Edition: BP 2025 (Ph. Eur. 11.6 update)

Human Coagulation Factor IX (rDNA) Powder for Solution for Injection

General Notices

(Ph. Eur. monograph 2994)

Ph Eur

DEFINITION

Sterile, freeze-dried preparation of closely related glycoproteins, which have the same amino acid sequence (415 amino acids) as the naturally occurring Ala 148 allelic form analogue (plasma-derived coagulation factor IX). The preparation is intended for intravenous injection.

Potency

80 per cent to 125 per cent of the potency stated on the label when determined using the conditions described under Assay.

Human coagulation factor IX (rDNA) powder for solution for injection complies with the monograph on Parenteral preparations (0520) and with the following additional requirements.

PRODUCTION

Human coagulation factor IX (rDNA) powder for solution for injection is prepared from *Human coagulation factor IX (rDNA) concentrated solution (2522)*. The concentrated solution is diluted with a formulation buffer containing excipients such as histidine, sucrose, glycine and polysorbate 80. This preparation is passed through a bacteria-retentive filter, distributed aseptically into the final containers and freeze-dried. The containers are closed under vacuum or under an inert gas. Where excipients other than those mentioned above are used, their compatibility with the tests described hereafter must be confirmed.

CHARACTERS

Appearance (before reconstitution)

White, hygroscopic powder or friable mass, free from visible foreign matter.

IDENTIFICATION

A. It complies with the limits of the assay.
B. Polyacrylamide gel electrophoresis (*2.2.31*).

Examine the electropherograms obtained in the test for impurities with molecular masses differing from that of human coagulation factor IX (rDNA).

Calculate the relative mobility (in per cent) of the main band in the electropherogram obtained with the test solution with reference to the mobility of the main band in the electropherogram obtained with reference solution (a), using the following expression:

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M_1 = molecular mass of the main band in the electropherogram obtained with the test solution;

M_2 = molecular mass of the main band in the electropherogram obtained with reference solution (a).

Results:

— the electropherogram obtained with the test solution is similar to the electropherogram obtained with reference solution (a);

— the mobility of the main band in the electropherogram obtained with the test solution is within 10 per cent of that of the main band in the electropherogram obtained with reference solution (a).

TESTS

Reconstitute the preparation to be examined as stated on the label immediately before carrying out the identification, test (except those for solubility and water) and assay.

Formulation buffer

Dissolve 19.53 g of *glycine R*, 1.55 g of *histidine R* and 10.00 g of *sucrose R* in 1000 mL of *water R*. Add 50 µL of *polysorbate 80 R* and adjust to pH 6.8 with *hydrochloric acid R*.

Appearance of solution

After reconstitution, the solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*), without visible particles.

Solubility

To a vial of the preparation to be examined add the volume of the diluent stated on the label at the recommended temperature. The preparation dissolves completely with gentle swirling within 5 min.

pH (*2.2.3*)

The limit is approved by the competent authority.

Related proteins

Liquid chromatography (*2.2.29*).

Test solution Dilute the reconstituted preparation to be examined with the formulation buffer to obtain a concentration of 0.5 mg/mL.

Reference solution Dissolve the contents of a vial of *human coagulation factor IX (rDNA) CRS* in the formulation buffer to obtain a concentration of about 0.5 mg/mL.

Column:

— *size*: *l* = 0.10 m, Ø = 4.6 mm;

— *stationary phase*: *styrene-divinylbenzene copolymer R* (10 µm) with a pore size of 400 nm;

— *temperature*: 37 °C.

Mobile phase:

— *mobile phase A*: add 1 mL of *trifluoroacetic acid R* to 1000 mL of *water for chromatography R*;

— *mobile phase B*: mix 1 mL of *trifluoroacetic acid R*, 200 mL of *water for chromatography R* and 800 mL of *acetonitrile R1*;

Time (min)	Mobile phase A (per cent *V/V*)	Mobile phase B (per cent *V/V*)
0 - 0.5	75	25
0.5 - 30	75 → 20	25 → 80
30 - 31	20 → 0	80 → 100
31 - 33	0	100

Flow rate 2.0 mL/min.

Detection Spectrophotometer at 214 nm.

Injection 100 µL; perform at least 3 injections using an automatic injector.

Relative retention With reference to the 2nd peak of the double peak due to human coagulation factor IX (rDNA) (retention time = about 12-14 min): related protein A = about 0.75; related protein B = about 0.78; related protein C = about 0.80; related protein D = about 0.85; related protein E = about 0.93.

System suitability Reference solution:

— the chromatogram obtained is qualitatively similar to the chromatogram supplied with *human coagulation factor IX (rDNA) CRS*;

— *repeatability*: maximum relative standard deviation of 3 per cent for the total area of the peak due to human coagulation factor IX (rDNA), determined on 3 injections performed immediately before the run;

— *peak-to-valley ratio*: minimum 1.2, where H_p = height above the baseline of the peak due to related protein E and H_v = height above the baseline of the lowest point of the curve separating this peak from the peak due to human coagulation factor IX (rDNA).

Report individual relative peak areas considering the peak area of the entire chromatogram. Individual relative per cent peak areas are calculated as the average of the 3 injections of the test solution.

Peaks due to related proteins A and D are not always present.

Results:

— the profile of the chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with the reference solution, except for minor peaks due to impurities, that may be absent in the chromatogram obtained with the test solution.

Limits:

— *related protein C*: maximum 0.6 per cent.

Impurities with molecular masses differing from that of human coagulation factor IX (rDNA)

Polyacrylamide gel electrophoresis (*2.2.31*) using a gradient gel with the following modifications.

Gel dimensions 1.0 mm thick.

Resolving gel 3-15 per cent acrylamide gradient gel.

Acrylamide solution *30 per cent acrylamide/bisacrylamide (36.5:1) solution R*.

To prepare a 15 per cent acrylamide solution, add *sucrose R* to obtain a concentration of 142 g/L.

The following elements are provided as examples for loading the gel-forming apparatus to prepare the stacking gel.

Stacking gel The stacking gel solution is prepared by mixing:

— 100 volumes of *30 per cent acrylamide/bisacrylamide (36.5:1) solution R*;

— 125 volumes of *1 M tris-hydrochloride buffer solution pH 6.8 R*;

— 740.5 volumes of *water R*;

— 1.5 volumes of *tetramethylethylenediamine R*;

— 33 volumes of a 150 mg/mL solution of *ammonium persulfate R*.

Load the compartments of the gradient-forming apparatus with the acrylamide solutions and proceed as per the instructions of the equipment supplier to obtain the polymerised gradient gel.

After polymerisation is completed, rinse the gradient gel with *water R*. Remove any excess liquid. Pour the stacking gel solution into the equipment, insert a clean comb and allow for polymerisation.

Alternatively, commercially available gradient gels may be used.

Test solution Dilute the reconstituted preparation to be examined with the formulation buffer to obtain a concentration of about 1 mg/mL.

Reference solution (a) Dissolve the contents of a vial of *human coagulation factor IX (rDNA) CRS* in the formulation buffer to obtain a concentration of about 1 mg/mL.

Reference solution (b) A 0.01 mg/mL solution of *bovine albumin R* in the formulation buffer.

Reference solution (c) A solution of molecular mass markers suitable for calibrating SDS-polyacrylamide gels in the range of 5-200 kDa.

Sample buffer *Concentrated SDS-PAGE sample buffer for reducing conditions R* containing dithiothreitol as the reducing agent.

Sample treatment Incubate in a water-bath for 5 min.

Application 35 µL.

Use *SDS-PAGE running buffer R* for running the gel.

For each gel, run 1 lane with reference solution (b), 2 lanes with reference solution (c), 2 lanes with the incubated reducing buffer (as blank) and at least 1 lane with reduced reference solution (a); use the remaining lanes for reduced test solutions.

Detection By Coomassie staining.

Identification of bands Human coagulation factor IX (rDNA) has an approximate molecular mass of 55 kDa, and related protein bands with molecular masses of approximately 54 kDa, 44 kDa, 29-32 kDa, 27 kDa and 14 kDa are present.

System suitability:

— a clear background is obtained after destaining;

— the band in the electropherogram obtained with reference solution (b) is clearly visible;

— all expected bands in the electropherogram obtained with reference solution (c) are visible;

— the bands in the electropherogram obtained with reference solution (c) are clearly separated;

— no band is visible in the blank lanes.

Results:

— the electropherogram obtained with the test solution is similar to the electropherogram obtained with reference solution (a);

— no new band in the electropherogram obtained with the test solution has an intensity greater than that of the band in the electropherogram obtained with reference solution (b).

Impurities with molecular masses greater than that of human coagulation factor IX (rDNA)

Size-exclusion chromatography (*2.2.30*): use the normalisation procedure.

Test solution Dilute the reconstituted preparation to be examined with the formulation buffer to obtain a concentration of 400 µg/mL.

Reference solution Dissolve the contents of a vial of *human coagulation factor IX (rDNA) CRS* in the formulation buffer to obtain a concentration of about 400 µg/mL.

Resolution solution Dissolve the contents of a vial of *human coagulation factor IX (rDNA) CRS* in the formulation buffer to obtain a concentration of about 400 µg/mL. Desalt and concentrate the preparation to be examined using a suitably validated procedure. Reconstitute the recovered material in *0.1 M phosphate buffer solution pH 8.0 R* to obtain a concentration of 400 µg/mL. To 500 µL of this solution add 1.4 µL of a 250 mg/L solution of *glutaraldehyde R*. Mix and incubate at 37 °C for 120 min.

Blank solution The formulation buffer.

Precolumn:

— *size*: *l* = 0.04 m, Ø = 6 mm;

— *stationary phase*: *hydrophilic silica gel for chromatography R* (5 µm) of a grade suitable for the fractionation of globular proteins in the relative molecular mass range of 10 000 to 500 000.

Column:

— *size*: *l* = 0.30 m, Ø = 7.8 mm;

— *stationary phase*: *hydrophilic silica gel for chromatography R* (5 µm) of a grade suitable for the fractionation of globular proteins in the relative molecular mass range of 10 000 to 500 000.

Mobile phase Dissolve 7.10 g of *anhydrous disodium hydrogen phosphate R* and 8.77 g of *sodium chloride R* in 1000 mL of *water for chromatography R*. Adjust to pH 7.00 ± 0.05 with *phosphoric acid R*.

Flow rate 1.0 mL/min.

Detection Spectrophotometer at 214 nm.

Injection 50 µL; perform 3 injections using an automatic injector maintained at 2-8 °C.

Retention time Human coagulation factor IX (rDNA) = about 9 min.

System suitability:

— the chromatogram obtained with the reference solution is qualitatively similar to the chromatogram supplied with *human coagulation factor IX (rDNA) CRS*;

— *peak-to-valley ratio*: minimum 2.0, where H_p = height above the baseline of the peak due to the high molecular mass species and H_v = height above the baseline of the lowest point of the curve separating this peak from the peak due to human coagulation factor IX (rDNA) in the chromatogram obtained with the resolution solution.

Calculate the relative area (in per cent) of the sum of the peaks with retention times less than that of human coagulation factor IX (rDNA), with reference to the area of the peak due to human coagulation factor IX (rDNA). Any shoulder appearing on the descending part of the peak due to human coagulation factor IX (rDNA) is included in its area.

Result:

— the profile of the chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with the reference solution.

Limit:

— *sum of the peaks eluted before the principal peak*: maximum 1.8 per cent.

Activated human coagulation factor IX (rDNA) (*2.6.22*)

Dilute the reconstituted preparation to be examined with *tris(hydroxymethyl)aminomethane buffer solution pH 7.5 R* to obtain a concentration of human coagulation factor IX (rDNA) of 20 IU/mL. For each of the dilutions, the coagulation time is not less than 150 s.

Osmolality (*2.2.35*)

The limit is approved by the competent authority.

Water (*2.5.32*)

Maximum 2.0 per cent.

Sterility (*2.6.1*)

It complies with the test.

Bacterial endotoxins (*2.6.14*)

Less than 1 IU per 100 IU of factor IX activity.

ASSAY

Protein

Size-exclusion chromatography (*2.2.30*) as described in the test for impurities with molecular masses greater than that of human coagulation factor IX (rDNA) with the following modifications.

Prepare triplicate dilutions of the test solution.

Reference solutions Dissolve the contents of a vial of *human coagulation factor IX (rDNA) CRS* in the formulation buffer to obtain a concentration of 1 mg/mL. Further dilute the solution to prepare a standard curve with concentrations in the range of 100-800 µg/mL (5 concentrations, typically 100 µg/mL, 200 µg/mL, 400 µg/mL, 600 µg/mL and 800 µg/mL).

Plot peak areas versus injected protein content and perform linear regression to create a standard curve.

System suitability (in addition to those described in the test for impurities with molecular masses greater than that of human coagulation factor IX (rDNA)):

— the correlation coefficient (r^2) calculated for the standard curve is not less than 0.995.

Calculate the protein concentration of each replicate of the preparation to be examined using the standard curve and the assigned content in *human coagulation factor IX (rDNA) CRS*.

Potency

Assay of human coagulation factor IX (*2.7.11*). The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence limits (P = 0.95) are not less than 80 per cent and not more than 120 per cent of the estimated potency.

Human coagulation factor IX concentrate BRP is suitable for use as a reference preparation.

STORAGE

In an airtight container, protected from light, at a temperature of 2 °C to 8 °C.

LABELLING

The label states:

— the factor IX content in International Units;

— the name of any excipient and added substance;

— the composition and volume of the liquid to be used for reconstitution.