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

Erythropoietin Injection

General Notices

Action and use

Erythropoietin analogue.

DEFINITION

Erythropoietin Injection is a sterile solution of Erythropoietin Concentrated Solution in a suitable liquid. It is supplied as a ready-to-use solution.

PRODUCTION

The test for 'Dimers and substances of higher molecular weight' does not apply to those products containing human serum albumin that have been validated to show aggregation does not occur.

The injection complies with the requirements stated under Parenteral Preparations and, when supplied as a ready-to-use solution, with the following requirements.

Potency

The estimated potency is not less than 80% and not more than 125% of the stated potency.

CHARACTERISTICS

A clear, colourless solution virtually free from particles.

IDENTIFICATION

A. The result calculated under Assay complies with the specification stated under Potency.
B. Carry out the method for polyacrylamide gel electrophoresis, Appendix III F, using slab gels 0.75 mm thick and about 16 cm square (or 7 cm × 8 cm) and 12% *acrylamide* as the resolving gel and the following solutions:
(1) Concentrate or dilute, if necessary, the injection being examined to give a solution containing 2000 IU per mL in *water*; and then add 1 volume of SDS-PAGE sample buffer (concentrated).
(2) Dissolve the contents of a vial of *erythropoietin for physiochemical tests EPCRS* in water to obtain a 0.0017% solution and then add 1 volume of SDS-PAGE sample buffer (concentrated).
(3) A solution of pre-stained molecular weight markers suitable for calibrating SDS-polyacrylamide gels in the range of 10 to 70 kDa and suitable for the electrotransfer to an appropriate membrane.

Boil solutions (1), (2) and (3) for 2 minutes. Apply 20 µL (or 15 µL for 7 cm × 8 cm slab gels) of the solutions separately to the surface of the gel in the following order: solution (2), solution (1), solution (3). At the end of the separation, remove the gel-cassette from the apparatus.

Immunoblotting is carried out as follows. Transfer the gel onto a membrane suitable for the immobilisation of proteins, using commercially available electrotransfer equipment and following the manufacturer’s instructions. After electrotransfer,

incubate the membrane in a neutral isotonic buffer containing a suitable blocking agent (for example, 50 g per litre of dried milk or 10% v/v foetal calf serum), for 1 to 2 hours, followed by incubation for 1 to 14 hours in the same blocking solution with a suitable dilution of either a polyclonal or monoclonal anti-erythropoietin antibody. Detect erythropoietin-bound antibody using a suitable enzyme or radiolabelled antibody (for example, an alkaline phosphatase-conjugated second antibody). The precise details of blocking agents, concentrations and incubation times should be optimised using the principles set out in *Immunochemical methods*, Appendix XIV B.

SYSTEM SUITABILITY

The test is not valid unless, in the electrophoretogram obtained with solution (3), the molecular weight markers are resolved on the membrane into discrete bands, with a linear relationship between distance migrated and logarithm_{10} of the molecular weight.

CONFIRMATION

The electrophoretogram obtained with solution (1) shows a single broad band corresponding in position and intensity to the single band seen in the electrophoretogram obtained with solution (2).

TESTS

Acidity or alkalinity

pH, 6.6 to 7.4, Appendix V L.

Dimers and related substances of higher molecular weight

Use method A or method B.

A. Carry out the method for *size-exclusion chromatography*, Appendix III C, using the following solutions and the *normalisation* procedure.
(1) Dilute the injection, if necessary, with the mobile phase to give a solution containing 1,000 IU of Erythropoietin per mL.
(2) Dissolve the contents of a vial of *erythropoietin for SEC system suitability EPCRS* in the mobile phase to obtain a 0.0005% w/v solution.
(3) Dilute 1 volume of solution (2) to 50 volumes with mobile phase.

CHROMATOGRAPHIC CONDITIONS

(a) Use a stainless steel column (30 cm × 7.5 mm) packed with *hydrophilic silica gel for chromatography* (5 µm) of a grade suitable for fractionation of globular proteins in the molecular weight range of 20,000 to 200,000 (TSK G3000SWXL is suitable).
(b) Use isocratic elution and the mobile phase described below.
(c) Use a flow rate of 0.6 mL per minute.
(d) Use an ambient column temperature.
(e) Use fluorimetric detection with an excitation wavelength of 280 nm and an emission wavelength of 340 nm.
(f) Inject 100 µL of each solution.
(g) Allow the chromatography to proceed for twice the retention time of erythropoietin monomer.

MOBILE PHASE

0.115% w/v of *anhydrous disodium hydrogen orthophosphate*, 0.02% w/v of *potassium dihydrogen orthophosphate* and 2.34% w/v of *sodium chloride* in *water;* if necessary, adjust to pH 7.3.

SYSTEM SUITABILITY

The test is not valid unless:

When the chromatograms are recorded under the prescribed conditions, the retention time of the peak due to erythropoietin dimer is about 25 minutes, and the retention time of the peak due to erythropoietin monomer is about 28 minutes.

in the chromatogram obtained with solution (2) aggregates are present;

in the chromatogram obtained with solution (2) the *resolution* between the peaks due to erythropoietin dimer and erythropoietin monomer is at least 0.8 and the relative standard deviation of erythromycin monomer is less than 10%

LIMITS

In the chromatogram obtained with solution (1) the total area of any peaks eluting before the principal peak is not greater than 2.0%.

In the chromatogram obtained with solution (3) the area of the principal peak is 1.5 to 2.5% of the area of the principal peak in the chromatogram obtained with the solution (1).

B. Carry out the method for *size-exclusion chromatography*, Appendix III C, using the following solutions prepared in solution C.

Solution A 2.28% w/v Dipotassium Hydrogen Phosphate Trihydrate and 1.49% w/v *potassium chloride*, adjusted to pH 6.8 with 85% w/w *orthophosphoric acid*.

Solution B 0.43% w/v *sodium chloride*, 0.103% w/v *sodium dihydrogen orthophosphate monohydrate*, 0.223% w/v *disodium hydrogen orthophosphate dihydrate*, 0.5% w/v *glycine* and 0.03% w/v *polysorbate 80*, adjusted to pH 6.9.

Solution C 2 volumes of solution A and 1 volume of solution B

(1) Prepare the injection being examined as given in the Table below using the following solutions.
(2) Dissolve the contents of a vial of *erythropoietin for SEC system suitability EPCRS* in the sample dilution buffer to obtain a 0.001% w/v solution.
(3) Dilute 0.02 mL of solution (1) to 1 mL with solution C.

Sample solution IU per mL	µg per mL	Dilution	Diluent	Final concentration of polysorbate 80 w/v	Injection volume µL
2,000	16.67	Undiluted	Not applicable	0.03%	50
4,000	33.33	Undiluted	Not applicable	0.03%	50
10,000	83.33	1:3	Solution A	0.01%	100
40,000	333.33	1:6	Solution C	0.01%	50

CHROMATOGRAPHIC CONDITIONS

(a) Use a stainless steel column (30 cm × 7.8 mm) packed with *hydrophilic silica gel for chromatography* of a grade suitable for fractionation of globular proteins in the molecular weight range of 20,000 to 200,000 (TSK G 3000 SWxl is suitable).
(b) Use isocratic elution and the mobile phase described below.
(c) Use a flow rate of 0.3 mL per minute.
(d) Use an ambient column temperature.
(e) Use fluorimetric detection with an excitation wavelength of 280 nm and an emission wavelength of 345 nm.
(f) For solution (2) use an injection volume of 50 µL. For solutions (1) and (3) use injection volumes as stated in the sample preparation table.
(g) Allow the chromatography to proceed for twice the retention time of erythropoietin monomer.

MOBILE PHASE

25 volumes of *propan-2-ol*, 75 volumes of solution A

SYSTEM SUITABILITY

The test is not valid unless:

When the chromatograms are recorded under the prescribed conditions, the retention time of the peak due to erythropoietin dimer is about 25 minutes, and the retention time of the peak due to erythropoietin monomer is about 28 minutes

solution B shows no interfering peaks;

in the chromatogram obtained with solution (2) the *resolution* between the peaks due to erythropoietin dimer and erythropoietin monomer is at least 0.8 and the relative standard deviation of erythromycin monomer is less than 10%

LIMITS

In the chromatogram obtained with solution (1) the total area of any peaks eluting before the principal peak is not greater than the area of the principal peak in the chromatogram obtained with solution (3) (2.0%).

The retention time of the principal peak is about 30 minutes.

Bacterial endotoxins

Carry out the *test for bacterial endotoxins*, Appendix XIV C. The endotoxin limit concentration is less than 20 IU in a volume containing 10,000 IU of Erythropoietin.

ASSAY

Carry out the Assay using method A or method B.

The activity of the preparation is compared with that of *erythropoietin EPBRP* and expressed in International Units (IU).

The confidence limits of the estimated potency (P = 0.95) are not less than 64% and not more than 156% of the stated potency.

A. In polycythaemic mice

The activity of the preparation is estimated by examining, under given conditions, its effect in stimulating the incorporation of ^{59}Fe into circulating red blood cells of mice made polycythaemic by exposure to reduced atmospheric pressure.

The following schedule, using treatment in a hypobaric chamber, has been found to be suitable.

Induce polycythaemia in female mice of the same strain, weighing 16 to 18 g. Place the mice in a hypoxic chamber and reduce the pressure to 0.6 atmospheres. After 3 days at 0.6 atmospheres, further reduce the pressure to between 0.4 and 0.5 atmospheres and maintain the animals at this pressure for a further 11 days (the partial vacuum is interrupted daily for a maximum of 1 hour at about 11:00 a.m., in order to clean the cages and feed the animals). At the end of the specified period, return the mice to normal atmospheric conditions. Randomly distribute the mice into cages, each containing 6 animals, and mark them. Use the following solutions:

(1) Dilute the substance to be examined in *phosphate-albumin buffered saline pH 7.2 R1* to obtain a concentration of 0.2 IU per mL.
(2) Mix equal volumes of solution (1) and *phosphate-albumin buffered saline pH 7.2 R1*
(3) Mix equal volumes of solution (2) and *phosphate-albumin buffered saline pH 7.2 R1*
(4) Dissolve *erythropoietin EPBRP* in *phosphate-albumin buffered saline pH 7.2 R1*
(5) Mix equal volumes of solution (4) and *phosphate-albumin buffered saline pH 7.2 R1*
(6) Mix equal volumes of solution (5) and *phosphate-albumin buffered saline pH 7.2 R1*
(7) Use a commercially available solution of [^{59}Fe] ferric chloride (approximate specific activity: 100 to 1000 MBq per mg of Fe).

Radiolabelled [^{59}Fe] ferric chloride solution Dilute the concentrated radiolabelled [^{59}Fe] ferric chloride solution in *sodium citrate buffer solution pH 7.8* to obtain a solution with an activity of 3.7 × 10^4 Bq per mL.

The concentrations of the test solutions and reference solutions may need to be modified, based on the response range of the animals used.

Three days after returning the animals to atmospheric pressure, inject each animal subcutaneously with 0.2 mL of one of the solutions. The 6 animals in each cage must each receive one of the 6 different treatments (solutions 1 to 6); the order of injection must be separately randomised for each cage. A minimum of 8 cages is recommended. Two days after injection of solutions 1 to 6, inject each animal intraperitoneally with 0.2 mL of solution 7. The order of the injections must be the same as that of the erythropoietin injections, and the time interval between administration of the erythropoietin and the radiolabelled ferric chloride solution must be the same for each animal. After a further 48 hours, anaesthetise each animal by injection of a suitable anaesthetic, record body weights and withdraw blood samples (0.65 mL) into haematocrit capillaries from the bifurcation of the aorta. After determining the packed cell volume for each sample, measure the radioactivity.

Calculate the response (percentage of ^{59}Fe in total circulating blood) for each mouse using the expression:

$$\frac{A_s \times M \times 7.5}{A_t \times V_s}$$

A_s	=	radioactivity in the sample,
A_t	=	total radioactivity injected,
7.5	=	total blood volume as per cent body weight,
M	=	body weight, in grams,
V_s	=	sample volume.

Calculate the potency by the usual statistical methods for a parallel line assay. Eliminate from the calculation any animal where the packed cell volume is less than 54%, or where the body weight is more than 24 g.

B. In normocythaemic mice

The Assay is based on the measurement of stimulation of reticulocyte production in normocythaemic mice.

The Assay may be carried out using the following solutions:

(1) Dilute the preparation being examined in *phosphate-albumin buffered saline pH 7.2 R1* to obtain a concentration of 80 IU per mL.
(2) Mix equal volumes of solution (1) and *phosphate-albumin buffered saline pH 7.2 R1*
(3) Mix equal volumes of solution (2) and *phosphate-albumin buffered saline pH 7.2 R1*.
(4) Dissolve *erythropoietin EPBRP* in *phosphate-albumin buffered saline pH 7.2 R1* to obtain a concentration of 80 IU per mL.
(5) Mix equal volumes of solution (4) and *phosphate-albumin buffered saline pH 7.2 R1*.
(6) Mix equal volumes of solution (5) and *phosphate-albumin buffered saline pH 7.2 R1*.

The exact concentrations of the test solutions and reference solutions may need to be modified, based on the response range of the animals used.

At the beginning of the assay procedure, randomly distribute mice of a suitable age and strain (8-week old B6D2F1 mice are suitable) into 6 cages. A minimum of 8 mice per cage is recommended. Inject each animal subcutaneously with 0.5 mL of the appropriate treatment (one solution per cage) and put the animal in a new cage. Combine the mice in such a way that each cage housing the treated mice contains one mouse out of the 6 different treatments (solutions 1 to 6, 6 mice per cage). 4 days after the injections, collect blood samples from the animals and determine the number of reticulocytes using a suitable procedure.

The following method may be employed:

The volume of blood, dilution procedure and fluorescent reagent may need to be modified to ensure maximum development and stability of fluorescence.

Colorant solution, concentrated Use a solution of thiazole orange suitable for the determination of reticulocytes. Prepare at a concentration twice that necessary for the analysis.

Proceed with the following dilution steps. Dilute whole blood 500-fold in the buffer used to prepare the colorant solution. Dilute this solution 2-fold in the concentrated colorant solution. After staining for 3 to 10 minutes, determine the reticulocyte count microfluorometrically in a flow cytometer. The percentage of reticulocytes is determined using a biparametric histogram: number of cells/red fluorescence (620 nm).

Calculate the potency by the usual statistical methods for a parallel line assay.

STORAGE

When supplied as a ready-to-use solution, Erythropoietin Injection should be stored protected from light at a temperature of 2° to 8°.

Erythropoietin Injection prepared by dissolving the contents of a sealed container in the liquid stated on the label should be used immediately after preparation but, in any case, within the period recommended by the manufacturer when prepared

and stored strictly in accordance with the manufacturer's instructions.

LABELLING

The label states the number of IU (Units) in a suitable dose-volume.

ERYTHROPOIETIN FOR INJECTION

DEFINITION

Erythropoietin for Injection is a freeze-dried, sterile preparation prepared from Erythropoietin Concentrated Solution. It is supplied in a sealed container.

The contents of the sealed container comply with the requirements for Powders for Injections or Infusions stated under Parenteral Preparations and with the following requirements.

IDENTIFICATION

A. It gives the appropriate response when examined using the conditions under Assay.
B. Comply with Identification test B as described for the ready to use solution. Prepare solution (1) by dissolving a quantity of powder in *water* to contain 2000 IU per mL. Prepare solution (2) by dissolving the contents of a vial of *erythropoietin for SEC system suitability EPCRS* in *water* to obtain a 0.0017% w/v solution. Add 1 volume of *SDS-PAGE sample buffer (concentrated)* to each solution.

TESTS

Dimers and related substances of higher molecular weight

Comply with the tests A or B described for the ready to use solution using the following test solution.

(1) Dissolve a quantity of the powder in the mobile phase to give a solution containing 1,000 IU of erythropoietin per mL.

Water

Not more than 4.0% w/w, Appendix IX C, Method 1.

Bacterial endotoxins

Carry out the *test for bacterial endotoxins*, Appendix XIV C. The endotoxin limit concentration is less than 20 IU in a volume containing 10,000 IU of Erythropoietin.

ASSAY

Carry out the Assay as described for the ready to use solution using the following solutions:

A. In polycythaemic mice

(1) 0.2 IU per mL in *phosphate-albumin buffered saline pH 7.2 R1*.

B. In normocythaemic mice

(1) 80 IU per mL in *phosphate-albumin buffered saline pH 7.2 R1*

LABELLING

The label of the sealed container states the number of IU (Units) contained in it.