## Epoetin

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| **Type of Posting** | Revision Bulletin, Postponement |
| **Posting Date** | 27–Apr–2018 |
| **Official Date** | 01–May–2018 |
| **Expert Committee** | Biologics Monographs 2—Proteins |
| **Reason for Revision** | Compliance |

In accordance with the Rules and Procedures of the 2015–2020 Council of Experts, the Biologics Monographs 2—Proteins Expert Committee has postponed the Epoetin monograph.

USP has received comments regarding the implementation of the monograph. Additional input from stakeholders is required in order to resolve the concerns raised. Interested parties are invited to contact USP for additional information on this topic and to get involved in the dialog on the path forward for the USP Epoetin standards.

The Epoetin Revision Bulletin postpones the implementation of the monograph becoming official in *USP 41–NF 36*.

Should you have any questions, please contact Kevin Carrick, Director, Science and Standards, Science—Global Biologics (301-230-6349 or klc@usp.org).

***Change to read:***

# ▲Epoetin

**▲(Postponed on 1-May-2018)▲** (RB 1-May-2018)

APPRLICDSR VLERYLLEAK EAENITTGCA EHCSLNENIT VPDTKVNFYA
WKRMEVGQQA VEVWQGLALL SEAVLRGQAL LVNSSQPWEP LQLHVDKAVS
GLRSLTTLLR ALGAQKEAIS PPDAASAAPL RTITADTFRK LFRVYSNFLR
GKLKLYTGEA CRTGD

$C_{809}H_{1301}N_{229}O_{240}S_5$ 18,236.06 Da (amino acid sequence)

## DEFINITION

Epoetin is the recombinant form of human erythropoietin. It is a 165-amino acid glycoprotein manufactured using recombinant DNA technology. The presence of the impurities, host cell DNA, and host cell protein in Epoetin is process-specific, and is controlled through the purification process. The impurity levels are determined by validated methods and limits approved by the competent regulatory authority. It has a potency of NLT 140,000 and NMT 200,000 international units (IU) per absorbance unit (AU) at 280 nm.

## IDENTIFICATION

- **A. Erythropoietin Bioassays ⟨124⟩:** Meets the requirements
- **B. Peptide Mapping**

**Solution A:** Trifluoroacetic acid and water (1.5: 1000)
**Solution B:** Acetonitrile, trifluoroacetic acid, and water (900: 1.2: 100)
**Mobile phase:** See *Table 1*.

**Table 1**

| Time (min) | Solution A (%) | Solution B (%) |
|---|---|---|
| 0 | 90 | 10 |
| 30.0 | 78 | 22 |
| 110.0 | 58 | 42 |
| 130.0 | 35 | 65 |
| 130.1 | 10 | 90 |
| 140.0 | 10 | 90 |
| 140.1 | 90 | 10 |

**Phosphate buffer saline:** 137 mM sodium chloride, 2.7 mM potassium chloride, 8.1 mM disodium hydrogen phosphate, and 1.47 mM potassium dihydrogen phosphate
**Buffer A:** 20 mM sodium citrate and 100 mM sodium chloride, pH 6.9, prepared as follows. Dissolve 5.80 g of sodium citrate dihydrate, 0.057 g of citric acid, and 5.84 g of sodium chloride in 800 mL of water, and mix. Adjust with hydrochloric acid to a pH of 6.9 and dilute with water to 1 L. Pass through a nylon filter of 0.2-µm pore size.
**Digest stop solution:** 8 M guanidine hydrochloride
**Digest buffer stock:** 1.0 M tris(hydroxymethyl) aminomethane solution. Adjust with hydrochloric acid to a pH of 7.3.
**Lys-C stock solution:** Dissolve lysyl endopeptidase (Lys-C), containing approximately 4.5 AU/mg, in *Digest buffer stock* to a final concentration of 2 mg/mL. Expiry of reconstituted Lys-C is 2 years at –70 ± 10°, and 1 day at 5 ± 3°.
**Lys-C digest solution:** Add 20 µL of *Lys-C stock solution* to 180 µL of *Digest buffer stock*. Expiry is 1 day stored on ice or at 5 ± 3°.
**Standard solution:** 100 µg of USP Erythropoietin RS in 50 µL of water. Buffer exchange by a suitable method into *Buffer A*. Add 5 µL of *Lys-C digest solution* to 50 µL of buffer exchanged USP Erythropoietin RS. Incubate at 37° for 30 min. Add 50 µL of *Digest stop solution* and mix.
**Sample solution:** 100 µg of Epoetin in 50 µL of *Phosphate buffer saline*. If necessary, buffer exchange by a suitable method into *Buffer A*. Add 5 µL of *Lys-C digest solution*. Incubate at 37° for 30 min. Add 50 µL of *Digest stop solution* and mix.
**Blank solution:** 50 µL of *Phosphate buffered saline*. Add 5 µL of *Lys-C digest solution*. Incubate at 37° for 30 min. Add 50 µL of *Digest stop solution* and mix.
**Chromatographic system**
(See *Chromatography* ⟨621⟩*, System Suitability*.)
**Mode:** LC
**Detector:** UV 214 nm
**Column:** 3.0-mm × 25-cm; 5-µm packing L7
**Column temperature:** 30°
**Flow rate:** 0.2 mL/min
**Injection volume:** 50 µL
**System suitability**
**Sample:** *Standard solution*
**Suitability requirements:** 10 major peaks must be present as illustrated in the reference chromatogram provided with the lot of USP Erythropoietin RS being used. The change in retention times for each of peaks 5, 8, and 9 is NMT 1.2 min when compared to the first injection for three replicate injections. The ratio of relative heights percentage (RRH%) for each peak 8 and peak 9 in the *Standard solution* injections must be within 94%–106%.
**Signal-to-noise ratio:** NLT 3 for peak 6
**Analysis**
**Samples:** *Standard solution*, *Sample solution*, and *Blank solution*
Follow each injection of *Standard solution* with a *Blank solution* injection. Record the retention time and response (peak height) of each of the 10 major peaks for both the *Standard solution* and *Sample solution*.
Calculate the relative peak height percentage (RH%) of peak 8 and peak 9 as compared to peak 5 for both the initial *Standard solution* injection and *Sample solution* taken:

$$\text{Result} = (r_U/r_S) \times 100$$

$r_U$ = peak height of peak 8 or 9
$r_S$ = peak height of peak 5

Calculate the ratio of RRH% for peak 8 and peak 9:

$$\text{Result} = (RH_x/RH_S) \times 100$$

$RH_x$ = RH% of peak 8 or 9 for the *Standard solution*
$RH_S$ = RH% of peak 8 or 9 for the *Sample solution*

**Acceptance criteria:** The chromatographic profile of the initial *Sample solution* injection is similar to that of the *Standard solution*. The absolute difference between the retention times of peaks 5, 8, and 9 in the *Sample solution* must be within 1.2 min when compared to the initial *Standard solution* injection. The RRH% for each peak 8 and peak 9 in the *Sample solution* must be within 94%–106% when compared to the initial *Standard solution*. There must be no new peaks greater than peak 6 in

height in the *Sample solution* that are not present in the *Standard solution* injection.

## ASSAY

• **Erythropoietin Bioassays ⟨124⟩**

**Acceptance criteria:** Meets the requirements. It has a potency of NLT 140,000 and NMT 200,000 IU/AU at 280 nm.

## IMPURITIES

• **Limit of High Molecular Weight Proteins**

**Mobile phase:** 20 mM sodium citrate and 100 mM sodium chloride, pH 6.9, prepared as follows. Dissolve 5.80 g of sodium citrate dihydrate, 0.057 g of citric acid, and 5.84 g of sodium chloride in 800 mL of water and mix. Adjust with hydrochloric acid to a pH of 6.9 and dilute with water to 1 L. Pass through a nylon filter of 0.2-µm pore size.

**System suitability solution:** 2 mg/mL of USP Erythropoietin RS in *Mobile phase*. Heat at 80° for 30 min, and use immediately.

**Sample solution:** 2 mg/mL of Epoetin

**Chromatographic system**

(See *Chromatography* ⟨621⟩*, System Suitability*.)

**Mode:** LC

**Detector:** UV 230 nm

**Column:** 7.8-mm × 30-cm; packing L20

**Temperatures**

**Autosampler:** 2°–8°

**Column:** 25°

**Flow rate:** 1.0 mL/min

**Injection volume:** 40 µL

**Run time:** 30 min

**System suitability**

**Sample:** *System suitability solution*

[NOTE—The relative retention time for the aggregate is approximately 0.7.]

**Suitability requirements:** The monomer retention time must be within 8–10 min for *System suitability solution* injections. The relative retention times for the dimer and monomer are about 0.9 and 1.0, respectively.

**Relative standard deviation:** The precision of the monomer peak area must be <2%.

**Analysis**

**Sample:** *Sample solution*

[NOTE—Condition the *Chromatographic system* with the *Standard solution*. Inject the *Standard solution* in duplicate before, and in a single bracket after, all *Sample solutions* injected.]

Record the chromatogram, and measure the areas of the main peak and of the peaks eluting near the main peak, excluding the solvent peaks.

**Acceptance criteria:** The total impurities (aggregates and dimer) should be NMT 0.1%.

## SPECIFIC TESTS

• ***N*-Glycan Profiling**

**Solution A:** Water. Degas before use.

**Solution B:** Dissolve 41.0 g of anhydrous sodium acetate in 900 mL of water. Transfer the solution to a 1-L volumetric flask, and dilute with water to volume. Pass the solution through a nylon membrane filter of NMT 0.45-µm pore size, and degas before use.

**Solution C:** To 900 mL of water add 26 mL of 50% (w/w) sodium hydroxide solution. Dilute the solution with water to a final volume of 1000 mL. Pass the solution through an alkaline-resistant nylon membrane filter of NMT 0.45-µm pore size, and degas before use.

**Mobile phase:** See *Table 2*.

**Table 2**

| Time (min) | Solution A (%) | Solution B (%) | Solution C (%) |
|---|---|---|---|
| 0 | 80 | 10 | 10 |
| 15 | 80 | 10 | 10 |
| 70 | 60 | 30 | 10 |
| 94 | 0 | 90 | 10 |
| 99 | 0 | 90 | 10 |
| 105 | 0 | 10 | 90 |
| 110 | 0 | 10 | 90 |
| 111 | 80 | 10 | 10 |
| 130 | 80 | 10 | 10 |

**2-AB labeling solution:** Add 150 µL of glacial acetic acid to 350 µL of dimethyl sulfoxide (DMSO) and mix. Add 110 µL of the acetic acid–DMSO mixture to a tube containing 5 mg of 2-aminobenzamide acid (2-AB) labeling dye and mix. Transfer the complete acetic-acid/DMSO/2-AB mixture to a tube containing 6 mg of sodium cyanoborohydride and mix. Use immediately and protect from light.

**Buffer A:** 20 mM sodium citrate, 100 mM sodium chloride, pH 6.9. Dissolve 5.80 g of sodium citrate dihydrate, 0.057 g of citric acid, and 5.84 g of sodium chloride in 800 mL of water and mix. Adjust with hydrochloric acid to a pH of 6.9 and dilute with water to 1 L. Pass through a nylon filter of 0.2-µm pore size.

**0.5 M DTT:** Add 77 mg of dithiothreitol to 1 mL of water and mix. Use immediately.

**Enzyme reaction buffer:** 0.5 M sodium phosphate, pH 7.5

**Standard solution:** Reconstitute 100 µg of USP Erythropoietin RS in 50 µL of water. If necessary, buffer exchange by a suitable method into *Buffer A*. Transfer the entire contents to a 1.5-mL polypropylene tube (or equivalent). Add 12.0 µL of *Enzyme reaction buffer*, 2.5 µL of *0.5 M DTT*, and 38.5 IUB milliunits of peptide-*N*-glycosidase F (PNGase F). [NOTE—1 unit of PNGase F is defined as the amount of enzyme required to catalyze the release of *N*-linked oligosaccharides from 1.0 nmol of denatured ribonuclease B per minute at a pH of 7.5 at 37°, and is equal to 1 IUB milliunit.] Dilute with water to a final volume of 100 µL, mix, and briefly centrifuge. Incubate at 37° for 30 min. Briefly centrifuge the sample, then freeze on dry ice for at least 5 min. After the sample is frozen, uncap the tube and place it in the centrifugal evaporator and operate under vacuum without heat for approximately 70–80 min or until completely dry.

Add 5.0 µL of *2-AB labeling solution*, vortex, briefly centrifuge, and incubate at 60° for 3 h. Following incubation, add 120 µL of water, vortex, and centrifuge the sample.

Remove excess 2-AB using a microcentrifuge column filled with an appropriate amount of G-10 gel filtration stationary phase for desalting a 75–150-µL sample. To prepare the resin, add 0.5 mL of water to the microcentrifuge column and swell for 15 min. Centrifuge at maximum speed for 5–10 s. Repeat wash and remove residual water. Apply labeled glycans, place the column in the microcentrifuge and spin at 200 × g for 1 min. Apply the flow through to a second column and repeat centrifugation. Transfer the repeat flow through to an HPLC vial for analysis.

**Sample solution:** Prepare as directed in the *Standard solution* using 100 µg of Epoetin.

**Blank:** Water
**Chromatographic system**
(See *Chromatography* ⟨621⟩, *System Suitability*.)
**Mode:** LC
**Detector:** Fluorescence (330- and 420-nm emission wavelength)
**Columns**
**Guard:** 4.0-mm × 5-cm; 10-µm packing L46
**Analytical:** 4.0-mm × 25-cm; 10-µm packing L46
**Temperatures**
**Column:** 25°
**Autosampler:** 2°–8°
**Flow rate:** 0.5 mL/min
**Injection volume:** 25 µL
**System suitability**
**Samples:** *Standard solution* and *Blank*
**Suitability requirements:** The percentage interference of the *Blank* injection must be <1% of the total peak area of the initial *Standard solution*. The *Standard solution* chromatogram must be consistent with the reference chromatogram provided with the lot of USP Erythropoietin RS being used, including the presence of peaks 1–7. [NOTE—Peak 5 may exhibit splitting or shouldering but must be consistent for all *Standard solution* injections within a run.]
**Relative standard deviation:** Precision of total integrated peak area must be NMT 1.5%, *Standard solution*.
**Analysis**
**Sample:** *Sample solution*
Calculate the peak area response of each of the three peak groups, bi-sialylated (2 N), tri-sialylated (3 N), and tetra-sialylated (4 N) [NOTE—Refer to the reference chromatogram provided with the lot of USP Erythropoietin RS being used] :

$$\text{Result} = (r_U/r_T) \times 100$$

$r_U$ = total peak response for each of the three peak groups, bi-sialylated (2 N), tri-sialylated (3 N), and tetra-sialylated (4 N)
$r_T$ = sum of the total peak responses for the three peak groups, bi-sialylated (2 N), tri-sialylated (3 N), and tetra-sialylated (4 N)

**Acceptance criteria:** The *N*-glycan percentages must be within the following ranges.
**2 N:** 4.5%–6.0%
**3 N:** 18%–25%
**4 N:** 69%–77%

• **ISOFORM DISTRIBUTION**
**Cathode solution:** Measure 0.31 g of L-histidine and dilute with water to a final volume of 10 mL.
**Anode solution:** 0.2 N sulfuric acid
**Initiator:** Measure 0.072 g of potassium persulfate and dilute with water to a final volume of 10 mL.
**Gel:** Prepare a single horizontal gel using plate dimensions of 265-mm × 128-mm × 3-mm with a gel of 0.5-mm thickness prepared as follows. Combine 9.0 g of urea, 5.9 mL of acrylamide (30%), 2.4 mL of bis-acrylamide (2%), 450 µL of 3-10 ampholyte, and 1.1 mL of 3-5 ampholyte. Dilute with water to 28 mL, mix thoroughly, and pass the solution through a filter of 0.45-µm pore size. Add 2 mL of *Initiator* and mix by inversion. Transfer the solution to the gel cassette assembly.
**Fixing solution:** Prepare a solution containing 200 mL of 60% (w/v) of a mixture containing trichloroacetic acid and 17.5% 5-sulfosalicylic acid, 100 mL of glacial acetic acid, 400 mL of absolute methanol, and 300 mL of water.
**Gel wash solution:** Prepare a solution containing 400 mL of absolute methanol and 100 mL of glacial acetic acid. Dilute with water to 1 L.
**Stain solution:** Dissolve 1.25 g of Coomassie Brilliant Blue R-250 in 1 L of *Gel wash solution*. Pass through a filter of 0.45-µm pore size.
**Destain solution:** Prepare a solution containing 75 mL of absolute methanol and 100 mL of glacial acetic acid. Dilute with water to 1 L.
**Standard stock solution:** 2 µg/µL of USP Erythropoietin RS in water
**Standard solution A:** 0.13 µg/µL of USP Erythropoietin RS, prepared by diluting *Standard stock solution* in water
**Standard solution B:** 1.3 µg/µL of USP Erythropoietin RS, prepared by diluting *Standard stock solution* in water
**Sample solution:** 20 µg of Epoetin in 15 µL of water
**Electrophoretic system**
**Mode:** Horizontal electrophoretic system with integrated cooling apparatus capable of maintaining 2°–8°
**Temperature:** Cool to 2°–8° before applying voltage and maintain the temperature during electrophoresis.
**Prefocusing:** Prefocus the gel for 20–40 min at a constant 10 watts (maximum of 3000 V and 50 mA).
**Loading profile:** Load 15 µL of *Standard solution A*, *Standard solution B*, and *Sample solution* onto separate lanes of the gel at the cathode site.
**Focusing:** Focus the gel for 2.5 h at a constant 10 watts (maximum of 3000 V and 50 mA).
**Staining:** Incubate the gel in *Fixing solution* two times for 15 min each. Decant and incubate the gel in *Gel wash solution* for at least 30 min. Decant and incubate the gel in *Stain solution* for 15–60 min. Decant and rinse the gel with *Destain*. Decant and incubate the gel in *Destain* until the background is clear and *Standard solution A* is still visible.
**System suitability**
**Samples:** *Standard solution A* and *Standard solution B*
There is no artifact of staining that obscures visualization of protein lanes and the location and intensity of bands are appropriate in *Standard solution A* and *Standard solution B*. Isoforms 10, 11, 12, and 13 are present in *Standard solution A* and *Standard solution B*. No bands are present below isoform 9 in *Standard solution B*. Refer to the typical image provided with the lot of USP Erythropoietin RS being used.
**Analysis**
**Sample:** *Sample solution*
Scan the gel with a densitometer.
Using data from the densitometric scan, calculate the relative percentage of each individual isoform in the portion of the *Sample solution* taken:

$$\text{Result} = (I/T) \times 100$$

$I$ = individual band intensity
$T$ = total band intensity in the *Sample solution*

**Acceptance criteria:** Isoforms 10–13 must be present at the following percentages.
**Isoform 13:** NLT 13%
**Isoforms 12 + 13:** 46%–72%
**Isoforms 10 + 11:** NMT 52%
**Isoforms 10–13:** NLT 91%
Isoforms 9 and 14 may be present in the *Sample solution*. No isoform below isoform 9 is observed and any minor band present in the *Sample solution* must also be present at the same location in *Standard solution B*. Refer to the typical image provided with the lot of USP Erythropoietin RS being used.

• **PROTEIN CONTENT**
**Sample solution:** Epoetin, undiluted
**Blank:** Use an appropriate buffer solution consistent with the Epoetin under test.
**Instrumental conditions**
**Mode:** UV
**Analytical wavelength:** 280 nm
**Cell:** Quartz cuvette
**Analysis**
**Samples:** *Sample solution* and *Blank*
Calculate the protein concentration, in mg/mL, of the sample taken:

$$\text{Result} = (A/e) \times l$$

$A$ = absorbance at 280 nm, corrected with the *Blank*
$e$ = extinction coefficient, 0.74 mL/mg · $cm^{-1}$
$l$ = path length (cm)

• **STERILITY TESTS ⟨71⟩:** Meets the requirements
• **BACTERIAL ENDOTOXINS TEST ⟨85⟩:** It contains NMT 2.5 USP Endotoxin Units/mL.

## ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in fluorinated ethylene propylene (FEP) or equivalent containers between 2° and 8°. Protect from light.
• **LABELING:** Label it to indicate that the material is of recombinant DNA origin.
• **USP REFERENCE STANDARDS ⟨11⟩**
USP Erythropoietin RS▲ USP41

▲(Postponed on 1-May-2018)▲ (RB 1-May-2018)