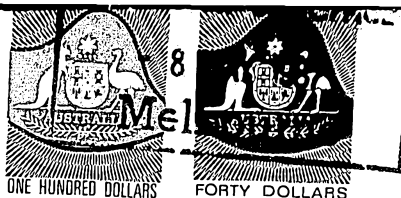


610823

COMMONWEALTH OF AUSTRALIA

Patents Act 1952-1969



CONVENTION APPLICATION FOR A PATENT

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MAIL OFFICER: *[Signature]*

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- 8 MAY 1987

Melbourne

(1) Here
Insert (in
full) Name
or Names of
Applicant or
Applicants,
followed by
Address (es).

Ix (1) BEHRINGWERKE AKTIENGESELLSCHAFT
We
of D-3550 Marburg, Federal Republic of Germany

(2) Here
Insert Title
of Invention.

hereby apply for the grant of a Patent for an invention entitled: (2)

A PROCESS FOR THE PREPARATION OF A FACTOR V CONCENTRATE

(3) Here insert
number(s)
of basic
application(s)

which is described in the accompanying complete specification. This application is a
Convention application and is based on the application numbered (3)

P36 15 558.6

(4) Here insert
Name of basic
Country or
Countries, and
basic date or
dates

for a patent or similar protection made in (4) Federal Republic of Germany
on 9th May 1986

~~My~~
Our address for service is Messrs. Edwd. Waters & Sons, Patent Attorneys,
50 Queen Street, Melbourne, Victoria, Australia.

DATED this 7th day of May 19 87

(5) Signa-
ture (s) of
Applicant (s)
or
Seal of
Company and
Signatures of
its Officer: as
prescribed by
its Articles of
Association.

(5)

BEHRINGWERKE AKTIENGESELLSCHAFT

by

James Murray

Registered Patent Attorney

To:

THE COMMISSIONER OF PATENTS.

COMMONWEALTH OF AUSTRALIAPatents Act 1952DECLARATION IN SUPPORT OF A CONVENTION APPLICATION UNDER PART XVI.
FOR A PATENT.

In support of the Convention application made under Part XVI. of the Patents Act 1952 by BEHRINGWERKE AKTIENGESELLSCHAFT of D-3550 Marburg, Federal Republic of Germany for a patent for an invention entitled:

A PROCESS FOR THE PREPARATION OF A FACTOR V CONCENTRATE

We, Heribert Bug of 7 Amselweg, D-3551 Niederweimar,
Philipp Stein of 28 Höhenweg, D-3550 Marburg,
Federal Republic of Germany

do solemnly and sincerely declare as follows:

1. We are authorized by BEHRINGWERKE AKTIENGESELLSCHAFT the applicant for the patent to make this declaration on its behalf.
2. The basic application(s) as defined by Section 141 of the Act was (were) made at München in the Federal Republic of Germany under P 36 15 558.6

on May 9, 1986

by BEHRINGWERKE AKTIENGESELLSCHAFT

3. a) Norbert Heimbürger, 10 Sonnenhang, D-3550 Marburg
b) Gerhardt Kume, 14 Aspherfeld, D-3552 Wetter
c) Wilfried Wormsbächer, 9 Blumenweg, D-3575 Kirchhain
a) - c) Federal Republic of Germany

~~XX~~/are the actual inventor(s) of the invention and the facts upon which BEHRINGWERKE AKTIENGESELLSCHAFT

is entitled to make the application are as follows:

The said BEHRINGWERKE AKTIENGESELLSCHAFT
is the assignee of the said

Norbert Heimbürger, Gerhardt Kume, Wilfried Wormsbächer

4. The basic application referred to in paragraph 2 of this Declaration was (were) the first application(s) made in a Convention country in respect of the invention the subject of the application.

DECLARED at Marburg, Federal Republic of Germany

this 27th day of March 1987

To the Commissioner of Patents

BEHRINGWERKE AKTIENGESELLSCHAFT

ppa Bug
Prokurist
ppa. Bug

ppa Stein
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ppa. Stein

(12) PATENT ABRIDGMENT (11) Document No. AU-B-72642/87
(19) AUSTRALIAN PATENT OFFICE (10) Acceptance No. 610823

(54) Title
PREPARATION OF FACTOR V CONCENTRATE

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3122

(57) Claim

1. A process for the preparation of a concentrate of coagulation factor V from an anticoagulant-containing human blood plasma or from an anticoagulant-containing fraction of human blood plasma, which comprises addition of a salt of a bivalent metal, in an amount such that the concentration of doubly charged cations which is reached is less than the concentration equivalent to the concentration of anticoagulant, to a plasma of this type or to a fraction of this type, and an F V concentrate being obtained in a known manner.
4. The process as claimed in claim 1, wherein the anticoagulant is citrate, heparin, phosphate-dextrose or citrate-dextrose or a mixture of such anticoagulants.

COMMONWEALTH OF AUSTRALIA

Form 10

PATENTS ACT 1952-69

COMPLETE SPECIFICATION

(ORIGINAL)

Application Number:
Lodged:

72642/87

Class

Int. Class

610823

Complete Specification Lodged:

Accepted:

Published:

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Related Art :

Name of Applicant : BEHRINGWERKE AKTIENGESELLSCHAFT

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50 QUEEN STREET, MELBOURNE, AUSTRALIA, 3000.

Complete Specification for the invention entitled:

A PROCESS FOR THE PREPARATION OF A FACTOR V CONCENTRATE

The following statement is a full description of this invention, including the best method of performing it known to : us

BEHRINGWERKE AKTIENGESELLSCHAFT

86/B 010 - Ma 556

Dr. Ha/Bn

A process for the preparation of a factor V concentrate

The invention relates to a process for the preparation of a factor V concentrate from human blood plasma or from a
5 fraction of blood plasma, which contain an anticoagulant.

There are contributions to the biological phenomenon of hemostasis by the vessels, the blood platelets and plasma factors. The primary wound closure is brought about by
10 the synergistic cooperation of vessels and blood platelets, which result in what is called primary wound closure, which is then reinforced by plasma coagulation which results in the formation of fibrin. The blood platelets form the reaction surface where plasma coagulation takes
15 place, in which 13 plasma factors are involved, not including the inhibitors. These plasma factors are proteins, mainly glycoproteins, and their action is predominantly that of serine proteases. The latter circulate in the blood in the form of inactive precursors and are
20 activated in response to stimuli such as are represented by an injury. The activation is based on limited proteolysis with each of the foregoing factors in the coagulation cascade activating the one which follows on the basis of an enzyme/substrate reaction. Essential for the activation are phospholipids, as are found in the form of a
25 mixture, which is called platelet factor 3 (PF 3), in the platelets, and calcium ions. Also important are so-called accelerators, proteins which are able to increase the rate of the coagulation process. The accelerators which act
30 in conjunction with the phospholipids and calcium, by forming what are called activation complexes, include factors V and VIII (F V and F VIII).

F V is a β -globulin which has a molecular weight of
35 330,000 dalton, is activated by limited proteolysis of thrombin and is inactivated by protein C_a. At the same time, specific activating peptides are produced.

The basis of the action of F V as accelerator is that it intensifies the interaction between F X a and prothrombin in the presence of calcium and platelet phospholipids to such an extent that the rate of thrombin formation is increased about 1,000 times. The complex composed of factors X a, phospholipids, calcium and FV_a is designated prothrombin activator or prothrombinase. As appropriate for the accelerator action of F V, a reduction in this coagulation factor results in a slowing down of thrombin formation, and thus of coagulation, and, as the biological consequence, in an increased risk of hemorrhage.

All coagulation factors, irrespective of their enzymatic characteristics, are, comparatively speaking, very unstable and sensitive to denaturation, at any rate in the form in which they are normally obtained from plasma which has been treated to prevent coagulation. This particularly applies to F VIII and F V, the two accelerator proteins.

Use is made of the instability of factor V in the preparation of F V-deficient plasma: human citrated plasma incubated in a water bath at 37°C loses its F V activity within 24 hours, whereas all the other factors remain stable.

Inborn and acquired F V-deficiency states are relatively rare. Nevertheless, there is a number of indications for which a F V concentrate appears indicated. However, owing to the instability and susceptibility to denaturation, no such concentrate is yet available. This is very much in contrast with other coagulation factors, all of which are available for therapy in the form of concentrates. This illustrates the particular instability of this coagulation factor, which is reflected in the designation "labile factor" for factor V.

For these reasons, there was a need for a process for obtaining F V from whole blood, which guarantees the

stability of this factor during removal, storage and processing of the blood in the presence of anticoagulants. Such a stabilization of F V would make it possible to prepare a stable F V plasma for coagulation tests and to prepare F V-containing plasma for replacement or as starting material for the preparation of an F V concentrate.

The object of the invention is to prepare an F V concentrate.

10

Although F V can easily be denatured, it has already been isolated in active form from plasma. This has been possible by adding to fresh citrated plasma, from which the prothrombin factors had been removed, synthetic protease inhibitors to protect against enzymatic breakdown of F V. Additions of this type are not suitable for the preparation of a standard plasma or for the preparation of pure F V since they are toxic and block the interaction of F V with other coagulation factors.

20

The obtaining of factor F V from plasma, from which the cryoprecipitate has been removed, by chromatography on an anion exchanger has also already been disclosed. The low yield is disadvantageous in this process.

25

It has been found, surprisingly, that the loss of F V activity when it is obtained from plasma is a function of the citrate concentration when citrate is used as anticoagulant. Whereas F V is relatively stable at 20°C and 5 mmol/l citrate, denaturation occurs as the citrate concentration is increased.

30

It has not been possible to use a citrate concentration below 5 mmol/l if coagulation was to be avoided.

35

It has also been found, surprisingly, that a loss of activity can be reduced by addition of doubly charged cations, preferably calcium ions. Whereas the F V activity in citrated plasma, which normally contains tri-Na citrate in a concentration of 11 mmol/l, falls to values near 0% of

normal after 21 hours at 37°C, the retention of activity is 35% if a solution of this type contains 2.5 mmol/l calcium ions, and is 70% if it contains 5 mmol/l calcium ions.

- 5 The invention relates to a process for the preparation of a concentrate of coagulation factor V from an anticoagulant-containing human blood plasma or from an anticoagulant-containing fraction of human blood plasma, which comprises addition of a salt of a bivalent metal, in an amount such that the concentration of doubly charged cations which is reached is less than the concentration equivalent to the concentration of anticoagulant, to a plasma of this type or to a fraction of this type, and an F V concentrate being obtained in a known manner.

- 15 Suitable bivalent metals are Ca, Mg or Mn.

- A metal salt of this type is preferably a soluble salt of calcium, in particular calcium chloride or acetate. The concentration of doubly charged cations is preferably at least 1 mmol/l, particularly preferably more than 2 mmol/l. The action does not necessarily increase continuously with the concentration, it may pass through a maximum. Of course, the concentration must not be so high that the mixture coagulates.

Anticoagulants are, in particular, salts of citric acid, phosphate dextrose, citrate dextrose or heparin.

- 30 It is not necessary to add the calcium immediately after removal of the blood. An advantageous effect is still achieved even if calcium is added up to 12 hours after the removal of blood, in particular if the plasma has been stored at 3-5°C for this time. Addition of calcium to frozen and rethawed plasma or to the supernatant after removal of cryoprecipitate likewise markedly stabilizes F V.

Fig. 1 shows the dependence of the F V activity on the time and the calcium concentration. The pH was as in



citrated plasma 7.5-7.8, and the temperature was 4°C.

This entailed frozen human citrated plasma obtained from 9 parts by volume of whole blood and one part by volume of sodium citrate, and having a final citrate concentration of 10 mmol/L, being thawed and dialyzed against a physiological buffer having a citrate concentration of 10 mmol/L and a calcium concentration of 0.25, 0.5, 1 or 2 mmol/L.

10

In Fig. 1, a corresponds to a calcium concentration of 0, b of 0.25, c of 0.5, d of 1 and e of 2 mmol/L. Whereas the F V activity remained virtually constant for 16 hours at a concentration of 2 mmol/L calcium in the citrate-containing F V solution, the activity fell as a function of time at lower calcium concentrations.

15

It is evident from the foregoing that a concentration of calcium ions of at least 2 mmol/L Ca^{2+} in a medium containing 10 mmol/L sodium citrate is sufficient to stabilize the F V in a freshly obtained citrated plasma, a frozen citrated plasma or a cryoprecipitate-depleted citrated plasma.

20

The citrated plasma mainly used for routine coagulation analyses is obtained by removing blood from a brachial vein into a tri-Na citrate solution.

25

A citrated plasma of this type which has been partially recalcified is suitable as starting material for obtaining an F V concentrate.

30

When 1 USP U/ml heparin was added to fresh human citrated plasma (final tri-Na citrate concentration: 11 mmol/L) and this solution was maintained at 20°C, the F V activity decreased to a considerably greater extent than in the control without addition of heparin. However, it is possible by addition of 5 mmol/L calcium chloride to reduce drastically this loss of activity even under these

35

conditions.

This shows that it is possible to replace citrate more or less by heparin and to stabilize the F V by calcium. Thus, it is also possible in this way to achieve an anticoagulant effect without removing the calcium which is necessary for the stabilization of F V.

However, with calcium it is possible to stabilize F V not only in plasma but also in fractions prepared therefrom. It is possible to use as anticoagulants for this all those which are normally used.

It is possible to use as F V-containing fraction, which can be employed as starting material for the preparation of a concentrate, the plasma supernatant after removal of cryoprecipitate. F V can be stabilized by calcium even in cryoprecipitate-free plasma. This also applies to the steps in the operation which can be used for obtaining an F V concentrate, for example chromatography of cryoprecipitate-free plasma on an anion exchanger.

F V determination: F V can be determined by the method of Koller, Loeliger and Duckert (Perlick, Gerinnungslaboratorium in Klinik and Praxis (The coagulation laboratory in hospitals and general practice), publisher: VEB Georg Thieme, Leipzig, 1960).

This entails 0.1 ml of F V-deficient plasma being mixed with 0.1 ml of a defined dilution of normal plasma. This mixture is incubated at 37°C for 30 sec. Then 0.2 ml of a calcium-containing thromboplastin is added, and the time until a clot appears is determined. A reference graph is constructed in this manner.

For the quantitative F V determination of a plasma or concentrate which is to be examined, dilutions thereof in barbitol buffer are used in place of normal plasma in the test which is described above, and the clotting time is

determined. The F V concentration can be found by reference to a calibration graph obtained with a normal plasma dilution series.

- 5 One unit of F V is equivalent to the F V activity of 1 ml of normal plasma.

The examples which follow illustrate the invention.

10 Example 1

Stabilization of F V in frozen plasma

- 15 Fresh human citrated plasma was obtained from 1 part of 110 mmol/l tri-Na citrate and 9 parts of whole blood by removal of the blood cells. The plasma was frozen at -25°C and, after 24 hours, thawed again, partially re-calcified and incubated at 37°C. The F V activity was measured at particular times during the incubation:

20

Table 1

F V activity in thawed plasma incubated without additions and with 2.5 mmol or 5 mmol/l Ca^{2+} .

25

Incubation time	Residual F V activity in %		
	Frozen plasma without additions	Frozen plasma + 2.5 mmol/l Ca^{2+}	Frozen plasma + 5 mmol/l Ca^{2+}
30 0	100	100	100
4 h	59	81	81
6 h	46	77	81
21 h	10	38	74

35

Example 2

Stabilization of F V while a plasma fraction is being obtained from citrated plasma

5 4.74 l of frozen human citrated plasma were thawed at 2-4°C, and the residue which remained insoluble, the cryo-precipitate, was removed by centrifugation. To remove the PPSB factors, 237 ml of an $\text{Al}(\text{OH})_3$ suspension containing 1 g/100 ml were added to the supernatant at 15°C, the
10 mixture was stirred for 15 min, the precipitate was removed by centrifugation, and 1 USP U/ml heparin was added to the solution.

The solution was divided into 2 portions each of 2350 ml.
15 CaCl_2 was added to portion 1 until the concentration was 5 mmol/l, and portion 2 was left as it was. Both were processed to give an F V concentrate under conditions which differed only in that all the solvents, namely the equilibration, washing and dialysis media, for portion 1 con-
20 tained 5 mmol/l CaCl_2 .

To obtain F V, 32 g of moist packed DEAE-Sephadex^(R) A 50 which had been equilibrated at pH 7.5 with a solution containing 60 mmol/l NaCl, 20 mmol/l tri-Na citrate, and for
25 portion 1 additionally 5 mmol/l CaCl_2 , were added to each portion, and the mixture was stirred at 15°C for 1 h.

The anion exchanger resin was removed from the supernatant, transferred into a column and washed free of plasma resi-
30 dues with 1.5 l of physiological NaCl solution.

Elution was carried out with a solution containing 1 mol/l NaCl, 1 USP U/ml heparin, 0.2 U/ml AT III, and for por-
tion 1 additionally 5 mmol/l CaCl_2 . The eluate was col-
35 lected in 3 fractions of 30 ml, 70 ml and 180 ml. Ammonium sulphate was added to fractions 2 and 3 until 60% saturation was reached, and the precipitate was dissolved in 5 ml of physiological saline, dialyzed and freeze-dried.

- 9 -

Whereas in the processing with the addition of calcium chloride the entire F V activity present after the aluminum hydroxide adsorption was recovered in the lyophilisate, it was only 20% of this without calcium.

XXXXXXXXXXXXXX
Patent claims:

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A process for the preparation of a concentrate of coagulation factor V from an anticoagulant-containing human blood plasma or from an anticoagulant-containing fraction of human blood plasma, which comprises addition of a salt of a bivalent metal, in an amount such that the concentration of doubly charged cations which is reached is less than the concentration equivalent to the concentration of anticoagulant, to a plasma of this type or to a fraction of this type, and an F V concentrate being obtained in a known manner.
2. The process as claimed in claim 1, wherein the metal salt is a soluble salt of calcium, in particular calcium chloride or acetate.
3. The process as claimed in claim 1, wherein the concentration of doubly charged cations is at least 2 mmol/l.
4. The process as claimed in claim 1, wherein the anticoagulant is citrate, heparin, phosphate-dextrose or citrate-dextrose or a mixture of such anticoagulants.
5. The process as claimed in claim 1, wherein the anticoagulant is heparin.
6. The process as claimed in claim 1, wherein the anticoagulant is a mixture of a salt of citric acid and heparin.

DATED this 7th day of May 1987.

BEHRINGWERKE AKTIENGESELLSCHAFT

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F.V. ACTIVITY
(% of the norm)

