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A Process for the Preparation of a Virus Inactivated Fraction Containing Factor VIII by means of Chromatographic Methods

This inventions is related to a process for the preparation of a virus inactivated fraction containing factor VIII by means of chromatographic methods as well as to a fraction containing factor VIII which can be obtained by the process according to the invention.

Factor VIII is a vital material that plays an important role in blood clotting. Blood clotting disorders can thus be treated by administration of factor VIII. Therefore, a large need exists for administerable factor VIII preparations. Numerous attempts have been made to isolate factor VIII in a highly enriched form from natural sources. Thus, chromatographic methods for the purification of factor VIII from cryoprecipitates are already known. The latter is a fraction which is available by treatment of plasma in the cold. EP 367 840 B1 pertains to a chromatographic method for the isolation of factor VIII from blood plasma without a preliminary cryoprecipitation. The fraction containing factor VIII is separated by chromatographic separation on a hydrophilic chromatographic material modified by ion-exchanging groups. EP 0 238 701 pertains to a method for the preparation of an ultrapure, infectious antihemophilic factor, where the pretreated fractions are a cryoprecipitate liberated from fibrinogen, globulin, albumins, and other interfering ingredients by means of ethanol precipitation. European Patent Application 88 108 458.6 describes chromatographic separation with ion exchangers after virus inactivation of the cryoprecipitate fraction. EP 0 173 242 A describes a method for obtaining factor VIII preparations by chromatography on anion-exchanging materials which are solely



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based on carbohydrates, the carbohydrate matrix being modified by DEAE groups. In particular, DEAE sepharose and DEAE cellulose are described as being useful. In GB-A-1,178,958, a purification of factor VIII using ECTEOLA cellulose columns is described. The modified cellulose contains basic substituents introduced by reaction of epichlorohydrin and triethanolamine. The above mentioned prior art makes use either of chromatographic separation in the form of batch processes or of column chromatography. Although fairly good results are obtained with these methods, it still remains desirable for economic as well as ethical reasons to increase the yield of biologically valuable factor VIII.

Hence, the technical problem underlying this invention consists in providing a method which, proceeding from the prior art, allows for an improved preparation of factor VIII with respect to yield and biological activity.

Surprisingly, the problem has been solved by a process proceeding from a cryoprecipitate or blood plasma, optionally followed by an aluminum hydroxide treatment, and employing a separation step using membrane chromatography after dissolution of the cryoprecipitate.

The process of the invention can be performed with commercial cryoprecipitate or blood plasma. Preferably, the thawed cryoprecipitate is treated with aluminium hydroxide for further prepurification of the sample in order to preconcentrate factor VIII.

Preferably, a virus inactivation is carried out prior to the actual chromatographic purification on materials arranged within or on membranes. This virus inactivation is performed according to the method described in EP 131 740 A1 by treatment with biocompatible organic solvents (detergents), Triton® X-100/TNBP, preferably Tween®/TNBP (tri-n-butyl phosphate). Good results will also be obtained with sodium cholate/TNPB. Preferably, detergents are employed in amounts of up to 15% by weight. However, virus

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inactivation may also be done after separation by membrane chromatography.

The chromatographic separation step for the purification of factor VIII in the sample may be carried out either on base materials modified by ion-exchanging groups, particularly anion exchangers, or on materials modified by immunoaffinity ligands. It is critical that the mentioned materials are arranged in membranes. Preferably, these membranes are made of a base material such as modified cellulose or plastics fiber. Particularly useful are membranes as well as compact disks made of porous poly(glycidyl methacrylates) and/or other porous hydrophilic polymers having a similar structure as well as hydrophilized polystyrene.

In the first case, a membrane suitable for separation either consists of stacked thin films made of cellulose or plastics fibers, or in the second case it consists of compact disks made of silica gel or polymer carriers. The base materials of the membranes or disks are provided with corresponding anion-exchanging groups or immunoaffinity ligands. Particularly anion-exchanging groups such as quaternary amines or diethylaminoethyl groups are considered as ion-exchanging groups. Suitable cation exchangers are in principle weakly and strongly acidic cation exchangers, such as materials which are modified by sulfonic acid or phosphoric acid groups.

The ion-exchanging groups can be bound to the base material fibers with or without a so-called spacer. Materials provided with spacers are also referred to as tentacle materials. In DE 42 04 694 corresponding spacers and ligands are mentioned. For example, a glucosamine residue can serve as a spacer as well. Anion-exchanging groups such as DEAE or quaternary amines may also be bound at the membranes made of porous poly(glycidyl methacrylate) or the other materials mentioned. The binding of the anion-exchanging groups takes place either directly to the

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material forming the membrane or else through a spacer as well, e.g. a glucosamine residue.

In another embodiment of the process of the invention affinity membrane chromatography with immobilized substances having a high affinity for factor VIII is used. In particular, monoclonal and/or polyclonal antibodies or factor VIII binding fragments of antibodies are suitable (immunoaffinity membrane chromatography). Preferably the antibodies are derived from humans or mice.

The substances having affinity for factor VIII are immobilized at the carrier by means of chemically reactive groups. Preferably, the reactive group will attack at the end of a spacer rather than directly at the carrier material. Immobilization of the substances for factor VIII takes place through binding at reactive groups such as tosyl, tresyl, hydrazid and others. Corresponding procedures are known from T.M. Phillips "Affinity chromatography" in "Chromatography" (E. Heftmann, ed.), 5th ed. Elsevier, Amsterdam 1992.

The antibodies may also be preadsorbed on membranes bearing protein A or protein G ligands. By subsequent covalent cross-linking, elution of the antibodies (leaching of the column) can be prevented. For cross-linking the antibodies on protein A or protein G membranes a similar procedure may be used as with loose carriers. The advantage of an immobilization at protein A or protein G is that the antibodies are immobilized solely at the constant segment of the molecule (F_c). Thus, the antigen binding portion (F_{ab}) remains free and its interaction with factor VIII is not hindered.

In another preferred embodiment materials are employed for the separation of factor VIII that can ensure a hydrophobic interaction. Hydrophobic materials which are employed are acyclic and/or cyclic alkyl chains, for instance C_1 to C_{18} alkyl chains, and aromatic substances. Suitable materials providing hydrophobic interaction preferably include those having graded hydrophobici-

ty. Hydrophobicity can be graded by introduction of polar groups, such as protic-polar or aprotic-polar groups, for example hydroxy, amino, cyano groups. Preferably, it is adapted corresponding to the respective separation conditions.

Virus inactivation may also take place by heat treatment. Preferably, the eluated sample containing factor VIII is subjected to a pasteurization step following a first membrane chromatographic step. A corresponding procedure is proposed in P 43 18 435.9. Therein, fraction enriched with factor VIII are contacted with di- or trialkyl phosphates and optionally wetting agents in the presence of stabilizing agents and simultaneously or subsequently treated for a period of 5 hours to 30 hours at elevated temperatures ranging from 55°C to 67°C. It may be of advantage to combine the two methods of virus inactivation, the treatment with detergents and with heat.

In order to remove the chemicals employed in the pasteurization step, a second membrane chromatography may follow. Preferably, the separation of the added stabilizing agents takes place by means of a membrane modified by DEAE or quaternary ammonium compounds that are arranged on the surface of the chromatographic carrier material through a spacer. In addition, it is possible to arrange the corresponding ligands on the surface of the carrier material without a spacer. The stabilizing agents are not retarded by this anion exchange material under the conditions chosen, whereas factor VIII is adsorbed on the chromatographic material.

Thereafter, factor VIII is eluated with an aqueous solvent system exhibiting gradually increasing salt concentrations.

The fraction containing factor VIII thus obtained is concentrated, filled and optionally lyophilized by conventional methods.

Preferably, factor VIII is applied from a solution with low ionic strength in the first membrane chromatographic separation step.

Preferably, the aqueous system has an ionic strength corresponding to a 0 to 150 mM sodium chloride solution. At such ionic strengths, factor VIII is still adsorbed on the chromatographic material, whereas more weakly binding impurities can be washed out with aqueous systems of the same ionic strength.

In another embodiment of the process according to the invention, purification of the adsorbed material may be performed with an aqueous system having an ionic strength corresponding to a 200 to 400 mM sodium chloride solution. Desorption of factor VIII and elution of this fraction then are carried out with an aqueous system having an ionic strength corresponding to a 500 to 1500 mM sodium chloride solution, while the pH value is maintained within a range of 4 to 9. If cation-exchange chromatography is performed, it preferably takes place at a pH value < 6, whereas anion-exchange chromatography is rather carried out at higher pH values of above 6.

If purification of factor VIII is performed by immunoaffinity membrane chromatography, then, unlike with the above mentioned method using anion-exchange materials, elution is carried out with chaotropic reagents or highly concentrated salt solutions. Preferably, elution takes place with concentrations of chaotropic reagents or salts which are sufficient to disrupt the binding between the substance having high affinity to factor VIII and factor VIII itself. The concentrations of the mentioned materials in the respective elution systems depends on the strength of the affinity between factor VIII and the corresponding binding component. Preferably, antibodies having not too high an affinity are employed as immunoaffinity ligands. As a result, elution can take place with aqueous solutions with lower denaturating potency. Preferably, aqueous solutions having concentrations of from 1 to 6 M of urea, especially from 2 to 4 M of urea, or correspondingly highly concentrated salt solutions are employed for elution of factor VIII from the immunoaffinity membrane.

In hydrophobic interaction chromatography, the sample is applied in an aqueous solution with very high ionic strength, such as, for example, highly concentrated ammonium sulfate (up to a concentration of 4 M) or sodium chloride (up to a concentration of up to 5 M). In particular, elution is carried out gradually or continuously with salt solutions of lower ionic strengths. An aqueous solution with organic solvents, particularly a diluted alcoholic solution, may also be used as solutions with lower ionic strengths for the elution of the samples in hydrophobic interaction membrane chromatography.

The procedure according to the invention surprisingly ensures a quick and uncomplicated purification of factor VIII which is obtained at the same time in high purity and high yields. In addition, the specific activity of the factor VIII obtained in this way is quite high which may be accounted for by the low denaturation of the active factor in the process according to the invention. Hence, a factor VIII containing fraction obtainable through the process according to the invention is also an object of the invention.

C l a i m s :

1. A process for the preparation of a virus inactivated fraction containing factor VIII by means of chromatographic methods, wherein - proceeding from a cryoprecipitate or blood plasma - optionally followed by aluminum hydroxide treatment, after dissolution of the cryoprecipitate at least one separation step using membrane chromatography is performed.

2. The process according to claim 1, wherein said separation step takes place on an ion-exchange material, particularly anion-exchange material, arranged within or on a membrane.

3. A process according to claim 1 and/or 2, wherein a virus inactivation is performed by treatment of said fraction with a di- or trialkyl phosphate and a non-ionic surfactant.

4. A process according to any of claims 1 to 3, wherein virus inactivation is performed by a pasteurization step, optionally followed by an additional separation step using membrane chromatography.

5. A process according to any of claims 1 to 4, wherein said membrane chromatography takes place on a material having a high affinity to factor VIII.

6. A process according to any of claims 1 to 5, wherein the material having a high affinity to factor VIII is modified by ligands having high and/or low molecular weights.

7. The process according to claim 6, wherein said material is modified with antibodies against factor VIII.

8. A process according to any of claims 5 to 7, wherein the modified material having a high affinity to factor VIII

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bears immobilized ligands having a high affinity to factor VIII.

9. A process according to any of claims 1 to 8, wherein the chromatographic material allows for hydrophobic interaction with the factor VIII to be separated or bears appropriate ligands mediating hydrophobic interaction.

10. A process according to any of claims 1 to 9, wherein the sample to be purified is applied to the membrane containing ion-exchange material in an aqueous system having low ionic strength, corresponding to 0 to 150 mM sodium chloride, optionally washed with an aqueous system having higher ionic strength, corresponding to a 200 to 400 mM sodium chloride solution, followed by elution with an aqueous system having high ionic strength, corresponding to a 500 to 1,500 mM sodium chloride solution, while the pH value is maintained at from 4 to 9.

11. A process according to any of claims 1 to 10, wherein the sample to be purified is applied from a solution which allows for binding of factor VIII to antibodies against factor VIII, the affinity membrane with factor VIII adsorbed thereon is optionally washed, followed by elution with chaotropic reagents at a concentration corresponding to, for example, 1 to 6 M of urea or with a correspondingly more highly concentrated salt solution.

12. A process according to any of claims 1 to 10, wherein the sample to be purified is applied from a solution having very high ionic strength to a membrane bearing hydrophobic ligands on its surface and is eluted with solvent systems having lower ionic strengths.

13. A process according to any of claims 1 to 12, wherein the eluted fraction containing factor VIII is concentrated, filled and/or lyophilized.

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14. A process according to any of claims 1 to 13, wherein said virus inactivation is performed by treatment with up to 15% by weight of a detergent.

15. A fraction containing factor VIII obtained by employing the process according to any of claims 1 to 14.

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