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(54) **Method of separating a factor IX preparation from blood plasma.**

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Method of separating a factor IX preparation from blood plasma

This invention relates to blood fractionation and more particularly to the separation of a blood coagulation Factor IX preparation from plasma by the selective adsorption of Factors II, IX and X.

The process of blood coagulation is a complicated physiological activity that involves the interaction of numerous substances found in normal whole blood. It is known that certain factors associated with the blood coagulation mechanism are absent or seriously deficient in certain individuals. In those patients suffering from classical hemophilia, antihemophilic factor A (AHF, Factor VIII) is deficient. In those patients afflicted with hemophilia B, plasma thromboplastin component (PTC, Factor IX) is missing from the blood.

Several other factors which are important in the coagulation mechanism are Factors II, VII and X. As with Factors VIII and IX, these other factors also are deficient or absent in certain individuals. Factors II, VII and X are usually associated with Factor IX in the fractionation of blood plasma into various fractions, and a concentrate of these four factors has come to be known as the prothrombin complex.

In the development of modern blood banking programs involving the collection and storage of large quantities of blood and blood components, the establishment of adequate preservation systems is critical. Since World War II it has been common practice to collect blood in a solution of citric acid, sodium citrate and dextrose known as ACD blood.

The problem of preserving blood is much simplified, however, when it is reduced to preservation of various blood components since it is easier to meet the environmental requirements of the separate components than of whole blood.

Moreover, it is wasteful and even detrimental to the patient to administer more blood components than required. Thus, the hemophiliac needing certain blood coagulation factors ideally should be given only those factors required or at least a purified concentrate of these factors containing a reduced level of unneeded factors.

The fractionation of blood to obtain blood coagulation Factors VIII and IX and the prothrombin complex is well known. Most fractionation methods require the separation of Factor VIII from the plasma or other starting material prior to the separation of Factor IX or the prothrombin complex. For example, Factor VIII is frequently first separated from plasma as a cryoprecipitate or by precipitation with glycine or polyethylene glycol as described in U.S. Patents 3,631,018 and 3,652,530 and references cited therein.

Various prior methods of blood fractionation for the preparation of the prothrombin complex include the barium sulfate adsorption method described by Fowell in U.S. Patent 2,999,791 and the tricalciumphosphate adsorption method disclosed by Soulier et al., *La Presse Medicale* 72, 1223—28 (1964). Tullis discloses the use of DEAE-cellulose ion exchanger for the production of a prothrombin complex, *New England Journal of Medicine* 273, 667—74 (1965) while the corresponding use of DEAE-Sephadex ("Sephadex" is a Registered Trade Mark) is described by Wado and Mozen in U.S. Patent 3,717,708. Andersson et al. in U.S. Patent 3,920,625 further describe the use of DEAE-Sephadex specifically for the preparation of Factor IX concentrates. Use of polyethylene glycol for the production of prothrombin complex is taught by Fekete and Shanbrom in U.S. Patents 3,560,475 and 3,682,881. Aluminum hydroxide and other such gel materials also are known as useful in the concentration of prothrombin complex factors as seen from Bidwell, U.S. Patent 2,867,567.

Casillas et al. in *Revista de la Associacion Bioquimica Argentina* 40 (222—223), 147—157 (1975) describe techniques for obtaining clotting factors (or groups of factors which do not contaminate one another) for clinical use and characterisation studies. In preparation 3, entitled "Concentrado de factores II—IX—X para uso clinico", Casillas et al. describes the production of a concentrate of factors II, IX and X by fractionating plasma with cold ethanol (−5°C.) according to Cohn fractionation procedures followed by addition of moistened and equilibrated DEAE-cellulose to the Cohn I supernatant at 5—10°C. in an amount of 1 gram of the ion exchange resin (dry basis) for each 100 ml of the Cohn I supernatant. The concentrate of factors II, IX and X is then recovered by further column chromatographic procedures.

In preparation 4, entitled "Concentrado de factor VII humano para uso de laboratorio" Casillas et al. describes the production of a concentrate of factor VII by column chromatographic treatment with DEAE-cellulose in proportions of 3 grams of the ion exchange resin for each 100 ml of citrated plasma. Factor VII is then recovered from an effluent complex of factors I, V, VII and VIII by adsorption with $Al(OH)_3$ gel, Na_2HPO_4 elution, and cold ethanol (−5°C) precipitation. Factors II, IX and X are indicated as adsorbed by the initial treatment with DEAE-cellulose but no recovery or assay of said factors is disclosed.

G. W. R. Dike *et al.*, in *Brit. j. Haematol* 1972, *22(4)*, 469—90, describe the use of DEAE-cellulose for the column chromatographic separation of Factor VII from Factors IX, II and X. However, the present invention involves neither column chromatography nor the use of DEAE cellulose resins, but the selective adsorp-

tion of Factors II, IX and X to the substantial exclusion of Factors VII and VIII. In the Dike *et al.* article, Factor VII is chromatographed as a separate concentrate and is the first fraction to be eluted. In the present invention, Factor VII remains unabsorbed together with Factor VIII and remains in the liquid plasma. In conventional practice, Factor VIII is removed from plasma prior to Factors II, VII, IX and X by cryoprecipitation. In the present invention, Factors II, IX and X are separated from the plasma without separation of Factors VII and VIII, which is substantially different from the Dike *et al.* method.

The method of the present invention is one of separating a Factor IX preparation from plasma, comprising contacting liquid blood plasma with a water-insoluble, cross-linked polyelectrolyte copolymer of ethylene and maleic anhydride containing pendant diloweralkylamino-loweralkyl functional groups linked to the copolymer via imide linkages, wherein "loweralkyl" has from 1 to 4 carbon atoms, characterised in that the contacting is carried out at a pH of from 7.4 to 8.5 and the amount of the copolymer is from 0.025% to 0.1% by weight of the plasma, whereby Factors II, IX and X are selectively adsorbed by the polyelectrolyte copolymer to the substantial exclusion of Factors VII and VIII which are unadsorbed and remain in the liquid plasma.

In accordance with the present invention, a Factor IX preparation containing Factors II, IX and X is separated from liquid plasma with a water-insoluble, cross-linked polyelectrolyte copolymer of ethylene and maleic anhydride containing pendant diloweralkylamino-loweralkyl functional groups. By use of the polyelectrolyte copolymer at a relatively low concentration of from 0.025% to 0.1% by weight of the plasma and a pH of from 7.4 to 8.5, a Factor IX preparation containing Factors II, IX and X surprisingly is selectively adsorbed by the polyelectrolyte copolymer to the substantial exclusion of Factors VII and VIII which are unadsorbed and remain in the liquid plasma.

If desired, the adsorbed Factor IX preparation can then be eluted from the polyelectrolyte by washing with an aqueous solution of a physiologically acceptable salt such as NaCl, for example a solution of about one to three molar NaCl. The elution preferably is carried out at a pH of from about 5.5 to about 6.5 although higher pH's also can be used.

The starting plasma used in the fractionation method of this invention is generally obtained fresh frozen. This plasma should be thawed before fractionation with the polyelectrolyte copolymer, preferably by heating to a temperature of at least about 35°C. The appropriate polyelectrolyte copolymer can then be admixed with the plasma at a concentration of from 0.025% to 0.1% and preferably 0.035% to 0.05%, and the pH adjusted to a range of from 7.4 to 8.5. The mixture can be stirred for a suitable time, for example at least about 10 minutes, during which time the Factor IX preparation is selectively adsorbed by the polyelectrolyte copolymer and the remaining liquid plasma is made deficient in Factors II, IX and X.

In general, the water-insoluble, cross-linked polyelectrolyte copolymers employed in this invention are copolymers of ethylene and maleic anhydride containing pendant diloweralkylaminoloweralkyl functional groups. By the term "loweralkyl" is meant an alkyl having from 1 to 4 carbon atoms.

The base copolymer of ethylene and maleic anhydride (EMA) can be prepared, for example, by reacting ethylene and maleic anhydride in the presence of a peroxide catalyst in a suitable solvent. The copolymer will preferably contain substantially equimolar quantities of the ethylene residue and the anhydride residue.

The base EMA copolymer can be reacted with a loweralkyliminobisloweralkylamine which has two primary amine groups and leads to a cross-linked EMA copolymer. The desired pendant diloweralkylaminoloweralkyl functional groups can then be incorporated into the cross-linked copolymer by reaction of diloweralkylaminoloweralkylamine with part of all of the remaining anhydride groups of the EMA polymer. The polyelectrolyte copolymer also desirably is converted to the HCl salt form to provide better handling characteristics. Further details on the preparation of these polyelectrolyte copolymers can be had by reference to the disclosure in U.S. Patent 3,554,985 which is incorporated herein by reference. Use of these polyelectrolyte copolymers in blood fractionation is described in U.S. Patent 3,555,001.

A preferred diloweralkylaminoloweralkyl functional group is dimethylaminopropyl and a preferred cross-linking agent is methyliminobispropylamine.

A preferred polyelectrolyte copolymer for use in this invention contains about five methyliminobispropylamine cross-linking groups and about 90 pendant dimethylaminopropylamine functional groups per 100 maleic anhydride units in the EMA copolymer.

Other cross-linking agents, for example, divinylbenzene and ethylene diamine, and other functional groups, for example, dimethylaminoethyl and diethylaminobutyl, also can be used in the polyelectrolyte copolymers which are employed in the method of separating the Factor IX preparation herein.

Following the adsorption of the Factor IX preparation, the Factor VIII remaining in the plasma solution can be further concentrated and recovered by known techniques.

In a preferred embodiment of the invention, about 0.035% by weight of the polyelectrolyte copolymer containing about five methyliminobispropylamine cross-linking groups and about 90 dimethylaminopropylamine functional groups per 100 maleic anhydride units in the

EMA copolymer is employed for selective adsorption of the Factor IX preparation at a pH of about 8. The adsorbed Factor IX preparation is then eluted from the polyelectrolyte copolymer by washing with 1.7 molar NaCl at pH 6. The eluant can then be dialyzed against 0.1 molar NaCl at 4°C and freeze dried for storage.

The following examples will further illustrate the invention although it will be appreciated that the invention is not limited to these specific examples.

Example I

In this example, the polyelectrolyte copolymer consisted of the reaction product of substantially equimolar parts of ethylene and maleic anhydride (EMA) cross-linked with methyliminobispropylamine (MIBPA) and then further reacted with dimethylaminopropylamine (DMAPA) such as to provide about five MIBPA cross-linking groups and about 90 DMAPA pendant groups per 100 maleic anhydride units in the EMA copolymer and converted to the HCl salt form. One liter of normal human plasma was adjusted to pH 8 with 1 molar NaOH and 0.35 grams of the aforesaid polyelectrolyte copolymer was added thereto and the mixture was stirred for 20 minutes. The mixture was then filtered and the filtrate was retained as a Factor IX depleted plasma. The filter cake was washed with distilled water to remove entrained protein.

A Factor IX preparation containing Factors II, IX and X was then eluted from the polyelectrolyte copolymer by washing with 25 ml. of 1.7 molar NaCl at pH 6.0 (the pH being adjusted with 0.1 molar citric acid) for 20 minutes. The copolymer slurry was then filtered and the filtrate was retained as the desired Factor IX preparation. In a series of seven one-liter replicate fractionations using the above procedure, an average of 483 ± 48 units of Factor IX per liter were obtained having a purification index of 178 ± 33. One unit of Factor IX is defined as the amount of said factor in one ml of pooled normal whole plasma. The purification index is calculated as the ratio of the amount of total protein in the starting plasma to the amount of total protein in the final Factor IX preparation multiplied by the ratio of the units of Factor IX in the final Factor IX preparation to the units of Factor IX in the starting plasma.

Example 2

Using 0.4 mg/ml of the polyelectrolyte copolymer of Example 1 for admixture with normal human plasma for 20 minutes at pH 7.4 to separate a Factor IX preparation, as in Example 1, the adsorption of Factors II, VII, VIII, IX and X was measured with the following results:

Factor	% Adsorbed*
II	83
VII	0
VIII	5
IX	96
X	83

* Based on amount in starting plasma.

These results show a high selectivity for adsorption of Factors II, IX and X to the substantial exclusion of Factors VII and VIII based on the corresponding amounts of these factors in the starting plasma.

Conventional one-stage assays were used for determining the coagulation factors in the foregoing examples. The one-stage assay system for Factor VIII sold commercially by Dade Division of American Hospital Supply Corporation was employed in these examples. This assay system is based on the activated partial thromboplastin time (PTT) used to determine deficiencies in factors necessary for the intrinsic method of clot formation. The PTT test was devised by Brinkhous and co-workers and reported in *J. Lab. Clin. Med. 41*, 637 (1953). In these assays for the various coagulation factors, the unknown sample was reacted with a partial thromboplastin reagent and the appropriate factor-deficient substrate plasma which did not contain the factor to be determined, and the time for clotting was observed. The partial thromboplastin reagent contains crude cephalin obtained from rabbit brain which is known to clot normal plasma faster than its clots hemophilic plasma. Such reagents are well-known and described, for example, in U.S. Patents 3,395,210, 3,486,981 and 3,522,148.

Claims

1. A method of separating a Factor IX preparation from plasma comprising contacting liquid blood plasma with a water-insoluble, cross-linked polyelectrolyte copolymer of ethylene and maleic anhydride containing pendant diloweralkylaminoloweralkyl functional groups linked to the copolymer via imide linkages, wherein "loweralkyl" has from 1 to 4 carbon atoms, characterised in that the contacting is carried out at a pH of from 7.4 to 8.5 and the amount of the copolymer is from 0.025% to 0.1% by weight of the plasma, whereby Factors II, IX and X are selectively adsorbed by the polyelectrolyte copolymer to the substantial exclusion of Factors VII and VIII

which are unadsorbed and remain in the liquid plasma.

2. A method of Claim 1 characterized in that the diloweralkylaminoloweralkyl functional group is dimethylaminopropyl.

3. A method of Claim 1 or Claim 2 characterized in that the copolymer of ethylene and maleic anhydride is cross-linked with methyliminobispropylamine.

4. A method of Claim 1 characterized in that the polyelectrolyte copolymer contains about five methyliminobispropylamine cross-linking groups and about 90 dimethylaminopropyl pendant groups per 100 maleic anhydride groups.

5. A method of any of the preceding claims characterized in that the absorbed Factor IX preparation is eluted from the polyelectrolyte copolymer by washing with an aqueous solution of NaCl having a molarity of from one to three.

6. A method of any of the preceding claims characterized in that the concentration of the polyelectrolyte copolymer is from 0.035% to 0.05% by weight of the plasma.

Revendications

1. Procédé de séparation d'une préparation de facteur IX d'un plasma comprenant la mise en contact d'un plasma sanguin liquide avec un copolymère polyélectrolytique réticulé, insoluble dans l'eau, d'éthylène et d'anhydride maléique contenant des groupes fonctionnels dialkyl(inférieur)aminoalkyle inférieur pendants liés au copolymère par des liaisons imides, où le groupe "alkyle inférieur" a entre 1 et 4 atomes de carbone, caractérisé en ce que le contact est exécuté à un pH compris entre 7,4 et 8,5 et la quantité de copolymère est comprise entre 0,025% et 0,1% en poids de plasma, à la suite de quoi les facteurs II, IX et X sont sélectivement adsorbés par le copolymère à l'exclusion importante des facteurs VII et VIII qui ne sont pas adsorbés et restent dans le plasma liquide.

2. Procédé selon la revendication 1, caractérisé en ce que le groupe fonctionnel dialkyl-(inférieur)aminoalkyle inférieur est le diméthylaminopropyle.

3. Procédé selon l'une des revendications 1 ou 2, caractérisé en ce que le copolymère d'éthylène et d'anhydride maléique est réticulé avec la methyliminobispropylamine.

4. Procédé selon la revendication 1, caractérisé en ce que le copolymère polyélectrolytique contient environ 5 groupes de réticulation méthyliminobispropylamine et environ 90 groupes diméthylaminopropyle pendants par 100 groupes d'anhydride maléique.

5. Procédé selon l'une des revendications 1 à 4, caractérisé en ce que la préparation de facteur IX absorbée est soumise à une élution pour la séparer du copolymère polyélectrolytique par lavage avec une solution aqueuse de NaCl ayant une molarité comprise entre 1 et 3.

6. Procédé selon l'une des revendications 1 à 5, caractérisé en ce que la concentration du copolymère est comprise entre 0,035% et 0,05% en poids du plasma.

Patentansprüche

1. Verfahren zur Abtrennung eines Faktor IX-Präparats aus Plasma, bei dem man flüssiges Blutplasma mit einem wasser unlöslichen, vernetzten Polyelectrolytmischpolymerisat aus Äthylen und Maleinsäureanhydrid, das seitenständige, funktionelle Niedrigalkylaminoniedrigalkyl-Gruppen enthält, die an das Mischpolymerisat über Imid-Bindungen gebunden sind, worin "Niedrigalkyl" 1 bis 4 Kohlenstoffatome bedeutet, in Kontakt bringt, dadurch gekennzeichnet, daß das Inkontaktbringen bei einem pH von 7,4 bis 8,5 ausgeführt wird und die Menge des Mischpolymerisats von 0,025 bis 0,1 Gewichtsprozent des Plasmas beträgt, wodurch die Faktoren II, IX und X durch das Polyelectrolytmischpolymerisat selektiv adsorbiert werden unter praktisch vollständigem Ausschluß der Faktoren VII und VIII, die nicht adsorbiert werden und in dem flüßigen Plasma verbleiben.

2. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß die funktionelle Diniedrigalkylaminoniedrigalkyl-Gruppe Dimethylaminopropyl ist.

3. Verfahren nach Anspruch 1 oder Anspruch 2, dadurch gekennzeichnet, daß das Mischpolymerisat aus Äthylen und Maleinsäureanhydrid mit Methyliminobispropylamin vernetzt ist.

4. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß das Polyelectrolytmischpolymerisat etwa 5 vernetzende Methyliminobispropylamin-Gruppen und etwa 90 seitenständige Dimethylaminopropyl-Gruppen pro 100 Maleinsäureanhydridgruppen enthält.

5. Verfahren nach einem der vorangehenden Ansprüche, dadurch gekennzeichnet, daß das absorbierte Faktor IX-Präparat aus dem Polyelectrolytmischpolymerisat durch Auswaschen mit einer wässrigen NaCl-Lösung mit einer Molarität von 1 bis 3 eluiert wird.

6. Verfahren nach einem der vorangehenden Ansprüche, dadurch gekennzeichnet, daß die Konzentration des Polyelectrolytmischpolymerisats von 0.035 bis 0.5 Gewichtsprozent des Plasmas beträgt.