⟨124⟩ ERYTHROPOIETIN BIOASSAYS

INTRODUCTION

This chapter provides guidance about the use of bioassays to measure the biological activity of erythropoietin (EPO). Based on a relative potency approach described in general chapter *Design and Development of Biological Assays* ⟨1032⟩, the biological activity of an unknown EPO preparation typically is determined by comparison to the biological activity of a Reference Standard (RS). For this purpose, a USP Erythropoietin for Bioassays RS has been developed. The assays described in the chapter are not applicable to forms of EPO that have been engineered to prolong their half-life.

The USP Erythropoietin for Bioassays RS has been calibrated against the World Health Organization (WHO) International Standard for EPO using both the normocythemic mouse bioassay and the exhypoxic polycythemic mouse bioassay. Accordingly, the USP Erythropoietin for Bioassays RS has an assigned unitage in terms of International Units (IU) of EPO as defined by WHO. Of these two in vivo bioassays, the normocythemic mouse bioassay is less cumbersome and is more widely used for the determination of EPO potency. The assay is based on the measurement of EPO-stimulated reticulocyte maturation. Because the USP Erythropoietin for Bioassays RS has been calibrated against the WHO International Standard for EPO by in vivo bioassay, it can be used directly in the normocythemic mouse bioassay for the calibration of any process-specific EPO preparation.

However, if the intent is to use a validated in vitro bioassay to transfer the unitage from the USP Erythropoietin for Bioassays RS to a process-specific EPO preparation, then it is necessary to demonstrate that the USP Erythropoietin for Bioassays RS and the process-specific EPO preparation exhibit an equivalent ratio of in vitro to in vivo potency. This is because the biological activity of EPO shows a complex relationship between structure and function. In particular, the ratio of in vitro to in vivo activity is inversely correlated with type and degree of terminal sialylation. Highly sialylated products have a higher level of in vivo activity; thus, they have a relatively lower ratio of in vitro to in vivo activity. Because of differences in the manufacturing processes used in the preparation of EPO, the type and degree of terminal sialylation can be variable from one EPO preparation to another, including EPO preparations used as Reference Standards. As a result, the use of an in vitro assay to measure the biological activity of an EPO preparation requires a full understanding of the relationship between the EPO's in vivo and in vitro activity. This understanding is best achieved by comparing the test EPO preparation with the USP Erythropoietin for Bioassays RS in both the in vivo assay and in a specific in vitro assay.

The USP Erythropoietin for Bioassays RS is assigned a unitage that represents its activity in both in vivo assays and in vitro assays. If the ratios of in vitro to in vivo potency for the material being tested and the USP Erythropoietin for Bioassays RS are equivalent, then the USP Erythropoietin for Bioassays RS can be used directly in the in vitro assay to calibrate the material being tested. However, if the ratios are not equivalent, then the material being tested and the USP Erythropoietin for Bioassays RS have a different ratio of in vitro to in vivo potency, and the standard cannot be used with its assigned potency in the in vitro assay. Instead, this ratio determined for the material being tested should be used to assign a process-specific in vitro assay unitage to the USP Erythropoietin for Bioassays RS. The USP Erythropoietin for Bioassays RS, with its adjusted in vitro assay unitage, then can be used in the in vitro assay to transfer the unitage from the USP Erythropoietin for Bioassays RS to the material being tested.

ASSAY

• **Normocythemic Mice Assay**

Solution A: Dissolve 10.75 g of disodium hydrogen phosphate dodecahydrate, 7.6 g of sodium chloride, and 10 g of bovine albumin in 1000 mL of water. Immediately before use adjust with sodium hydroxide solution or phosphoric acid to a pH of 7.2.

Standard solution 1: Dissolve USP Erythropoietin for Bioassays RS in *Solution A* to a concentration of 80 IU/mL.

Standard solution 2: Mix equal volumes of *Solution A* and *Standard solution 1*.

Standard solution 3: Mix equal volumes of *Solution A* and *Standard solution 2*.

Sample solution 1: Prepare the sample to be tested in *Solution A* to a concentration of 80 IU/mL.

Sample solution 2: Mix equal volumes of *Solution A* and *Sample solution 1*.

Sample solution 3: Mix equal volumes of *Solution A* and *Sample solution 2*.

The exact concentrations of each *Standard solution* and *Sample solution* may require adjustment based on the response range in the animals used.

Preparation of animals: At the beginning of the assay procedure, randomly distribute mice of a suitable age and strain (8-week-old B6D2F1 mice are suitable) into six cages. A minimum of eight 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 six different treatments (each *Standard solution* and each *Sample solution*; six mice per cage).

Analysis: Four days after the injections, collect blood samples from the animals, and determine the number of reticulocytes by using a suitable procedure. The following method can be employed.

The volume of blood, dilution procedure, and fluorescent reagent may require modification to ensure maximum development and stability of fluorescence.

For concentrated colorant solution, 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 two-fold in the concentrated colorant solution. After staining for 3–10 min, 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. For additional information, see general chapter *Analysis of Biological Assays* ⟨1034⟩.

Acceptance criteria: The activity of each *Sample solution* is compared with that of the USP Erythropoietin for Bioassays RS and is expressed in IU. The confidence limits of the estimated potency ($P = 0.95$) are NLT 64% and NMT 156% of potency as determined by the method given above. The estimated potency is NLT 80% and NMT 125% of the stated potency.

ADDITIONAL REQUIREMENTS

- **USP REFERENCE STANDARDS** ⟨11⟩

  USP Erythropoietin for Bioassays RS