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2,989,438

PROCESS OF PURIFYING HEPARIN, AND PRODUCT PRODUCED THEREFROM

Gerard Nominé, Noisy-le-Sec, Lucien Penasse, Paris, and
Pierre Barthelemy, Saint Germain en Laye, France,
assignors to UCLAF, Paris, France, a corporation of
France

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The present invention relates to a process of purifying heparin and to products obtained thereby.

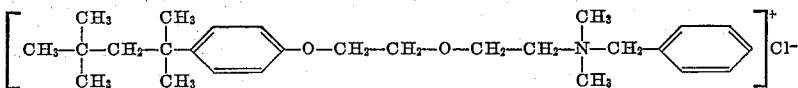
Heparin is a polysaccharide containing acid groups. It is present in various animal tissues and is characterized by its ability of prolonging the clotting time of blood. It is used clinically in preventing formation of blood clots (thrombosis) after surgery.

It is one object of the present invention to provide a simple and effective process of producing heparin.

Another object of the present invention is to provide new salts of heparin which are valuable as intermediates in the process of producing heparin.

These and other objects of the present invention and advantageous features thereof will become apparent as the description proceeds.

In principle, the present invention relates to a process of isolating and purifying heparin by conversion into its insoluble salts with quaternary ammonium compounds containing a long chain. Such quaternary ammonium compounds which are used as surface active agents and as antiseptics are, for instance, the benzyl dimethyl-2-[2-(p-1,1,3,3-tetramethyl butyl phenoxy) ethoxy] ethyl ammonium chloride of Formula I:



(I)

which is sold under the trademark "Hyamine 1622." Another quaternary ammonium compound of a similar nature as Hyamine 1622, which may be used in the process according to the present invention, is "Hyamine 2389." These long chain quaternary ammonium compounds are listed and characterized in the "Index des huiles sulfonées et détergents modernes" by J. Sisley (Paris, France, 1954), vol. II. According to p. 373 of said Index, "Hyamine 2389" is a mixture of alkylated tolyl methyl trimethyl ammonium-chloride wherein the alkyl radicals are radicals from C_9H_{19} to $\text{C}_{16}\text{H}_{31}$. Said quaternary ammonium chlorides have an average molecular weight of 331. All the heparin salts with such quaternary ammonium bases as given hereinabove are soluble in butanol and, sometimes, also in lower alcohols. As they are insoluble in water, they permit precipitation of heparin from its aqueous solutions in dilutions between $\frac{1}{8},000$ and $\frac{1}{12},000$.

The heparin salt of Hyamine 1622 is soluble in lower alkanols, such as methanol, ethanol, propanol, isopropanol, butanol, isoamyl alcohol, in lower alkanones, such as acetone, methyl ethyl ketone, methyl isobutyl ketone, in aromatic hydrocarbons, such as benzene, in heterocyclic bases, such as pyridine, in dioxane, tetrahydrofuran, acetonitrile, chloroform, methylal, acetic acid, and dimethyl formamide. It is insoluble in water, ethyl acetate, amyl acetate, ethyl ether, isopropyl ether, cyclohexane, petroleum ether, and carbon tetrachloride.

Likewise the salts of heparin with other quaternary ammonium salts mentioned hereinabove are not soluble in the last mentioned solvents.

According to the present invention, these particular solubilities of the quaternary ammonium salts of heparin

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may be made use of for purifying said salts from impurities which have been carried along in the extraction process of the manufacture of heparin. For this purpose the salts are treated with a solvent wherein they are not soluble, or the heparin salts are dissolved in a water-immiscible solvent and the resulting solution is washed with water or with other solvents wherein said quaternary ammonium salts of heparin are insoluble.

When treating alcoholic solutions of the quaternary ammonium salts of heparin with an aqueous solution of the potassium salt of a lower fatty acid, the heparin salt readily undergoes metathesis and the crystalline potassium salt of heparin is obtained. It may subsequently be converted into the sodium salt of heparin by passing the aqueous solution of said potassium salt over a column of a cation exchange resin, neutralizing the resulting solution with aqueous sodium hydroxide solution, and precipitating the salt by the addition of a water-miscible solvent wherein the desired sodium salt is insoluble.

The precipitation of heparin in the form of its quaternary ammonium salt is carried out preferably in a highly acid medium in order to avoid formation of quaternary ammonium salts of compounds of lower acidity than heparin which are present in products obtained by diastatic digestion of lung tissue. Operation in acid medium has the further advantage that the precipitate readily flocculates and, as a result thereof, can readily be filtered. This is an important feature of the present invention as the quaternary ammonium salts tend to precipitate from a neutral medium in the colloidal state which renders filtration rather difficult.

According to a preferred embodiment of the present invention, the quaternary ammonium salt of heparin, ob-

tained as described hereinabove, is dissolved in butanol and the resulting solution is washed with an aqueous solution of an alkali metal salt of a weak acid, for instance, sodium acetate or potassium acetate. Metathesis takes place and the corresponding alkali metal salt of heparin which is formed thereby, passes into the aqueous layer wherefrom, after separation of the butanol layer by decanting, the potassium salt of heparin is separated in pure and crystalline form by the addition of potassium acetate whereby said heparin salt is salted out, or by the addition of a water-miscible solvent wherein it is only slightly soluble, for instance, acetone, dioxane, or ethanol.

Furthermore, in case the quaternary ammonium salt is soluble in a water-miscible solvent, metathesis and precipitation of potassium heparinate may be carried out simultaneously by adding to such a solution the calculated amount of potassium acetate dissolved in water. However, purification of heparin is not as complete as when using butanol which retains certain impurities in the metathesis and extraction step. The potassium salt of the sodium salt by passing the solution over a column of heparin crystallizes soon and can be converted into of a cation exchange resin, destroying pyrogenic compounds, if necessary, and neutralizing the resulting solution by means of aqueous sodium hydroxide solution.

It is understood that the process according to the present invention may also be applied to a product obtained by subjecting lung tissue to the action of a proteolytic enzyme, as well as to other impure heparin preparations or pyrogenic commercial heparin preparations.

According to the present invention the quaternary ammonium salt of heparin is precipitated at a pH-value between about 1.0 and about 3.0. This pH-value is at-

tained by the addition of a mineral acid, such as hydrochloric acid or sulfuric acid, before, during, or after the addition of the quaternary ammonium chloride to the digestion product of lung tissue or to a solution of impure heparin. The preferred amounts of Hyamine 1622 to be added in order to precipitate heparin from dilute solutions of its sodium salt are between about 2 g. and about 3 g. per g. of impure sodium heparinate, and between about 2 g. and about 3 g. per kg. of fresh lung tissue to cause precipitation of heparin from the digestion mixture. These amounts correspond to amounts of about 2.85 g. and about 4.3 g. of Hyamine 1622 for 100,000 international units. The content of the solution used as starting material, i.e., the solution of impure sodium heparinate or digestion mixture, can readily be determined by biological titration.

When using Hyamine 1622 in an amount higher—as well as lower—than indicated hereinabove, a product is obtained the heparin content of which is considerably lower. Likewise, the yields are also reduced. The optimum amounts for other quaternary ammonium compounds mentioned hereinabove can readily be determined in preliminary tests by the biological titration method.

The following examples serve to illustrate the present invention without, however, limiting the same thereto. More particularly, the quaternary ammonium salts of heparin may be purified and converted into the potassium salt in the presence of other solvents than indicated. Metathesis may also be carried out by means of other potassium salts than potassium acetate, for instance, by means of di- or tri-potassium phosphate. Heparin may be transferred from its Hyamine 1622 salt solution in a water-immiscible solvent, such as butanol, into the aqueous phase by means of an electrolyte, such as a dilute acid. Metathesis of the quaternary ammonium salt of heparin may be carried out directly with a sodium salt of a weak acid, such as sodium acetate or di-sodium phosphate when complete purification of the heparin is not required. These and other changes and variations in the procedure followed according to the present invention may be made by those skilled in the art in accordance with the principles set forth herein and in the claims annexed hereto.

EXAMPLE 1

Preparation of the Hyamine 1622 salt of heparin from digested lung tissue and conversion of said salt into the sodium salt of heparin

2 kg. of ground fresh lungs are mixed with 1 l. of water and subjected to the action of papain by adding thereto about 9 g. of commercial papain and 100 cc. of toluene as antiseptic. After digestion is completed, the mixture is heated to boiling, insoluble material formed thereby is filtered off, and the solution is allowed to cool. 4 g. of commercial Hyamine 1622 are added with stirring. Without ceasing to stir the pH-value of the solution is adjusted to a pH of 2.0 by the addition of $\frac{1}{10}$ N sulfuric acid. In order to facilitate filtration, 8 g. of a filter aid such as the product sold under the trademark "Clarcel" is added. The mixture is filtered with suction whereby about 22 g. of the wet Hyamine 1622 salt of heparin mixed with the filter aid are obtained. The precipitate is dissolved in 60 cc. of butanol and the insoluble filter aid is removed from the butanol solution of the Hyamine salt of heparin. The butanol solution is extracted successively first with 2 cc. of a saturated aqueous solution of sodium acetate and then twice with 6 cc. of an aqueous 20% solution of sodium acetate. The aqueous extracts are combined and potassium acetate is added in such an amount that the concentration of this salt in the extract is about 30%. The potassium salt of heparin crystallizes. It is filtered off and dried. The yield is 0.6 g. of the potassium salt of heparin with a content of 110 international units of heparin per mg. of the compound. In order to convert the potassium salt of heparin

into the non-pyrogenic sodium salt of heparin, it is dissolved in 18 cc. of water, the pH-value of the solution is adjusted to a pH of 8.5 by the addition of aqueous sodium hydroxide solution. 0.2 g. of potassium permanganate are added in order to destroy pyrogenic compounds, and the mixture is heated to 80° C. for 30 minutes. After cooling, the reaction mixture is passed through a column of a cation exchange resin of the type sold under the trademark "Dowex," i.e., a phenol formaldehyde resin containing sulfonic acid groups for demineralization. The resulting filtrate is neutralized by the addition of aqueous sodium hydroxide solution. Sodium acetate is added in an amount of 2% by weight of the volume of the solution and ethanol is added in an amount of twice the volume of the solution. After filtration and drying, 0.4 to 0.5 g. of pure, colorless, and non-pyrogenic sodium salt of heparin are obtained which has an activity of 115 international units per mg.

EXAMPLE 2

Preparation of the Hyamine 1622 salt of heparin from digested lung tissue and conversion of said salt into the potassium salt of heparin

The Hyamine 1622 salt of heparin is precipitated and dissolved by following the procedure described in Example 1. The resulting butanol solution is then extracted three times with 20 cc. of a 2% aqueous potassium acetate solution. Ethanol is added to the combined extracts until precipitation is completed. The resulting potassium salt of heparin is filtered off with suction and dried.

EXAMPLE 3

Preparation of the Hyamine 1622 salt of heparin from an impure heparin preparation and conversion of said salt into the sodium salt of heparin

500 mg. of impure sodium heparinate containing 70 international units per mg. are dissolved in 500 cc. of water. 1.35 g. of Hyamine 1622 are added, the solution is acidified by the addition of $\frac{1}{10}$ N sulfuric acid, and a filter aid of the "Clarcel" type is added. The precipitate is filtered off with suction, dried at 40° C., and extracted with ethanol. After removing the filter aid by filtration, 5 cc. of a 10% aqueous potassium acetate solution are added. The resulting precipitate is separated by centrifuging, washed with alcohol and with acetone, and then dried. 0.35 g. of the potassium salt of heparin are obtained containing 98 international units per mg.

When subjecting a solution of said potassium heparinate to the action of a cation exchange resin, neutralizing the resulting solution with aqueous sodium hydroxide solution, and isolating the resulting sodium heparinate from its aqueous solution by following the procedure described in Example 1, 0.30 g. of the sodium salt of heparin containing 111 international units per mg. are obtained.

EXAMPLE 4

Preparation of the Hyamine 1622 salt of heparin from a solution of sodium heparinate

1 g. of sodium heparinate containing 70 international units per mg., are dissolved in 1,000 cc. of water. 2.7 g. of Hyamine 1622 are added and the mixture is acidified by the addition of $\frac{1}{10}$ N sulfuric acid. After standing overnight, the precipitated insoluble material is separated by centrifuging, washed three times by pasting it each time with 10 cc. of water, and dried in a vacuum. 2.640 g. of the Hyamine 1622 salt of heparin are obtained.

To convert said salt into the sodium salt, it is dissolved in butanol. The butanol solution is extracted three times with a 2% aqueous sodium acetate solution. The combined aqueous extracts are mixed with 2 parts by volume of ethanol and the precipitated material is filtered off and dried. The yield is 0.6 g. of the sodium salt of heparin containing 115 international units per mg.

which corresponds to a recovery of 98.5% of the starting material.

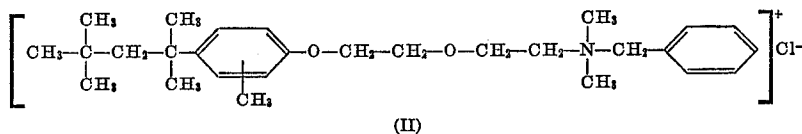
EXAMPLE 5

Preparation of the hyamine 2389 salt of heparin from a solution of sodium heparinate

1 g. of impure sodium heparinate containing 70 international units per mg. are dissolved in 1000 cc. of water. 1.96 g. of Hyamine 2389 are added and the solution is acidified by the addition of $\frac{1}{10}$ N sulfuric acid. The reaction mixture is allowed to stand for one night and the precipitated material is separated by centrifuging, washed as described in the previous example and dried. The yield is the same as in Example 4 for the Hyamine 1622 salt of heparin.

Other long chain quaternary ammonium salts than those given in the preceding examples may also be used for precipitating heparin from its dilute impure solutions whereby the procedure is substantially the same as described in said examples. For instance, the following groups quaternary ammonium compounds may be used in the process according to the present invention:

Compounds containing an aromatic ring in the long chain. A typical representative of this group of compounds is "Hyamine 1622," also designated as benzethonium chloride. The methyl benzethonium chloride of the Formula II



and other benzyl dimethyl-2-[2-(p-higher alkyl phenoxy) ethoxy] ethyl ammonium halogenides may also be used in the present invention.

The potassium salt of heparin is characterized by following properties.

Molecular weight: 35,000.

Analysis:—Calculated: 20.57% C, 2.19% H, 2.00% N, 12.59% S, 20.93% K. Found: 20.6% C, 2.5% H, 2.0% N, 13.0% S, 21.3% K (flame spectrum), 21.0% K (sulphuric ash).

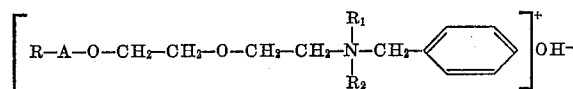
Anticoagulating activity: 155 international units per mg.

We claim:

1. The benzyl dimethyl-2-[2-(p-1,1,3,3-tetramethyl butyl phenoxy) ethoxy] ethyl ammonium salt of heparin.

2. The heparin salt of a mixture of alkyl tolyl methyl trimethyl ammonium bases, the alkyl radical thereof having 9 to 15 carbon atoms.

3. The heparin salt of the benzyl di-(lower) alkyl-2-[2-(p-higher alkyl phenoxy) ethoxy] ethyl ammonium base of the formula



wherein

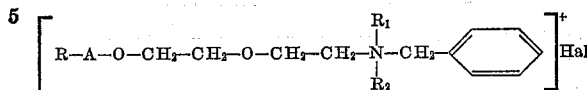
R indicates a higher alkyl radical,

A indicates an aromatic radical selected from the group consisting of the phenyl radical and the cresyl radical, and

R₁ and R₂ are lower alkyl radicals.

4. In the process of preparing heparin by precipitating an impure aqueous solution of heparin with a quaternary ammonium halogenide and separating heparin from the precipitated quaternary ammonium salt, the improvement

which consists in adding to an impure aqueous solution of heparin a benzyl di-(lower)alkyl-2-[2-(p-higher alkyl phenoxy) ethoxy] ethyl ammonium halogenide of the formula



wherein

10 R indicates a higher alkyl radical,

A indicates an aromatic radical selected from the group consisting of the phenyl radical the cresyl radical, and R₁ and R₂ are lower alkyl radicals,

15 converting the resulting water-insoluble and butanol-soluble quaternary ammonium salt of heparin into the corresponding crystalline potassium salt by metathesis, passing the aqueous solution of said potassium heparinate through a column of a strongly acidic exchange resin, 20 neutralizing the filtered solution by means of aqueous sodium hydroxide solution, and precipitating sodium heparinate by the addition of an organic water-miscible solvent wherein said sodium salt is insoluble.

5. The process according to claim 4, wherein the 25 quaternary ammonium halogenide is benzyl dimethyl-2-[2-(p-1,1,3,3-tetramethyl butyl phenoxy) ethoxy] ethyl ammonium chloride.

6. The process according to claim 4, wherein the 40 quaternary ammonium salt of heparin is formed and precipitated in an acid medium of a pH-value between about 1.0 and about 3.0.

7. The process according to claim 4, wherein conversion of the quaternary ammonium salt of heparin by 45 metathesis carried out by dissolving the quaternary ammonium salt of heparin in butanol, extracting the resulting solution with an aqueous solution of an alkali metal salt of a weak acid, adding potassium acetate to the extracts in an amount sufficient to yield solutions containing between 30% and 50% of potassium acetate, and separating the precipitated crystalline potassium heparinate 50 from the mixture.

8. The process according to claim 4, wherein conversion of the quaternary ammonium salt of heparin by 55 metathesis is carried out by dissolving the quaternary ammonium salt of heparin in butanol, extracting the resulting solution with an aqueous solution of a potassium salt of a weak acid, adding a water-miscible organic solvent to the extracts, and separating the precipitated crystalline potassium heparinate from the mixture.

9. In the process of preparing heparin by precipitating an impure aqueous solution of heparin with a quaternary ammonium halogenide and separating heparin from the precipitated quaternary ammonium salt, the improvement 60 which consists in adding to an impure aqueous solution of heparin a mixture of (alkyl tolyl methyl) trimethyl ammonium halogenides, the alkyl residue thereof having 9 to 15 carbon atoms, converting the resulting water-insoluble and butanol-soluble quaternary ammonium salt of heparin into the corresponding crystalline potassium 65 salt by metathesis, passing the aqueous solution of said potassium heparinate through a column of a strongly acidic exchange resin, neutralizing the filtered solution by means of aqueous sodium hydroxide solution, and 70 precipitating sodium heparinate by the addition of an

organic water-miscible solvent wherein said sodium salt is insoluble.

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UNITED STATES PATENT OFFICE
CERTIFICATE OF CORRECTION

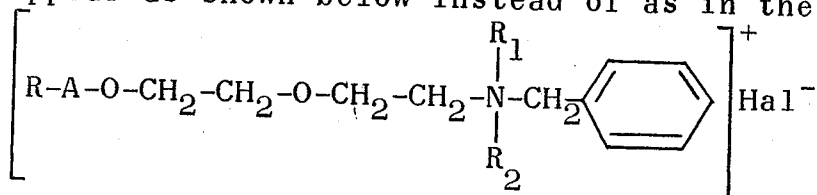
Patent No. 2,989,438

June 20, 1961

Gerard Nominé et al.

It is hereby certified that error appears in the above numbered patent requiring correction and that the said Letters Patent should read as corrected below.

Column 2, line 29, for "inveniion" read -- invention --; lines 59 and 60, for "the sodium salt by passing the solution over a column of heparin crystallizes soon and can be converted into" read -- of heparin crystallizes soon and can be converted into the sodium salt by passing the solution over a column --; column 3, line 13, for "interntional" read -- international --; line 74, for "internaional" read -- international --; column 6, lines 5 to 8, the formula should appear as shown below instead of as in the patent:



same column 6, line 43, for "clami" read -- claim --.

Signed and sealed this 27th day of February 1962.

(SEAL)

Attest:

ERNEST W. SWIDER

Attesting Officer

DAVID L. LADD

Commissioner of Patents