Office de la Propriété Intellectuelle du Canada
Un organisme d'Industrie Canada

Canadian Intellectual Property Office
An agency of Industry Canada

CA 2083243 C 2005/02/22

(11)(21) 2 083 243

(12) BREVET CANADIEN
CANADIAN PATENT

(13) C

(22) Date de dépôt/Filing Date: 1992/11/18

(41) Mise à la disp. pub./Open to Public Insp.: 1993/05/20

(45) Date de délivrance/Issue Date: 2005/02/22

(30) Priorité/Priority: 1991/11/19 (P 41 37 996.9) DE

(51) Cl.Int.^{5}/Int.Cl.^{5} C12N 9/74, A61K 38/48

(72) Inventeurs/Inventors:
KARGES, HERMANN, DE;
NAUMANN, HORST, DE

(73) Propriétaire/Owner:
ZLB BEHRING GMBH, DE

(74) Agent: BERESKIN & PARR

(54) Titre : PROCEDE DE FABRICATION D'UN CONCENTRE DE THROMBINE EXEMPT DE VIRUS
(54) Title: PROCESS FOR THE PRODUCTION OF A VIRUS-FREE CONCENTRATE OF THROMBIN

(57) Abrégé/Abstract:
A process that in a simple manner allows a virus-free concentrate of thrombin to be obtained from pasteurized solutions of a prothrombin complex, and the use of the concentrate as pharmaceutical, are described.

2083243

BEHRINGWERKE AKTIENGESELLSCHAFT

91/B 034 - Ma 889
Dr. Ha/Sd

Abstract of the disclosure

A process for the production of a virus-free concentrate of thrombin

---

A process that in a simple manner allows a virus-free concentrate of thrombin to be obtained from pasteurized solutions of a prothrombin complex, and the use of the concentrate as pharmaceutical, are described.

2083243

BEHRINGWERKE AKTIENGESELLSCHAFT

91/B 034 - Ma 889
Dr. Ha/Sd

A process for the production of a virus-free concentrate of thrombin

---

The invention relates to a process which makes it possible to produce a virus-free preparation of thrombin in a simple manner from a pasteurized solution of a prothrombin complex.

Several processes have been described for obtaining thrombin that start from partially-purified prothrombin and then transform this into thrombin by addition of tissue thromboplastin and Ca ions.

Methods are also known for transforming a crude prothrombin concentrate into thrombin using high salt concentrations. This transformation only takes place if all the factors of the prothrombin complex are present in the mixture in sufficient quantity. A prothrombin complex that has been purified on an anion exchanger can be activated with salt if autoprothrombin C (F X) is added. The presence of F VII is also essential.

Because of the danger of transmission of pathogens of viral origin (e.g. hepatitis, AIDS, BSE) with proteins of human or animal origin, procedures for inactivating pathogens are required when producing concentrates containing such proteins.

A number of processes for the production of thrombin are known that contain a step for inactivating pathogens, for example using dry heat.

Processes are also known for inactivating viruses in aqueous solutions of thrombin (DE 38 09 991).

EP 0 378 798, corresponding to DE 38 43 126, describes a process in which the prothrombin complex is bound to an anion exchanger and activated with Ca ions, tissue thromboplastin or activated F X.

All processes that employ tissue thromboplastin for activation of the prothrombin complex have the disadvantage that the former cannot subsequently be removed and represents a source of product contamination.

Activation with high concentrations of salts complex Ca ions, such as sodium citrate, has the advantage that the prothrombin complex is not additionally contaminated with tissue proteins. However, a prothrombin concentrate that has been purified on DEAE exchangers cannot be activated to thrombin without the addition of activated F X or tissue thromboplastin. It would also be advantageous to carry out virus inactivation even on the prothrombin complex so that the enzyme thrombin, which is labile in comparison with prothrombin, is not exposed to the harsh methods of virus inactivation and does not lose native characteristics as a result of structural changes.

It has now been found, surprisingly, that a prothrombin complex, that has been purified on anion exchangers and pasteurized, can be activated to thrombin by addition of a soluble salt that has an anion that forms a sparingly-soluble salt or a soluble complex with calcium, in a concentration of at least 0.5 mol/l, if the mixture contains a catalytic amount of thrombin (Tab. 1, column b). Without the addition of the salt, the same quantity of thrombin does not activate the prothrombin suf-

ficiently (Tab. 2, column c). Additionally, the activation is temperature-dependent.

The invention therefore relates to a process for producing a purified and virus-free preparation of thrombin, which comprises a soluble salt which has an anion that forms a sparingly-soluble salt or a soluble complex with calcium being added in a concentration of at least 0.5 mol/l to a solution of a prothrombin complex which has been purified on an anion exchanger and subjected to virus inactivation, and the solution being treated with a catalytic quantity of thrombin.

The catalytic quantity of thrombin can have arisen during the purification process. Otherwise thrombin is added.

A catalytic quantity of thrombin is intended to mean thrombin in a concentration of greater than zero and up to 200, preferably 10 to 50, units per ml.

The prothrombin complex can have been obtained from animal plasma.

Preferably used is a prothrombin complex that has been purified on DEAE ion exchangers and pasteurized by, for example, the method of EP 0 137 428 at 60°C for 10 h.

Instead of pasteurization, however, the viruses can also be inactivated in any other suitable way.

Preferably the sulfate, citrate, phosphate or oxalate anion, particularly the citrate anion, is used as calcium-binding anion. The corresponding salt, preferably an alkali metal or ammonium salt, is used at a concentration of from 0.5 mol/l to the particular

saturation limit.

Further preferred embodiments comprise using prothrombin complex from animal plasma, or treating with thrombin at 0°C to 50°C, preferably at 28°C, for 2 - 100 hours, preferably 5 - 20 hours.

The process according to the invention allows the production in a simple manner of a native, highly-purified and virus-free concentrate of thrombin that can be used as an hemostatic agent or in a tissue adhesive that is based on fibrinogen.

Accordingly, the present invention provides a process for the production of a purified and virus-free preparation of thrombin, comprising the steps of:

(i) subjecting a prothrombin containing solution to an anion exchanger chromatography;

(ii) subjecting the solution of step (i) to virus inactivation;

(iii) adding to the solution a soluble salt which has an anion that forms a sparingly-soluble salt or a soluble complex with calcium in a concentration of at least 0.5 mol/l, wherein the anion that binds calcium is sulfate, citrate, phosphate or oxalate anion; and

(iv) treating the solution with a catalytic quantity of thrombin.

Examples

Example 1

16 ml of pasteurized human prothrombin concentrate with 65 U of F II/ml were mixed with 20 U/ml human thrombin and 4 g (25% w/v) of trisodium citrate and incubated at 28°C once the salt had dissolved. The thrombin activity that was obtained was determined at different times (Table 1b), and at the end of the activation the citrate was removed by dialysis, and the thrombin was lyophilized following stabilization and adjustment to the required activity.

Table 1

Activation of prothrombin complex, purified on anion exchangers, with saturated citrate solution as a function of temperature

Thrombin activity (IU/ml) of the mixtures

Time (h)	a) at 4°C	b) at 28°C	c) at 37°C
0	15	20	20
1	n.d.	61	63
3	13	350	1084
5	n.d.	1695	3351
10	18	5858	5141
21	n.d.	8492	7393
25	14	8978	6991
45	94	8109	7575
70	6800	n.d.*	n.d.
96	7700	n.d.	n.d.
Highest thrombin activity per 1 IU of F II	138		117

*n.d. = not determined

Example 2

18 ml of pasteurized human prothrombin concentrate with 65 U of F II/ml were mixed with 20 U/ml human thrombin and 4.5 g of trisodium citrate and incubated at 37°C once the salt had dissolved. The thrombin activity that was obtained was determined at different times (Table 1c).

Example 3

8 ml of pasteurized human prothrombin concentrate with 70 U of F II/ml were mixed with 20 U/ml human thrombin/ml and incubated at 37°C without addition of a salt with calcium-binding anion. The thrombin activities that were obtained after different times are shown in Table 2, column c.

Example 4

10 ml of pasteurized human prothrombin concentrate with 80 U of F II/ml were mixed with 2.5 g of trisodium citrate without addition of thrombin and incubated at 28°C once the salt had dissolved. Samples were taken after different times and the thrombin activity determined (Table 2, column b).

Table 2

Activation of prothrombin complex, purified on anion exchangers, as a function of the species of the prothrombin, of thrombin and addition of a salt with Ca-binding anion

Time (h)	Thrombin activity (IU/ml) of the mixtures a) Bov.prothr., 28°C, citr.	b) Citr., without thrombin, 28°C	c) Thrombin, without addition of salt, 37°C
0	11	< 0.1	20
2	224	n.d.*	36
4	767	< 0.1	52
6	2833	n.d.	93
11	4718	< 0.1	222
25	5870	< 0.1	846
45	5921	< 0.1	1090
70	n.d.	< 0.1	1409
101	n.d.	n.d.	1511

Highest thrombin activity per 1 IU of F II 118

22

n.d.* = not determined

Example 5

20 ml of pasteurized prothrombin concentrate from bovine plasma with 50 U of F II/ml were mixed with 10 U/ml bovine thrombin and 5 g of trisodium citrate and incubated at 28°C. The thrombin activity that was obtained after different times was determined (Table 2, column a).

Example 6

500 ml of virus-inactivated human prothrombin concentrate with 75 U of F II/ml were mixed with 15 U/ml thrombin and 125 g of trisodium citrate. Once the salt had dissolved at room temperature the solution was incubated at 4°C. Samples were taken after different times and the thrombin activity determined (Table 1, column a).

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A process for the production of a purified and virus-free preparation of thrombin, comprising the steps of:

(i) subjecting a prothrombin containing solution to an anion exchanger chromatography;

(ii) subjecting the solution of step (i) to virus inactivation;

(iii) adding to the solution a soluble salt which has an anion that forms a sparingly-soluble salt or a soluble complex with calcium in a concentration of at least 0.5 mol/l, wherein the anion that binds calcium is sulfate, citrate, phosphate or oxalate anion; and

(iv) treating the solution with a catalytic quantity of thrombin.

2. The process as claimed in claim 1, wherein thrombin is used in a concentration of greater than zero up to 200 units per ml.

3. The process as claimed in claim 2, wherein the thrombin is used in a concentration of 10 to 50 units per ml.

4. The process as claimed in claim 1, wherein the solution comprises prothrombin complex from animal plasma.

5. The process as claimed in claim 1, wherein the solution comprises prothrombin complex from human plasma.

6. The process as claimed in claim 1, wherein the treatment with thrombin takes place at 0°C to 50°C.

7. The process as claimed in claim 6, wherein the treatment with thrombin takes place at 28°C.

8. The process as claimed in claim 1, wherein the prothrombin solution is purified on DEAE ion exchangers.

9. The process as claimed in claim 1, wherein the salt is used in a concentration of from 0.5 mol/l up to saturation limit of the solution.

10. The process as claimed in claim 1, wherein the catalytic quantity of thrombin has been generated during step (i) or step (ii) of the process.