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Preparation, Optimization, and in Vitro Evaluation of Alendronate Sodium Encapsulated Nanoparticles for the Treatment of Osteoporosis

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ABSTRACT:

This study aimed to prepare, optimize, and in vitro assessment of alendronate sodium (ALD) encapsulated poly (DL-lactide-co-glycolide) (PLGA) nanoparticles, which is prepared by $W_1/O/W_2$ solvent evaporation method for the first time to overcome the issues related with the treatment of osteoporosis. Nanoparticles loaded with ALD were synthesized by varying the volumes of the organic phase (O) and the water phase (W2), as well as the percentages of PVA and the quantities of PLGA. These parameters were optimized to assess their impact on encapsulation efficiency and loading percentage, employing a central composite experimental design (CCD) for the evaluation. Particle size, shape surface morphology, and in vitro drug release were assessed using the optimized formulation. A two-factor interaction (2FI) model was suggested to describe the relationships between the variables and EE %, while a quadratic regression model was suitable for loading percentage. The optimized formulation showed an average diameter of 236 nm with a polydispersity index of 0.06 %, encapsulation efficiency of 34.6%±2.26, and loading % of 23.9% ±1.32. The prepared nanoparticles had a spherical shape, and the in vitro drug release profile appeared biphasic, showing an initial burst release of 10.33% in the first hour, followed by a sustained release for up to 24 hours. The optimization of ALD-loaded PLGA nanoparticles, achieved by applying the central composite design (CCD) methodology, facilitated the forecasting of the characteristics of nanoparticles produced by the $W_1/O/W_2$ solvent evaporation process employing PLGA polymer. Consequently, this improved model presents significant prospects for application in a controlled release system, especially within the framework of osteoporosis treatment.

Keywords: Alendronate sodium; PLGA nanoparticles; Central composite design; Loading percentage; Encapsulation efficiency.

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1. Introduction

Alendronate sodium (ALD) (4-amino-1-hydroxybutane-1, 1-diphosphonic acid sodium salt) is a nitrogen-containing bisphosphonate that is extensively recommended for the treatment of osteoporosis. Osteoporosis is a common bone disease characterized by low bone mass and increased bone fragility, putting patients at risk of fractures, disability, and mortality [1, 2].
ALD belongs to BCS Class III, meaning it has high solubility and low permeability. The absorption rate of commercially available tablets is approximately 0.6%, which decreases when food or divalent ions, such as calcium, are present. The poor systemic bioavailability (<1%) may result from its high hydrophilicity and the negative charges that hinder crossing the lipoid biomembrane of the gastrointestinal tract. Also, oral administration of alendronate sodium may result in esophageal side effects (e.g., esophagitis, bleeding, and erosions), osteonecrosis of the jaws, and musculoskeletal pain. Therefore, the efficacy and safety of alendronate sodium are strictly dependent on the complicated process

of the rational use of medicines (RUM) specified by the world health organization (WHO), including the availability of efficient and quality medicine as well as appropriate prescription and special conditions of use [3-8].
In order to obtain more edificant drugs with improved bioavailability and fewer side effects, nanomedical strategies such as encapsulating the therapeutic compounds into nanostructures have been well appreciated [9]. The nanocarriers for oral administration can be designed with different materials and architectures, such as liposomes and other lipidic nanostructures, niosomes, polymeric nanoparticles, dendrimers, and cubosomes. Optimization of some factors such as encapsulation rate, physical and chemical stability, biodegradability, cytotoxicity, and production costs is required to achieve well-engineered nanoparticle-based delivery systems [10, 11].
Poly-lactide-co-glycolide (PLGA) is a versatile polymer that has been approved as a drug delivery system by the Food and Drug Administration (FDA) and the European Medicine Agency (EMA) as a result of its biodegradability, biocompatibility, and immune neutral properties. Both hydrophobic and polar groups in their structures have made PLGA nanoparticles good candidates for encapsulating hydrophobic and hydrophilic drugs [12]. Factors such as initial molecular weight, the ratio of lactide to glycolide, exposure to water, and storage temperature may affect the physical properties of PLGA. Molecular weight and polydispersity index may influence the mechanical properties of PLGA and its performance as a drug carrier [13]. PLGA-based delivery systems have been studied as versatile carriers for the encapsulation of therapeutic molecules such as oxaliplatin, curcumin, paclitaxel, risperidone, paeonol, micronized triamcinolone acetonide, bone morphogenetic protein (BMP-2), trolamine salicylate, antisense oligonucleotide [14, 15].
PLGA-based nanoparticles for delivering ALD were previously developed using the nanoprecipitation method, with optimization conducted for various parameters in earlier research [16, 17]. The current study introduces a novel approach by employing the double emulsion solvent evaporation technique to fabricate PLGA nanoparticles while optimizing various formulation parameters.

2. Materials & Methods

2.1. Materials

The alendronate standard was provided by Rosamed Group (IRAN) as a gift sample. Resomer®RG502, Poly (D, L-lactide-co-glycolide) (50:50), MW 7000-17000 g/mol and polyvinyl alcohol (PVA) (MW 13000-23000 g/mol, 87-89 % hydrolyzed) was obtained from Sigma-Aldrich (United States). Water used in the experiments was prepared by reverse osmosis and passed through a 0.22 µm Millipore® filter (Millipore Company, USA) before use. The dialysis bag with a 12,400 g/mol cut-off size was from Sigma-Aldrich (USA). All additional chemical substances utilized were of pharmaceutical quality.

2.2. Fabrication of ALD-Loaded Nanoparticles

ALD-loaded nanoparticles were produced by the $W_1/O/W_2$ solvent evaporation method. First, an aqueous solution of sodium alendronate was made (W_1). Then, known amounts of PLGA polymer were dissolved in a certain amount of dichloromethane (O). The aqueous and organic solutions were emulsified by homogenizing (T 18 digital ULTRA-TURRAX®, IKA, Germany) at 21000 rpm for 5 minutes (initial emulsion W_1/O). Then, specific concentrations of the aqueous polyvinyl alcohol (PVA) solution were prepared (W_2). The initial emulsion was added to W_2 under homogenization at 14000 rpm for 4 minutes to form a $W_1/O/W_2$ emulsion. Then, the evaporation of dichloromethane was done at ambient temperature and by a rotary evaporator (Hei-VAP Core, Heidolph, Germany) within a few hours. The resulting suspension was centrifuged by ultracentrifuge (Optima L90 K, Beckman Coulter, USA) at 40000 rpm and 4 °C for 30 min. Nanoparticles were centrifuged three times in distilled water to eliminate free drugs and excess surfactants. Finally, the particles were freeze-dried and stored in the refrigerator under vacuum desiccators for investigation.

2.3. Experimental design

Response Surface Methodology (RSM) with a central composite design (CCD) was applied to the experiments [18, 19]. The selection of variables was informed by prior research examining the influence of process variables on the optimization of PLGA nanoparticles [20]. The screening was used to determine the ranges for the organic phase volume (O), water phase volume 2 (W_2), PVA %, and PLGA quantity (mg) and find out the maximum and minimum concentrations in the formulation. The desired design (alpha; $\alpha=\pm1.5$) with the factors (independent variables) investigated in the current paper are shown in Table 1. The volume of W_1 was considered 1 ml. The response functions (dependent variables) associated with encapsulation efficiency (%) and loading % were also studied. Design-Expert® (version 7.0.0, Stat Ease, Inc, MN, USA) was applied for data regression analysis, assuming a quadratic model within which the factors interacted.
The significance of individual factors, binary interactions, and quadratic terms associated with the impact on the investigated responses was identified by analysis of variance (ANOVA).

Table 1. Basic characteristics of participants with and without NAFLD**.**

Levels	Organic Phase Volume (O)	Water Phase Volume 2 (W_2)	PVA %	PLGA Quantity (mg)
Minus alpha (α= -1.5)	2.5	0	0.25	0
Low	5	1	0.5	20
Central point	7.5	25.5	0.75	110
High	10	50	1	200
Plus alpha (α= +1.5)	12.5	74.5	1.25	290

The coefficient $p<0.05$ represented the significance of the factors. The non-significant variables were removed from the model unless necessary to maintain the hierarchy as a parent term for significant interactions. The mathematical model design focused on maximizing the encapsulation efficiency (%) and loading %.

2.4. Freeze-drying of Alendronate-Loaded Nanoparticles

The nanoparticles underwent freeze-drying using a Christ Alpha 1-2 LD plus apparatus (Germany) to obtain a white, cotton-like product. This procedure was adapted from the methodology established by Holzer et al. [21]. Initially, the samples were frozen at -50 °C for 3 hours. Subsequently, primary drying was carried out at a shelf temperature of -40 °C and a pressure of 5×10^{-2} mbar for 5 days. This was followed by a secondary drying phase, where the shelf temperature was raised to 20 °C and the pressure adjusted to 2.5×10^{-2} mbar for an additional 2 days. After a total duration of 7 days, the vials were sealed and removed from the apparatus.

2.5. Characterization of Alendronate-Loaded Nanoparticles

2.5.1. Determination of ALD encapsulation efficiency and loading percentage

Initially, 20 mg of freeze-dried nanoparticle powder was dissolved in 1 mL of acetonitrile. 2 mL of methanol was added to precipitate the polymer. Centrifuging the prepared nanoparticle samples was then carried out for 5 min at 14000 rpm, and 500 µL of aliquot taken from the supernatant was evaluated using the UV method described by Asad Gulzar et al. [22].
A linear calibration curve for ALD measurement was obtained over the range of standard concentrations of ALD at 1-200 µg/mL with a correlation coefficient of R2 = 0.9993. The encapsulation efficiency and loading percentage were obtained using the following equations:

Eq. 1: $$\text{EE \%} = \frac{\text{amount of ALD in nanoparticles}}{\text{amount of ALD used in the nanoparticle preparation}} \times 100$$

Eq. 2: $$\text{Loading \%} = \frac{\text{amount of ALD in nanoparticles}}{\text{amount of nanoparticles}} \times 100$$

2.5.2. Size measurement

The systems ' particle size and polydispersity index were assessed through photon correlation spectroscopy (PCS) utilizing a Zetasizer, Nano-ZS (Malvern Instruments, United Kingdom). Three measurements were done for all freeze-dried samples initially diluted with ultrapure water and placed in quartz cuvettes.

2.5.3. Particle morphology

Scanning electron microscopy (SEM) (Tescan Mira, Czechoslovakia) was utilized to study the microstructure of the ALD-loaded nanoparticles. The samples were prepared and gold-coated on aluminum stabs before examination by SEM.

2.5.4. In vitro drug release

Dialysis-based drug release testing utilized a cellulose acetate dialysis membrane on freeze-dried nanoparticles and the free form of ALD in triplicates. Initially, the samples were prepared by suspending the optimum formulation of ALD nanocarriers in phosphate buffer solution (pH 7.4) (2 mL) inside a dialysis bag. Controlled magnetic stirring at 37 °C was done on samples placed in 50 mL of the buffer solution (sink condition). 1 mL aliquots of the medium were collected periodically for analysis with high-performance liquid chromatography (HPLC).

2.6. HPLC analysis

An HPLC method was employed to analyze ALD using an Agilent 1100 system from the United States. The stationary phase comprised a L21 column (5 µm, 250 mm × 4.1 mm, Phenomenex, CA, USA). The mobile phase was formulated from a buffer solution containing 14.7 g/L of sodium citrate dihydrate and 7.05 g/L of anhydrous dibasic sodium phosphate, along with acetonitrile and methanol in a ratio of 70:25:5. The analysis was performed at a wavelength of 266 nm, with

a flow rate set at 1.2 mL min−1 and an injection volume of 10 µL. The column temperature was maintained at 35 ºC, and the quantification of the analyte was based on the peak area obtained at the specified retention time.

3. Results & Discussion

3.1. Encapsulation efficiency (EE %) and loading percentage of the formulations

Based on RSM (CCD), thirty formulations were designed indicating the amounts of organic phase volume (O), water phase volume 2 (W_2), PVA %, and PLGA quantity (mg) (Table 2).

Figures 1 and 2 illustrate the perturbation plots for the effects of independent factors on a specific response, with all other characteristics maintained constant. A steep slope or curve indicates that the reaction to a given element is very sensitive, while flat lines are indicators of insensitivity to change in that specific factor.

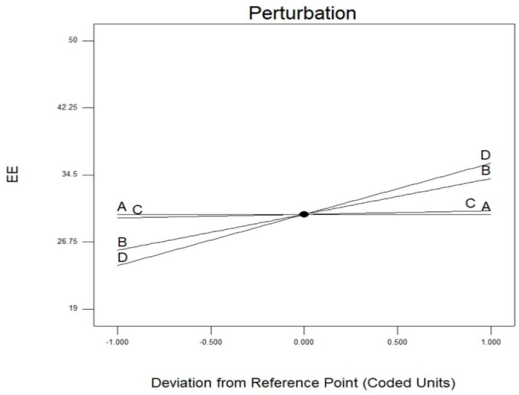

Figure 1. Perturbation plot for the effect of independent variables on encapsulation efficiency, where A, B, C and D are organic phase volume, water phase volume 2, PVA % and PLGA quantity, respectively.

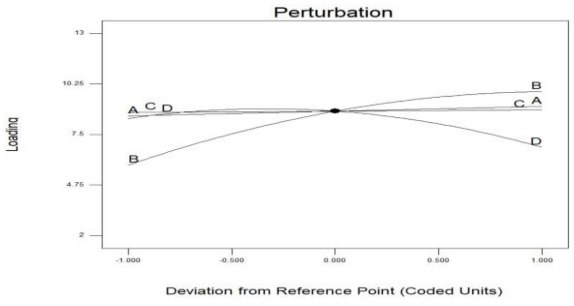

Figure 2. Perturbation plot for the effect of independent variables on loading %. Where A, B, C and D are organic phase volume, water phase volume 2, PVA % and PLGA quantity, respectively.

The results of response functions, including EE % and loading %, are shown in Table 2. EE % and loading % were 19.13-49.98 % and 2.65-12.65%, respectively. The EE% obtained was higher than the amount reported in some previous studies on the formulation of Alendronate-loaded nanoparticles. Design of polycaprolactone (PCL)-based noncarriers as the alendronate delivery system resulted in 15.12-34.21 % encapsulation efficiencies using the double emulsion technique and 0.36-18.8% with the nanoprecipitation method. Also, the highest drug loading reported for PCL-based nanoparticles produced with nanoprecipitation was 8.2% [23].

Therefore, applying PLGA and W/O/W solvent evaporation methods may result in a superior system for alendronate incorporation.

The results of ANOVA for the responses are tabulated in Tables 3 and 4. The F values for both models generated for EE % and loading % were statistically significant, with 6.67 and 6.30, respectively.

When considering EE% as the response function, the linear coefficients of water phase volume (B) and PLGA quantity (D), interaction coefficients of organic phase volume and PLGA quantity (AD), as well as that of the water phase volume and PLGA quantity (BD) were statistically significant ($p<0.05$) (3).

Regarding the loading % as the response, Table 4 shows the statistical significance of the linear coefficients of water phase volume (B) and PLGA quantity (D) and their squared coefficients. Also, the interaction coefficients of water phase volume and PLGA quantity (BD) were significant ($p<0.05$).

A two-factor interaction (2FI) model was suggested to describe the relationships between the variables and EE %, while a quadratic regression model was suitable for loading %. The regression models obtained for EE % and Loading % based on ANOVA of the independent variables were as follows:

Eq.3: $EE\ \% = 14.40+1.16x_1-0.01\ x_2+0.10x_3-0.01\ x_1x_3+1.70x_2x_3$

Eq. 4: $Loading\ \% = 2.54+0.25x_2+0.04x_3-8.25x_2x_3 - 1.56x_{22}-1.46x_{32}$

X_1, x_2, and x_3 are the organic phase volume, water phase volume (2), and PLGA quantity, respectively.

3.1.1. Effect of PVA concentration

The data presented in Figures 1 and 2 demonstrate that variations in PVA concentration have minimal impact on encapsulation efficiency and loading percentage. This is in good accordance with the previous study [24]. PVA was previously demonstrated to serve as an emulsifier in the external aqueous phase (W_2), aiding in forming the $W_1/O/W_2$ double emulsion. At this initial emulsification stage, PVA was absent and, therefore, could not

Table 2. CCD-based formulation designs and the response functions comprising of EE % and Loading %**.**

Run	Organic Phase Volume (A)	Water Phase Volume 2 (B)	PVA % (C)	PLGA Quantity (mg) (D)	EE %	Loading %
1	5	1	0.5	200	21.16	4.35
2	7.5	25.5	0.75	110	30.67	8.71
3	7.5	-23.5	0.75	110	---	---
4	10	1	0.5	20	20.97	3.45
5	10	1	1	200	29.98	5.9
6	5	50	0.5	200	49.98	5.43
7	5	1	1	200	27.09	5.72
8	7.5	25.5	0.75	110	35.54	9.12
9	7.5	25.5	0.75	110	32.33	8.99
10	7.5	25.5	1.25	110	25.61	8.18
11	7.5	25.5	0.75	290	47.13	3.56
12	10	50	1	200	40.19	6.8
13	2.5	25.5	0.75	110	28.25	8.93
14	10	50	0.5	200	30.94	4.93
15	7.5	25.5	0.75	110	32.18	9.07
16	5	50	1	200	47.72	4.78
17	5	1	1	20	20.65	2.67
18	10	50	0.5	20	27.42	12.45
19	10	50	1	20	25.9	12.05
20	7.5	25.5	0.75	110	33.65	9.16
21	7.5	25.5	0.25	110	35.32	9.56
22	10	1	0.5	200	24.78	5.64
23	10	1	1	20	28.72	2.65
24	12.5	25.5	0.75	110	29.06	9.12
25	7.5	74.5	0.75	110	35.67	10.12
26	5	50	1	20	24.25	12.65
27	5	1	0.5	20	20.7	5.67
28	7.5	25.5	0.75	110	33.67	9.06
29	7.5	25.5	0.75	-70	---	---
30	5	50	0.5	20	19.13	6.76

influence the process, which primarily involved the entrapment of aqueous ALD within the organic droplets composed of PLGA [24].

3.1.2. Effect of PLGA concentration

The data in Figures 1 and 2 indicate that the quantity of PLGA present in the intermediate organic phase significantly affects the encapsulation efficiency.
Figures 3a and 3b illustrate the interaction effects between the volume of the organic phase and the quantity of PLGA, as well as the volume of the aqueous phase and the quantity of PLGA, respectively. The highest EE% was obtained with the highest amount of PLGA and the lowest amount of organic phase volume. The positive effect of higher amounts of PLGA on EE % has been reported in previous studies on PLGA-based nanoencapsulation of cinnamic acid [14].
The results indicate that elevating the concentration of the polymer while simultaneously decreasing the volume of the organic phase results in an increase in the viscosity of the organic phase and a reduction in net shear stress. This change contributes to increased particle size and provides a greater surface area for the active molecule to be incorporated. Conversely, the increased viscosity impedes the diffusion of drug molecules into the aqueous phase during the homogenization process, which subsequently improves the entrapment of the drug within the nanoparticle matrix [20].

Table 3. Analysis of variance for EE %**.**

Source	Sum of Squares	Df	Mean Square	F- Value	Prob > F
Model	1349.13	10	143.13	6.67	0.0001<
A	1.06E-003	1	1.06E-003	4.96E-005	0.9943
B	341.36	1	341.36	15.90	0.0005
C	4.17	1	4.17	0.19	0.6651
D	696.66	1	696.66	32.44	<0.0001
AB	61.94	1	61.94	2.88	0.1077
AC	8.91	1	8.91	0.41	0.5281
AD	91.87	1	91.87	4.28	0.0450
BC	4.24	1	4.24	0.20	0.6623
BD	226.20	1	226.20	10.53	0.0034
CD	2.91	1	2.91	0.14	0.7175

Table 4. Analysis of variance for loading %**.**

Source	Sum of Squares	Df	Mean Square	F- Value	Prob > F
Model	193.28	14	13.81	6.30	0.0001<
A	1.61	1	1.61	0.74	0.4066
B	66.41	1	66.41	30.31	0.0001
C	0.13	1	0.13	0.060	0.8099
D	11.05	1	11.05	5.04	0.0245
AB	3.40	1	3.40	1.55	0.2346
AC	0.45	1	0.45	0.2	0.6583
AD	1.22E-003	1	1.22E-003	5.59E-004	0.9815
BC	4.93	1	4.93	2.25	0.1576
BD	53.07	1	53.07	24.22	0.0001
CD	0.084	1	0.084	0.038	0.8477
A^2	0.35	1	0.35	0.16	0.6957
B^2	13.48	1	13.48	6.15	0.0131
C^2	0.62	1	0.62	0.29	0.6024
D^2	21.38	1	21.38	9.76	0.0025

Regarding the interaction effects of water phase volume and PLGA quantity, EE % increased with an increase in water phase volume and PLGA quantity. As the volume of the external water phase (W_2) grows, the oil droplets absorb larger water droplets, increasing the sizes of the droplets in W/O/W emulsions and enhancing the EE% [25].

Research demonstrates that the ratio of internal to external phases substantially impacts the percentage of encapsulation efficiency (EE%). A decrease in this ratio is associated with an increase in EE% across different concentrations of polymers.

In particular, a reduced volume of the internal aqueous phase, when compared to a constant volume of the organic phase, lessens the likelihood of drug diffusion from the polymeric matrix into the external aqueous phase, which may improve drug loading. Abdelkader et al. emphasized in their research that the effectiveness of protein entrapment is intricately related to the thickness of the organic phase surrounding the drug's aqueous solution during the initial emulsion formation. An increase in the volume of the internal aqueous phase could jeopardize the stability of the organic phase, thus promoting the release of drug molecules into the external aqueous medium [20].

Figure 4 shows the interactions between water phase volume and PLGA quantity when loading percentage was considered the response. With increased water phase volume, the loading percentage initially increased and then decreased at higher amounts of the water phase volume and PLGA quantity. Some researchers have shown that the volume of the internal aqueous phase affects the microstructure (porosity) of the microspheres, resulting in increased porosity of the matrix and a reduction in loading percentage [26].

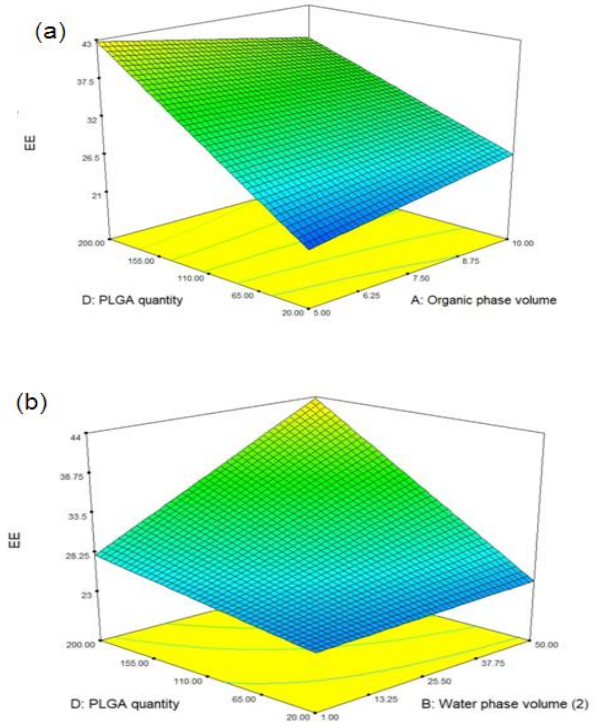

Figure 3. Response surface plots showing the interaction effects of (a) organic phase volume and PLGA quantity and (b) water phase volume 2 and PLGA quantity on EE.

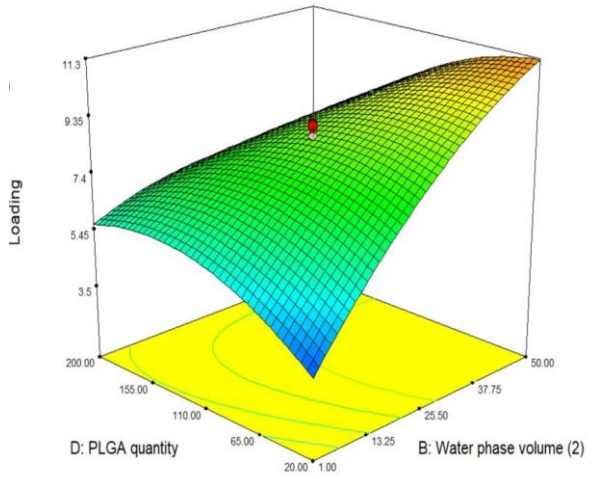

Figure 4. Response surface plots showing the interaction effects of water phase volume 2 and PLGA quantity on loading %.

3.1.3. Result of Optimization

The optimization process was based on achieving the highest EE % and loading % using equations 3 and 4. Therefore, the final optimized formula was specified with the following characteristics: organic phase volume=10 mL, water phase volume=50 mL, PVA%=0.86%, and PLGA quantity=127.56 mg. The finished formula was then reproduced in five verification experiments, and nanoparticles with EE % of 34.6%±2.26 and loading % of 23.9% ±1.32 were obtained. High similarity (>90%) between observed and predicted values indicated the validity and precision of the model. The optimized formula was then used for further studies.

3.2. Characterization of the optimized ALD-loaded nanocarriers regarding shape and particle size

SEM image of ALD-loaded nanoparticles is shown in Figure 5. Particles were spherical with smooth surfaces. A good homogeneity regarding particle size distribution was observed.

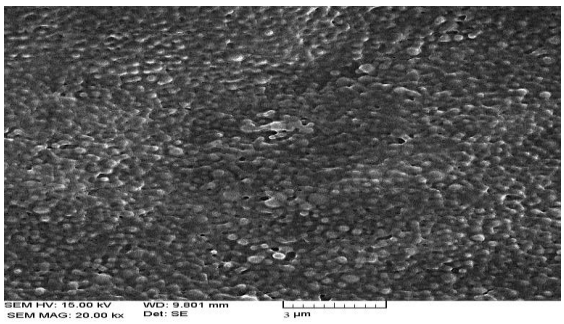

Figure 5. SEM image of ALD-loaded PLGA nanoparticles.

The analysis of the size and polydispersity index by PCS using Zetasizer revealed an average diameter of 236 nm (Fig. 6). This agrees with the particle size results obtained in a previous study on azithromycin-encapsulated PLGA nanoparticles (183±1.8 nm) [27].

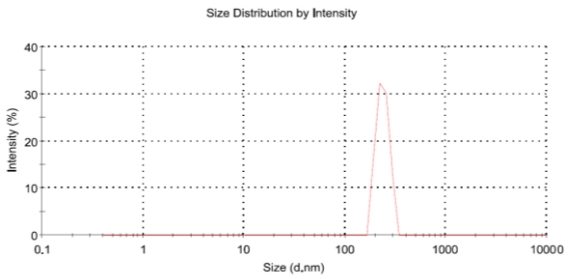

Figure 6. Size distribution diagram of ALD-loaded PLGA nanoparticles.

3.3. In vitro drug release

Nanoparticle release mechanisms can be categorized into two primary types: "burst release" and "sustained release.

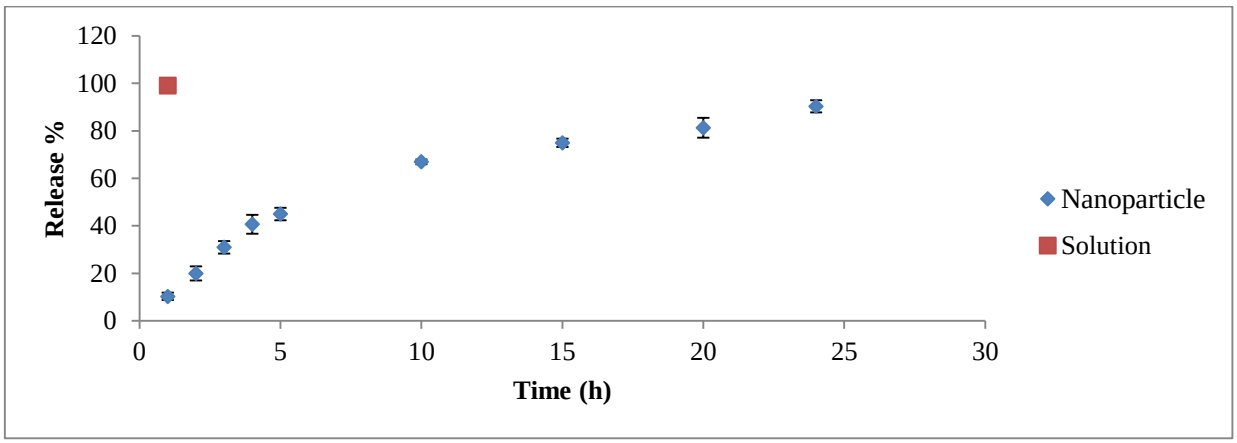

Figure 7. In vitro release profile of ALD from PLGA nanoparticles and free form of ALD powder, data presented mean (n = 3).

Burst release refers to the swift liberation of a drug from the nanoparticle surface or its diffusion from the polymer matrix, enabling the drug to achieve an effective concentration within the bloodstream quickly. In contrast, sustained release involves the gradual release of a drug encapsulated within nanoparticles as they undergo bio-degradation, thereby maintaining the drug at an effective concentration over an extended period [28]. Figure 7 illustrates ALD's in vitro release profiles from PLGA nanoparticles and the free-form ALD during 24 h. The release profile of ALD from free-form ALD showed that 99.1 ± 0.75 % of ALD was released during the first hour. The release profiles of ALD from PLGA nanoparticles exhibit both burst and sustained release characteristics. The burst release phase, which accounted for approximately 10.33% of the ALD, occurred within the first hour. Subsequently, the sustained release phase took place from 1 to 24 hours, releasing around 90.33% of the ALD. From a clinical point of view, the achieved release pattern, including a longer slow-release phase, indicated a successful design of the ALD-loaded delivery system.

4. Conclusion

In this research, we successfully synthesized and optimized ALD-loaded PLGA nanoparticles utilizing the double emulsion solvent evaporation method for osteoporosis treatment. These nanoparticles demonstrated advantageous properties, such as appropriate size, morphology, encapsulation efficiency, and prolonged drug release, which were reproducible. Our findings indicate that the optimization of process parameters during the fabrication of nanoparticles is essential for regulating their characteristics. It is recommended that additional research be conducted on the safety and efficacy of these optimized nanoparticles in vivo in future investigations.

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Conflict of interest

There is no conflict of interest.

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