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(54) ARTERIAL POLYSACCHARIDE COMPLEX, PROCESS FOR ITS PREPARATION AND ITS USE IN HUMAN THERAPY

(71) We, MEDIOLANUM FARMACEUTICI S.r.l., of Via San Giuseppe Cottolengo, 31, Milan, Italy, a Company organised and existing under the laws of Italy, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates to a new polysaccharide complex produced by extraction from the aorta of young mammals, and its use in the cure and prevention of arteriopathies.

In recent years, numerous drugs have been proposed for aiding the return to normal concentrations of the various lipid components of the serum, in consideration of the particular seriousness of hyper and dislipidemias in the etiopathogenesis of numerous illnesses, including illnesses of social interest. These drugs can be distinguished schematically in the following manner by their action mechanism:

1) those which inhibit the intestinal absorption of cholesterol
2) those which act on the secretion of bile acids
3) those which inhibit the synthesis of cholesterol and the mobility of the fatty acids of the adipose tissue
4) those which activate fibrinolysis and thrombolysis
5) those which favour increase in the removal of lipoproteins.

Among these latter drugs, heparin and analogous substances generally known as heparinoids have assumed special importance in the treatment of hyper and dislipidemias.

Among the various pharmacological actions of heparin, one of particular importance is the so-called clarifying action which consists essentially of inducing the appearance in the bloodstream of lipoproteinlipase, a physiological enzyme which separates the ester bonds of triglycerides to transform them into monoglycerides and free fatty acids. This action is entirely independent of the anticoagulating action, which represents the negative side and determines the limit of use of heparin in this type of therapy.

Again, within the field of antiarteriosclerotic therapy, it has been stated in published works (Comi and Coll.—Boll. Chim. Farm. 106/5/309/21 (1967); French patent M 4,871 filed 24 Nov. 1965) that by administering total aorta extracts to rabbits made hypercholesterolemic by means of cholesterol-based diets, a strong reduction in atheromatosis formations has been obtained. On the other hand, it has been shown (Dall'Occhi and Coll. Adv. Exi. Med. Biol. 1967 1,468/83) that the total aorta extract leads to reactions of immunity with harmful effects on the pulmonary arteries.

We have now discovered, and the present invention relates thereto, that it is possible to extract a specific polysaccharide complex of anionic character from the aorta of young mammals, which has demonstrated surprising antiarteriosclerotic properties in addition to a complete lack of side-effects.

This new complex, which for simplicity will be indicated hereinafter by the letters FAPA (Arterial Antiarteriosclerotic Polysaccharide Factor), is characterised unambiguously by the following chemical-physical properties:

a) on hydrolysis it liberates hexosamine, uronic acids and sulphates in the molar ratios of approximately 1/1/1 with variable quantities of nucleosides.

After acid hydrolysis of the FAPA, analysis shows the following average composition of the polysaccharide components.

— hexosamine (glucosamine): specific colorimetric reaction of the amine in hexoses with p-dimethylaminobenzaldehyde =30±2.25%
— uronic acid: specific colorimetric reaction with carbazole =30.5±5%
— organic SO_4: in accordance with FU 8th ed. Vol. I page 106 paragraph B =16.5±3%
— Na: determined by atomic absorption (not routine) =10±2%
— ash =15±3%

b) electrophoretically, it separates into three anionic bands distinguishable with Alcian Blue and Toluidine Blue.

The following table shows the relative electrophoretic mobility of the three anionic bands

Band	Anodic mobility	Average densitometric ratios of positive Toluidine blue bands
1	U = 2.2 ± 0.5 . 10^{-4} $cm^2V^{-1}sec.^1$	14 ± 3%
2	U = 2.09 ± 0.5 . 10^{-4} $cm^2V^{-1}sec.^1$	9 ± 2%
3	U = 1.93 ±0.05 . 10^{-4} $cm^2V^{-1}sec.^1$	77 ± 8%

c) it reacts with alkaline and alkaline earth metals to form salts which are very soluble in water, whereas the salts which it forms with the aliphatic ammonium ion are insoluble.

d) the sodium salt is 2% soluble in 50% ethyl alcohol, whereas the acid form is 2% soluble in 70% ethyl alcohol

e) it reacts with Toluidine Blue to give a minimum metachromatic reaction. The term "minimum metachromatic reaction" as used herein and in the claims hereof means a very small change in colour which is approximately 1/10 of that given by heparin,

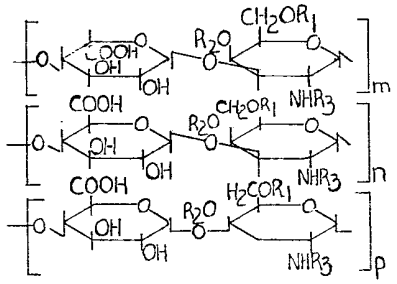
f) spectrophotometrically, it shows a maximum at 260±1 nm

g) it shows a rotatory power on polarised light of −11° to −22°

h) the infrared spectrum of the substance determined in KBr, to which the graph of the accompanying drawing refers, shows the following functions:

—COOH
—NHR
—$COCH_3$
—OSO_3H
—CH_2OH

From the total analytical data it can be established that FAPA has a structure of the following type:



where R_1 and R_2 are —H or —SO_3H, R_3 is —SO_3H or —CO—CH_3, and m, n, p are whole numbers which can vary over a wide range and define a molecular weight of 10,000 to 30,000 daltons for the polysaccharide chains.

These characteristics, and in particular the molecular ratios of the components obtained after hydrolysis, indicate that FAPA may be defined chemically as a molecular complex consisting of hexosamine glycanmonosulphates (chondroitin sulphates B and C and heparin sulphates of the arterial wall).

The process for producing FAPA according to the present invention is characterised essentially by the following operations:

1) removing and immediately grinding the aortas of young mammals which have just been slaughtered
2) homogenising and suspending said tissues in water or suitable saline solutions
3) attack by proteolytic enzymes such as trypsin, chymotrypsin, pancreatin, papain, ficin or bromelin, under the respective optimum pH, temperature and time conditions
4) separating the digested liquid by filtration, and collecting it
5) precipitating FAPA from the digested liquid by solvents miscible with water and/or by forming insoluble salts with the aliphatic ammonium ion
6) purifying by salinifying with alkaline or alkaline earth metal bases, decolouration with permangenate, treatment with alkali at 70°C for eliminating the terminal parts of the residual peptide chains, separation of the desired salt form with solvents (sodium, potassium, calcium salts etc.), filtration, sterilisation and lyophilisation.

With regard to its pharmacological properties, FAPA has been examined in comparison with certain antiarteriosclerotic preparations of the aforesaid type; in particular it has been examined in comparison with heparin, the clarifying drug for antonomasia, and with the total aorta extract.

The following basic characteristics were determined by in vivo experimentation:

1. *Action on experimental arteriosclerosis*

This study was carried out on rabbits divided into batches of 5 animals for treatment and fed with a heterogeneous diet rich in cholesterol.

The blood of the animals was tested for determining the esterified fatty acids (EFA), the total cholesterol, the betalipoproteins and the coagulation time. The cholesterol content of the aortic tissue was determined, together with the incidence of atheromas.

Table 1 summarises the results obtained using FAPA, heparin and total aorta extract, after 40 days of treatment.

TABLE I

	EFA mg/100 ml of serum	Cholesterol mg/100 ml of serum	B-lipoproteins mg/100 ml of serum	Coag. time log. of minutes	Aort. cholesterol mg/100 g of dry substance	Atheromas
Controls	456 ± 14	215 ± 19	78 ± 4	0.78 ± 0.06	2.85 ± 0.12	++++
FAPA 1 mg/kg	180 ± 5	90 ± 15	40 ± 2.5	0.81 ± 0.04	1.98 ± 0.08	++
FAPA 10 mg/kg	162 ± 8	76 ± 13	35 ± 13	0.9 ± 0.04	1.7 ± 0.04	+
Heparin 1 mg/kg	171 ± 11	78 ± 13	38 ± 1	1.9 ± 0.4	2 ± 0.05	+
Heparin 10 mg/kg	138 ± 8	76 ± 15	30 ± 2	2.3 ± 0.8	1.8 ± 0.03	+
Total aorta extract 1 mg/kg	430 ± 14	190 ± 14	79 ± 3	0.75 ± 0.1	2.9 ± 0.2	++++
Total aorta extract 10 mg/kg	218 ± 4	97 ± 1	36 ± 4	0.78 ± 0.1	1.6 ± 0.04	++

4+ = marked occurrence of atheromas in terms of number and extension

2++ = about 50% of occurrence in controls

1+ = occurrence approximately equal to that in normal rabbits

2. *Biological activity*

The aspects of biological activity determined were the anticoagulating activity (in accordance with USP) and the clarification activity on rats (Morton Grosman method J. Lab. and Clin. Med. March 1954 page 445). With regard to this latter determination, modifications were introduced in the present case to enable the effect to be expressed in international lipasemic units (ULI), in accordance with the UIB definition (International Biochemical Union), where one international unit (ULI) is equal to the activity which transforms the substrate at a speed of 1 micromole per minute.

The results of the biological activity aspects of FAPA are shown in Table 2, in comparison with heparin and aorta extract.

TABLE 2

	Heparin	Aorta extract	FAPA
Anticoagulating activity (USP/mg)	135–148	About 0.5	5–15
Clarification activity (ULI/1 of plasma) at a dose of 0.5 mg/kg	480 ±100	15 ±7	270 ±80

3. *Product tolerance*

A) — Toxicity: FAPA proved to be well tolerated by rats, rabbits and guinea pigs treated either by parenteral administration or by oral administration with per diem doses of 200 mg/kg.

b) — Immunological tests: In guinea pigs treated with FAPA, immunological tests on antibody and anaphylaxis production gave no antibody or anaphylactogenic response.

c) — Vasokinetic investigations: no effect, either of histamine or adrenaline type, was noted even with doses of 50 mg/kg.

All the aforesaid tests show that FAPA can be chosen as a drug for antiarteriosclerotic use as its effectiveness is of the order of magnitude of heparin, but without giving rise to the risks of haemorrhage typical of this latter substance.

Furthermore, the biological activity of FAPA is approximately ten times greater than that of total aorta extract, but without the immunoreactive side-effects, and thus with considerable advantage with regard to posology and the risk of sensitisation during therapy. Some practical examples of preparation of the new arterial polysaccharide complex according to the invention are given hereinafter, in order to enable the process to be easily reproduced, but without in any way limiting it.

EXAMPLE I.

50 kg of calf aorta were finely ground and homogenised in 66 l of water. After slowly heating the mass to 50°C, 0.6 kg of papain were added, previously suspended in 9 l of water containing 10 g of sodium metabisulphite.

The entire mass was heated to 65°C and kept under these conditions for three hours, taking care that the pH did not descend below 6 by suitably adding alkali. After this time had passed, the mass was heated to 95°C for 15 minutes.

After standing for 30 minutes, it was filtered through cloth with a precoat of filter earth.

0.6 kg of cetyltrimethylammonium chloride dissolved in 35 l of water were added to the filtrate.

After leaving overnight, the overlying liquid was syphoned off. The precipitate was collected by filtration and dissolved in 25 l of water containing 3 kg of sodium chloride, by heating to 70°C. This solution was clarified by filtration. The filtrate was treated with an equal volume of acetone. The precipitate, namely the crude sodium salt of FAPA, was washed several times with 70% acetone and then dried. 85 g of the crude sodium salt of FAPA were obtained in this manner.

This precipitate was washed in 1500 ml of water, its pH was then adjusted to 8 and it was then treated with 1.5 g of potassium permanganate, and gradually heated to 90°C. It was filtered in order to eliminate the precipitate formed. The pH of the filtrate was adjusted to 11 with sodium hydrate and kept at 70°C for 10 hours.

After this time, the liquid was cooled, its pH adjusted to 7 with hydrochloric acid, and filtered through a 0.8 micron membrane. The liquid obtained was treated with 2 volumes of ethyl alcohol. After 2 hours of standing, the precipitate formed was collected by filtration and washed with 500 ml of 75% alcohol.

The precipitate was dissolved in 800 ml of water and concentrated until the residual alcohol was eliminated.

The concentrate was filtered through a 0.2 micron membrane, and the clear filtrate was lyophilised.

In this manner 42 g of the sodium salt of FAPA were obtained, having the following characteristics:

uronic acid	35%
hexosamine	30.5%
— SO_3Na	18.1%
— nucleosides	5.2%
inorganic salts	8% (mainly chlorides)

clarification activity 210±20 ULI/l
anticoagulation activity 6 UI/mg

EXAMPLE 2.

50 kg of pig aorta were processed as in Example 1. 36 g of the sodium salt of FAPA were obtained, with chemical-physical characteristics which were closely analogous to those of the previous example, and a clarification induction activity of 180±30 ULI/l.

EXAMPLE 3.

50 kg of calf aorta were lysised with ficin instead of papain, and the process then continued as in Example 1. The yield was 35 g of the sodium salt of FAPA with chemical-physical characteristics closely analogous to those of Example 1, and a clarification activity of 280+40 ULI/l.

EXAMPLE 4.

50 kg of calf aorta were homogenised in 150 l of water and lysised with 2 kg of pancreatin at pH 8 and 40°C for 24 hours, in the presence of toluene as the bacteriostatic.

After filtering, the clear liquid was treated with 3 volumes of acetone. The precipitate obtained was hydrolysed with alkali at pH 11 and 70°C for 15 hours. After filtering, the liquid was treated with 400 g of cetylpiridinochloride to give the corresponding insoluble quaternary ammonium salt of FAPA.

The precipitate was then dissolved in 1500 ml of water containing 90 g of calcium chloride. After filtering, the liquid was treated at pH 8 with 1 g of potassium permanganate, heated to 90°C and filtered. The filtrate was dialysed for 15 hours against a stream of water to eliminate the excess of salts, and then filtered through a 0.45 micron membrane and lyophilised.

In this manner 35.5 g of the calcium salt of FAPA were obtained, with chemical-physical characteristics closely analogous to those of the product of Example 1, and a clarification induction activity of 315±30 ULI/l.

WHAT WE CLAIM IS:—

1. An arterial polysaccharide complex of anionic character consisting of hexosamine glycanmonosulphates (herein referred to as FAPA) derived from the aorta of young mammals, and which has the following characteristics: a) on acid hydrolysis it liberates hexosamine, uronic acids and sulphates in the molar ratio of approximately 1/1/1 together with variable quantities of nucleosides; b) electrophoretically, it separates into three anionic bands which can be seen with Alcian Blue and Toluidine Blue; c) it reacts with alkaline and alkaline earth metal bases to form salts soluble in water, whereas with the aliphatic ammonium ion it forms salts insoluble in water; d) its sodium salt is soluble to the extent of 2% in 50% ethyl alcohol, whereas its acid form is soluble to the extent of 2% in 70% ethyl alcohol; e) it reacts with Toluidine Blue to give a minimum metachromatic reaction; f) spectrophotometrically it shows a maximum at 260±1 nm; g) it has a rotatory power in polarised light of −11° to −22°.

2. A process for preparing an arterial polysaccharide complex (FAPA) of anionic character constituted by hexosamine glycanmonosulphates, derived from the aorta of young mammals, and wherein

1) the aorta of young mammals are removed and ground immediately after slaughter;
2) the ground substance is homogenised and suspended in water or a saline solution;
3) the suspension is subjected to attack by proteolytic enzymes under optimum pH, temperature and time conditions;
4) the digested liquid is separated by filtration and collected;
5) the FAPA is precipitated from said liquid by solvents miscible with water and/or by the formation of insoluble ammonium salts;

6) the FAPA is purified.

3. A process for preparing an arterial polysaccharide complex (FAPA) according to claim 2, wherein the proteolytic enzyme to which the suspension is subjected to attack by comprises trypsin, chymotrypsin, pancreatin, papain, ficin or bromelin.

4. An arterial polysaccharide complex of anionic character, constituted by hexosamine glycanmonosulphates, when obtained by the process as claimed in claim 2 or claim 3.

5. Therapeutic compositions effective in curing and preventing arteriopathies, which contain as their active principle the arterial polysaccharide complex as claimed in claims 1 and 4.

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1564592 COMPLETE SPECIFICATION

1 SHEET *This drawing is a reproduction of the Original on a reduced scale*

