
A Multi-Methodological Approach to Evaluating the Biological Activity of Erythropoietin Marketed in Thailand

Boontarika Boonyapiwat^{1*}, Yanika Rattasuwan² and Phannarak Khruaplao³

^{1*,2,3} Bureau of Drug and Narcotic, Department of Medical Sciences, Ministry of Public Health

Email: ^{1*}boontarika.b@dmisc.mail.go.th, ²yanika.r@dmisc.mail.go.th,

³phannarak.k@dmisc.mail.go.th

Abstract

This study evaluated and validated two analytical methods, an enzyme-linked immunosorbent assay (ELISA) and a cell-based bioassay, for the determination of erythropoietin in pharmaceutical formulations marketed in Thailand. Method validation was carried out following ICH guidelines. The ELISA method demonstrated high specificity, as the erythropoietin standard and formulated samples produced clear absorbance signals that were clearly different from those of the excipient matrix. The excipients alone showed minimal response, confirming that formulation components did not interfere with the assay. A strong relationship between absorbance and erythropoietin concentration was observed, and the method showed excellent linearity over the tested range, with coefficients of determination greater than 0.99 across three consecutive days. Accuracy results were acceptable for a biological assay, with recovery values within the expected range. Although some variability was observed in repeatability testing, this was consistent with the nature of immunoassays. Intermediate precision results were consistent across different days and analysts, indicating that the ELISA method is reliable for routine analysis. The cell-based bioassay also showed good specificity. Parallel dilution curves of the erythropoietin standard and sample demonstrated consistent relationships between response and concentration, while formulation excipients produced only weak signals. The assay was linear over a concentration range of 0.0625 to 1 IU/mL, with strong correlations observed over three days. Accuracy testing showed recovery values within acceptable limits, and precision results were within the expected range for cell-based assays, where higher variability is commonly observed. The precision of the assay across different days and analysts confirmed its robustness.

In conclusion, both the ELISA and cell-based bioassay methods were successfully validated and shown to be suitable for erythropoietin determination. The ELISA method provides reliable quantitative measurement, while the cell-based assay effectively assesses biological activity and potency. Together, these complementary methods offer a practical and reliable approach for routine quality control of erythropoietin products.

Keywords: Erythropoietin, ELISA, Cell-based assay, Potency, Method validation

* Corresponding author, e-mail: boontarika.b@dmisc.mail.go.th

1. Introduction

Erythropoietin is a sialoglycoprotein hormone that consists of a single polypeptide chain of 165 amino acids [1]. It is mainly produced by the renal cortex and stimulates the production of red blood cells [2,3]. Due to its structural complexity the potency cannot be determined by molecular weight; on the other hand, it must be evaluated through functional assays using in vivo or in vitro systems [4]. Potency assays are critical for quality control for erythropoietin as they reflect biological activity and ensure batch-to-batch consistency and comply with the regulatory requirements [5]. Conventionally, the measurement of erythropoietin potency has been performed using in vivo animal bioassays, such as the normocythemic or polycythemic mouse models [4]. However, these methods are gradually replaced by alternative methods. This substitution is based primarily on the 3Rs principle: Replacement, then Reduction and Refinement of animal tests [6]. The conventional animal models are not only expensive and time consuming, but also variable, so that it is a challenge for releasing batch precision [3].

Thus, to address this limitation and to reduce the use of animal models, a number of alternative approaches including enzyme-linked immunosorbent assay (ELISA) and cell-based assays have been explored as potential options. The mechanism of ELISA for determining erythropoietin potency is based on sandwich ELISA, in which the signal intensity is directly proportional to the erythropoietin concentration [7,8]. In contrast, cell-based assay mechanism measures the proliferation response, which is induced by erythropoietin binding to its receptor [9,10]. Integrating both methods to determine erythropoietin potency can provide a more robust assessment of the erythropoietin efficacy than any single method alone [11]. Hence, the objective of this study is to provide validated ELISA and cell-based assay methods for erythropoietin potency determination marketed in Thailand using a multi-methodological approach.

2. Methodology

2.1 Reagents and materials

Potassium chloride, sodium chloride, disodium hydrogen phosphate, sodium dihydrogen phosphate, potassium dihydrogen phosphate and sulfuric acid were obtained from Carlo Erba, Italy. Mannitol, human serum albumin, polysorbate 80, glycine, sodium citrate, trypan blue and bovine serum albumin (BSA) were purchased from Sigma, United States. Tween 20 was obtained from Scharlau, Spain. Human erythropoietin antibody (primary antibody; clone: 9C21D11), rabbit anti-human erythropoietin and polyclonal antibody (secondary antibody) were purchased from R&D Systems, United States. Peroxidase conjugated affinity purified Goat Anti-Rabbit IgG (IgG-HRP) was obtained from Jackson ImmunoResearch Laboratories, United States. TMB solution, TF-1 erythroblasts (CRL-2003), RPMI-1640 Medium, and fetal bovine serum (FBS) were acquired from Thermo Fisher Scientific Inc., United States. WHO International Standard for erythropoietin recombinant for bioassay was obtained from NIBSC, United Kingdom. CellTiter-Blue® reagent was procured from Promega Corporation, United States. *m*-cresol was purchased from Acros organics, Belgium. Ultrapure water was used throughout the study.

2.2 Validation of enzyme-linked immunosorbent assay and cell-based methods for erythropoietin determination

Method validation was performed according to ICH Q2(R1) guidelines, which included evaluation of four core parameters: specificity, linearity and range, accuracy, and precision [12]. Specificity testing confirmed that excipients present in the erythropoietin formulation did not interfere with the detection or biological response of erythropoietin in either the ELISA or the cell-based assay. Linearity and range were assessed to demonstrate that the assay responses, measured as ELISA absorbance and cell-based bioassay signals, were directly proportional to erythropoietin concentrations across the tested range. Accuracy was evaluated to determine the agreement between measured values and the true erythropoietin concentration, while precision was assessed by examining the repeatability and intermediate precision. The method for determining erythropoietin using ELISA and cell-based techniques were performed as follows.

2.3 Determination of erythropoietin by enzyme-linked immunosorbent assay

Primary antibody was dissolved in 1x phosphate buffered saline (PBS), prepared by diluting a 10 x PBS stock solution 1:10. The 10 x PBS solution contained 2 g of potassium chloride, 2 g of potassium dihydrogen phosphate, 80 g of sodium chloride and 11.5 g of disodium hydrogen phosphate in 1000 mL of ultrapure water. The primary antibody (100 μ L) was dispensed into each well of 96-well plate, and the plate was incubated at 4 °C for 24 h. The solution in each well was then aspirated off and the plate was washed four times using plate washer. Blocking buffer (200 μ L), consisting of 10 g of BSA, 100 mL of 10 X PBS stock solution and 5 mL of 10% (v/v) Tween 20 in 1,000 mL of ultrapure water, was dispensed into each well. The plate was incubated at ambient temperature for 30 min, and then washed four times. The WHO international standard, sample and blank solutions were pipetted at 50 μ L per well. Subsequently, blocking buffer was dispensed at 50 μ L per well. The plate was incubated at ambient temperature for 1 h and then washed four times. The IgG-HRP solution was added at 100 μ L per well, and the plate was incubated at ambient for 30 min, followed by four washing steps. TMB solution was added at 100 μ L per well, and the plate was incubated at ambient for 10 min. The reaction was stopped by adding 100 μ L/well of 1 N sulfuric acid, and the absorbance of each well was measured at 450 nm. During incubation, the 96-well plate was sealed with adhesive film and protected from light by covering it with aluminum foil.

2.4 Determination of erythropoietin by cell-based assay

TF-1 cells were prepared 3-4 days prior to the assay to ensure growth rate. On the day of experiment, cells were and centrifuged at $130 \times g$ for 5 minutes. The cell pellet was resuspended in assay medium consisting of RPMI-1640 supplemented with 10% heat-inactivated FBS (56 °C for 30 minutes). Cell viability was confirmed to be more than 80% by Trypan blue using a hemocytometer, and the cell suspension was subsequently adjusted to a final concentration of 2.0×10^5 cells/mL. Serial dilutions of WHO international standard and sample were prepared directly to the plate with each well containing 50 μ L. The prepared cell suspension (50 μ L) was added into each well, bringing the total volume to 100 μ L per well. The plates were then incubated at 37.0 °C under 5.0% CO₂ for 44 hours. After incubation, 20 μ L of CellTiter-Blue® reagent was added to each well. The plates were agitated at 500 rpm for 30 seconds and returned to the incubator for a final 4 hours. Fluorescence was measured at an excitation wavelength of 560 nm and an emission

wavelength of 590 nm using a microplate reader. Finally, potency evaluation was conducted using CombiStats software (EDQM), in accordance with the European Pharmacopoeia.

3. Results

3.1 The validation of ELISA method for determining erythropoietin

Specificity of the method was performed by comparing the absorbance of a standard solution against both the formulated sample and a common excipient matrix used in erythropoietin products marketed in Thailand. This matrix consisted of mannitol, human serum albumin, sodium chloride, sodium dihydrogen phosphate, disodium hydrogen phosphate, polysorbate 80, glycine, m-cresol, and sodium citrate. Each group was tested in quadruplicate. At a final concentration of 0.3 IU/mL, the standard and sample yielded absorbance values of 0.3638 and 0.3328, respectively, whereas the excipient matrix showed only a minimal response of 0.0206 (Figure 1).

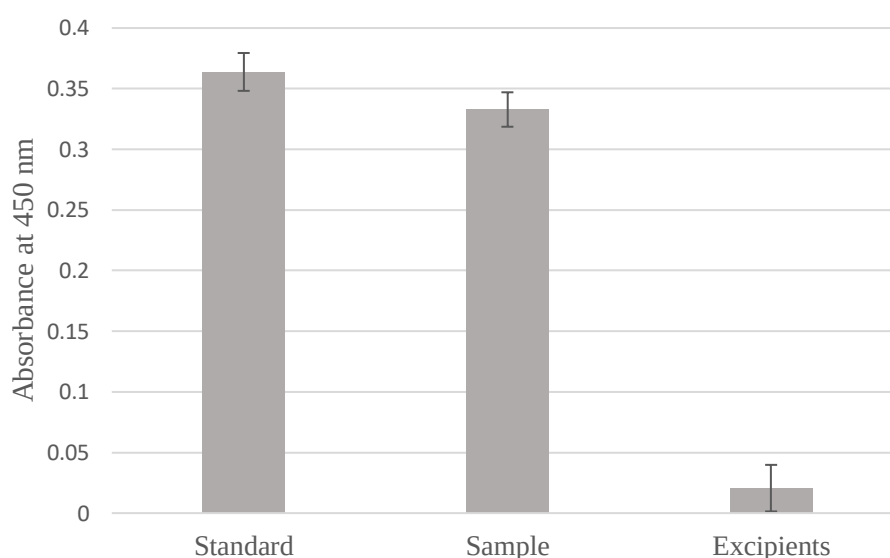


Figure 1 Specificity of the ELISA method using erythropoietin standard, sample, and excipients

Linearity was assessed using five erythropoietin concentrations ranging from 0.2 to 0.4 IU/mL over three consecutive days. The calibration curve was constructed by plotting the logarithm of erythropoietin concentration against absorbance. The assay showed good linearity, with R^2 values of 0.9952, 0.9962, and 0.9953 (Figure 2).

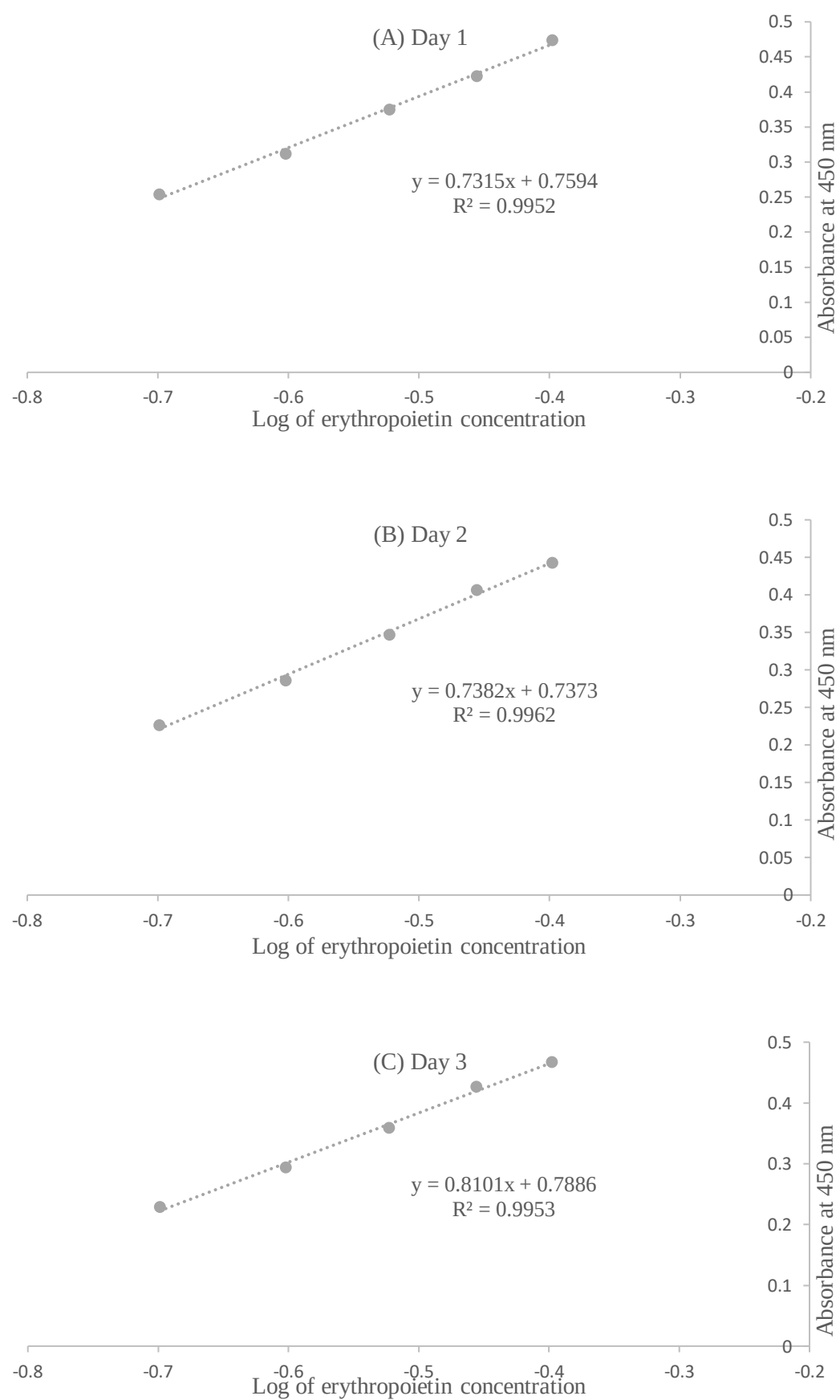


Figure 2 Linearity evaluation of the erythropoietin ELISA method over the concentration range of 0.2–0.4 IU/mL

Accuracy was determined by analyzing five erythropoietin concentrations between 0.2 and 0.4 IU/mL at 70% (SAM70), 100% (SAM100), and 130% (SAM130) of the target concentration, with four replicates at each concentration. The percent recovery ranged from 87.18% to 122.81%, with average recoveries of 101.47%, 116.77% and 101.9% for SAM70, SAM100, and SAM130, respectively (Table 1). Repeatability was evaluated under the same conditions, and the %RSD values obtained were 4.76%, 4.49%, and 12.75% for SAM70, SAM100, and SAM130, in the same order (Table 1).

Table 1 Accuracy (% recovery) and repeatability (%RSD) of the erythropoietin ELISA at 70%, 100%, and 130% of the target concentration (0.2–0.4 IU/mL)

Erythropoietin concentration (IU/mL)	% Recovery		
	SAM 70	SAM100	SAM130
0.2-0.4	96.62	122.81	106.73
	106.27	113.97	87.18
	101.53	113.52	111.8
Average of % Recovery	101.47	116.77	101.9
%RSD	4.76	4.49	12.75

Intermediate precision was assessed by performing the assay at the same concentration levels used for accuracy and repeatability across three different days and by different analysts. The inter-day precision showed %RSD values ranging from 6.62% to 15.11% over the three-day study, while the inter-analyst precision produced %RSD values between 6.15% and 13.57% (Table 2).

Table 2 Inter-day and inter-analyst precision (%RSD) of the erythropoietin ELISA assay

Precision	% Recovery		
	SAM 70	SAM100	SAM130
<i>Inter-day precision</i>			
Day 1	96.62	122.81	106.73
Day 2	92.83	97.23	109.42
Day 3	105.50	93.90	94.79
Average of % Recovery	98.32	104.65	103.64
% RSD	6.62	15.11	7.51
<i>Inter-analyst precision</i>			
	107.81	118.59	103.15
Average of % Recovery	100.69	108.13	103.52
% RSD	7.07	13.57	6.15

3.2 The validation of cell-based method for determining erythropoietin

Specificity was evaluated by comparing parallel dilutions of the standard solution, sample and formulation excipients alone, the composition of which is described above. The curves, obtained from cells treated with logarithmic concentrations, were compared to assess the interference from excipients. The graphs showed a linear relationship between fluorescence

intensity and the logarithm of erythropoietin concentration for standard and sample solutions. A five-point concentration range of 0.0625 to 1 IU/mL was studied and yielded the coefficients of determination (R^2) of 0.9985 for the standard, 0.9981 for the sample and 0.7328 for the excipients as shown in Figure 3.

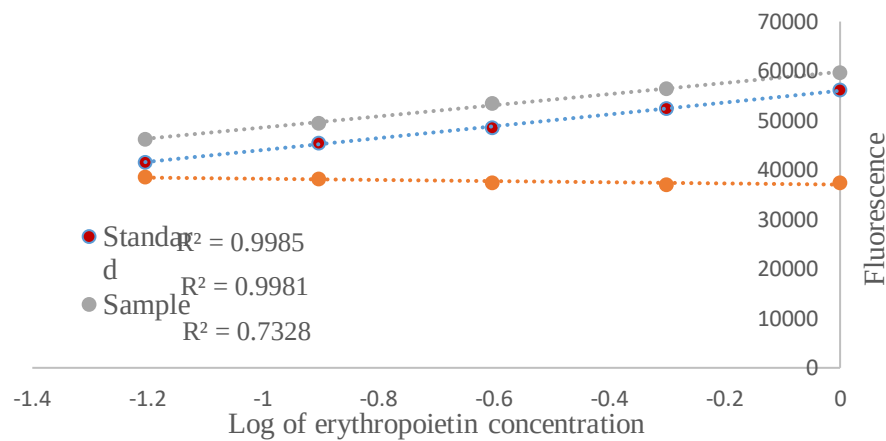
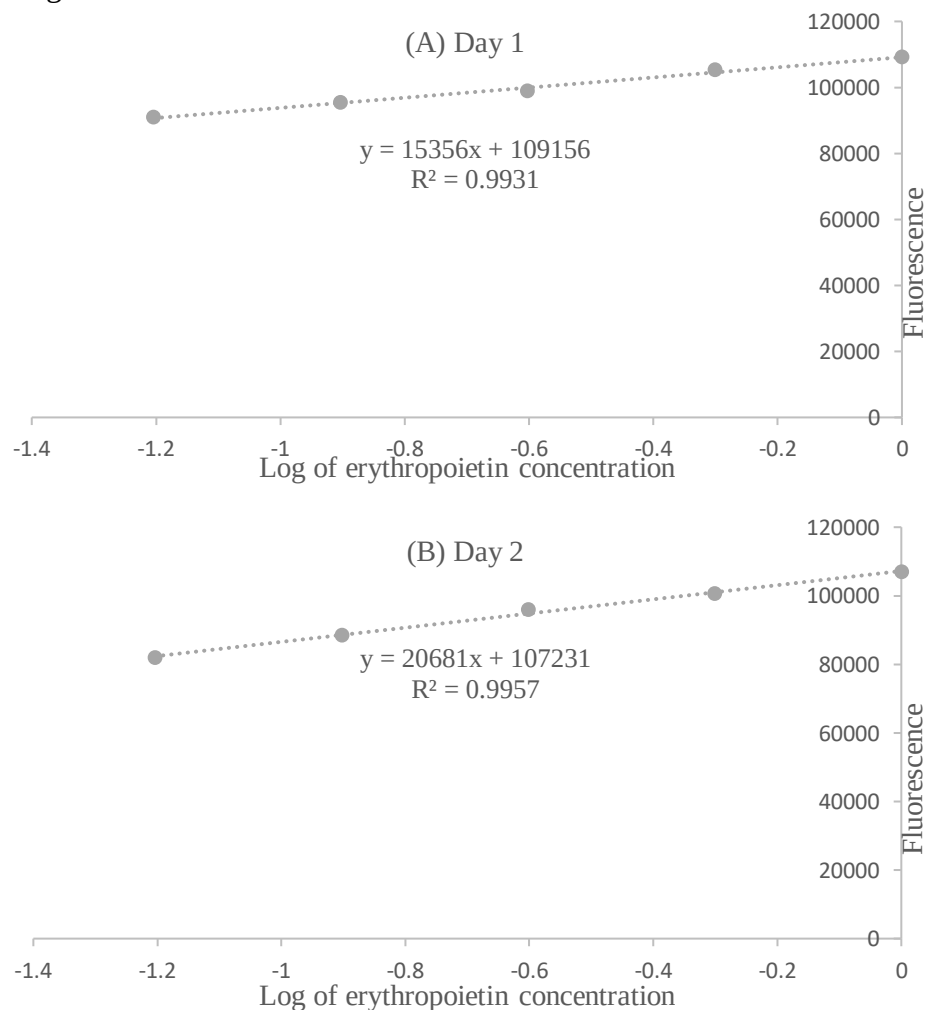


Figure 3 Specificity of the erythropoietin cell-based bioassay assessed by parallel dilution

Linearity was evaluated using five erythropoietin concentrations ranging from 0.0625 to 1 IU/mL over three consecutive days, resulting in R^2 values of 0.9931, 0.9957 and 0.9966, as shown in Figure 4.



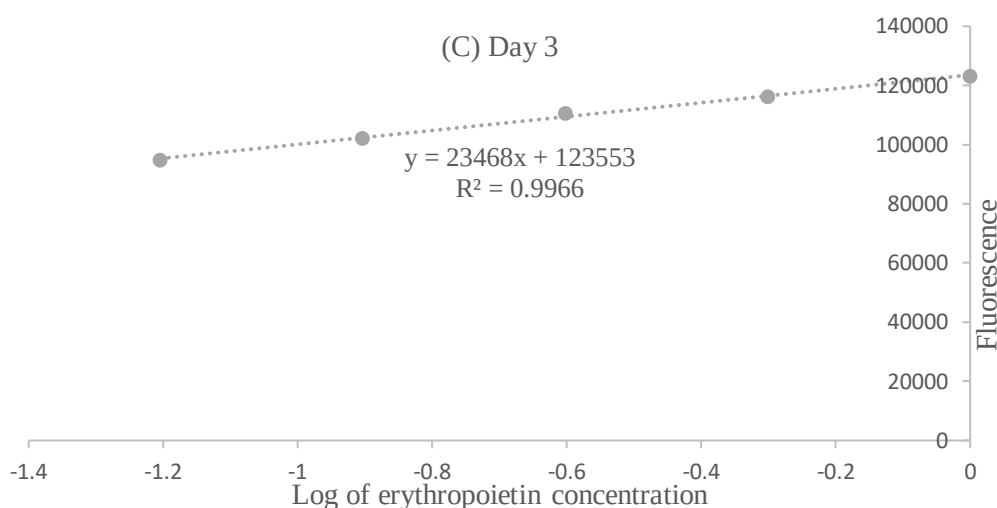


Figure 4 Linearity evaluation of erythropoietin cell-based bioassay over the concentration range of 0.0625–1 IU/mL

The accuracy of the method was evaluated using five erythropoietin concentrations ranging from 0.0625 to 1 IU/mL and expressed as percent recovery at 70% (SAM70), 100% (SAM100), and 130% (SAM130) of the target concentration, with each level tested in triplicate. Percent recovery ranged from 81.74 to 116.66% with the average values of 100.56%, 103.11% and 104.98% for SAM70, SAM100 and SAM130, respectively (Table 3). The repeatability was assessed using the same concentration range and levels as those used for the accuracy study, yielding % relative standard deviation (RSD) values of 6.95, 18.16 and 10.79 for SAM 70, SAM100 and SAM130, respectively (Table 3).

Table 3 Accuracy (% recovery) and repeatability (%RSD) of the erythropoietin cell-based bioassay at 70%, 100%, and 130% of the target concentration (0.0625–1 IU/mL)

Erythropoietin concentration (IU/mL)	% Recovery		
	SAM70	SAM100	SAM130
0.0625-1	108.61	110.94	112.00
	96.03	116.66	111.02
	97.06	81.74	91.92
Average of % Recovery	100.56	103.11	104.98
%RSD	6.95	18.16	10.79

Intermediate precision was conducted by consistent assay at the same concentration levels used for the accuracy and repeatability studies when evaluated over three different days and by different analysts. The inter-day precision showed the % RSD range of 3.20% to 10.02% across three-day study, while the inter-analyst precision yielded %RSD values between 3.83% to 8.39% (Table 4).

Table 4 Inter-day and inter-analyst precision (%RSD) of the erythropoietin cell-based assay

Precision	% Recovery		
	SAM70	SAM100	SAM130
<i>Inter-day precision</i>			
Day 1	99.17	98.84	104.38
Day 2	96.03	116.66	111.02
Day 3	112.02	119.47	109.42
Average of % Recovery	102.41	111.66	108.27
% RSD	8.28	10.02	3.20
<i>Inter-analyst precision</i>			
Average of % Recovery	104.08	108.23	114.51
% RSD	6.78	8.39	3.83

4. Discussion and Conclusion

For validation of the ELISA method for erythropoietin determination, the results demonstrate that the method is highly specific. The erythropoietin standard and the sample showed clear response charts that were distinctly different from those of the excipient. A strong correlation between absorbance and concentration was observed for both the standard and the sample, whereas the excipients alone produced only minimal responses. This confirms that formulation components do not interfere with the assay. The assay exhibited linearity across the tested concentration range, with R^2 values greater than 0.99 over three consecutive days, indicating reliable analytical performance [13]. The method also showed good accuracy, with mean recovery values ranging from 101.47% to 116.77%, which are acceptable for biological assays (75-125%) and confirm accurate quantification of erythropoietin, as shown in Table 1 [14]. Although some variability was observed in the repeatability assessment, with %RSD values up to 12.75%, this level of variation is expected for bioassays. Furthermore, the intermediate precision results were consistent across different days and analysts, with %RSD values meeting the acceptance criteria ($RSD < 20\%$) [13], demonstrating that the method is robust and suitable for routine laboratory use.

For the cell-based bioassay validation, the specificity of the assay was confirmed by comparing the parallel dilution curves of the erythropoietin standard, sample, and the formulation excipients. Both the standard and sample showed a consistent relationship between fluorescence intensity and the logarithm of erythropoietin concentration. In contrast, the excipients generated a weak response, indicating that these components did not contribute to the assay signal. Across the five concentration levels (0.0625-1 IU/mL), the standard and sample demonstrated linearity, with R^2 values exceeding 0.99 [13], while the excipients showed lower linearity. As illustrated in Figure 3, the result confirmed the specificity of the method for potency determination. The linearity results with R^2 values of 0.9931, 0.9957, and 0.9966 were in acceptance criteria [13] and demonstrated a fit to the linear model across three days. By testing three levels at 70%, 100%, and 130% of the target, the recovery percentages, which averaged between 100.56% and 104.98%, stayed within the acceptable analytical limits of accuracy for bioassay (75-125%) [14,15]. These values indicate that the method is highly effective at recovering the target analyte. Regarding precision, the repeatability

study yielded %RSD values ranging from 6.95% to 18.16%. The results of repeatability were in line with the acceptance criteria ($RSD < 20\%$) [13]. The SAM100 level showed slightly higher variability (18.16%), which is normal for cell-based assay because of natural biological differences. This level of RSD is acceptable for this type of study. However, using robotic automation may improve consistency by reducing human error and ensuring that all wells are treated in the same way and at the same time [16]. Intermediate precision was confirmed by evaluating the assay over three different days and by different analysts using the same concentration levels applied in the accuracy and repeatability studies. Both inter-day (%RSD at 3.20%-10.02%) and inter-analyst (%RSD at 3.83%–8.39%) precision remained within acceptable limits ($RSD < 20\%$) [13], as shown in Table 4.

In summary, the validation results demonstrate that both the ELISA and cell-based methods are reliable as multi-methodology approaches for erythropoietin determination. The ELISA method provided high specificity, strong linearity, and acceptable accuracy and precision, ensuring reliable quantitative measurement of erythropoietin concentration. In parallel, the cell-based assay reliably measured biological activity and potency, with acceptable variability typical of bioassays. The use of multiple validation approaches, including specificity, linearity, accuracy, repeatability, and intermediate precision, strengthens confidence in the analytical performance of both methods. These findings confirm that the combined use of ELISA and cell-based assays offers a robust and comprehensive strategy for the accurate determination and potency evaluation of erythropoietin in routine laboratory analysis.

5. References

- [1] Lin, F. K., Suggs, S., Lin, C. H., Browne, J. K., Smalling, R., Egrie, J. C., Chen, K. K., Fox, G. M., Martin, F., & Stabinsky, Z. (1985). Cloning and expression of the human erythropoietin gene. *proceedings of the national academy of sciences of the united states of America*, 82(22): 7580-7584. <https://doi.org/10.1073/pnas.82.22.7580>
- [2] Murakami, M., Kiuchi, T., Nishihara, M., Tezuka, K., Okamoto, R., Izumi, M., & Kajihara, Y. (2016). Chemical synthesis of erythropoietin glycoforms for insights into the relationship between glycosylation pattern and bioactivity. *Science Advances*, 2(1). <https://doi.org/10.1126/sciadv.1500678>
- [3] Nascimento, M. C., Abreu, C. L., Costa, R. N., Delgado, W. C., & Fernandes, I. (2015). Potency evaluation of recombinant human erythropoietin in brazil: assessment of reproducibility using a practical approach. *Brazilian Archives of Biology and Technology*, 58(4), 562-568. <https://doi.org/10.1590/s1516-8913201500228>
- [4] Barth, T., Abreu, C. L., Albani, S. M. V., Dalmora, S. L., & Schwingel, I. C. (2008). Validation of the normocythemmic mice bioassay for the potency evaluation of recombinant human erythropoietin in pharmaceutical formulations. *Journal of AOAC International*, 91(2), 285-291. <https://doi.org/10.1093/jaoac/91.2.285>
- [5] Rey, G., Schuetz, F., Schroeder, D., Kaluschke, C., Wendeler, M. W., Hofmann, I., Dumbliauskas, E., & Obrdlik, P. (2024). Automated ELISA for potency measurements of therapeutic antibodies and antibody fragments. *Journal of Pharmaceutical and Biomedical Analysis*, 245, 116141. <https://doi.org/10.1016/j.jpba.2024.116141>

- [6] Hendriksen, C. F. M. (2002). Refinement, reduction, and replacement of animal use for regulatory testing: Current best scientific practices for the evaluation of safety and potency of biologicals. *ILAR Journal*, 43(Suppl. 1), S43–S48. https://doi.org/10.1093/ilar.43.Suppl_1.S43
- [7] Noé, G., Riedel, W., Kubanek, B., & Rich, I. N. (1992). A sensitive sandwich elisa for measuring erythropoietin in human serum. *British Journal of Haematology*, 80(3), 285-292. <https://doi.org/10.1111/j.1365-2141.1992.tb08134.x>
- [8] Okano, M., Suga, H., Masuda, S., Nagao, M., Narita, H., Ikura, K., & Sasaki, R. (1993). Characterization of erythropoietin isolated from rat serum: biochemical comparison of rat and human erythropoietins. *Bioscience, Biotechnology, and Biochemistry*, 57(11), 1882-1885. <https://doi.org/10.1271/bbb.57.1882>
- [9] Kitamura, T., Tange, T., Terasawa, T., Chiba, S., Kuwaki, T., Miyagawa, K., Piao, Y.-F., Miyazono, K., Urabe, A., & Takaku, F. (1989). Establishment and characterization of a unique human cell line that proliferates dependently on GM-CSF, IL-3, or erythropoietin. *Journal of Cellular Physiology*, 140(2), 323-334. <https://doi.org/10.1002/jcp.1041400219>
- [10] Suresh, S., Rajvanshi, P. K., & Noguchi, C. T. (2020). The many facets of erythropoietin physiologic and metabolic response. *Frontiers in Physiology*, 10, 1534. <https://doi.org/10.3389/fphys.2019.01534>
- [11] Ramos, A. S., Schmidt, C. A., Andrade, S. S., Fronza, M., Rafferty, B., & Dalmora, S. L. (2003). Biological evaluation of recombinant human erythropoietin in pharmaceutical products. *Brazilian Journal of Medical and Biological Research*, 36(11), 1561-1569. <https://doi.org/10.1590/s0100-879x2003001100014>
- [12] International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use. (2005). *Validation of analytical procedures: text and methodology Q2(R1)*. Search at <https://database.ich.org/sites/default/files/Q2%28R1%29%20Guideline.pdf>
- [13] United Nations Office on Drugs and Crime. (2009). *Guidance for the validation of analytical methodology and calibration of equipment used for testing of illicit drugs in seized materials and biological specimens*. United Nations Office on Drugs and Crime. Search at https://www.unodc.org/documents/scientific/validation_E.pdf
- [14] Lee, J. W., Devanarayan, V., Chen Barrett, Y., Weiner, R., Allinson, J., Fountain, S., Keller, S., Weinryb, I., Green, M., Duan, L., Rogers, J. A., Millham, R., O'Brien, P. J., Sailstad, J., Khan, M., Ray, C., & Wagner, J. A. (2006). Fit-for-purpose method development and validation for successful biomarker measurement. *Pharmaceutical Research*, 23(2), 312–328. <https://doi.org/10.1007/s11095-005-9045-3>
- [15] Fang, Z., Tao, X., Liu, S., Liu, Q., Zhang, M., Yang, N., Hu, Z., Jin, T., Tsao, E., Yu, P., & Zhu, W. (2026). Validation of the rapid fluorescent focus inhibition test for rabies virus neutralizing antibodies — China, 2025. *China CDC Weekly*, 8(3), 64–70. Search at <https://pmc.ncbi.nlm.nih.gov/articles/PMC12982118/>
- [16] Markalunas, E. G., Harold, S. E., Arnold, D. H., Martin, J. C., Edenfield, W. J., & Blenda, A. V. (2025). Comparative analysis of manual elisa and ella, an automated instrument for elisa, in measuring serum galectin-3 levels in breast cancer patient samples. *Cancers*, 17(19), 3206. <https://doi.org/10.3390/cancers17193206>