IU/mg and no, or virtually no, anti-IIa activity,

- they contain from 30 to 60% of hexasaccharide fraction, which contains from 25% to 55% of Δ IIa-II_s-Is fraction.

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The activity of the oligosaccharide mixtures disclosed is obtained by means of a very particular process that is described hereinbelow. It is well known to those skilled in the art that the physicochemical characteristics of polysaccharide mixtures and the activity deriving therefrom are linked to the production process (J. Med. Chem. 33(6) 1639-2093 (1990)).

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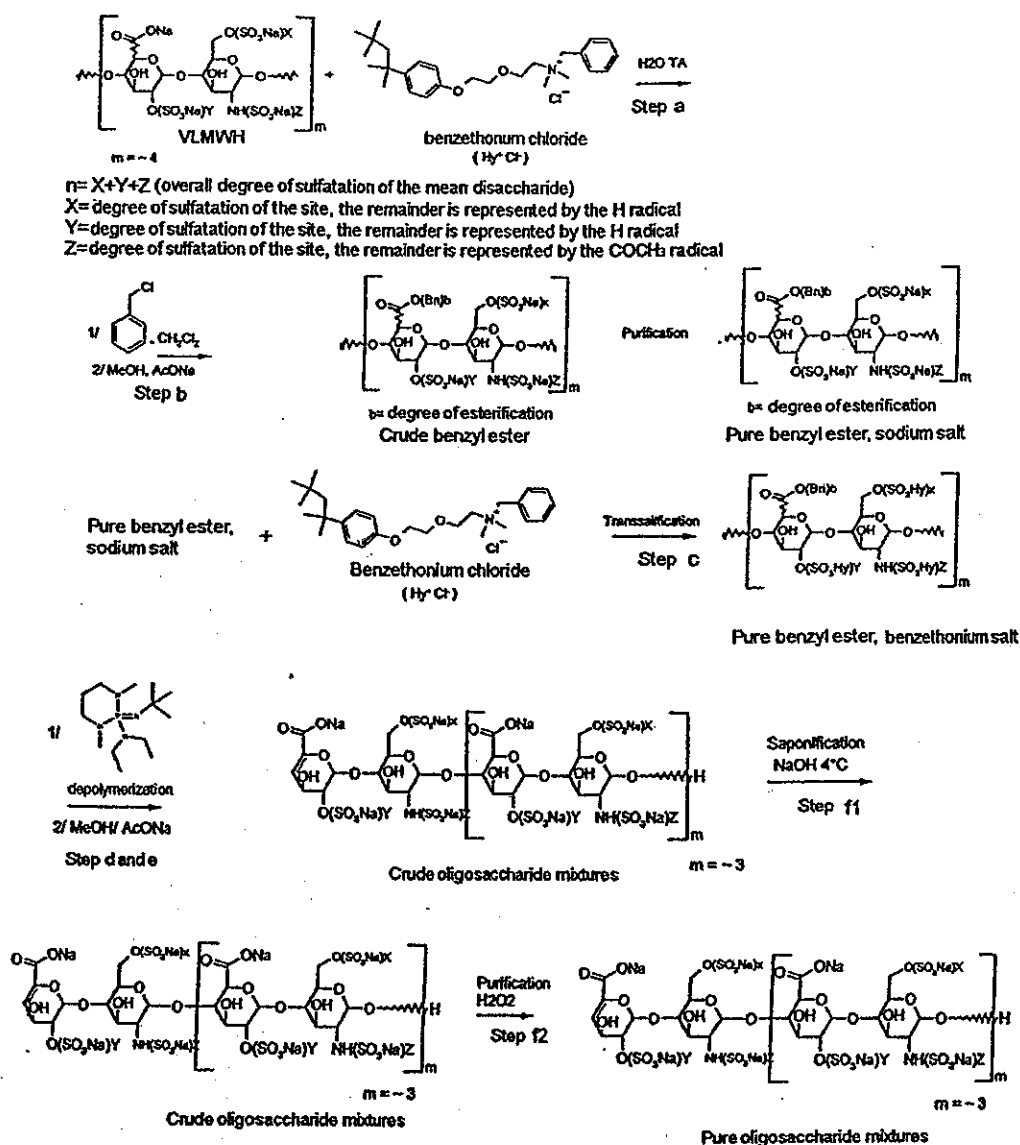
The oligosaccharide mixtures disclosed are prepared by depolymerization of a quaternary ammonium salt of the benzyl ester of a Very Low Molecular Weight Heparin (VLMWH) in organic medium, this (VLMWH) itself being prepared according to the

teaching of patent applications WO 02/08295 and
WO 2004/033503. It is generally a matter of re-
depolymerizing a very low molecular weight heparin
which has itself been specifically obtained by
5 depolymerization of esterified heparin in the presence
of a strong base, preferably in dichloromethane, and in
the presence of a percentage of water of less than 3%.

The VLMWHs used as starting material in this invention
10 were prepared in particular according to the processes
described in patent applications WO 02/08295 and
WO 2004/033503.

The VLMWHs used as starting material especially have
15 aXa activity of greater than 140 IU/mg, aIIa activity
of less than 5 IU/mg and average molecular masses of
between 2000 and 3000 daltons. The aXa activities are
measured relative to a standard VLMWH with a measured
activity of 158 IU/mg. The activity of the standard
20 VLMWH is measured relative to the international low
molecular weight heparin standard.

The starting VLMWHs obtained according to the process
as described above are re-depolymerized using a strong
25 organic base with a pKa value preferably of greater
than 20 (preferred phosphazene-family-related
properties defined, for example, by Schwesinger et al,
Angew. Chem. Int. Ed. Engl. 26, 1167-1169 (1987) or R.
Schwesinger et al, Angew. Chem. 105, 1420 (1993)).
30 Next, the quaternary ammonium salt of the benzyl ester
of the depolymerized VLMWH is converted into the sodium
salt, the residual esters are saponified and the
product obtained is optionally purified. The reaction
scheme below illustrates the present invention:



In one embodiment, there is provided a process for preparing the oligosaccharide mixtures as defined above, wherein a very low molecular weight heparin with aXa activity of greater than 140 IU/mg, aIIa activity of less than 5 IU/mg and an average molecular mass of between 2000 and 3000 daltons is subjected to the following chemical reactions:

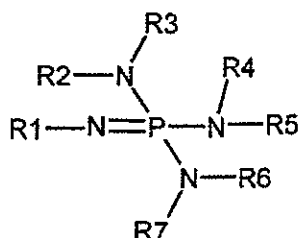
- transsulfation by the action of benzethonium chloride to obtain benzethonium heparinate,
- esterification of the benzethonium heparinate obtained by the action of benzyl chloride, and treatment with alcoholic sodium acetate solution

- to obtain the sodium salt of the benzyl ester of the very low molecular weight heparin,
- c) transsalification of the benzyl ester obtained and production of the quaternary ammonium salt, preferably as the benzethonium, cetylpyridinium or cetyltrimethylammonium salt,
 - d) depolymerization by means of a strong organic base with a pKa value preferably of greater than 20, so as to obtain a depolymerized very low molecular weight heparin,
 - e) conversion of the quaternary ammonium salt of the depolymerized very low molecular weight heparin into the sodium salt,
 - f) saponification of the residual esters and optional purification.

The high selectivity of the phosphazene base during the depolymerization step (step d) is most particularly used to enrich, unexpectedly, the oligosaccharide mixture in sequences with affinity for ATIII. Preferably, the strong base/ester mole ratio is between 0.2 and 5 and more particularly between 0.6 and 2.

For optimum selectivity and maximum preservation of the sequences with affinity for ATIII, it is preferable to work at water contents of less than 0.3% when working with 1 molar equivalent of phosphazene base relative to the benzyl ester of the VLMWH, benzethonium salt.

The bases of the phosphazene family are preferably those of formula:



in which the radicals R_1 to R_7 , which are identical or different, represent linear, branched or cyclic alkyl radicals containing from 1 to 6 carbon atoms it being possible for R_3 and R_4 , where appropriate, to form with the -N-P-N-group which carries them, a 6-membered heterocycle. In particular, one subject of the invention is the process as defined above, wherein the base used in the depolymerization step d) is 2-tert-butylimino-2-diethylamino-1,3-dimethylperhydro-1,3,2-diazaphosphorine (official nomenclature: 1,3,2-diazaphosphorin-2-amine, 2-[(1,1-dimethylethyl)imino]-N,N-diethyl-1,2,2,2,3,5,6-octahydro-1,3-dimethyl).

The reaction of the transsalification step a) is preferably performed by the action of excess benzethonium chloride on the sodium VLMWH, at a temperature in the region of 15 to 25°C. Advantageously, the salt/sodium heparin mole ratio is between 2.5 and 3.5.

The esterification step b) is preferably performed in an organic chlorinated solvent (such as chloroform or dichloromethane), at a temperature of between 25 and 45°C and preferably between 30 and 40°C. The ester in the form of the sodium salt is then recovered by precipitation using 10% by weight of sodium acetate in an alcohol such as methanol. 1 to 1.2 volumes of alcohol per volume of reaction medium are generally used. The amount of benzyl chloride and the reaction time are adapted to obtain a degree of esterification of between 40% and 100% and preferably between 70% and

90%. 0.5 to 1.5 parts by weight of benzyl chloride per 1 part by weight of the benzethonium salt of heparin are preferably used. Similarly, the reaction time will preferably be between 10 and 35 hours.

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Consequently, the process according to the invention uses a degree of esterification of the quaternary ammonium salt of the benzyl ester of heparin of between 40% and 100% and preferably between 70 and 90%.

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The conversion of the quaternary ammonium salt of the benzyl ester of the depolymerized heparin into the sodium salt is generally performed by treating the reaction medium with alcoholic sodium acetate solution and preferably with a 10% solution of sodium acetate in methanol (weight/volume), at a temperature of between 15 and 25°C.

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The weight equivalent of acetate added is preferably 3 times as great as the mass of quaternary ammonium salt of the benzyl ester of heparin subsequently used in the depolymerization reaction. The quaternary ammonium salt of the benzyl ester of the VLMWH obtained is preferably the benzethonium, cetylpyridinium or cetyltrimethylammonium salt.

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The transsalification step c) is performed using a quaternary ammonium chloride, preferably using benzethonium chloride, cetylpyridinium chloride or cetyltrimethylammonium chloride, in aqueous medium, at a temperature of between 10 and 25°C. Advantageously, the quaternary ammonium chloride/sodium salt of the benzyl ester of heparin mole ratio is between 2.5 and 3.5.

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The saponification is generally performed using an alkali metal hydroxide such as sodium hydroxide, potassium hydroxide or lithium hydroxide, in aqueous medium, at a temperature of between 0 and 20°C and

preferably between 0 and 10°C. From 1 to 5 molar equivalents of alkali metal hydroxide will generally be used. The saponification will preferably be performed in the presence of 1 to 2 molar equivalents of alkali metal hydroxide.

The final product may optionally be purified by any known method for purifying depolymerized heparins (for example EP 0 037 319 B1). Preferably, the purification is performed using hydrogen peroxide, in aqueous medium, at a temperature of from 10 to 50°C. This operation will preferably be performed at between 20 and 40°C.

The mixtures according to the invention in sodium salt form may be converted into a salt of another alkali metal or alkaline-earth metal. The conversion from one salt to the other is optionally performed using the method described in patent FR 73/13580.

The present invention especially allows a high enrichment in hexasaccharide ΔIIa-II_s-Is. When a low molecular weight heparin is re-depolymerized by the process that generated it, it is well known to those skilled in the art that the anti-Xa activity of the product obtained decreases greatly until it is zero. In the case where the processes for obtaining Enoxaparin, Fraxiparin, Fragmin, Innohep (or Logiparin), Normiflo, Embollex (or Sandoparin), Fluxum (or Minidaltol), Clivarin and Hibor are used, this phenomenon may be observed if these LMWHs are re-depolymerized with their original process. This is the consequence of the low selectivity of these processes with respect to the preservation of the ATIII sites.

In the present invention, if a VLMWH derived from the phosphazene depolymerization process is used as starting material, exactly the reverse phenomenon takes place. The aXa activity of the oligosaccharide mixture

increases and even exceeds that of the heparin that was used to prepare the VLMWH. This is the probable consequence of the noteworthy selectivity of the phosphazene bases on the preservation of the sequences with affinity for ATIII.

This characteristic of the process can also be observed through the average molecular masses of the oligosaccharide mixture obtained. By way of example, when a VLMWH with an average molecular mass of 2400 daltons is depolymerized, an oligosaccharide mixture with an average molecular mass of 2000 Da is obtained. It is found that the sequences with affinity for ATIII (hexasaccharides and octasaccharides) are preserved from the action of the phosphazene base, which results in the destruction and elimination of the other sequences, and consequently an average molecular mass that tends toward the average molecular mass of the non-depolymerizable species, ie the hexasaccharide AIIa-IIIs-Is (1834 g/mol). It should be pointed out that, in the step of heparin depolymerization with a phosphazene base, the average molecular mass changes from about 15 000 Da to about 2400 Da.

As an alternative method, the process disclosed for increasing the activity and selectivity toward factor Xa is also applicable to low molecular weight heparins in general. By way of example, mention will be made, for example, of Enoxaparin, Fraxiparin, Fragmin, Innohep (or Logiparin), Normiflo, Embollex (or Sandoparin), Fluxum (or Minidaltin), Clivarin and Hibor. They may also be certain very low molecular weight heparins as described in US 6 384 021 (2000-4000 Da) or WO 02/08295 (1500 to 3000 Da), with anti-Xa activity of less than 140 IU/mg. (in particular between 100 and 140 IU/mg).

This characteristic of the process is reflected by the production of anti-Xa activities that are unexpected

with regard to the average molecular weight of the oligosaccharide mixtures ($190 \text{ IU/mg} < \alpha\text{Xa} < 450 \text{ IU/mg}$; $1800 \text{ Da} < \text{MW} < 2400 \text{ Da}$).

- 5 According to one particular embodiment of the invention, the selectivity toward factor Xa of the oligosaccharide mixtures may be further increased by removing the disaccharide and tetrasaccharide fractions (fractions not specifically binding to ATIII). In this
- 10 case, the mixture is chromatographed on columns filled with gel of polyacrylamide agarose type or a polyacrylamide gel. The mixture is eluted with a sodium hydrogen carbonate solution. Preferably, the sodium hydrogen carbonate solution is a solution of from
- 15 0.1 mol/l to 1 mol/l . Even more preferably, the separation is performed at a concentration of 1 mol/l . The detection is performed by UV spectrometry (254 nm). After removal of the disaccharide and tetrasaccharide fractions, the oligosaccharide mixture in solution in
- 20 sodium hydrogen carbonate is neutralized with glacial acetic acid. The solution is then concentrated under reduced pressure so as to obtain a sodium acetate concentration of greater than 20% by weight. The oligosaccharide mixture is precipitated by addition of
- 25 3 to 5 volumes of methanol. The high-affinity oligosaccharide mixture is recovered by filtration. If necessary, it may be purified by desalting on a suitable column. Example 6 illustrates this alternative method and allows very low molecular weight heparins
- 30 with anti-Xa activity of greater than 400 IU/mg to be obtained.

Disclosed is also a process as defined above for preparing oligosaccharide mixtures

35 having increased selectivity toward factor Xa of the oligosaccharide mixture, wherein the disaccharide and tetrasaccharide fractions are also removed by chromatography, especially on columns filled with gel of polyacrylamide agarose type.

The mixtures according to the invention may be used as medicinal products.

- 5 The oligosaccharide mixtures of the present invention may be used as antithrombotic agents. In particular, they are useful for treating or preventing venous and arterial thrombosis, deep vein thrombosis, pulmonary embolism, unstable angina, myocardial infarction, 10 cardiac ischemia, occlusive diseases of the peripheral arteries and atrial fibrillation. They are also useful in preventing and treating smooth muscle cell proliferation, atherosclerosis and arteriosclerosis, for treating and preventing cancer by modulating 15 angiogenesis and growth factors, and for treating and preventing diabetic disorders such as diabetic retinopathy and diabetic nephropathy.

- Disclosed are also pharmaceutical 20 compositions containing, as active principle, a mixture of formula (I) optionally combined with one or more inert excipients.

- The pharmaceutical compositions are, for example, 25 solutions for subcutaneous or intravenous injection. Other pharmaceutical compositions according to the invention are also used for pulmonary administration (inhalation) or oral administration.

- 30 The dosage may vary as a function of the age, weight and state of health of the patient. For an adult, it is generally between 20 and 100 mg per day via the intramuscular or subcutaneous route.

- 35 The examples that follow illustrate the invention without, however, limiting it.

Preparation 1: Production of a starting very low molecular weight heparin having aXa activity equal to 158.8 IU/mg

5 The very low molecular weight heparin (VLMWH) used as starting material for example 1 is prepared according to patent application WO 2004/033503 from sodium heparin, by performing steps a to f as defined above, the depolymerization step being performed in the presence of 2-tert-butylimino-2-diethylamino-1,3-dimethylperhydro-1,3,2-diazaphosphorine in the presence
10 of a percentage of water of less than 0.6%.

Characteristics of the very low molecular weight heparin obtained

15 The characteristics of the depolymerized heparin thus obtained are as follows:

Average molecular weight: 2400 daltons

Anti-Xa activity: 158.8 IU/mg

Anti-IIa activity: 3.1 IU/mg

20 Anti-Xa activity/anti-IIa activity ratio: 51

Preparation 2: Production of a starting very low molecular weight heparin having aXa activity equal to 158 IU/mg

25 The very low molecular weight heparin (VLMWH) used as starting material for examples 2, 3, 4 and 5 is prepared according to patent application WO 2004/033503, from sodium heparin, by performing steps a to f as defined above, the depolymerization
30 step being performed in the presence of 2-tert-butylimino-2-diethylamino-1,3-dimethylperhydro-1,3,2-diazaphosphorine in the presence of a percentage of water of less than 0.6%.

35 **Characteristics of the very low molecular weight heparin obtained**

The characteristics of the depolymerized heparin thus obtained are as follows:

Average molecular weight: 2450 daltons

Anti-Xa activity: 158 IU/mg

Anti-IIa activity: 2.1 IU/mg

Anti-Xa activity/anti-IIa activity ratio: 75

5 **Preparation 3: VLMWH, benzethonium salt**

Transsalification of the VLMWH to the benzethonium salt (corresponding to step a) of the process):

10 12.53 g (20.7 mmol) of the VLMWH sodium salt obtained according to preparation 1 are placed in a 500 ml conical flask A and dissolved in 85 ml of water (yellow solution).

15 31.62 g (70.5 mmol) of benzethonium chloride are placed in a 100 ml conical flask B with 250 ml of water (colorless solution).

The content of B is poured into A and the mixture is
20 stirred for about 1 hour at room temperature. The resulting mixture is left to sediment for about 1 hour. The supernatant is discarded and then replaced with the same volume of water (250 ml). The mixture is stirred for about 15 minutes and left to sediment for
25 approximately 30 minutes. The supernatant is discarded and then replaced with the same volume of water (250 ml). The mixture is stirred for about 15 minutes and then filtered. The cake is washed with 3 times 200 ml of water. The wet beige-colored solid is drained
30 by suction and then dried at 80°C for about 18 hours in an oven under reduced pressure (6 kPa). 35.56 g of VLMWH benzethonium salt are obtained.

The yield obtained is 89%.

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Preparation 4

Transsalification of the VLMWH to the benzethonium salt (corresponding to step a) of the process):

17.93 g (30.2 mmol) of VLMWH sodium salt obtained according to preparation 2 are placed in a 1 l conical flask A and dissolved in 120 ml of water (yellow solution).

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45 g (0.1 mol) of benzethonium chloride are placed in a 500 ml conical flask B with 360 ml of water (colorless solution).

10 The content of B is poured into A and the mixture is stirred for about 1 hour at room temperature. The resulting mixture is left to sediment for about 1 hour. The supernatant is discarded and then replaced with the same volume of water (500 ml). The mixture is stirred
15 for about 15 minutes and left to sediment for approximately 30 minutes. The supernatant is discarded and then replaced with the same volume of water (500 ml). The mixture is stirred for about 15 minutes and then filtered. The cake is washed with 3 times
20 200 ml of water. The wet beige-colored solid is drained by suction and then dried at 80°C for about 48 hours in an oven under reduced pressure (6 kPa). 49.5 g of VLMWH benzethonium salt are obtained.

25 The yield obtained is 87%.

Example 1:

Very low molecular weight heparin (VLMWH) obtained by the process according to the invention, comprising a
30 step of 77% esterification and a step of depolymerization with a base derived from phosphazene, in anhydrous medium

.Esterification of the VLMWH (step b of the process):

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35.39 g (18.3 mmol) of VLMWH benzethonium salt obtained according to preparation 3 (with a water content of 0.20%) are dissolved in 183.3 g of dry dichloromethane and placed in a 500 ml three-necked flask. 29.5 ml

(25.7 mmol) of benzyl chloride are added at a temperature of 30°C. The degree of esterification is 77% after about 23 hours of reaction at 30°C. After cooling to room temperature (22±3°C), the reaction mixture is poured into 490 ml of a 10% solution of sodium acetate in methanol. The mixture is stirred for about 1 hour and then left to sediment for approximately 1 hour. The supernatant is discarded and then replaced with the same volume of methanol (250 ml). The mixture is stirred for approximately 30 minutes and then left to sediment for about 45 minutes. The supernatant is discarded and then replaced with the same volume of methanol (250 ml). This mixture is left to sediment for about 16 hours. The supernatant is discarded and then replaced with the same volume of methanol (350 ml). The mixture is stirred for about 5 minutes and the suspension is filtered. The cake is washed with twice 50 ml of methanol, drained by suction and then dried at 40°C under reduced pressure (6 kPa) for about 18 hours. 34.48 g of crude VLMWH benzyl ester sodium salt with a degree of esterification of 77% are obtained.

.Purification of the VLMWH benzyl ester sodium salt (step b) of the process):

The 34.48 g of crude VLMWH benzyl ester sodium salt are dissolved in 350 ml of aqueous 10% NaCl solution. The solution is poured into 1.57 l of methanol. The suspension is stirred for about 40 minutes and is then left to sediment for about 16 hours. The supernatant is discarded and replaced with the same volume of methanol (1.5 l). This mixture is stirred for about 1 hour and is left to sediment for about 1.5 hours. The supernatant is discarded and replaced with the same volume of methanol (1.2 l). This mixture is stirred for approximately 15 minutes and then filtered. The cake is washed with 3 times 50 ml of methanol. The wet white solid is drained by suction and then dried at 40°C

under reduced pressure (6 kPa) for about 18 hours.
6.07 g of VLMWH benzyl ester sodium salt are obtained.

The esterification yield is 50%.

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.Transsalification of the VLMWH benzyl ester sodium salt to the benzethonium salt (step c) of the process):

6 g (9.14 mmol) of VLMWH benzyl ester sodium salt are
10 dissolved in 40 ml of water in a 250 ml conical flask A.

In parallel, 13.93 g (31 mmol) of benzethonium chloride
15 are placed in 110 ml of water in a 250 ml conical flask B.

The content of B is poured into A. The suspension is stirred for about 1 hour at room temperature ($22\pm 3^{\circ}\text{C}$) and then left to sediment for 1 hour. The supernatant
20 is discarded and replaced with the same volume of water (140 ml). This mixture is stirred for about 15 minutes and left to sediment for 1 hour. The supernatant is discarded and replaced with the same volume of water (140 ml). This mixture is stirred for approximately
25 15 minutes and left to sediment for about 30 minutes. The supernatant is discarded and replaced with the same volume of water (140 ml). This mixture is stirred for about 5 minutes and then filtered. The cake is washed with 3 times 50 ml of water, drained by suction and
30 then dried at 80°C under reduced pressure (6 kPa) for about 18 hours. 17.43 g of VLMWH benzyl ester, benzethonium salt are obtained.

The yield is 100%.

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.Depolymerization of the VLMWH benzyl ester, benzethonium salt in anhydrous medium: undetectable water content < 0.01% (step d) of the process)

17.43 g (9.14 mmol) of VLMWH according to preparation 2 are placed in a 250 ml three-necked flask with 122 ml of dry dichloromethane. 17.4 g of 4 Å molecular sieves are added. The mixture is stirred for about 18 hours at
5 room temperature ($22\pm3^{\circ}\text{C}$) under an argon atmosphere.

The sieves are separated from the mixture by transferring the solution into a 250 ml three-necked flask. 2.64 ml (9.14 mmol) of 2-tert-butylimino-2-
10 diethylamino-1,3-dimethylperhydro-1,3,2-diazaphosphorine are added and the mixture is stirred for 24 hours at $22\pm3^{\circ}\text{C}$ under an argon atmosphere.

**.Conversion of the quaternary ammonium salt into the
15 sodium salt (step e) of the process)**

In parallel, 730 ml of methanolic 10% sodium acetate solution are prepared in a 2 l conical flask. 8.71 g of Hyflo supercel Celite are added to the solution. The
20 reaction mixture is poured into the methanolic solution, while maintaining the temperature at about 4°C . The suspension is stirred for about 15 minutes at this temperature. The mixture is left to sediment for approximately 45 minutes at room temperature and the
25 supernatant is then discarded and replaced with the same amount of methanol (450 ml). This mixture is stirred for 15 minutes and left to sediment for approximately 45 minutes. The supernatant is again discarded and replaced with the same amount of methanol
30 (420 ml). This mixture is stirred for about 15 minutes and then filtered through a No. 3 sinter funnel. The cake is washed with twice 70 ml of methanol, drained by suction and then dried for about 18 hours at 50°C under reduced pressure (6 kPa). 4.35 g of crude depolymerized
35 VLMWH (sodium salt) in Celite (8.71 g) are obtained.

The yield is 72.5%.

.Saponification of the crude depolymerized VLMWH, sodium salt (step f1) of the process):

4.35 g (6.63 mmol) of crude depolymerized VLMWH (sodium salt) in Celite are dissolved in 46 ml of water and then filtered through a No. 3 sinter funnel. The Celite is rinsed with 2 portions of 30 ml of water. The filtrate is placed in a 500 ml conical flask. 823 μ l (9.94 mmol) of 35% sodium hydroxide solution are introduced at a temperature in the region of 4°C. This mixture is stirred for about 3 hours at this temperature. The medium is neutralized by adding 1N HCl solution, followed by addition of 11.5 g of NaCl and 80 ml of methanol. After stirring for approximately 15 minutes, 210 ml of methanol are added. The suspension is stirred for about 1 hour and then left to sediment for 30 minutes. The supernatant is discarded and replaced with the same amount of methanol (230 ml). This mixture is stirred for about 15 minutes and left to sediment for 30 minutes. The supernatant is discarded and replaced with the same amount of methanol (210 ml). This mixture is stirred for approximately 15 minutes and then filtered. The cake is washed with twice 9 ml of methanol, drained by suction and then dried for about 18 hours at 50°C under reduced pressure (6 kPa). 2.95 g of crude depolymerized VLMWH (sodium salt) are obtained.

The yield is 73.7%.

f) Purification of the crude depolymerized VLMWH, sodium salt (step f2) of the process):

1.5 g of crude depolymerized VLMWH, sodium salt, are placed in a 50 ml three-necked flask with 16 ml of water. The solution is maintained at 40°C for about 10 minutes. The pH is brought to about 9.7 by addition of 0.1N sodium hydroxide solution. The solution is filtered through a 0.45 μ m membrane, and 84 μ l of

aqueous 30% hydrogen peroxide solution are then added. The mixture is stirred for 2 hours at room temperature, while keeping the pH constant at 9.7 ± 0.1 by adding 0.1N sodium hydroxide solution. The reaction mixture is then neutralized with 0.1N HCl, and 2 g of NaCl are then added. After stirring for about 10 minutes, the solution is filtered through a 0.45 μ m membrane. 14 ml of methanol are added at a temperature in the region of 4°C. The solution is stirred for approximately 15 minutes at room temperature. 36 ml of methanol are then added and the suspension is stirred for about 1 hour. The stirring is then stopped and the mixture is left to sediment for about 30 minutes. The supernatant is then taken up and discarded (40 ml). 40 ml of methanol are added to the sedimented precipitate and this mixture is stirred for about 10 minutes. The precipitate is left to resediment for approximately 30 minutes. The supernatant is taken up and discarded (45 ml). 45 ml of methanol are added and the precipitate in suspension is then filtered off. The white cake obtained is then washed with 2 portions of 3 ml of methanol. The wet solid is drained by suction and then dried under reduced pressure (6 kPa) at a temperature in the region of 50°C. After drying for about 18 hours, 1.303 g of pure depolymerized VLMWH (sodium salt) are obtained.

The yield obtained is 86.8%.

g) Characteristics of the depolymerized VLMWH thus obtained

Average molecular weight: 1950 daltons

Polydispersity index: 1.1

Anti-Xa activity: 283 U/mg

aIIa activity: undetectable (<0.2 U/mg)

Example 2:

Very low molecular weight heparin (VLMWH) obtained by the process according to the invention, comprising a

step of 49% esterification and a step of depolymerization with a base derived from phosphazene, in anhydrous medium

5 **.Esterification of the VLMWH (step b) of the process):**

13.29 g (7.6 mmol) of VLMWH benzethonium salt obtained according to preparation 4 are dissolved in 70.43 g of anhydrous dichloromethane and placed in a 100 ml three-necked flask (the water content of the reaction medium is 0.073%). 12.3 ml (107 mmol) of benzyl chloride are added at a temperature of 30°C. The degree of esterification is 49% after reaction for about 7 hours at 30°C. After cooling, the reaction mixture is poured into 160 ml of a 12% solution of sodium acetate in methanol. The mixture is stirred for 1 hour at room temperature and then left to sediment for about 16 hours. The supernatant is discarded and then replaced with the same volume of methanol (100 ml). This mixture is stirred for about 1 hour and left to sediment for about 1 hour. The supernatant is again discarded and replaced with the same volume of methanol (100 ml). This mixture is stirred for about 5 minutes and then filtered. The cake is washed with 2 x 40 ml of methanol, drained by suction and then dried in an oven at 40°C under reduced pressure (6 kPa) for about 18 hours. 3.90 g of crude VLMWH benzyl ester sodium salt with a degree of esterification of 49% are obtained.

30

.Purification of the VLMWH benzyl ester (49% esterified) sodium salt (step b) of the process)

3.90 g of crude VLMWH benzyl ester sodium salt are dissolved in 39 ml of aqueous 10% NaCl solution. The solution is poured into 176 ml of methanol. The suspension is stirred for about 15 minutes and then left to sediment for 2 hours. The mixture is filtered. The cake is resuspended in 175 ml of methanol and

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stirred for 10 minutes. The mixture is filtered and the cake is washed with 2 portions of 10 ml of methanol. The wet white solid is drained by suction and dried in an oven at 40°C under reduced pressure (6 kPa) for about 18 hours. 2.62 g of VLMWH benzyl ester sodium salt are obtained.

The overall yield for the esterification phase is 57.3%.

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.Transsalification of the VLMWH benzyl ester to the benzethonium salt (step c) of the process):

2.62 g (4.37 mmol) of VLMWH benzyl ester sodium salt are dissolved in 20 ml of water (conical flask "A"). In parallel, 5.92 g (13.2 mmol) of benzethonium chloride are placed in 60 ml of water in a conical flask "B".

The content of "B" is poured into "A". The suspension is stirred for about 1 hour at room temperature and then left to sediment for 1 hour. The supernatant is discarded and replaced with the same volume of water (70 ml). This mixture is stirred for about 15 minutes and left to sediment for 1 hour. The supernatant is discarded and replaced with the same volume of water (70 ml). This mixture is stirred for a further 5 minutes approximately and filtered. The cake is washed with 3 portions of 50 ml of water, drained by suction and then dried in an oven at 80°C under reduced pressure (6 kPa) for about 18 hours. 6.85 g of VLMWH benzyl ester benzethonium salt are obtained.

The yield is 99%. The water content of the benzethonium salt is 0.6%.

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.Depolymerization of the VLMWH benzyl ester, benzethonium salt:

6.80 g (4.3 mmol) of VLMWH are placed in a 100 ml three-necked flask with 54 ml of dry dichloromethane. The mixture is brought to 30°C and then stirred until dissolution is complete. The estimated water content of the reaction mixture is about 0.05%. 1.25 ml (4.3 mmol) of 2-tert-butylimino-2-diethylamino-1,3-dimethylperhydro-1,3,2-diazaphosphorine are added and the mixture is stirred for 24 hours at 30°C under an inert atmosphere.

10

.Conversion of the quaternary ammonium salt into the sodium salt (step e) of the process)

In parallel, 270 ml of methanolic 10% sodium acetate solution are prepared in a 1 l conical flask. The reaction mixture is poured into the methanolic solution, while maintaining the temperature at about 4°C. The suspension is stirred for about 1 hour at room temperature. This mixture is left to sediment for 1 hour. The supernatant is discarded and then replaced with the same amount of methanol (165 ml). This mixture is stirred for about 1 hour and left to sediment for 1 hour. The supernatant is again discarded and replaced with the same amount of methanol (170 ml). This mixture is stirred for about 15 minutes and filtered. The cake is washed with 3 portions of 40 ml of methanol, drained by suction and then dried for about 18 hours in an oven at 50°C under reduced pressure (6 kPa). 2.29 g of crude depolymerized VLMWH, sodium salt, are obtained.

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The yield obtained is 89%.

.Saponification of the crude VLMWH, sodium salt (step f1) of the process):

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2.29 g (3.8 mmol) of crude depolymerized VLMWH, sodium salt, are dissolved in 23 ml of water. The solution is filtered through a 0.8 µm membrane and then placed in a 100 ml three-necked flask. 575 µl (5.73 mmol) of 30%

sodium hydroxide solution are introduced at a temperature in the region of 3°C. The mixture is stirred for about 2 hours at this temperature.

- 5 Half of the reaction mixture is neutralized by adding glacial acetic acid, followed by addition of 367 mg of solid sodium acetate and 13 ml of methanol. The solution is stirred for about 15 minutes and 65 ml of methanol are then added. The suspension obtained is
10 stirred for about 30 minutes and is then left to sediment for about 16 hours. The supernatant is discarded and replaced with the same amount of methanol (36 ml). This mixture is stirred for a further 30 minutes approximately and is left to sediment for
15 about 30 minutes. The supernatant is discarded and replaced with the same amount of methanol (16 ml). This mixture is stirred for about 15 minutes and filtered through a 0.22 µm membrane. The cake is washed with twice 5 ml of methanol, drained by suction and then
20 dried under reduced pressure (6 kPa) for about 18 hours in an oven at 50°C. 563 mg of crude depolymerized VLMWH (sodium salt) are obtained.

The yield is 52.6%.

25

.Purification of the crude depolymerized VLMWH (sodium salt) precipitated with NaOAc (step f2) of the process):

- 30 560 mg of crude depolymerized VLMWH (sodium salt) are placed in a 100 ml three-necked flask with 5.6 ml of water. The brown solution is maintained at 40°C for 10 minutes. The pH is brought to 9.7 by adding 0.1N sodium hydroxide solution. The solution is filtered
35 through a 0.45 µm membrane and 28 µl of aqueous 30% hydrogen peroxide solution are added. The mixture is stirred for 2 hours at room temperature, while keeping the pH constant at 9.5 ± 0.1 by adding 0.1N sodium hydroxide solution. The reaction mixture is neutralized

with 0.1N HCl and 620 mg of NaCl are added. After stirring for 10 minutes, the solution is filtered through a 0.45 μ m membrane. 4.35 ml of methanol are added at a temperature in the region of 4°C. The solution is stirred for 15 minutes at room temperature. 11.2 ml of methanol are added. The suspension is stirred for 1 hour. The stirring is then stopped and the mixture is left to sediment for 1 hour. The supernatant is then taken up and discarded (13.5 ml). 13.5 ml of methanol are added to the sedimented precipitate and the mixture is stirred for 15 minutes. The precipitate is left to resediment for about 30 minutes. The supernatant is taken up and discarded (13 ml). 13 ml of methanol are added and the precipitate in suspension is then filtered off. The white cake obtained is then washed with 2 portions of 5 ml of methanol. The wet solid is drained by suction and then dried under reduced pressure (6 kPa) at a temperature in the region of 50°C. After drying for 18 hours, 376 mg of pure depolymerized VLMWH (sodium salt) are obtained. The yield obtained is 67%.

Characteristics of the depolymerized VLMWH thus obtained

Anti-Xa activity: 191 IU/mg
Average molecular weight: 2100 Da

Example 3:

Very low molecular weight heparin (VLMWH) obtained by the process according to the invention, comprising a step of 73% esterification and a step of depolymerization with a base derived from phosphazene, in anhydrous medium

.Esterification of the VLMWH (step b) of the process):

13.7 g (7.3 mmol) of VLMWH benzethonium salt obtained according to preparation 4 are dissolved in 73.67 g of anhydrous dichloromethane and placed in a 100 ml three-

necked flask (the water content of the reaction medium is assayed as 0.23%). 13 ml (113 mmol) of benzyl chloride are added at a temperature of 30°C. The degree of esterification is 73% after reaction for about 20 hours at 30°C. After cooling to room temperature, the reaction mixture is poured into 210 ml of a 12% solution of sodium acetate in methanol. The mixture is stirred for 30 minutes at room temperature and then left to sediment for about 1.5 hours. The supernatant is discarded and then replaced with the same volume of methanol (140 ml). This mixture is stirred for 15 minutes and the suspension is filtered. The cake is washed with 2 portions of 100 ml of methanol, drained by suction and then dried for approximately 18 hours in an oven at 40°C under reduced pressure (6 kPa). 13.3 g of crude VLMWH benzyl ester sodium salt, with a degree of esterification of 73%, are obtained.

.Purification of the VLMWH benzyl ester (73% esterified), sodium salt (step b) of the process):

The 13.3 g of crude VLMWH benzyl ester sodium salt are dissolved in 133 ml of aqueous 10% NaCl solution. The solution is poured into 600 ml of methanol. The suspension is stirred for about 15 minutes and then left to sediment for approximately 1 hour. The supernatant is discarded and then replaced with the same volume of methanol (400 ml). This mixture is stirred for about 5 minutes and then filtered. The cake is washed with 3 times 100 ml of methanol. The wet white solid is drained by suction and then dried for approximately 18 hours in an oven at 40°C under reduced pressure (6 kPa). 2.33 g of VLMWH benzyl ester sodium salt are obtained.

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The esterification yield is 49.6%.

.Transsalification of the VLMWH benzyl ester, benzethonium salt (step c) of the process):

2.27 g (3.53 mmol) of VLMWH benzyl ester sodium salt are dissolved in 15 ml of water in a 100 ml conical flask "A". In parallel, 5.22 g (11.6 mmol) of benzethonium chloride are placed in 55 ml of water in a 100 ml conical flask "B".

The content of "B" is poured into "A". The suspension is stirred for about 1 hour at room temperature and then left to sediment for approximately 1 hour. The supernatant is discarded and replaced with the same volume of water (50 ml). The mixture is stirred for about 15 minutes and left to sediment for approximately 1 hour. The supernatant is discarded and replaced with the same volume of water (50 ml). The mixture is stirred for a further 5 minutes and then filtered. The cake is washed with 3 portions of 50 ml of water, drained by suction and then dried for about 18 hours in an oven at 80°C under reduced pressure (6 kPa). 5.67 g of VLMWH benzyl ester benzethonium salt are obtained.

The yield obtained is 98%. The water content of the product obtained is 1%.

.Depolymerization of the VLMWH benzyl ester, benzethonium salt (step d) of the process):

5.45 g (3.3 mmol) of VLMWH are placed in a 100 ml three-necked flask with 40 ml of dry dichloromethane. The estimated water content of the mixture is about 0.1%. The mixture is brought to 30°C. 958 µl (3.3 mmol) of 2-tert-butylimino-2-diethylamino-1,3-dimethylperhydro-1,3,2-diazaphosphorine are added and the mixture is stirred for 24 hours at 30°C under an argon atmosphere.

.Conversion of the quaternary ammonium salt into the sodium salt (step e) of the process)

In parallel, 200 ml of methanolic 10% sodium acetate solution are prepared in a 500 ml conical flask. The reaction mixture is poured into the methanolic solution, while maintaining the temperature at about 4°C. The suspension is stirred for about 1 hour at room temperature. This mixture is left to sediment for approximately 1 hour. The supernatant is discarded and then replaced with the same amount of methanol (150 ml). This mixture is stirred for about 30 minutes and left to sediment for about 30 minutes. The supernatant is again discarded and replaced with the same amount of methanol (150 ml). This mixture is stirred for approximately 15 minutes and then filtered. The cake is washed with 3 times 50 ml of methanol, drained by suction and then dried for about 18 hours at 50°C under reduced pressure (6 kPa). 1.40 g of crude depolymerized VLMWH, sodium salt, are obtained. The yield obtained is 65.8%.

.Saponification of the crude depolymerized VLMWH, sodium salt (step f1) of the process):

1.40 g (2.18 mmol) of crude depolymerized VLMWH (sodium salt) are dissolved in 14 ml of water. The solution is placed in a 100 ml three-necked round-bottomed flask. 351 µl (3.5 mmol) of 30% sodium hydroxide solution are introduced at a temperature in the region of 4°C. This mixture is stirred for about 2 hours at this temperature. The solution is neutralized by adding glacial acetic acid (100%). 7 g of solid sodium acetate and 130 ml of methanol are then added. The suspension is stirred for 30 minutes and is then left to sediment for about 1 hour. The supernatant is discarded and replaced with the same amount of methanol (80 ml). This mixture is stirred for a further 30 minutes approximately and is left to sediment for approximately 16 hours. The supernatant is discarded and replaced with the same amount of methanol (80 ml). This mixture is stirred for about 15 minutes and is then filtered

through a 0.45 μm membrane. The cake is washed with twice 10 ml of methanol, drained by suction and then dried for about 18 hours at 50°C under reduced pressure (6 kPa). 1.15 g (yield: 89.4%) of crude depolymerized
5 VLMWH (sodium salt) are obtained.

The yield obtained is 89.4%.

**.Purification of the crude depolymerized VLMWH, sodium
10 salt (step f2) of the process):**

373 mg of crude depolymerized VLMWH (sodium salt) are placed in a 10 ml three-necked flask with 3.7 ml of water. The solution is maintained at 40°C for
15 10 minutes. The pH is brought to about 9.5 by adding 1N sodium hydroxide solution. The solution is filtered through a 0.45 μm membrane and 18 μl of aqueous 30% hydrogen peroxide solution are then added. The mixture is stirred for about 2 hours at room temperature, while
20 keeping the pH constant at 9.5 ± 0.1 by adding 0.1N sodium hydroxide solution. The reaction mixture is neutralized with 0.1N HCl and 430 mg of NaCl are then added. After stirring for about 10 minutes, the solution is filtered through a 0.45 μm membrane. 3 ml
25 of methanol are added at a temperature in the region of 4°C. The solution is stirred for 15 minutes at room temperature. 7.7 ml of methanol are then added. The suspension is stirred for about 1 hour. The stirring is then stopped and the mixture is left to sediment for
30 approximately 40 minutes. The supernatant is then taken up and discarded (10 ml). 10 ml of methanol are added to the sedimented precipitate and the mixture is stirred for 15 minutes. The precipitate is left to resediment for about 30 minutes. The supernatant is
35 taken up and discarded (10 ml). 10 ml of methanol are added and the precipitate in suspension is then filtered off on a 0.45 μm membrane. The white cake obtained is washed with 4 portions of 5 ml of methanol. The wet solid is drained by suction and then dried

under reduced pressure (6 kPa) at a temperature in the region of 50°C. After drying for about 18 hours, 199 mg of pure depolymerized VLMWH (sodium salt) are obtained. The yield obtained is 54%.

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Characteristics of the depolymerized VLMWH thus obtained

Average molecular weight: 2000 daltons.

10 Polydispersity index: 1.1

Anti-Xa activity: 252 IU/mg

Example 4:

15 VLMWH obtained by the process according to the invention, comprising a step of 96% esterification and a step of depolymerization with BEMP

.Esterification of the VLMWH (step b) of the process)

20

14.45 g (7.7 mmol) of VLMWH benzethonium salt obtained according to preparation 4 are dissolved in 75.79 g of anhydrous dichloromethane and placed in a 250 ml three-necked flask (the water content of the reaction medium is 0.20%). 12.4 ml (108 mmol) of benzyl chloride are added at a temperature of 30°C. The degree of esterification is 96% after reaction for about 26 hours at 30°C. After cooling to room temperature, the reaction mixture is poured into 180 ml of a 12% solution of sodium acetate in methanol. The mixture is stirred for about 30 minutes at room temperature and is then left to sediment for approximately 30 minutes. The supernatant is discarded and then replaced with the same volume of methanol (150 ml). This mixture is stirred for about 15 minutes and then filtered. The cake is washed with 2 portions of 100 ml of methanol, drained by suction and then dried for about 18 hours at 40°C under reduced pressure (6 kPa). 3.67 g of crude

VLMWH benzyl ester, sodium salt, with a degree of esterification of 96%, are obtained.

.Purification of the VLMWH benzyl ester (96% esterified) sodium salt (step b) of the process):

The 3.67 g of crude VLMWH benzyl ester, sodium salt are dissolved in 37 ml of aqueous 10% NaCl solution (3.7 g of NaCl in 37 ml of water). The solution is poured into 10 167 ml of methanol. The suspension is stirred for about 15 minutes and is then left to sediment for approximately 1 hour. The supernatant is discarded and then replaced with the same volume of methanol (38 ml). This mixture is stirred for about 5 minutes and 15 filtered. The cake is washed with twice 30 ml of methanol. The wet white solid is drained by suction and dried for about 18 hours at 40°C under reduced pressure (6 kPa). 2.76 g of VLMWH benzyl ester, sodium salt are obtained.

20

The esterification yield is 54.4%.

. Transsalification of the VLMWH benzyl ester to the benzethonium salt (step c) of the process):

25

2.83 g (4.29 mmol) of VLMWH benzyl ester, sodium salt, are dissolved in 20 ml of water in a 100 ml conical flask "A". In parallel, 6.35 g (14.2 mmol) of benzethonium chloride are placed in 50 ml of water in a 30 100 ml conical flask "B".

The content of "B" is poured into "A". The suspension is stirred for about 1 hour at room temperature and is then left to sediment for approximately 1 hour. The 35 supernatant is discarded and replaced with the same volume of water (60 ml). This mixture is stirred for about 15 minutes and left to sediment for approximately 1 hour. The supernatant is discarded and replaced with the same volume of water (60 ml). This mixture is

stirred for a further 5 minutes and then filtered. The cake is washed with 4 times 50 ml of water, drained by suction and then dried for about 18 hours at 80°C under reduced pressure (6 kPa). 7.0 g of VLMWH benzyl ester, benzethonium salt, are obtained.

The observed yield is about 100%. The water content is 0.23%.

10 **.Depolymerization of VLMWH benzyl ester, benzethonium salt (step d) of the process):**

3.67 g (2.3 mmol) of VLMWH are placed in a 50 ml three-necked round-bottomed flask with 27 ml of dry dichloromethane. The mixture is brought to 30°C. 676 µl (2.3 mmol) of 2-tert-butylimino-2-diethylamino-1,3-dimethylperhydro-1,3,2-diazaphosphorine are added and the mixture is stirred for 24 hours at 30°C.

20 **.Conversion of the quaternary ammonium salt into the sodium salt (step e) of the process):**

In parallel, 150 ml of methanolic 10% sodium acetate solution are prepared in a 250 ml conical flask. The reaction mixture is poured into the methanolic solution, while maintaining the temperature at about 4°C. The suspension is stirred for about 1 hour at room temperature. This mixture is left to sediment for approximately 1 hour. The supernatant is discarded and then replaced with the same amount of methanol (100 ml). This mixture is stirred for 30 minutes and left to sediment for 30 minutes. The supernatant is again discarded and replaced with the same amount of methanol (100 ml). This mixture is stirred for 15 minutes and filtered. The cake is washed with 3 times 40 ml of methanol, drained by suction and dried for about 18 hours at 50°C under reduced pressure (6 kPa). 966 mg of depolymerized VLMWH, sodium salt, are obtained.

The yield obtained is 64%.

**.Saponification of the VLMWH benzyl ester, sodium salt
5 (step f1) of the process):**

942 mg (1.43 mmol) of depolymerized VLMWH sodium salt are dissolved in 9.5 ml of water. The solution is placed in a 100 ml three-necked round-bottomed flask.
10 236 μ l (2.35 mmol) of 30% sodium hydroxide solution are introduced at a temperature in the region of 4°C. This mixture is stirred for about 2 hours at room temperature. The solution is neutralized by addition of glacial acetic acid (100%). 4.5 g of solid sodium
15 acetate and 85 ml of methanol are then added. The suspension is stirred for about 30 minutes and then left to sediment for about 1 hour. The supernatant is discarded and replaced with the same amount of methanol (40 ml). This mixture is stirred for a further
20 30 minutes approximately and is left to sediment for about 16 hours. The supernatant is discarded and replaced with the same amount of methanol (40 ml). This mixture is stirred for about 30 minutes and filtered through a 0.45 μ m membrane. The cake is washed with
25 twice 10 ml of methanol, drained by suction and then dried for about 18 hours at 50°C under reduced pressure (6 kPa). 776 mg of crude depolymerized VLMWH (sodium salt) are obtained.

30 The yield obtained is 91.4%.

.Purification of the crude depolymerized VLMWH, sodium salt (step f2) of the process):

35 758 mg of crude depolymerized VLMWH (sodium salt) are placed in a 25 ml three-necked flask with 7.6 ml of water. The solution is maintained at 40°C for 10 minutes. The pH is brought to about 9.5 by addition of 0.1N sodium hydroxide solution. The solution is

filtered through a 0.45 μ m membrane, and 38 μ l of aqueous 30% hydrogen peroxide solution are then added. The mixture is stirred for about 2 hours at room temperature, while keeping the pH constant at 9.5 ± 0.1 by addition of 0.1N sodium hydroxide solution. The reaction mixture is neutralized with 1N HCl and 880 mg of NaCl are added. After stirring for about 10 minutes, the solution is filtered through a 0.45 μ m membrane. 6.2 ml of methanol are added at a temperature in the region of 4°C. The solution is stirred for approximately 15 minutes at room temperature. 16 ml of methanol are added and the suspension is stirred for about 1 hour. The stirring is then stopped and the suspension is filtered. The cake is washed with 2 portions of 15 ml of methanol. The wet solid is drained by suction and then dried under reduced pressure (6 kPa) at a temperature in the region of 50°C. After drying for approximately 18 hours, 490 mg of pure depolymerized VLMWH (sodium salt) are obtained. The yield obtained is 65%.

Characteristics of the depolymerized VLMWH thus obtained

Average molecular weight: 2000 daltons

Polydispersity index: 1.1

Anti-Xa activity: 205 IU/mg

Example 5:

VLMWH obtained by the process according to the invention, comprising a step of 96% esterification and a step of depolymerization with tert-butyliminotris(dimethylamino)phosphorane

.Depolymerization of VLMWH benzyl ester, benzethonium salt (step d) of the process):

3.67 g (2.3 mmol) of VLMWH benzyl ester, benzethonium salt, obtained according to example 4 (96% esterified), with a water content of 0.23%, are placed in a 50 ml

three-necked flask with 30 ml of dry dichloromethane. The mixture is brought to 30°C. 595 µl (2.3 mmol) of tert-butyliminotris(dimethylamino)phosphorane are added and the mixture is stirred for 24 hours at 30°C.

5

.Conversion of the quaternary ammonium salt into the sodium salt (step e) of the process):

10 In parallel, 160 ml of methanolic 10% sodium acetate solution are prepared in a 250 ml conical flask. The reaction mixture is poured into the methanolic solution, while maintaining the temperature at about 4°C. The suspension is stirred for approximately 1 hour at room temperature. This mixture is left to sediment
15 for about 1 hour. The supernatant is discarded and then replaced with the same amount of methanol (120 ml). This mixture is stirred for about 30 minutes and left to sediment for 30 minutes. The supernatant is again discarded and replaced with the same amount of methanol
20 (125 ml). This mixture is stirred for about 15 minutes and filtered. The cake is washed with 3 times 40 ml of methanol, drained by suction and dried for about 18 hours at a temperature in the region of 50°C under reduced pressure (6 kPa). 982 mg of crude depolymerized
25 VLMWH, sodium salt, are obtained. The yield obtained is 65%.

.Saponification of the crude depolymerized VLMWH, sodium salt (step f1) of the process):

30

980 mg (1.49 mmol) of crude depolymerized VLMWH, sodium salt, are dissolved in 10 ml of water. The solution is placed in a 100 ml three-necked round-bottomed flask. 246 µl (2.45 mmol) of 30% sodium hydroxide solution are
35 introduced at a temperature in the region of 4°C. This mixture is stirred for about 2 hours at this temperature. The solution is neutralized by adding glacial acetic acid (100%). 4.9 g of solid sodium acetate and 95 ml of methanol are then added. The

suspension is stirred for 30 minutes and then left to sediment for about 1 hour. The supernatant is discarded and then replaced with the same amount of methanol (60 ml). This mixture is stirred for a further
5 30 minutes approximately and is then left to sediment for about 16 hours. The supernatant is discarded and then replaced with the same amount of methanol (60 ml). This mixture is stirred for about 30 minutes and filtered through a 0.45 μ m membrane. The cake is washed
10 with twice 10 ml of methanol, drained by suction and then dried for approximately 18 hours at 50°C under reduced pressure (6 kPa). 809 mg of crude depolymerized VLMWH, sodium salt, are obtained.

15 The reaction yield is 91.6%.

.Purification of the crude depolymerized VLMWH, sodium salt (step f2) of the process):

20 792 mg of crude depolymerized VLMWH (sodium salt) are placed in a 25 ml three-necked flask with 8 ml of water. The solution is maintained at 40°C for 10 minutes. The pH is brought to about 9.5 by adding 0.1N sodium hydroxide solution. The solution is
25 filtered through a 0.45 μ m membrane, and 39.6 μ l of aqueous 30% hydrogen peroxide solution are then added. The mixture is stirred for 2 hours at room temperature, while keeping the pH constant at 9.5 ± 0.1 by adding 0.1N sodium hydroxide solution. The reaction mixture is
30 neutralized with 1N HCl and 1.04 g of NaCl are added. After stirring for about 10 minutes, the solution is filtered through a 0.45 μ m membrane. 7.3 ml of methanol are added at a temperature in the region of 4°C. The solution is stirred for 15 minutes at room temperature.
35 18.8 ml of methanol are added and the suspension is stirred for about 1 hour. The stirring is then stopped and the mixture is filtered. The cake is washed with 3 portions of 15 ml of methanol. The wet solid is drained by suction and then dried under reduced

pressure (6 kPa) at a temperature in the region of 50°C. After drying for 18 hours, 538 mg of pure depolymerized VLMWH (sodium salt) are obtained. The yield obtained is 67.9%.

5

Characteristics of the depolymerized VLMWH thus obtained

Average molecular weight: 2100 daltons

Polydispersity index: 1.1

10 Anti-Xa activity: 209 IU/mg

Example 6:

VLMWH obtained by the process according to the invention, comprising an additional step of separation
15 by chromatography to remove the disaccharide and tetrasaccharide fractions

The oligosaccharide mixture described in example 1 (286 mg) is dissolved in 20 ml of mobile phase (aqueous
20 sodium bicarbonate solution at a concentration of 0.2 mol/l).

The chromatographic conditions are as follows:

- 25 - Mobile phase: sodium bicarbonate solution at a concentration of 0.2 mol/l
- Stationary phase: biogel P6 gel
- Column: length 1 m, diameter 5 cm
- Detection wavelength: 240 nm.

30 The fractions greater than or equal to a hexasaccharide are collected and pooled. They are neutralized with acetic acid and then concentrated until a solution containing 200 g/l of sodium acetate is obtained. 5 volumes of methanol are added to the solution
35 obtained with stirring. The suspension is stirred for about 18 hours and then filtered through a 0.45 µm membrane. The cake is dried for about 6 hours at a temperature in the region of 40°C under reduced pressure (6 kPa). The product obtained is

reprecipitated and then dissolved in a minimum amount of water and desalified on a Sephadex G10 column. After concentrating the desalified fractions and then freeze-drying, 109 mg of product are obtained. The yield is 5 38%.

The characteristics of the oligosaccharide mixture thus obtained are as follows:

10 Anti-Xa activity: 403 IU/mg

The oligosaccharide percentage is as follows:

Mw (Da)	Poly- dispersity	Di %	Tetra %	Hexa %	Octa %	Deca %	>Deca %
2150	1.0	0	0	53.83	32.58	10.5	3.5

15 Pharmacological activity of the compounds according to the invention:

Examples	Average molecular weight	Anti-Xa activity IU/mg	Anti-IIa activity
1	1950	283	<0.2
2	2100	191	0
3	2000	252	0
4	2000	205	0
5	2100	209	0
6	2150	403	0

Percentage of hexasaccharide Δ IIa-II_s-Is in the 20 compounds according to the invention:

Examples	Percentage of hexasaccharide fraction	Percentage of hexasaccharide Δ IIa- II _s -Is in the hexasaccharide fraction
1	31%	46%
2	30%	26%
3	33%	33.5%
4	32%	30.8%
5	31.5%	28.7%
6	53.8%	46%

CLAIMS

1) An oligosaccharide mixture having the general structure of the constituent polysaccharides of heparin and having the following characteristics:

- an average molecular weight of from 1800 to 2400 daltons,

- anti-Xa activity of between 190 IU/mg and 450 IU/mg

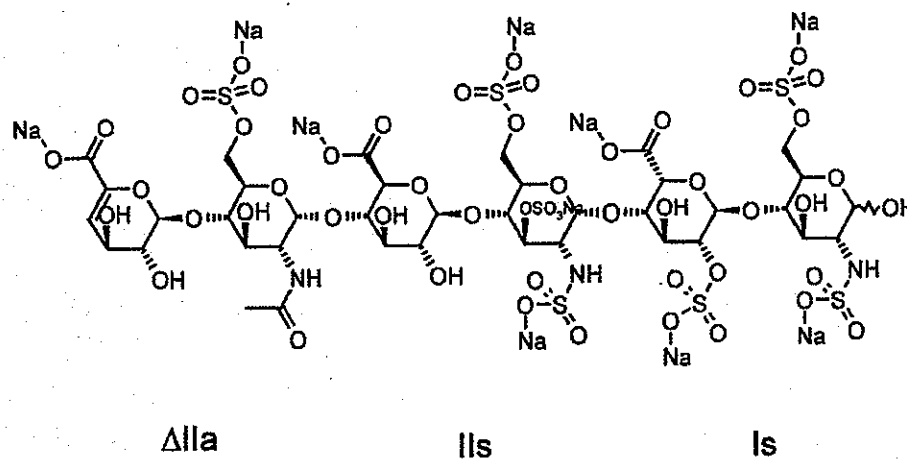
- no, or virtually no, anti-IIa activity,

- it being understood that the constituent oligosaccharides of the mixtures

- contain from 2 to 16 saccharide units,

- have a 4,5-unsaturated uronic acid 2-O-sulfate unit at one of their ends,

- and contain the hexasaccharide of the following formula:



in the form of an alkali metal or alkaline-earth metal salt.

2) The oligosaccharide mixture as claimed in claim 1, wherein the alkali metal or alkaline-earth metal salts are the sodium, potassium, calcium and magnesium salts.

3) The oligosaccharide mixture as claimed in claim 1 or 2, which contains from 20% to 100% and in particular from 30% to 60% of hexasaccharide fraction.

4) The oligosaccharide mixture as claimed in claim 3, wherein the hexasaccharide fraction contains from 20% to 70% and in particular from 25% to 50% of the hexasaccharide Δ IIa-II $\underline{s}$ -Is as defined in claim 1.

5) The oligosaccharide mixture as claimed in any one of claims 1 to 4, which has an average molecular weight of between 1900 and 2200 and in particular from 1950 to 2150 daltons.

6) The oligosaccharide mixture as claimed in any one of claims 1 to 5, which has anti-Xa activity of between 190 IU/mg and 410 IU/mg and in particular between 200 and 300 IU/mg, while at the same time having no, or virtually no, anti-IIa activity.

7) The oligosaccharide mixture as claimed in any one of claims 1 to 6, which has the following characteristics:

- an average molecular weight of between 1950 and 2150 daltons,

- anti-Xa activity of between 190 IU/mg and 410 IU/mg and no, or virtually no, anti-IIa activity,

- it contains from 30% to 60% of hexasaccharide fraction, which contains from 25% to 55% of Δ IIa-II $\underline{s}$ -Is fraction.

8) A process for preparing the oligosaccharide mixture as claimed in any one of claims 1 to 7, wherein a very

low molecular weight heparin with aXa activity of greater than 140 IU/mg, aIIa activity of greater than 5 IU/mg and an average molecular mass of between 2000 and 3000 daltons is subjected to the following chemical reactions:

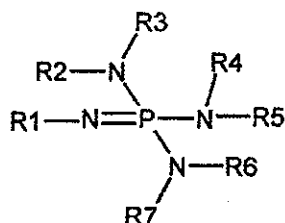
- a) transsalification by the action of benzethonium chloride to obtain benzethonium heparinate,
- b) esterification of the benzethonium heparinate obtained by the action of benzyl chloride, and treatment to obtain the sodium salt of the benzyl ester of the very low molecular weight heparin,
- c) transsalification of the benzyl ester obtained and production of the quaternary ammonium salt,
- d) depolymerization by means of a strong organic base with a pKa value preferably of greater than 20, so as to obtain a depolymerized very low molecular weight heparin,
- e) conversion of the quaternary ammonium salt of the depolymerized very low molecular weight heparin into the sodium salt,
- f) saponification of the residual esters and optional purification.

9) The preparation process as claimed in claim 8, wherein the strong base/ester mole ratio used during the polymerization step d) is between 0.2 and 5 and preferably between 0.6 and 2.

10) The preparation process as claimed in claim 8 or 9, wherein the depolymerization step d) is performed using a phosphazene derivative as base.

11) The preparation process as claimed in any one of claims 8 to 10, wherein the depolymerization step d) is performed with water contents of less than 0.3% when the process is performed with 1 molar equivalent of phosphazene base relative to the benzyl ester of the VLMWH, benzethonium salt.

12) The preparation process as claimed in any one of claims 8 to 11, wherein the bases of the phosphazene family used during the depolymerization step d) are preferably those of formula:



in which the radicals R_1 to R_7 , which are identical or different, represent linear, branched or cyclic alkyl radicals containing from 1 to 6 carbon atoms, it being possible for R_3 and R_4 , where appropriate, to form, with the $-\text{N}-\text{P}-\text{N}$ group which carries them, a 6-membered heterocycle.

13) The preparation process as claimed in any one of claims 8 to 12, wherein the base of the family of phosphazenes used in the depolymerization step is 2-tert-butylimino-2-diethylamino-1,3-dimethylperhydro-1,3,2-diazaphosphorine.

14) The preparation process as claimed in claim 8, wherein the degree of esterification of the quaternary ammonium salt of the benzyl ester of heparin during step b) is between 40% and 100% and preferably between 70% and 90%.

15) The preparation process as claimed in claim 8, wherein the conversion of the quaternary ammonium salt of the benzyl ester of the very low molecular weight heparin as prepared according to step b) of the process as claimed in claim 8, into the sodium salt, is performed by treating the reaction medium with alcoholic sodium acetate solution and preferably with a 10% solution of sodium acetate in

methanol (weight/volume), at a temperature of between 15 and 25°C.

16) The preparation process as claimed in claim 15, wherein the weight equivalent of sodium acetate added during the esterification step b) is 3 times as great as the mass of quaternary ammonium salt of the benzyl ester of heparin used in the depolymerization reaction.

17) The process as claimed in claim 8 for preparing oligosaccharide mixtures, wherein the quaternary ammonium salt of the benzyl ester of the very low molecular weight heparin obtained during step c) is preferably the benzethonium, cetylpyridinium or cetyltrimethylammonium salt.

18) The preparation process as claimed in claim 8, wherein the saponification (according to step f) is performed using an alkali metal hydroxide such as sodium hydroxide, potassium hydroxide or lithium hydroxide, in aqueous medium, at a temperature of between 0 and 20°C and preferably between 0 and 10°C.

19) The preparation process as claimed in claim 18, wherein from 1 to 5 molar equivalents of alkali metal hydroxide and more particularly from 1 to 2 molar equivalents of alkali metal hydroxide are used.

20) The process, as claimed in any one of claims 8 to 19, for preparing the oligosaccharide mixture as described in any one of claims 1 to 5 having increased selectivity toward factor Xa of the oligosaccharide mixture, wherein the disaccharide and tetrasaccharide fractions are also removed by chromatography, especially on columns filled with gel of polyacrylamide agarose type.

21) The process as claimed in any one of claims 8 to 20 for preparing the oligosaccharide mixture as claimed in

any one of claims 1 to 7, wherein a low molecular weight heparin is used as starting material, or a very low molecular weight heparin (1500 to 3000 Da) with an anti-Xa activity of between 100 and 140 IU/mg instead of the very low molecular weight heparin as defined in claims 8.

22) The process as claimed in any one of claims 8 to 20 for preparing the oligosaccharide mixture as claimed in any one of claims 1 to 7, wherein a very low molecular weight heparin (1500 to 4000 Da) with an anti-Xa activity of between 100 and 140 IU/mg is used as starting material instead of the very low molecular weight heparin as defined in claim 8.

23) The preparation process as claimed in claim 21, wherein the low molecular weight heparin is chosen from Enoxaparin, Fraxiparin, Fragmin, Innohep (or Logiparin), Normiflo, Embollex (or Sandoparin), Fluxum (or Minidaltol), Clivarin and Hibor.

24) The oligosaccharide mixture as defined in any one of claims 1 to 7, which may be obtained by the process as defined in any one of claims 7 to 23.

25) As a medicinal product, the oligosaccharide mixture as claimed in any one of claims 1 to 7.

26) As a medicinal product with antithrombotic activity, the oligosaccharide mixture as claimed in any one of claims 1 to 7.

27) The medicinal product as claimed in claim 25 or 26, for treating or preventing venous and arterial thrombosis, deep vein thrombosis, pulmonary embolism, unstable angina, myocardial infarction, cardiac ischemia, occlusive diseases of the peripheral arteries and atrial fibrillation, smooth muscle cell proliferation, atherosclerosis and arteriosclerosis,

cancer by modulating angiogenesis and growth factors, and also diabetic disorders such as diabetic retinopathy and diabetic nephropathy.

28) A pharmaceutical composition containing at least one medicinal product as defined in claim 25 and one or more pharmaceutically inert excipients or vehicles or additives.

29) The pharmaceutical composition as claimed in claim 28, which consists of a solution for subcutaneous or intravenous injection.

30) The pharmaceutical composition as claimed in claim 28, which consists of a pulmonary formulation for inhalation.

31) The pharmaceutical composition as claimed in claim 28, which consists of an oral formulation.

32) A method for determining the anti-Xa activity of the oligosaccharide mixture as claimed in any one of claims 1 to 7, wherein an amidolytic method is used on a chromogenic substrate in which the reconstitution buffer is Polyethylene Glycol 6000 (PEG 6000).