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A novel reporter gene assay for Recombinant Human Erythropoietin (rHuEPO) pharmaceutical products

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ABSTRACT

Accurate determination of in vitro biological activity of therapeutic erythropoietin is essential in quality control of recombinant human erythropoietin (rHuEPO) pharmaceutical products. However, most of currently-used methods leave much to be desired so that a simpler, quicker and more accurate method is urgently needed. The bioassay described here utilizes a sub clone of UT-7/epo cell line stably transfected with luciferase gene under the control of sis inducible element and interferon γ-activated sequence element promoter. Active erythropoietin could induce the expression of luciferase by signaling through the erythropoietin receptor and the dose-response curve showed good linearity, yielding a coefficient of determination of 0.99 or higher. The optimized assay was simpler with the operation completed within 24 h and more sensitive with EC_{50} being 0.077 IU/mL. The accuracy estimates ranged from 81.7% to 102.4%, and both intra-assay and inter-assay precision was below 15.0%. The robustness of the assay was demonstrated by no effect of passage levels of the cells on the performance of the assay (*p* values: 0.772 for sample 1 and 0.943 for sample 2). Besides, Bland–Altman analysis showed a high consistency of the new assay with in vivo reticulocyte assay in results. These results suggested that the new reporter gene assay can be a viable supplement to the traditional reticulocyte assay and employed in potency determination of rHuEPO pharmaceutical products.

1. Introduction

Erythropoietin (EPO) is mainly secreted by the kidney and involved in the growth and maturation of erythroid cells from precursors. Decreased bioactive production of EPO could result in anemia [1–3]. Since recombinant human erythropoietin (rHuEPO) was successfully developed in the 1980s, it has been widely used in the treatment of anemia caused by chronic kidney disease, blood loss anemia, and myelodysplasia induced by chemoradiotherapy of cancer as the first hematopoietic growth factor applied clinically [4–7].

Accurate determination of in vivo and in vitro biological activities of therapeutic EPO is crucial to quality control of rHuEPO pharmaceutical products [8,9]. In vivo bioactivity of EPO is closely related to its serum half-life, which is dependent on carbohydrate structure, especially on the number of sialic acid residues at the termini of the tri- and tetra-antennary sugar chains [10,11]. Traditional ^{59}Fe incorporation assay is considered the gold standard assay for EPO, but the procedure has such drawbacks as the use of a radioisotope and elaborate animal preparation [8]. Recently, reticulocyte assay based on the proliferation of reticulocytes in normocythemic mice has been widely applied in standardization studies and potency evaluation of rHuEPO pharmaceutical preparations [12,13]. In vitro bioassay could estimate EPO's receptor-activating ability that is associated with the protein structure of EPO molecules [14]. The various in vitro bioassays that have been developed so far, including immunoassays, affinity assays and cell proliferation assays, have some limitations: immunoassays and affinity assays can confirm nothing more than antigenic sites and binding activities respectively while cell proliferation assays such as murine 32D cell line, NFS60, TF1, UT-7 and UT-7/EPO usually require extended experimental cycles [15–19]. Considering the huge market demand for rHuEPO products, a more accurate and convenient in vitro assay needs to be explored to facilitate the rapid batch release of rHuEPO formulations.

The reporter gene assay (RGA) has been increasingly used to determine the bioactivities of cytokines based on their

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individual signal pathway for its simplicity, reliability and high efficiency [20–23]. EPO signal pathway has been extensively explored over the past decades. It has been confirmed that EPO works by binding to its receptor EpoR which will undergo a conformational change that activates Janus kinase 2 (JAK2). The activated JAK2 will in turn activate tyrosine phosphorylation of signal transducer and activator of transcription 5 (STAT5) that will bind to the sis inducible element (SIE) and the interferon γ-activated sequence (GAS) element, activate the expression of downstream genes and finally promote erythropoiesis [24–26]. But till today, there has been no report on the potency determination of EPO based on its signal pathway using RGA.

In this report, a novel RGA for EPO was developed based on a UT-7/epo cell line stably transfected with luciferase gene under the control of SIE and GAS promoter. The new RGA was optimized and fully validated in accordance with the Guidelines of the International Conference on Harmonization (ICH) using good laboratory practices (GLPs), followed by comparison with in vivo reticulocyte assay in terms of results.

2. Materials and methods

2.1. Reagents

IMDM (12440), RPMI 1640 (11835) and fetal bovine serum (10099) were purchased from Gibco, Bright Glo luciferase assay reagent (E2620) from Promega, and hygromycin B (K547) from Amersco.

2.2. RHuEPO and other cytokines

National Standard for rHuEPO was obtained from National Standardization Study Center of Pharmaceutical and Biological Products. RHuEPO products and other cytokines including recombinant human growth hormone (rhGH), recombinant human epidermal growth factor(rhEGF), recombinant human basic fibroblast growth factor(rhBFGF), interferon α-2a(IFNα-2a), interleukin-11(IL-11), interleukin-2(IL-2), recombinant human granulocyte colony-stimulating factor(rhG-CSF) and recombinant human granulocyte-macrophage colony-stimulating factor(rhGM-CSF) were provided by different manufacturers. All rHuEPO products used in this study were produced in Chinese hamster ovary cells.

2.3. Construction of the reporter gene vector pGL4-SG-Luc

PGL4.26 vector (Promega) contains a minimal promoter followed by a luciferase gene. Three tandem repeats of consensus SIE (5′-GTCGACATTTCCCGTAAATC-3′) and GAS (5′-GTATTTCCCAGAAAAGGAAC-3′) were sequentially connected and inserted into the multiple cloning site of pGL4.26 vector and the positive clone was verified by DNA sequencing.

2.4. Development of UT-7-SG-Luc cells stably transfected with pGL4-SG-Luc

The plasmid pGL4-SG-Luc was introduced into UT-7/epo cells (ATCC) by electroporation. Cells were selected beginning at 48 h after transfection in selective media (IMDM containing 10% fetal bovine serum, 1 IU/mL EPO and 500 μg/mL hygromycin B). After about 4 weeks' selection, hygromycin-resistant cells were then cloned by limited dilutions to obtain single cell clones and screened for the induction of luciferase by treating cells with gradient concentrations of EPO. After that the positive clones were routinely maintained in selective media.

2.5. RGA procedure

UT-7-SG-Luc cells were washed three times by phosphate buffer solution and incubated at 37 °C with 5% CO_2 in assay medium (RPMI 1640 containing 5% fetal bovine serum and 10 mM HEPES) for 17–20 h. Cells were then collected and seeded into 96 well Costar plates, with 6×10^4 cells in the presence of rHuEPO standards or test samples in a total volume of 100 μL per well, followed by incubation at 37 °C with 5% CO_2 for 4 h. Then 100 μL of Promega Bright Glo Luciferase Assay reagent was added, and the plate was subsequently shaken for 5 min on a titre-plate shaker. Luciferase activity was finally determined by a Luminoscan Ascent plate reader.

2.6. Reticulocyte assay for EPO potency determination

Eight-week-old BALB/c mice were allocated to standard and sample groups in fully randomized order, with 5 mice per treatment group. The standard and test samples were diluted to appropriate concentrations with saline containing 0.1% bovine serum albumin. A single dose of 7.5, 15 or 30 IU EPO/0.2 mL per mouse was injected subcutaneously into the respective animal on day 1. On day 4, blood was taken from the orbital venous sinus of each mouse and reticulocytes were counted by the Sysmex R-500 Hematology Analyzer. The study was approved by the Ethic Committee of National Institute for Food and Drug Control.

2.7. Specificity

The specificity of the RGA was evaluated by determining whether the luciferase output could be affected by the presence of aggregated rHuEPO and other therapeutic cytokines including rhGH, rhEGF, rhBFGF, IFNα-2a, IL-11, IL-2, rhG-CSF and rhGM-CSF. The aggregated rHuEPOs were prepared by incubating rHuEPO samples at 90 °C for 1 h. All samples were diluted to gradient concentrations of 10, 2.5, 0.63, 0.16, 0.039, 0.0098, 0.0024 and 0.00061 μg/mL in assay medium, and then used to treat the cells under the same condition as described above for the analyses of rHuEPO. The levels of luciferase activity were compared to those obtained with intact rHuEPO treatment.

2.8. Bland–Altman analysis

The agreement between the results of RGA and reticulocyte assay was evaluated by a Bland-Altman plot. Thirty-four batches of qualified rHuEPO injections or powder injections that ranged from 2500 to 45,000 IU per vial were obtained from different manufacturers. Both assays were performed on each sample, and the relative potency was calculated respectively, resulting in 68 data points. Each of the 34 samples was then represented on the graph by assigning the log-transformed average of two measurements as the abscissa (x-axis) value, and the ratio of RGA vs. reticulocyte assay as the ordinate (y-axis) value.

2.9. Statistical analyses

Data analyses of reticulocyte assays were carried out according to the protocol of Chinese Pharmacopeias by parallel line methods. A four-parameter logistic model (Section 5.3 of European Pharmacopoeia) was used to calculate the relative potency, dose response and linear range for RGA. Statistical techniques for method validation involved the coefficient of variation (CV), recovery rate, ANOVA and Bland–Altman plot. Analyses were carried out using Sigmaplot 12.0 for dose response and linear range determination, SoftMax Pro software (Molecular Devices, USA) for relative potency estimation and SPSS 19.0 for method validation.