Development of In-Vitro Bio-Assay for The Bioactivity Determination of Human Erythropoietin (rHuEPO) Bio-Pharmaceutical Products. (November 2020 - August 2021)

By

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A thesis submitted to the Department of Mathematics and Natural Science in partial fulfillment of the requirements for the degree of
Master of Science in Biotechnology

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Declaration

It is hereby declared that

1. The thesis submitted is my/our own original work while completing degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. I/We have acknowledged all main sources of help.

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Approval

The thesis/project titled “Development of In-Vitro Bio-Assay for The Bioactivity Determination of Human Erythropoietin (rHuEPO) Bio-Pharmaceutical Products. (November 2020 - August 2021)” submitted by Ayeasha Siddika Lamia (ID:20376007)
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Abstract:

Erythropoietin is mainly secreted by the kidney and involved in the growth and maturation of erythroid cells from precursors (Jelkmann, et al, 2013). Insufficient production of EPO could result in anemia. Considering this, recombinant human erythropoietin (rHuEPO) was profoundly generated in 1980s. Therefore, it has been clinically approved 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 (Moore, et al. 2011). Thus, the quality control process and accurate determination of bioactivity is pivotal for the safety and efficacy of rHuEPO. Since, in-vivo bioassay, based on rHuEPO-induced increases in swiss albino mice reticulocyte count is the only method accepted by pharmacopoeias which is complex, expensive and time consuming.

This proliferating bioassay will utilize a sub clone of TF-1 cell line. TF-1 cell line proliferates in the presence of GMCSF and IL-3. Active erythropoietin will induce the dose-response curve might show good linearity, yielding a coefficient of determination of 0.99 or higher.
This new in-vitro bio-assay is simpler, faster and viable substitution of in-vivo reticulocyte assay and employed in potency determination of rHuEPO bio-pharmaceutical products.

INTRODUCTION AND REVIEW LITERATURE

Introduction:

Erythropoietin (EPO) is a glycoprotein involved in maintaining sufficient red blood cells (RBC) production [1]. In adult humans, EPO is produced mainly by the renal cortex, which contributes to ~ 90% of the blood level of this hormone. Other organs producing EPO include liver, spleen, lungs, testis, brain, and erythroid progenitor cells. In a healthy human, the blood concentration of EPO is 10 pM (< 5–25 IU/l) [2]. It is similar to the baseline value reported for rats (5.4 IU/l) [3]. Erythropoietin is a main regulator of erythropoiesis, a process in which new erythrocytes origins from pluripotent stem cells in the bone marrow. In humans and other mammals, erythropoiesis is a slow process in which old RBCs are replaced with young reticulocytes. In some pathologic conditions, like hemorrhage or severe hemolysis, the rate of reticulocyte production may increase even eight times and an increase in EPO concentration might be 1000-fold. [2, 4] Erythropoietin binds to the specific erythroid progenitor cell surface receptor (EPO-R) to regulate bone marrow erythroid cell proliferation, differentiation, and survival. Binding to EPO-R expressed on the erythroid progenitor cells in the bone marrow has been reported as an important pathway of EPO elimination [5, 6]

The fundamental purpose of EPO is to support the segregation of erythroid in hematopoietic cell lineages and support the proliferation of erythroid cells [2]. In the bone marrow resident, beside extra cytokines, EPO works on the progenitors of erythroid in order to support the survival of erythroid progenitor, and endorse the proliferation and segregation of progenitors into mature erythrocytes (Fig. 1). The main regulator of human erythropoiesis is EPO. The sialoglycoprotein is made of a single polypeptide chain which consists of 165 amino acids, including Cys7-161 and Cys29-33; it contains disulfide bonds and forms four possible sites of glycosylation: O-bonded Ser126, N-bonded Asn24, Asn38, and Asn83 [3, 4]. The EPO molecular mass is 30–34 kDa and the glycosylated EPO molecule consists of 40 percent carbohydrate with glycans of intricate N-bonded-type [5]. Glycans take part in the evaluation of bioactivity of EPO, which is likely to be dependent on the sialic acid residue count [6–8]. It is believed that the carbohydrate component and the degree of sialyation of glycoproteins play an important role in cell signaling and are responsible for transmitting different physiologic signals to the target cells upon binding of the glycoprotein to its cell-surface receptor. Sialylation imparts a negative charge on the glycoprotein molecule and the different degrees of sialylation gives rise to many different isoforms of glycoproteins. All these isoforms have different half-lives and consequently different pharmacodynamic behavior. For clinical purposes, many pharmaceutical organizations are producing rHuEPO. It is a fact that the final bioactivity of rHuEPO is influenced by the glycosylation profile of the preparations due to a variety of host cell lines used, the environment of fermentation culture, and the applied purification processes of rHuEPO. Hence, to regulate the assurance of therapeutic efficacy and to increase the quality, a properly described method should be used by different manufacturers to guarantee set to set constancy [7, 8]. The present authors have developed a biological assay for rHuEPO. In this research study, we demonstrated the variation in bioactivity between intact sialoEPO and asialoEPO by neuraminidase enzyme digestion. In fact, the concoction of carbohydrates in N-linked chains of rHuEPO are composite,

hence the differences of the extensions and the degree of branching of N-acetyllactosamine, O-acetylation of the N-acetylneuraminic acid moieties, terminal Neu5Ac content [10, 11]. Hence, the final rHuEPO characteristics, including micro heterogeneity generation in the glycosylation, can be affected by changes in cell lines, condition of growth, and processes of manufacturing [12]. In the present work, the intact EPO digested with the neuraminidase enzyme at different time intervals of 10th min, 30th min, 2nd hr, 4th hr, 10th hr and 24th hr at 37°C. After the enzymatic treatment, the molecular weight difference between intact EPO and asialoEPO was identified by using SDS PAGE analysis. Upon confirmation of proper sialidase treatment by SDS–PAGE results, we analyzed the above treated samples for the determination of proliferative stimulation induced by rHuEPO. The TF-1 cell line has a large number (7,200 to 13,000/cell) of EPO binding sites [13, 14] and is one of the best available human cell lines to study the expression and function of EPO-R. The concentrations of active drugs were quantified using bio-sample collected from the studies of pharmacokinetics (PK) or toxicokinetics (TK), using in vitro cell-based bioassay performed with biological proteins. While in vivo generated metabolites, active and inactive drugs were measured by immunoassays [15, 16], biologically active drug levels were estimated by cell based bioassays [17–19]. A cell line with measurable response (OD, counts, etc.) was used by a typical bioassay for quantitative purpose [17–20]. The potency calculation of manufactured drugs and measurement of the active drug concentrations of PK or TK experiments were included in the in vitro bioassays productive applications. Measurement of modifications in cell proliferation should be done by titrated thymidine [15,19] or using a selected type of dyes with non-radioactivity including MTS or methylthiotetrazole (Promega CellTiter96®, Madison, WI) [14], Alamar Blue (BioSource International, Inc., USA 542 Flynn Road, Camarillo, CA 93012) [18,19], or measuring the ATP live cells using the reagents, which are Cell titer-Glo or ViaLight (Cambrex Corp., East Rutherford, NJ) [17-20].

Potency readings obtained by cellular stimulation with rHuEPO were balanced against analysis of the separated glycoprotein isoforms. In a light of such assays, using sugar-trimmed hormone, evidence was furnished that the bioassay can discriminate between functionally satisfactory and suboptimal rHuEPO preparations as regards structural features pivotal for the pharmacokinetic properties. The bioassay used erythropoietin induced proliferation of the TF-1 cell line, quantified by fluorescence units readout. The method was subsequently used to determine the activity of rHuEPO sample solutions.

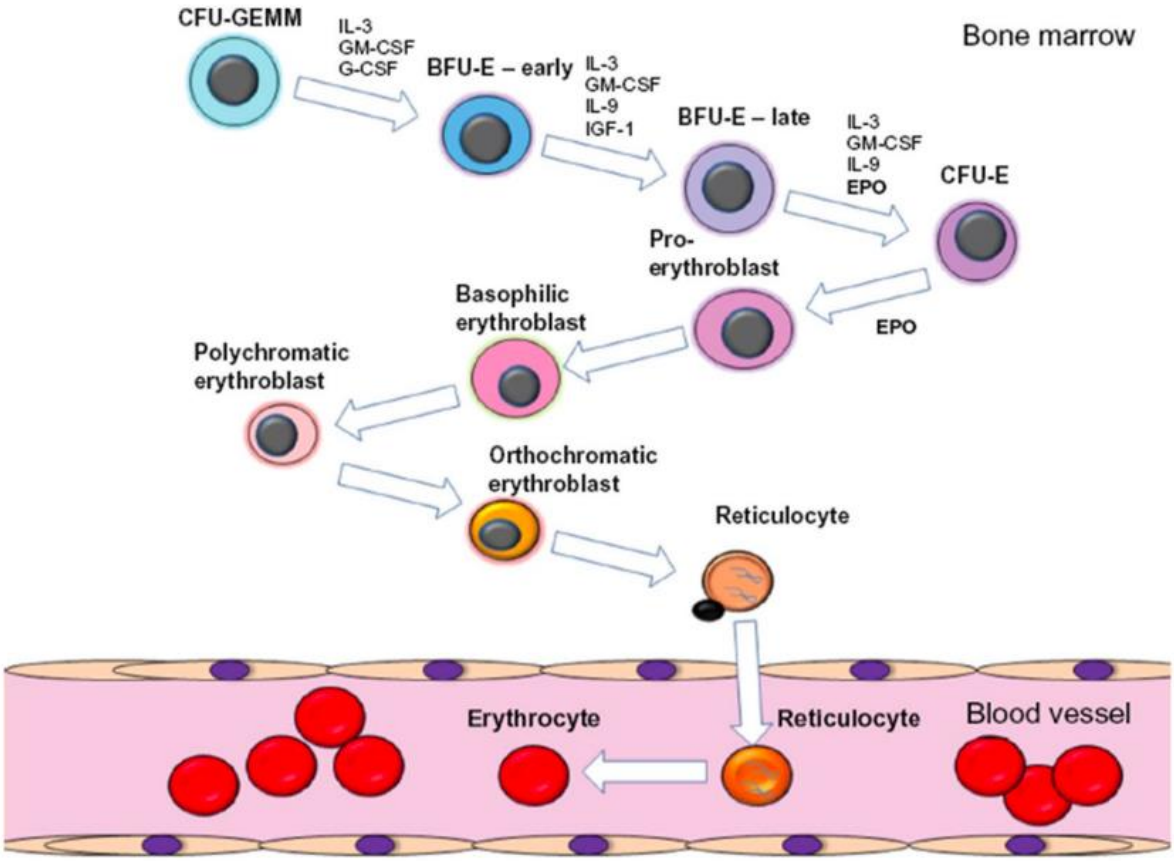

Fig. 1 Diagrammatic representation of the method of erythropoiesis. Different phases of erythroid segregation, proliferation, survival, and differentiation of erythroid progenitors were shown

rhEpo is produced with the use of cells transfected with human EPO complementary DNA (cDNA) (the coding sequence of the gene) linked to an expression vector (‘recombinant DNA’). The therapeutic rhEpo drug substances must be manufactured in mammalian host cells because Epo is a complex glycoprotein [14, 15]. Transformed bacteria such as Escherichia coli are primarily useful for the production of non-glycosylated recombinant proteins like insulin or somatotropin. In mammalian cells, the synthesis of N-glycans starts in the cytosol with the anchoring of sugar molecules to dolichol, which is then transferred to the growing polypeptide in the endoplasmic reticulum. There, several sugars are removed, and the protein is folded and moved to the Golgi complex, where further mannose elimination occurs followed by the addition of N-acetylglucosamine, galactose and sialic acid.

Chinese hamster ovary (CHO) cells deficient in the dihydrofolate reductase gene are most commonly used for the large-scale manufacture of rhEpo or other therapeutic glycoproteins. This is because gene amplification can be achieved in these cells by co-selection in the presence of methotrexate [8] . The structures of the sialylated N- and O-glycans of rhEpo expressed in

CHO cells have been studied by means of fast protein and high-performance liquid chromatography, high-pH anion-exchange chromatography and nuclear magnetic resonance spectroscopy [15]. There are di-, tri-, tri'- and tetra-antennary N-acetyllactosamine-type oligosaccharides, which can be completely or partially sialylated. Three different types of α2-3-linked sialic acids are present, namely N-acetylneuraminic (95%), N-glycolylneuraminic (2%) and N-acetyl-9-O-acetylneuraminic (3%) acid. CHO cell lines express the α2,3-sialyltransferase but not a functional α2,6-sialyltransferase. This explains why CHO cells add sialic acid exclusively in an α2-3-linkage, whereas the α2-6-linkage seen in human oligosaccharides is missing. On the other hand, CHO cells add N-glycolylneuraminic acid residues (Neu5Gc) to the glycans, which human cells cannot synthesize.

According to the World Health Organization, eukaryotic cell-derived rhEpo, whose peptide core is identical with that of the native human Epo, is given the international non-proprietary drug name (INN) 'epoetin'. Differences in the amino acid residues chain are indicated by a random prefix (e.g. 'darbepoetin'). Differences in the glycosylation pattern were supposed to be indicated by a specific Greek letter added to the name (e.g. 'epoetin alfa'), but this was not always applied (see 10.4.2). Two innovator CHO cell-derived epoetins have been used for over two decades in clinical routine, namely epoetin alfa and epoetin beta. Epoetin alfa is marketed primarily under the trade names Epogen® (Amgen), Procrit® (Johnson & Johnson) and—outside the USA—Eprex® or Erypo® (Ortho Biotech). Epoetin beta is marketed under the brand names NeoRecormon® (F. Hoffmann-LaRoche) and Epogin® (Chugai). Epoetin alfa and epoetin beta are alike in their four glycosylation sites and their secondary structure. However, they exhibit distinct differences in their glycosylation patterns. For example, there are less N-glycans with non-sialylated outer Galβ1–4GlcNAc moieties and O-glycans with Galβ1–3GalNAc in epoetin alfa compared to epoetin beta. Even so, clinically, the two epoetins are considered equivalent. In 2009, another original CHO cell-derived rhEpo (epoetin theta) has been launched in the European Union (EU), which is marketed as Biopoin® (CT Arzneimittel) and Eporatio® (Ratiopharm).

In some East European, Asian and Latin American countries, CKD patients have been treated with epoetin omega, which is expressed in EPO cDNA-transfected baby hamster kidney (from Syrian hamster) cells. Epoetin omega has been traded as Repotin® (Bioclones) and Epomax® (Lek; Cryopharma Pizzard y Salud). Although epoetin omega is probably non-inferior to epoetin alfa in correcting renal anaemia in CKD patients on dialysis, this medicine is not widely used. In contrast to CHO cell-derived rhEpo, epoetin omega has an N-glycan with phosphorylated oligomannoside chains and it possesses less O-glycans (8).

Another recombinant product earlier marketed in the EU was epoetin delta (Dynepo®, Shire Pharmaceutical). This unique rhEpo was homologously expressed on gene activation in a human fibrosarcoma cell line (HT-1080) into which a DNA fragment was transfected that activated the native EPO promoter (8). Contrasting the other epoetins, epoetin delta lacked Neu5Gc residues as it was human cell-derived. In 2009 Shire Pharmaceutical decided to withdraw the marketing authorization for commercial reasons (not affording profits).

The epoetins are purified by a number of chromatographic procedures to remove xenogenic proteins, oncogenes, pyrogens and microorganisms. The medicinal products are formulated as

dry powders or as isotonic sodium chloride/sodium citrate buffered solutions for intravenous (IV) or subcutaneous (SC) administration. They are stabilized by the addition of either polyoxyethylene sorbitan (Tween-20 or Tween-80) or human albumin (2.5 mg/ml) and benzyl alcohol (1%).

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], [2], [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], [5], [6]

Accurate determination of in vivo and in vitro biological activities of therapeutic EPO is crucial to quality control of rHuEPO pharmaceutical products [8], [9].

Aim of this project is to construct in-vitro based bioassay of recombinant human erythropoietin to replace in-vivo based bio-assay, for accurate potency and the quality control process determination, for measure the safety and efficacy.

Specific objectives:

1. To gather available data of rHuEPO for both in-vivo and in-vitro potency determination test.

2. To prepare the TF-1 cell line with granulocyte macrophage colony stimulating factor (GMCSF) and prepare them with rHuEPO instead of GMCSF for bio-assay.

3. Figure out the potency of in-vitro bio-assay of rHuEPO and compare them with in-vivo bio-assay of rHuEPO, at routine mannar to check whether the potency is higher or equal than in-vivo bioassay.

Expected outcomes:

- The quality control process and accurate determination of bioactivity of in-vitro based bioassay could be establishes for the safety and efficacy of rHuEPO.
- This new in-vitro reporter gene assay could be simpler, faster and viable substitution of in-vivo reticulocyte assay and employed in potency determination of rHuEPO bio-pharmaceutical products.
- The robustness of the assay will be demonstrated by no effect of passage levels of the cells on the performance of the assay.
- This project is to construct in-vitro based bioassay of recombinant human erythropoietin to replace in-vivo based bio-assay, for accurate potency and the quality control process determination, for measure the safety and efficacy.

MATERIALS AND METHODS

2 Materials and Methods

2.1 Materials

Iscove's Modified Dulbecco's Medium (IMDM), Fetal Bovine Serum (Non heat inactivated), L Glutamine (100X), Penicillin–streptomycin solution 10,000 units penicillin/ mL with 10,000 µg Streptomycin /mL in 0.85% Sodium Chloride (NaCl), Roswell Park Memorial Institute (RPMI) 1640, Recombinant human EPO (rHuEPO), Resazurin (7-Hydroxy-3H-phenoxazin-3-one 10-oxide), Trypan blue, Neuraminidase (from Clostridium perfringens (C. welchii) from Sigma

2.2 Cells and Culture Conditions

TF-1 is a human megakaryoblastic leukemia cell line with absolute dependence on interleukin-3, granulocyte-macrophage colony-stimulating factor (GM-CSF), or EPO for growth and survival. Using vented cap, canted neck and cell culture flasks, the cells were maintained at 37 °C, 5% CO2; furthermore, a 95% r.h. 2 mM I-glutamine, 15% inactivated FBS, and 1% (v/v) penicillin/streptomycin mixture was supplemented in growth medium containing RPMI 1640. The growth medium was supplemented with 10 U/ mL of rHuEPO, as the cells were dependent on rHuEPO for growth. To maintain cell densities between 5×104 and 1×106 cells/mL, the cells were sub-cultured two to three times a week. Using the Trypan Blue exclusion method, the viability of healthy growing cells were calculated as 98–100%. At passage number 9, the cells were banked and used for up to 40 days in assays after thawing.

2.3 Asialoerythropoietin Recombinant human erythropoietin was purified to homogeneity from a conditioned medium of CHO cells transfected with a cDNA clone for human erythropoietin [27, 33]. Various partially or fully desialylated EPO were prepared by neuraminidase digestion of the hormone with a specific activity of 110% in vivo. Six test tubes containing 1 mg EPO (1 mg/mL) were added to 1.5 mL 0.5 M sodium acetate, pH 5.5, and 50 µL of 0.1 M calcium acetate. The six reaction mixtures were added to neuraminidase Neuraminidase from Clostridium perfringens (C. welchii) and incubated at 37°C. At fixed time intervals (10 min, 30 min, 2 h, 4 h, 10 h, and 24 h), EPO samples were withdrawn for SDS PAGE analysis to confirm the desialylation.

2.4 SDS Polyacrylamide Gel Electrophoresis

To confirm the completion of each glycosidase reaction, the preparations containing digested EPO were analyzed by SDS PAGE with a separating gel containing 12% acrylamide. After electrophoresis, protein bands were detected by silver staining

2.5 Culture Medium 1 RPMI with 20% heat inactivated FBS and 5 ng/mL of GM-CSF.

2.6 Culture Medium 2 IMDM with 2 mM glutamine, 2 g/L of sodium bicarbonate, 4.5 g/L of glucose, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1 mM sodium pyruvate, 10% heat inactivated FBS, and 10 ng/mL EPO.

2.7 Assay Medium RPMI 1640 basal medium and 1% heat inactivated FBS.

2.8 Cell Suspension 2.0–2.5×105 TF-1 cells/mL in culture medium. Maintained the prepared cell suspension during the time of addition to assay plate.

2.9 Resazurin Sodium (Alamar Blue)
10 mg of Resazurin in 100 mL of sterile phosphate buffered saline. The above solution was sterilized, filtered, and stored at 4°C and was protected from light.

2.10 Cell Viability and Density Determination

Cell viability and density of culture samples were measured based on trypan blue staining.

2.11 Cell Adaptation

Revived cells in RPMI medium with 20% heat inactivated FBS and 5 ng/mL of GM-CSF, and passaged TF-1 cells in RPMI medium with 2 mM glutamine, 2 g/L of sodium bicarbonate, 4.5 g/L of glucose, 10 mM HEPES, 1 mM sodium pyruvate, 10% heat inactivated FBS, and 10ng/mL Pen-Strep solution.

2.12 Cell Proliferation Assay

TF-1 cells were incubated with varying dilutions of sample solutions and standard solutions of EPO. To a 96-well microtitre plate, 90 µL each of standard solutions and sample solutions of mentioned concentrations (100, 33.33, 11.11, 3.70, 1.23, 0.41, 0.137, 0.046, 0.015, ng/mL) were added to the wells designated as standard and sample solutions. 100 and 50 µL of culture medium was added to the wells

designated as medium control and cell control, respectively. 50 µL of cell suspension was added to each standard, sample, and cell control well. A suitable plate designed to distribute the standard and sample solutions to achieve randomization was used. The plate was mixed and incubated at 37 °C for a period of 46±2 hr in a humidified incubator with 5% CO2. Later 50 µL of pre-warmed Resazurin sodium was added to each well and the plate was incubated for 7±1 h. The test plate was cooled to room temperature and the relative potency was determined by measuring the fluorescence (relative fluorescence units, RFU) using a suitable 96-well plate reader using an excitation wavelength of 530 nm and an emission wavelength of 590 nm [30, 31]. The potency calculated is not 125% of the declared potency. The confidence intervals (p=0.95) limit of the calculated potency is not 136% of the declared potency

Procedure Determined the biological activity of rHuEPO based on the stimulation of proliferation of TF-1 cells. The following method uses the conversion of Resazurin as a staining method.

- Aseptically removed 100 µL rHuEPO sample and place in sterile polypropylene micro-centrifuge tube.

- Label the aliquots and store at 2-8 °C for further use, do not freeze.
- The assay media for the experiment should be freshly prepared at room temperature during the entire experiment.
- Wet all fresh pipette tips at least once before aliquoting samples.
- Prepare dilution of standard accurately in assay media as given in Table-1
- Prepare dilutions of the sample to 1500 ng/mL as per their concentration in assay media, followed by 3 fold dilutions as shown in Table-1
  (To prepare sample dilution to 1500 ng/mL always keep 10 µL drug volume constant and change assay medium volume)
- Prepare 1st dilution in sterile 1.5 mL microcentrifuge tubes/ centrifuge tubes remaining dilutions in 96 well deep well block and into sterile 96 well plate as discussed in setting up assay plate.
- From dilution step 1, add 133 µL of 1500 ng/mL solution into the first well of deep well block containing 867 µL of assay medium. Mix gently by pipetting up and down 4-6 times taking care not to allow forth formation. To the rest of the wells add 500 µL of assay medium per well.
- Withdraw an aliquot of 250 µL for next dilution and mix gently. Repeat this for next 7 dilutions.

Dilution Step	From Step	Conc. (ng/mL)	Fold Dilution (X)	Volume (µL)	Vol. of assay medium	Final Conc. (ng/mL)	Final Conc. In Plate (ng/mL)
1	N/A	1500	7.5	133	867	200	100
2	1	200	3	250	500	66.66	33.33
3	2	66.66	3	250	500	22.22	11.11
4	3	22.22	3	250	500	7.407	3.70
5	4	7.407	3	250	500	2.469	1.23
6	5	2.469	3	250	500	0.823	0.41
7	6	0.823	3	250	500	0.274	0.137
8	7	0.274	3	250	500	0.091	0.046
9	8	0.091	3	250	500	0.03	0.015

2.13 Setting up of Assay plate

- Using 8 tip multichannel pipette after wetting it twice, aliquot 90 µL standard/ samples in triplicate from the deep well block into sterile 96 well plate as shown in plate layout 2.12
- Add 90 µL of assay media in each assay control wells.
- The assay will be performed in triplicates for each dilution of sample and standard.
- Keep the assay plate in the laminar hood at room temperature till the cell suspension is ready.

2.14 Preparation of cell suspension

- Subculture cells before proceeding to the next step unless another flask is available for cell maintenance.
- Pipette out cell suspension from the culture flask into sterile 50 mL centrifuge tube and centrifuge at 200 g for 5 minutes at 25 °C. 6.6.5.3 Decant the supernatant, disturb the cell pellet by gentle tapping and add 15 -20 mL PBS and centrifuge at 200 g for 5 minutes at 25 °C. Repeat the procedure two more times.
- Re-suspend the cells in assay medium.
- Perform cell count and adjust the cell density to 2 x 105 cells/mL
- Add 90 µL of cell suspension (2 x 105 cells/mL) to each well so that the final volume in each well so the final volume is 180 µL.
- Incubate the plate for 48 ± 4 hours at 37 °C in a humidified CO2 incubator.

  Note: Perform cell washing and dilution preparation simultaneously, so that the cells are ready by the time the dilution in the plate is completed. It is not advisable to leave cells at room temperature for more than 60 minutes.

2.15 Plate Layout

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	Media	Media	Media	Media	Media	Media	Media	Media	Media	Media	Media	Media
B	Media	STD 100	STD 33.33	STD 11.1	STD 3.7	STD 1.23	STD 0.41	STD 0.13	STD 0.046	STD 0.015	AC	Media
C	Media	STD 100	STD 33.33	STD 11.1	STD 3.7	STD 1.23	STD 0.41	STD 0.13	STD 0.046	STD 0.015	AC	Media
D	Media	STD 100	STD 33.33	STD 11.1	STD 3.7	STD 1.23	STD 0.41	STD 0.13	STD 0.046	STD 0.015	AC	Media
E	Media	SP 100	SP 33.33	SP 11.1	SP 3.7	SP 1.23	SP 0.41	SP 0.13	SP 0.046	SP 0.015	AC	Media
F	Media	SP 100	SP 33.33	SP 11.1	SP 3.7	SP 1.23	SP 0.41	SP 0.13	SP 0.046	SP 0.015	AC	Media
G	Media	SP 100	SP 33.33	SP 11.1	SP 3.7	SP 1.23	SP 0.41	SP 0.13	SP 0.046	SP 0.015	AC	Media
H	Media	Media	Media	Media	Media	Media	Media	Media	Media	Media	Media	Media

Where: STD- standard, SP- sample, AC- Assay control and the number of concentrations in ng/mL

2.16 Measurement of cell proliferation

- After 48 ± 4 hours of incubation, check the plate for any visible contamination under the microscope. 6.6.7.2 If any visible contamination is observed microscopically discard the plates otherwise follow the steps given below for termination of the plate.
- Transfer 3.0 mL aliquot of Alamar Blue per plate to a 15 mI, tube, wrap in an aluminium foil and keep at room temperature (one hour prior to use).
- Add 50 µL of Alamar Blue solution per well using a multichannel pipette.
- Incubate the plate in CO2 incubator for another 24 ± 4 hours, so that the total period of incubation of the assay plate is 72 hours.
- After 24 ± 4 hours of incubation with Alamar Blue read theplate by the Biotek Reader at 530/590 nm excitation/emission.

2.17 Data Analysis

- Calculate Average, Standard Deviation (SD) and %CV of sub sampling for the observed Relative Fluorescence Unit (RFU) at all drug concentrations as well as for the assay control.
- Calculate mean corrected RFU at each drug concentration by subtracting the mean assay control RFU at the particular drug concentration.
- Enter the mean corrected RFU and doses for every run into PLA 3.0 program for further analysis. The analysis is done using a 4-parameter logistic regression function. The 4-parameter values (A,B,C and D) for standard and sample were captured from PLA software.

Where,
A = Highest estimated value (max)
B = Slope C = Estimated mid-point between A and D [Effective concentration to induce 50% effect (EC50)]
D = Lowest estimated value (min)

- PLA 3.0 software program setting were as follows

Dose settings
• Number of treatments (dilution steps): 9
• Number of replicates: 3
• Dilution scale: Direct dose input

Analysis settings

- Model: 4-parameter logistic curve (Full curve)
- Outlier Detection: No outlier detection
- Hypothesis Testing: ANOVA based on pure error separation
- Test for parallelism: Hypothesis based on F-test or Equivalence based on ratio of slopes or Equivalence test based on difference of slopes
- Significance of the regression: 95%
- Significance of deviations from Linearity: 95%
- Significance of parallelism test: 95%
- Fiducial limits of potency estimation: 95%

Configuration settings

Linear detection type: Full range

2.18 Rounding off and Reporting results

- The in-vitro activity of Darbepoetin alfa will be reported as 103 U/mcg.
- To the ROA attach the print outs of gen5 readouts, Calculation sheet PLA results report.
- Every sample will be analyzed three independent times by different analyst or by the same analyst on three different days. The average of three independent analysis for any given sample will be reported as the potency of the sample.

2.19 Validation Recommendation

2.19.1 Assay acceptance criteria

Acceptance for standard

- All the response value in the DRC should have %CV as <15
- The 4-parametric fit should be R2 > 0.98
- Max value should be > 2029
- Max response /min response from the raw RFUs should be > 1.2
- Slope should be > 0.7

2.19.2 Acceptance criteria for sample

- All the response values in the DRC should have %CV as <15
- The 4-parametric fit should be R2> 0.98
- The sample must pass Test of Non-parallelism in PLA 3.0
- Slope ratio (Sample slope/standard slope) should be between 0.8 to 1.2 6.9.1.3 The %CV criterion for the three (03) independent potencies should be less than 30%

2.19.3 The CV = Standard deviation of the (03) independent potencies/ Mean of the %Relative potencies multiply by 100

2.19.4 Any failures in %CV criteria in between the potency estimates will be handle through discrepancy management system. In such cases, the assay stands invalid and the sample will be re-analyzed.

2.20 Sub-visible particulate matter

2.20.1 Perform the test as per by a calibrated liquid particle counter.

2.20.2 Acceptance criteria

- For particles > 10µ: Not more than 6000/container
- For particles > 25µ: Not more than 600/container

2.21 Bacterial Endotoxin

Perform the Bacterial Endotoxin test as per SOP of Bacterial Endotoxin Test of Raw Material/ Finished Product.

2.22 Sterility

Perform the sterility test as per SOP of Sterility Test Procedure of Raw Material, Finished Product & Packaging Materials

METHODS VALIDATION

3.0 Method Validation

The parameters to be fulfilled for validation were established according to the ICH guideline Q2(R1) [9] and the USP Biological Assay Validation Chapter [1] considering the parameters below. The acceptance criteria for each evaluated parameter of the validation exercise was established according to the method capabilities observed during the standardization stage and its intended purpose, which is to evaluate in vitro the binding of adalimumab to mTNFα express on recombinant CHO cells for QC purposes.

3.1. Specificity (Curve fitting)

Specific recognition and binding of adalimumab to mTNFα was tested against matrix components (excipients). A dose-response curve (model curve) constructed from the Fold increase in MFI values generated from independent triplicates at 9 dilution levels of adalimumab (in a range from 3.2 to 700 ng/mL) with respect to the matrix prepared under the same dilution scheme and replicates. In this context, specificity is given by the fitting of the model curve into the assay data through a non-linear regression model. Curve fitting was tested under four or five-parameter logistic models (4PL and 5PL, respectively) using the software Graph Pad Prism.

3.2. Accuracy (dilutional linearity)

Accuracy several factors such as buffer components and sample matrix can impact on binding of antibodies in bioassays, hence influence the accuracy of the method. Accordingly, accuracy is usually measured through dilutional linearity that accounts for such variations. We evaluated accuracy at all the dilution levels of the dose-response curve in a concentration range from 60% to 140%. The pre-defined acceptance criteria for acceptable linearity was $r\ 2 \geq 0.90$ and slope in a range from 0.80 to 1.25.

3.3. Precision

The precision describes how the method is capable to reproduce independent results with variations within an acceptable given distribution of single measurements. We estimate precision through the coefficient of variation (CV) from three independent replicates at the nine concentration levels of the dose-response curve. The pre-defined acceptance criteria were CV ≤ 20% among replicates at all the evaluated levels.

3.4. System

Suitability According to the manufacturer's guides, before setting up an experiment, we run a performance check using the Cytometer Setup and Tracking (CST) application (BDTM). This performance check ensures that the cytometer is performing consistently for automatic characterization, tracking, and measurement acquisition. Additionally, the system suitability should be determined by specificity using dye controls capable to unambiguously identify specific populations. In this work the system suitability was determined considering the capability of the flow cytometer to detect a differential dose response among the samples containing darbepoetin mTNFα complex and the negative controls. The parameters evaluated were CV (from precision) and r2 of the dose-response curve within the concentration levels range

Experimental Design

The schematic presentation of the experimental procedures performed in this study is summarized below (Figure 2.1)

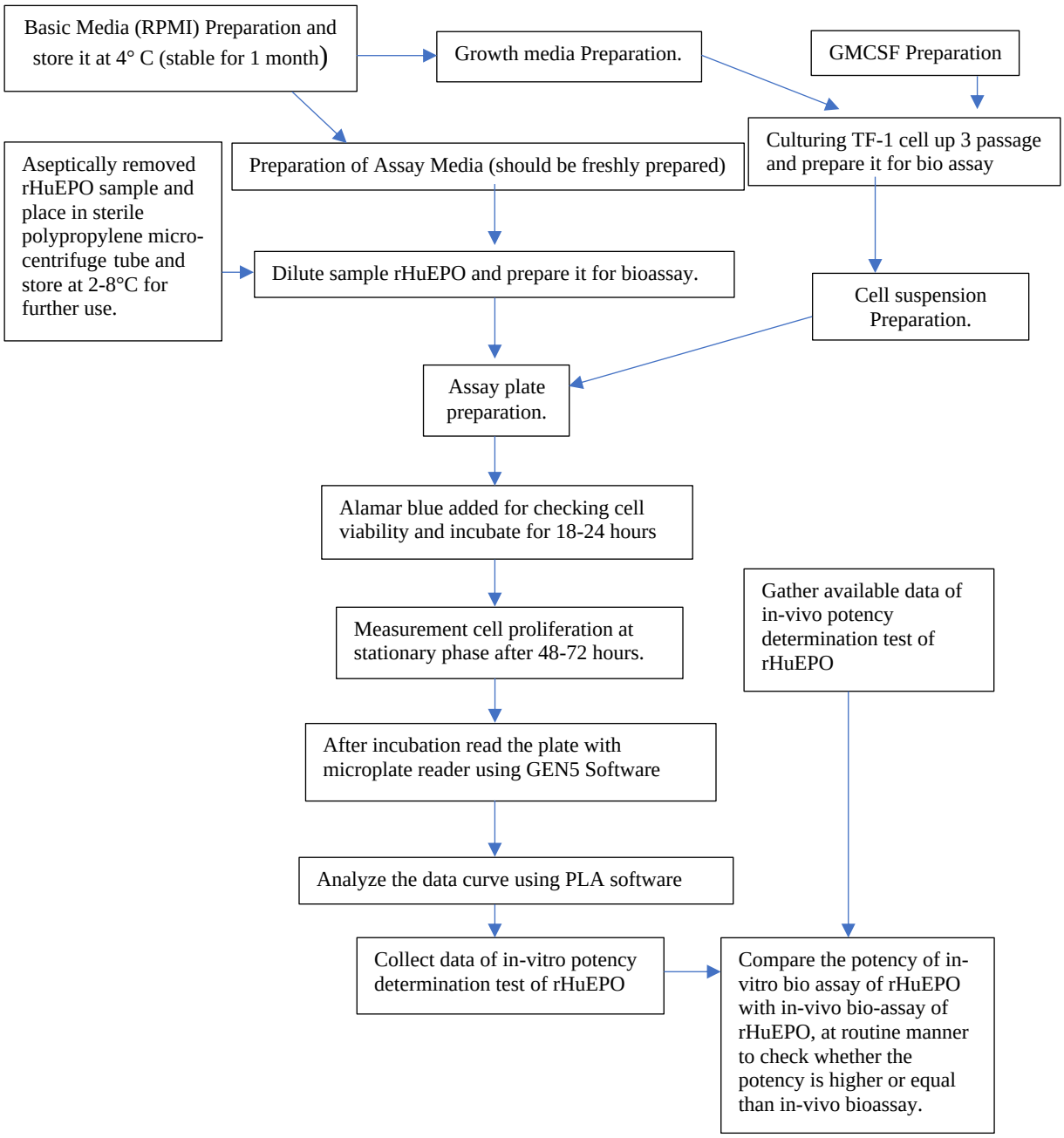

Figure 2.1. Schematic diagram of laboratory experimental design

4.0 Results and Discussion

Method Development Proliferation inhibition of Jurkat clone E6.1 T leukemia cells is induced by a purine analogue, azathioprine, which interferes with the DNA replication. Conversely, the treatment of the cells with both Transferon® and azathioprine avoided this effect in a concentration range from 1 to 180 µg/mL. The obtained dose–response curve exhibited a sigmoidal behavior with a half-maximal effective concentration (EC50) of 13.07 µg/mL, which fitted into the four-parameters logistic model.

From different batches of standard rHuEPO which has been tested prior through western blotting to check its purification, sterility and stability, data has been generated over the time through invitro bioassay.

The table below shown the regression, CV, slope and the potency of the standard sample in experimented method over particular time.

Date	Batch	Initial concentration	Mean	SD	CV	Regression	Potency
07-02-2021	1901928	100 ng/ml	9.46667	0.823	0.087	96.01%	118%
	1900806	100 ng/ml	9.5789	0.962	0.032	98.99%	126%
	1901377	100 ng/ml	9.8937	0.922	0.043	95.42%	97.47%
11-02-2021	1900826	100 ng/ml	9.9225	0.877	0.028	96.11%	106.21%
	1901241	100 ng/ml	9.787	0.721	0.053	95.48%	96.09%
	1902890	100 ng/ml	9.924	0.844	0.068	97.53%	105.02%
14-02-2021	1900723	100 ng/ml	9.448	0.738	0.058	97.21%	98.77%
	1901377	100 ng/ml	9.5447	0.867	0.038	98.34%	111.89%
	1901240	100 ng/ml	9.8937	0.922	0.044	96.59%	98.47%
17-02-2021	1901377	100 ng/ml	9.9225	0.877	0.025	97.37%	109.21%
	1900826	100 ng/ml	9.8937	0.922	0.043	96.46%	98.42%
	1901241	100 ng/ml	9.9225	0.877	0.028	98.78%	108.11%
25-02-2021	1902890	100 ng/ml	9.5789	0.962	0.032	98.85%	126.33%
	1900723	100 ng/ml	9.8937	0.922	0.043	97.93%	126.47%
	1901928	100 ng/ml	9.9225	0.877	0.045	98.34%	123.09 %
04-03-2021	1900806	100 ng/ml	9.46667	0.823	0.087	97.52%	118%
	1901241	100 ng/ml	9.5789	0.962	0.032	97.71%	120.02%
	1902890	100 ng/ml	9.924	0.844	0.068	96.15%	105.02%
15-04-2021	1900723	100 ng/ml	9.58	0.738	0.058	96.39%	98.77%
	1902890	100 ng/ml	9.6447	0.867	0.038	98.76%	111.89%
	1900806	100 ng/ml	9.46667	0.823	0.077	96.93%	110.58%
06-05-2021	1900826	100 ng/ml	9.5789	0.962	0.032	97.72%	117.49%
	1901241	100 ng/ml	9.8937	0.922	0.047	95.91%	98.47%
	1901377	100 ng/ml	9.9225	0.877	0.035	97.24%	108.51%

Over the time it has been observed that, the different batches of rHuEPO shown consistent in above criteria curve in the above method.

To validate the established method, the methods and the standard shown similar consistency in specificity, accuracy and precision test.

For different batches, two triplicate result of standard rHuEPO has been performed. Later, In-vivo bioassay or reticulocyte assay using flow cytometry also performed of the same batches and data generated using PLA software and compare the data with our constructed assay.

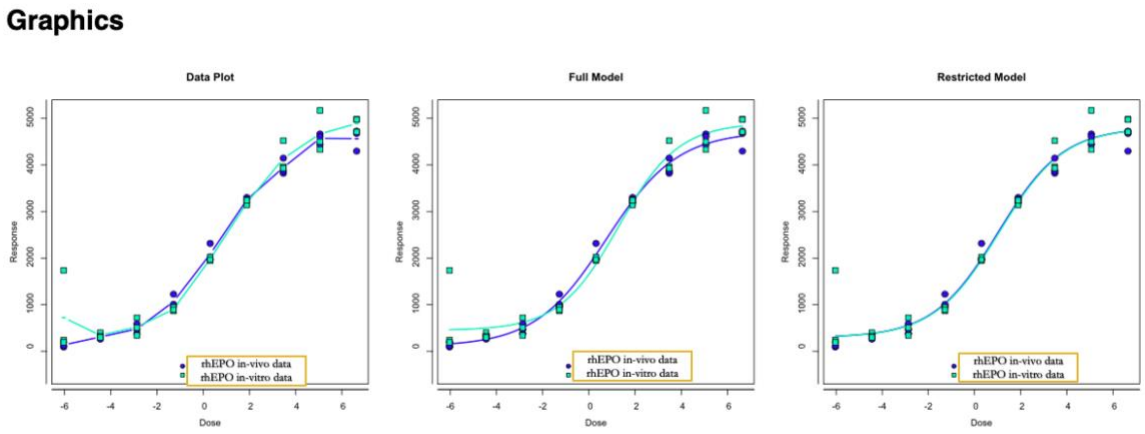

Fig 3.1 : Comparison of In-vivo and In-vitro bio-assay data generated by PLA software of same batch of rhEPO

Statistical analysis of all data, including assessment of assay validity (i.e acceptable parallelism, linearity) and estimation of relative potencies between the study samples and standard was performed. Using the approach for assessment of parallelism described in section 2.3, acceptance ranges of [-0.076, 0.076] and [-0.116, 0.116]. Adopting these limits, acceptable parallelism was observed between coded duplicate samples. Several assays were excluded due to non-linearity of dose-response curves, and few assays was excluded due to poor agreement between coded duplicates. A complete list of relative potency estimates for samples including both valid and invalid assays, is provided in above supplementary table.

4.0 Conclusion

The pharmacological use of rHuEPO has been well established and has had a dramatic impact on the quality of life of patients with renal disease [5], [6]. EPO is a glycoprotein, with approximately 40% of the molecular mass of the mature molecule made up of four carbohydrate chains (three N-linked and one O-linked). The carbohydrate moieties of EPO contain at least 10 molecules of sialic acid, which is an important determinant of the pharmacokinetic behavior of EPO in vivo by preventing degradation and delaying clearance of EPO from circulation [10], [11]. The protein structure of EPO molecules is associated with the receptor-activating ability of EPO, which is always estimated by in vitro bioassays. Only when in vitro bioactivities are consistent with in vivo bioactivities can the rHuEPO products be used clinically [8]. Since rHuEPOs produced in mammalian cells contain multiple isoforms with heterogeneous carbohydrate moieties, potency determination of sialic acid and isoelectric focusing electrophoresis are also essential test items in routine quality control of rHuEPO pharmacological products (Chinese Pharmacopoeia (2010)).

In this study, an in-vitro bio assay for rHuEPO was developed employing a TF-1 cell line subsequently validated for its precision, specificity, robustness and agreement with reticulocyte assay. The constructed method showed strong reactivity to rHuEPO and excellent curve-fitting compared to in-vivo bio-assay. The optimized assay could be completed within 24 h and proved to be more sensitive with an EC50 of 0.077 IU/mL. Our analyses resulted in both intra- and inter-assay variation less than 15.0%, and recovery rates ranged from 81.7% to 102.4%, demonstrating good precision and accuracy, which were even better than most of currently-used methods [8], [13].
The quality control process and accurate determination of bioactivity of in-vitro based bioassay could be establishes for the safety and efficacy of rHuEPO. This new in-vitro bio-assay could be simpler, faster and viable substitution of in-vivo reticulocyte assay and employed in potency determination of rHuEPO bio-pharmaceutical products. The robustness of the assay demonstrated by no effect of passage levels of the cells on the performance of the assay.
The new assay also showed a high consistency with reticulocyte assay in terms of results. However, it has many advantages over reticulocyte assay, such as simpler operation, higher efficiency and no need of animal test. This assay could be used as a viable supplement to reticulocyte assay and employed in potency determination of rHuEPO pharmaceutical products.

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