Indonesian Journal of Pharmacy

VOL 37 (2) 2026: 345–354 | RESEARCH ARTICLE

Application of D-Optimal Mixture Design to Optimization and Formulation of Erythropoietin in Nanoparticles Drug Delivery System

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Article Info

Submitted: 03-03-2022
Revised: 24-02-2026
Accepted: 24-02-2026

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ABSTRACT

Erythropoietin (EPO) is a therapeutic glycoprotein widely used in the management of anemia; however, its clinical application is limited by the need for parenteral administration, short biological half-life, and potential systemic adverse effects. Oral delivery of EPO remains challenging due to enzymatic degradation and poor intestinal permeability. This study aimed to optimize and characterize chitosan–pectin-based erythropoietin nanoparticles for oral drug delivery using a D-optimal mixture design approach. Nanoparticles were prepared by ionic gelation, with chitosan and pectin concentrations as independent variables, while particle size, polydispersity index (PDI), zeta potential, and entrapment efficiency served as response parameters. Eight formulations were generated and statistically evaluated using Design-Expert® software. The optimized formulation consisted of 0.097% (w/v) chitosan and 0.013% (w/v) pectin, achieving a desirability value of 0.855, particle size of 360.3 ± 2.80 nm, PDI of 0.307 ± 0.01, zeta potential of 30.83 ± 1.31 mV, and entrapment efficiency of approximately 79.45%. Morphological analysis confirmed the presence of spherical nanoparticles with porous surfaces. In vitro release studies demonstrated an initial burst release followed by sustained release behavior. Release kinetics were best described by the Gomperzl model in acidic medium (pH 1.2) and phosphate-buffered saline (pH 7.4), indicating a non-linear, sigmoid release profile. Overall, chitosan–pectin nanoparticles optimized through D-optimal mixture design show significant potential as an oral delivery system for erythropoietin.

Keywords: Erythropoietin, nanoparticles, D-Optimal mixture design, chitosan, pectin.

INTRODUCTION

Erythropoietin (EPO) is a glycoprotein hormone that plays a crucial role in stimulating red blood cell production in the bone marrow. Recombinant human erythropoietin (rhEPO) has become the standard therapy for anemia associated with chronic kidney disease, chemotherapy-induced anemia, and other conditions that impair erythropoiesis (Yanuartono et al. 2019). Beyond its hematopoietic effects, EPO also exhibits tissue-protective properties, including anti-apoptotic, angiogenic, and anti-inflammatory activities, leading to expanding therapeutic applications. However, conventional administration routes (intravenous or intramuscular) present several challenges, such as short plasma half-life, enzymatic degradation, the need for frequent dosing, and an increased risk of systemic adverse effects including polycythemia and thrombosis (Zhu et al. 2021).

Oral delivery of protein and peptide drugs remains highly challenging due to biological barriers, including proteolytic degradation in the

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gastrointestinal tract, mucus and epithelial barriers, and limited intestinal permeability of macromolecules. Nanotechnology based strategies, particularly nanoencapsulation, have emerged as promising approaches to overcome these limitations by protecting proteins from enzymatic degradation, enabling controlled release, reducing dosing frequency, and enhancing mucosal interactions to improve local or trans-epithelial absorption (Zhu et al. 2021). Several studies have reported polymeric nanoparticles as a viable strategy for oral rhEPO delivery to reduce injection frequency and high-dose-related adverse effects. Nanoparticles improve protein stability, enhance bioavailability, and allow better modulation of drug release profiles (Dhapake and Avari 2019).

Chitosan and pectin are biocompatible polysaccharides extensively studied as nanoparticle-forming materials for oral drug delivery. Chitosan is a cationic polymer due to protonation of its amino groups under acidic conditions, facilitating electrostatic complexation with polyanions or cross-linkers via ionic gelation (Van Bavel et al. 2023). Pectin is an anionic polysaccharide composed mainly of galacturonic acid units, which carries a negative charge at pH values above 3.5 and can form polyelectrolyte complexes (PECs) with chitosan (Quiñones, Peniche, and Peniche 2018). The chitosan–pectin combination enables the formation of multilayer PEC nanoparticles, potentially reducing burst release, improving physicochemical stability, and providing pH- or enzyme-responsive behavior—features particularly relevant for oral protein delivery. Several studies have demonstrated that pectin-coated chitosan nanoparticles enhance gastrointestinal stability and local therapeutic effectiveness following oral administration(Hoang et al. 2022). The ionic gelation method between chitosan and cross-linking agents such as tripolyphosphate (TPP) or polyanions like pectin is widely used for protein-loaded nanoparticle preparation due to its simplicity. However, formulation parameters—including chitosan–pectin ratio, mixing pH, total polymer concentration, addition rate, and ionic strength critically influence particle size, polydispersity index (PDI), zeta potential, entrapment efficiency, and release behavior. Therefore, systematic formulation optimization is required to achieve nanoparticles with suitable stability and oral delivery performance for EPO (Nalini et al. 2022).

D-optimal mixture design is a specialized experimental design for mixture formulations that enables efficient exploration of constrained composition spaces while providing robust predictive models for target responses. This approach minimizes the number of experimental runs while maintaining strong statistical power to identify optimal compositions. The application of D-optimal design in pharmaceutical formulations, including nanoparticle systems, has been widely reported and proven effective for optimizing complex multicomponent mixtures(Mohamad Zen et al. 2015). Accordingly, this study aimed to optimize and evaluate chitosan–pectin-based EPO nanoparticles prepared via ionic gelation using a D-optimal mixture design for oral drug delivery applications.

MATERIALS AND METHODS

Erythropoietin bulk was obtained from PT Kalbio Global Medika. Chitosan and pectin were purchased from Sigma-Aldrich. Glacial acetic acid and hydrochloric acid were supplied by Merck. Deionized water was obtained from One Lab. Rat EPO ELISA kits were purchased from Elabscience. Bio-Rad Protein Assay reagent was used for protein quantification. All other solvents and chemicals were of analytical grade and used without further purification

Optimization and Preparation of Erythropoietin Nanoparticles

Optimization of the erythropoietin nanoparticle formulation was conducted using a D-optimal mixture design with two factors at two levels and three replicates, employing Design-Expert® software version 7.1.5. The independent variables were chitosan concentration (0.01–0.10%) and pectin concentration (0.01–0.10%), while the erythropoietin concentration was fixed at 0.834%. The evaluated responses included particle size, polydispersity index (PDI), zeta potential, and entrapment efficiency. Nanoparticles were prepared by ionic gelation. The chitosan solution was first mixed with erythropoietin using a vortex mixer for 60 seconds at 2–8°C. Subsequently, the pectin solution was added and mixed for an additional 60 second. The volume ratio of chitosan, erythropoietin, and pectin was maintained at 1:1:1 (Kusuma 2016; Syukri et al. 2020).

Table I. Composition and Response of Erythropoietin Nanoparticles

Formula	Factor and level		EPO(%)	Response ± SD (n=3)			
	Chitosan	Pectin (%)		Size (nm)	PDI	ZP (mV)	EE (%)
F1	0.100	0.010	0.046	369.9±3.37	0.447±0.14	28.9±1.86	79.5±1.05
F2	0.033	0.077	0.046	665.6±2.58	0.607±0.09	-14.0±0.66	74.5±2.37
F3	0.089	0.021	0.046	407.2±2.46	0.450±0.09	20.9±2.15	77.8±2.00
F4	0.044	0.066	0.046	630.6±1.37	0.391±0.05	17.1±0.69	79.2±1.57
F5	0.077	0.033	0.046	458.8±2.85	0.438±0.01	23.5±2.12	78.6±1.13
F6	0.010	0.100	0.046	975.9±3.52	0.682±0.13	-21.7±0.26	76.9±0.23
F7	0.055	0.055	0.046	577.3±4.26	0.625±0.09	24.9±0.82	80.3±0.73
F8	0.021	0.089	0.046	812.8±2.69	0.501±0.25	-16.2±1.26	73.6±0.73

Particle Size, PDI, and Zeta Potential Measurement

Particle size and PDI were measured using a Malvern Zetasizer (Malvern Instruments, UK) based on dynamic light scattering (DLS). Samples were placed in cuvettes and analyzed at 25 ± 1°C with a scattering angle of 90°. Zeta potential measurements were conducted using a dip cell accessory. All measurements were performed in triplicate, and results are reported as mean ± standard deviation (Agoston et al. 2016; Dara et al. 2019; Xue et al. 2020).

Entrapment Efficiency Determination

Entrapment efficiency of erythropoietin nanoparticles was quantified using an ELISA kit (Elabscience). Standard EPO solutions (62.5–4000 pg/mL) were prepared by serial dilution according to the manufacturer's protocol. Absorbance measurements were used to determine EPO concentration. Entrapment efficiency was calculated based on the amount of EPO encapsulated relative to the total amount added. All measurements were performed in triplicate and expressed as mean ± SD (Agoston et al. 2016; Dara et al. 2019; Xue et al. 2020).

Determination and Verification of the Optimum Formula

The optimum formulation was identified based on the highest desirability value predicted from contour plots generated using Design-Expert® software. Criteria were set for each response (particle size, PDI, and zeta potential) using defined goals and importance levels. Verification of the optimum formulation was performed by comparing experimental results with predicted values within a 95% confidence interval (Pertiwi et al. 2023).

Morphological Analysis

Nanoparticle morphology was examined using transmission electron microscopy (TEM) and scanning electron microscopy (SEM). For TEM analysis, samples were placed on copper grids, carbon-coated, stained with 2% phosphotungstic acid, and air-dried. SEM samples were gold- coated and observed at an accelerating voltage of 5–10 kV (Edam and Roomi 2024; Hwang 2020).

In Vitro Release Study

In vitro release studies were conducted using a controlled agitation method followed by ultracentrifugation. EPO nanoparticles (3 mL) were incubated in 6 mL of HCl buffer (pH 1.2) or PBS buffer (pH 7.4) at 37°C with shaking at 50 rpm for 4 hours. Samples were withdrawn at predetermined intervals and analyzed using visible spectrophotometry at 595 nm after reaction with Bio-Rad Protein Assay reagent (Bagherpour Zarchi et al., 2017; Dara et al., 2019).

RESULTS AND DISCUSSION

Optimization and Characterization of Erythropoietin Nanoparticles

The D-optimal mixture design generated eight experimental formulations with varying chitosan and pectin concentrations (Table I). The particle size of the EPO nanoparticles ranged from 369.9 ± 3.37 nm to 975.9 ± 3.52 nm. The smallest particle size was observed in formulation F1 (369.9±3.37 nm), while formulation F6 exhibited the largest particle size (975.9±3.52 nm). The lowest polydispersity index (PDI) was obtained for formulation F4 (0.391±0.05), indicating a relatively narrow particle size distribution. The highest zeta potential value was recorded for formulation F1 (28.9±1.86 mV), whereas the highest entrapment efficiency was achieved by formulation F7 (80.3±0.73%).

Table II. ANOVA summary for the measured responses (analyzed via Design Expert® v. 7.1.5).

Respond	Particle Size	Polydispersity Index	Zeta Potential	Entrapment Efficiency
p-value of the model	0.0001 (p-value < 0.05)	0.0383 (p-value < 0.05)	0.0001 (p-value < 0.05)	0.0070 (p-value < 0.05)
Lack of fit analysis	F 0.40 p-value 0.9256	F 1.04 p-value 0.4758	F 2.79 p-value 0.0563	F 6.11 p-value 0.0028
Equations model	Linier Y = 339.99A + 898.91B	Linier Y = 0.43A + 0.67B	Cubic Y = 26.95A + 21.01B – 15.39AB + 34.09AB(A-B)	Linier Y = 79.59A + 75.61B

Particle Size Analysis

The particle size range observed in the present study (369.9–975.9 nm) is consistent with previously reported EPO nanoparticle systems formulated using biodegradable polymers. Colloidal nanoparticle systems encapsulating drugs typically exhibit particle diameters between 1 and 1000 nm (Zielińska et al. 2020). The results obtained in this study closely resemble those reported for chitosan–carrageenan EPO nanoparticles (200–1000 nm) (Bulmer et al. 2012). Polynomial equations derived from the D-optimal mixture design indicated that a linear model best described the particle size response (Table II). ANOVA analysis demonstrated a highly significant model (p = 0.0001) with a lack-of-fit value of 0.40, confirming the adequacy of the model. Both chitosan and pectin concentrations positively influenced particle size, with pectin exerting a stronger effect, as reflected by its higher regression coefficient (898.91). Increasing pectin concentration resulted in larger particle sizes, likely due to enhanced polyelectrolyte complex formation and increased matrix density.

The dimensions of the synthesized EPO nanoparticles (369.9–975.9 nm) are comparable to those of chitosan–carrageenan systems (200–1000 nm), SLS-EPO nanoparticles (280 nm), and EPO-loaded poly(DL-lactide-co-glycolide) nanoparticles (225.9 nm). Conversely, the particles produced in this study are significantly larger than Gemini-EPO nanoparticles, which have been reported at 150 nm (Bagherpour et al. 2017; Dara et al. 2019; Fayed et al. 2012).

Polydispersity Index Analysis

The Polydispersity Index (PDI) serves as a critical indicator of particle size homogeneity within a dispersion. PDI values approaching zero represent a highly homogenous system, whereas values exceeding 0.5 indicate significant heterogeneity (Avadi et al. 2010). Theoretically, a PDI below 0.05 characterizes a monodisperse system, while a PDI greater than 0.7 suggests a very broad size distribution(Danaei et al. 2018). In this study, the eight EPO nanoparticle formulations exhibited PDI values ranging from 0.391 to 0.682 (Table I), indicating that all formulations maintained a relatively acceptable level of dispersion homogeneity.

Statistical optimization using a D-optimal mixture design identified a linear model as the most suitable fit for the data (Table II). ANOVA results demonstrated the model's significance (p = 0.0383) with a non-significant lack of fit (1.04), confirming that the model effectively represents the observed data without significant deviation. Furthermore, the normality test at a 95% confidence level displayed a linear pattern without specific outliers, reinforcing the model's reliability. The linear equation revealed that both chitosan and pectin proportions positively influence the PDI. However, pectin exerted a more substantial effect, evidenced by its higher coefficient of 0.67. Consequently, an increase in pectin concentration leads to an elevated PDI. Mechanistically, this phenomenon can be attributed to the interaction between opposing charges within the nanoparticle formulation, which leads to partial neutralization. This state facilitates the coalescence of colloidal particles, resulting in larger particle diameters and, subsequently, a broader polydispersity index.

Comparatively, previous studies have reported PDI values for EPO nanoparticles using chitosan-carrageenan (0.2–0.35) and solid lipid nanoparticles (SLN) (0.282), which are often categorized as monodisperse systems when the PDI is maintained at low thresholds(Bulmer, Margaritis, and Xenocostas 2012; Dara et al. 2019). Although the PDI in this chitosan-pectin system

(0.307 in optimized conditions) is slightly higher, it remains within the functional range for stable nanoparticulate drug delivery systems

Zeta Potential Analysis

Zeta potential is utilized to predict the stability of nanoparticle formulations and characterize the particle surface charge. A high particle charge prevents aggregation within the buffer solution. Specifically, zeta potential values exceeding ±30 mV indicate excellent stability, whereas values around ±20 mV provide short-term stability conversely, values near ±5 mV typically lead to particle aggregation. The zeta potential measurements for EPO nanoparticles ranged from 14.0 to 28.9 mV, exhibiting both positive and negative charges that suggest short-term stability (Table I). ANOVA results yielded a significance value of 0.0001 with a lack-of-fit value of 2.79, indicating a robust model fit with no significant discrepancies from the observed data. The normal distribution test, performed at a 95% confidence level, followed a cubic pattern.

The functional relationship between the factors (chitosan and pectin concentrations) and the zeta potential (Table II). Both chitosan and pectin concentrations exhibited a positive response toward the zeta potential, whereas the interaction between the two factors showed a negative response. Previous studies indicate that Gemini-EPO nanoparticles and recombinant human erythropoietin encapsulated in chitosan-carrageenan nanoparticles share a similar positive charge with the chitosan-pectin EPO nanoparticles developed in this study. However, the highest zeta potential values were observed in chitosan-carrageenan EPO nanoparticles (+40 to +55 mV), while the lowest was recorded for Gemini-EPO nanoparticles at +12.81 mV (Bagherpour et al. 2017; Bulmer et al. 2012).

The resulting zeta potential values are attributed to the formation of nanoparticles via the ionic gelation method, where the positive charge generated from chitosan protonation is neutralized through electrostatic interactions with the negative charge of pectin. The positive zeta potential is influenced by the concentration and ratio of chitosan; higher concentrations and ratios lead to increased protonated NH3+ groups, thereby elevating the zeta potential. The zeta potential of EPO nanoparticles shifts to a negative charge when the pectin concentration exceeds 0.077%. Nanoparticles synthesized with chitosan and its derivatives typically possess a positive surface charge and mucoadhesive properties, allowing them to adhere to mucous membranes for sustained drug release (Table II). Upon reaching the mucosal layer, these positively charged nanoparticles bindto the negatively charged mucus through electrostatic attraction, facilitating time-dependent drug delivery (Mohammed et al. 2017).

Entrapment Efficiency

The entrapment efficiency (EE) values range from 73.6% to 80.3% (Table I) Statistical analysis performed via D-Optimal Mixture Design software confirmed that the data fit a linear model, yielding a significant p-value of 0.0070 and a lack-of-fit value of 6.11. These results indicate that the model adequately represents the observed data with no significant deviations. Consequently, since the EE values were consistently high and showed minimal variation across the design space, this parameter was not utilized as a primary determinant for establishing the optimum formulation. Furthermore, a comparative analysis reveals that the EPO nanoparticles synthesized using the chitosan-pectin polymer matrix achieved significantly higher entrapment efficiency (73.6–80.3%) than those reported in previous studies. Specifically, our results surpass the EE of recombinant human erythropoietin encapsulated in chitosan-carrageenan nanoparticles (47.97 ± 4.10%), erythropoietin-loaded SLS nanoparticles (43.4%), and EPO-loaded poly(DL-lactide-co-glycolide) (PLGA) nanoparticles (33.3%) (Bulmer et al. 2012; Dara et al. 2019; Fayed et al. 2012). This substantial improvement suggests that the chitosan-pectin complex provides a superior matrix for the efficient loading of EPO.

Optimization and Verification of the Optimum Formulation

The selection of the optimum formula was based on the critical quality attributes (CQAs), including particle size, polydispersity index (PDI), and zeta potential. A numerical optimization approach was employed to determine the most favorable composition. The target values were evaluated using the desirability function, which ranges from 0 to 1. A desirability value approaching unity indicates that the formula effectively meets the predefined target criteria for each variable. The contour plots and the resulting desirability score were generated by establishing specific criteria for factors and responses, categorized by their respective goals and importance levels (Table III).

Table III. List of response criteria for optimization and optimum formula verification results of erythropoietin nanoparticles

Respond	**Goal**	**Importance**	**Lower Limit**	**Upper Limit**
Particle Size	Minimize	+++	367.4 nm	973 nm
PDI	Minimize	++	0.291	0.784
Zeta Potential	Maximize	++	13.3	30.6
Optimum formula verification results				
Respond	95% CI low	95% CI high	Test Value	
Particle Size	298.05	420.49	360.3± 2.80 nm	
PDI	0.33	0.53	0.307 ± 0.01	
Zeta Potential	24.52	30.08	30.833 ± 1.31 mV	

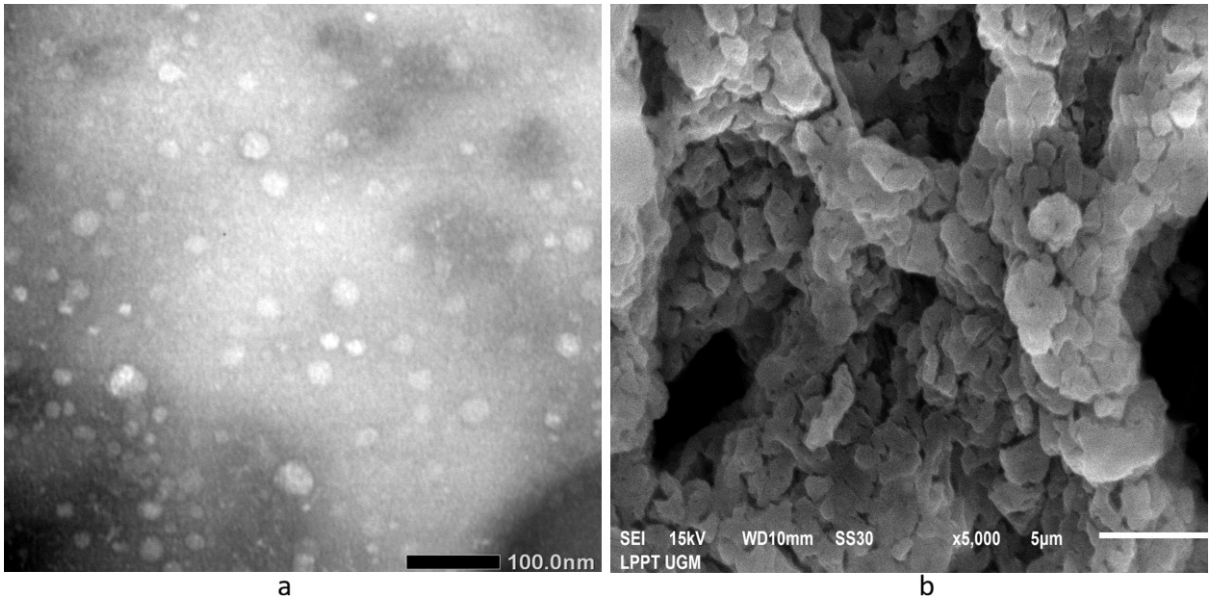


Figure 1. Morphological characterization of the nanoparticles: (a) TEM image at 40,000× magnification and (b) SEM image at 5,000× magnification.

Based on the analysis performed using Design-Expert® software (version(Bhumkar and Pokharkar 2006) 7.1.5), the recommended optimum composition consists of 0.097% w/v chitosan and 0.013% w/v pectin, yielding a desirability value of 0.855. This score implies that the predicted characteristics of the nanoparticles have an 85.5% degree of congruence with the idealized target responses.

To validate the reliability of the model, a verification study was conducted on the optimized formula. The experimental results for all responses fell within the 95% confidence interval (CI) low and high ranges (Table III). These findings demonstrate a high level of agreement between the predicted values from the Design-Expert software and the empirical experimental data, thereby confirming the robustness and predictive power of the mathematical model.

Morphological Analysis of EPO Nanoparticles

The morphological characteristics of the EPO nanoparticles were investigated using Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM). TEM analysis was performed to elucidate the internal structure and shape of the nanoparticles. The EPO nanoparticles exhibited a distinct spherical morphology (Figure 1a). This spherical symmetry indicates a successful entrapment of EPO within the chitosan matrix and suggests an efficient cross-linking process with pectin through robust electrostatic interactions.

Table IV. Comparative DDSolver Kinetic Analysis of Erythropoietin Release from Nanoparticles in HCl pH 1.2 and PBS pH 7.4.

Model	HCl pH 1.2			PBS pH 7.4		
	Adjusted R^2	AIC	MSC	Adjusted R^2	AIC	MSC
Korsmeyer–Peppas	0.9997	3.6546	5.6969	0.9929	32.5996	3.1548
Weibull	0.9998	0.7233	6.0226	0.9998	0.7233	6.0226
Logistic	0.9998	−0.0695	6.1107	0.9998	−0.0695	6.1107
Gompertz	**0.9998**	**−0.9394**	**6.2073**	**0.9998**	**−0.9394**	**6.2073**

Furthermore, SEM imaging was utilized to examine the surface topography of the nanoparticles. (Nandee et al. 2024) The results revealed that the EPO nanoparticles possess an irregular and porous surface (Figure 1b). The presence of a hollow or porous morphology in the nanoparticle powder suggests that the chitosan-pectin framework effectively encapsulates the EPO, creating a structured matrix through successful polymer cross-linking. These surface characteristics are often associated with a high surface-area-to-volume ratio, which is beneficial for controlled drug release applications.(Bhumkar and Pokharkar 2006).

In Vitro Release Study of EPO Nanoparticles

The release kinetics of erythropoietin (EPO) from nanoparticles were evaluated in **HCl pH 1.2** and **PBS pH 7.4** using DDSolver to elucidate the influence of environmental pH on the release mechanism. Multiple kinetic models were fitted to the experimental data, and model suitability was assessed using adjusted coefficient of determination (Adjusted R^2), Akaike Information Criterion (AIC), and Model Selection Criterion (MSC). Across both media, **sigmoidal-based models (**Korsmeyer–Peppas, **Gompertz, Logistic, and Weibull)** exhibited substantially higher goodness-of-fit compared to classical diffusion-based models (Table IV).

Under simulated gastric conditions (HCl pH 1.2), all models demonstrated exceptionally high Adjusted R^2 values (0.9997–0.9998), indicating an excellent agreement between the predicted curves and the experimental release data. However, the primary discriminatory parameters were the AIC and MSC values. The Gompertz model exhibited the lowest AIC value (−0.9394) and the highest MSC value (6.2073), followed by the Logistic and Weibull models. In contrast, the Korsmeyer–Peppas model showed the highest AIC value (3.6546) and the lowest MSC value (5.6969), suggesting that it was less representative compared to the other models. The predominance of the Gompertz model indicates that the release profile in the acidic medium follows an asymmetric sigmoidal pattern, which typically reflects a gradual release mechanism characterized by an initial lag phase, followed by an acceleration phase and subsequently a deceleration phase approaching a plateau. This behavior suggests that under acidic conditions, the matrix system maintains its structural integrity during the early stage of exposure. Consequently, erythropoietin release does not occur through simple diffusion alone but rather through a combined mechanism involving diffusion and matrix relaxation.

Furthermore, the consistently high goodness-of-fit values observed across all non-linear models indicate that drug release at pH 1.2 is relatively well-controlled and does not exhibit significant burst release, which is essential for preserving protein stability under gastric conditions.

In PBS medium at pH 7.4 (simulating intestinal conditions), the differences among models became more pronounced. The Korsmeyer–Peppas model exhibited reduced performance, with an Adjusted R^2 value of 0.9929, a markedly high AIC value (32.5996), and a low MSC value (3.1548). These results indicate that the release mechanism under neutral–alkaline conditions cannot be adequately described by a simple semi-empirical diffusion model alone. In contrast, the Gompertz, Logistic, and Weibull models again demonstrated excellent performance (Adjusted R^2 = 0.9998). Among them, the Gompertz model showed the lowest AIC value (−0.9394) and the highest MSC value (6.2073), confirming that it was statistically the most appropriate model to describe erythropoietin release in PBS pH 7.4. The suitability of a sigmoidal model in PBS suggests that erythropoietin release at pH 7.4 likely involves matrix swelling, polymer relaxation, and increased porosity resulting from the ionization of functional groups. The neutral pH environment facilitates

expansion of the matrix network, thereby accelerating protein diffusion following the initial lag phase. Therefore, the release mechanism is not governed by purely Fickian diffusion but rather by a more complex process dependent on dynamic structural changes within the matrix

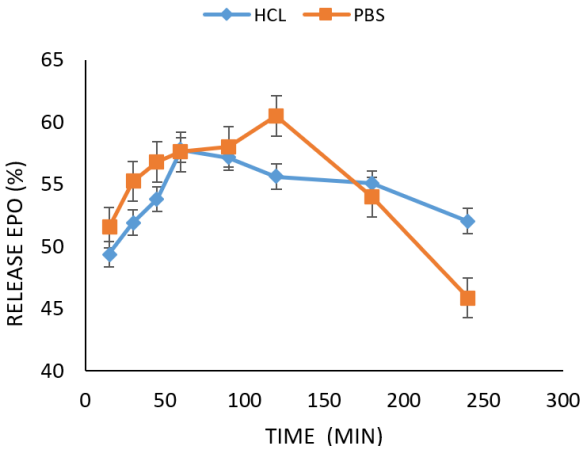


Figure 2. Release profiles of EPO from nanoparticles in HCl buffer (pH 1.2) and PBS (pH 7.4).

A significant initial burst release of Erythropoietin (EPO) from the matrix system was observed within the first 15 minutes (Figure 2). The cumulative release reached 51.51% in HCl buffer (pH 1.2) and 49.36% in PBS (pH 7.4). This rapid initial release is likely attributed to the fraction of EPO that is adsorbed on the surface or remains unencapsulated within the nanoparticle system. By the 240-minute mark, a distinct variation in the drug release profile emerged, with the amount of EPO released in HCl being approximately 6.17% higher than in PBS. This discrepancy is closely related to the pH-dependent solubility of both the drug and the polymer. Chitosan, which possesses a pKa of 6.5, contains amine groups that are readily protonated and soluble in acidic environments. Similarly, EPO, which has an isoelectric point (pI) of 5.1, exhibits high solubility at pH 1.2. Consequently, the acidic medium facilitates faster dissolution of the matrix and the cargo.

In contrast, the lower release rate observed in PBS (pH 7.4) can be explained by the reduced solubility of chitosan in alkaline media. In this environment, the chitosan-pectin matrix remains more intact, acting as a physical barrier that restricts medium diffusion into the polymer network. This results in a slower diffusion-controlled release of EPO. The release kinetics of EPO from the chitosan-pectin framework are fundamentally governed by the cross-linking density, morphological integrity, and particle size of the nanoparticles. The release mechanism from this hybrid matrix is hypothesized to involve drug diffusion through a swollen rubbery matrix, which accounts for the substantial cumulative release observed (60.50%). Despite the initial burst, the chitosan-pectin nanoparticle system remains a promising vehicle for enhancing the oral bioavailability of EPO by protecting it from premature enzymatic degradation and facilitating controlled intestinal uptake (Mohammed et al., 2017).

CONCLUSION

This study demonstrates that the oral delivery performance of erythropoietin-loaded chitosan–pectin nanoparticles is primarily governed by the structural integrity of the polyelectrolyte complex and its pH-responsive release behavior. The consistent dominance of sigmoidal release kinetics, best described by the Gompertz model under both gastric and physiological conditions, indicates effective modulation of protein release across gastrointestinal environments. Electrostatic interactions within the polymeric matrix play a critical role in maintaining matrix stability, limiting premature release in acidic conditions, and enabling controlled release under absorption-relevant conditions. Together with the rational formulation approach enabled by D-optimal mixture design, these findings support the translational potential of chitosan–pectin nanoparticles as a robust and controllable platform for oral protein delivery.

ACKNOWLEDGMENTS

This research was funded by the Ministry of Research, Technology, and Higher Education of the Republic of Indonesia through the BPPDN (Beasiswa Pendidikan Pascasarjana Dalam Negeri) scholarship program and PDD (Pendidikan Doktor Disertasi) scheme.

CONFLICT OF INTEREST

"The authors declare no conflict of interest".

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