



# Central composite design aided optimization and validation of developed an eco-friendly HPLC method for the quantification of Lenalidomide loaded mesoporous silica nanoparticles

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## ABSTRACT

Lenalidomide (LND) was encapsulated within mesoporous silica nanoparticles (MSNs), and we developed an reverse-phase (RP)-HPLC analytical method to quantitate the LND content in the formulation. Through a multivariate Central composite design (CCD), we systematically optimized key chromatographic parameters, including the flow rate, sample injection volume, and organic phase ratio. We evaluated the responses of retention time, peak area, and theoretical plate. Our enhanced chromatographic approach employed a Spherisorb ODS C18 column. To investigate the suitability of the mobile phase for isocratic elution, we adhered to International Conference on Harmonisation Q2(R1) standards. Suggesting the optimality of a methanol and ammonium acetate buffer combination (pH 5.5, adjusted with 1% v/v glacial acetic acid and ammonia solution). This validated RP-HPLC analytical method exhibited specificity for LND even in the presence of the matrix of MSNs. The fabricated MSNs were confirmed by evaluating the surface morphology of the formulation. We successfully applied the developed RP-HPLC method to quantify the amount of LND entrapped and to determine the drug loaded in the MSNs formulation. The % EE for LND in MSNs was found to be 76.66% and % DL for LND in MSNs was found to be 14.00%, respectively. The novelty of the Design of expert -based method development is that it reduces the number of trials, thereby reducing solvent wastage and is environmentally friendly, scoring eight green, six yellow, and one red.

## INTRODUCTION

Lenalidomide (LND) falls under the category of immunomodulatory drugs. It serves as a therapeutic agent for various medical conditions, including cancer types such as blood cancer and myelodysplastic syndromes (MDS), a group of disorders characterized by abnormal blood cell production. LND exerts its effect by slowing cancer progression and enhancing the production of healthy blood cells [1]. This is achieved through its ability to modify the immune system and the bone marrow microenvironment. Multiple mechanisms of action are at play, encompassing increased immune cell

activity, controlled production of cytokines and growth factors, and the inhibition of new blood vessel formation that sustains tumors (anti-angiogenic effects). Given its potency, LND necessitates meticulous administration and monitoring under the supervision of a healthcare professional well-versed in its usage and potential side effects. Possible adverse effects of LND include weakness, rash, diarrhea, neutropenia (a decrease in white blood cell count), thrombocytopenia, anemia, and an elevated risk of blood clots [2].

Nanopharmaceuticals are rapidly emerging as a groundbreaking and promising treatment approach [3,4]. Nanoformulations offer a more favorable drug release profile while also enhancing drug penetration and retention. Scientific literature has documented the enhanced efficacy of nanoformulated anti-cancer drugs, attributing this improvement to the enhanced targetability of the formulation and increased bioavailability [5,6].

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Mesoporous silica nanoparticles (MSNs) possess a structural resemblance to the cells of a honeycomb due to their silica (SiO<sub>2</sub>) framework [7]. This inherent porosity, along with their adaptability in terms of size and shape, biocompatibility, and expanded surface area, offers a range of advantages. Notably, their extensive surface area, derived from their porous structure, facilitates the attachment of functional groups to MSNs. Drug loading into the core or onto the surface of MSNs can be achieved through several feasible mechanisms, including electrostatic adsorption, hydrophobic interactions, and covalent binding. LND was loaded in the MSNs using the sol-gel-based method [8]. The structure of the drug is illustrated in Figure 1.

Numerous analytical techniques can be employed for drug quantification within the formulation, including UV spectrophotometry, reverse-phase (RP)-HPLC, HPTLC, and LC-MS/MS methods. However, a limited body of literature addresses the estimation of LND from MSNs. The objective and novelty of this study were to quantify LND by RP-HPLC method attached with a photodiode array (PDA) detector from the MSNs using the Design of expert (DoE) tool. We validated this method following the guidelines outlined in International Conference on Harmonization (ICH) Q2 R1 and further optimized it through the utilization of a Central Composite design (CCD). The method was developed specifically for the quantification of LND from MSNs. The application of DoEs involves statistical tools that aid in identifying and exploring critical factors during the development of analytical methods [9,10]. DoE plays a crucial role in the analytical process, beginning with the preliminary screening and determining important chromatographic characteristics and extending to optimization and the assessment of robustness, as documented in numerous instances. The properties of LND are shown in Table 1.

Prasad and their research group have previously reported a quantification method for LND using a DoEs-based analytical approach [11]. Notably, this represents the sole instance of utilizing a quality by design method for LND analysis. However, their analysis is primarily objective to resolving and evaluating impurities associated with LND, rather than quantifying the drug itself [12]. Consequently, a suitable HPLC analytical procedure for quantifying LND and other drug components within nanoformulations, employing DoE, was presumed to be unavailable. To establish optimal chromatographic conditions, a central composite response

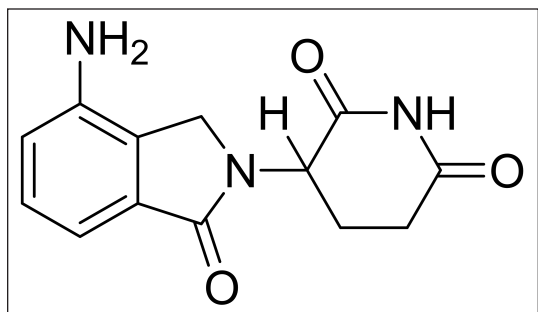


Figure 1. Pictorial representation of LND.

Table 1. Physicochemical properties of lenalidomide.

Parameters	Lenalidomide
BCS class	I
Molecular weight (g/mol)	259.261
Solubility	Soluble in organic solvent (Methanol and acetonitrile), <1.5 mg/ml water soluble
Half-life	3 hours
Melting point (°C)	270°C
Mechanism of action	Lenalidomide modifies the generation of cytokines, controls T cell co-stimulation, and increases NK cell-mediated cytotoxicity to provide immunomodulating effects.
Pka	2.31 (strong basic), 11.61 (strong acidic)
Log P	-0.71

surface design was used [13,14]. Furthermore, a stress-induced degradation test was conducted to check the stability and specificity of the developed analytical method in quantifying LND amidst potentially degraded products when the drug was exposed to various stressors. This optimized and validated analytical method was subsequently employed to quantify LND within MSNs.

## MATERIALS AND METHODS

### Material and reagents

LND (off-white powder; purity > 98%) was procured from Apicore Pharmaceutical Ltd (Vadodara, Gujarat). The supplier of sodium hydroxide pellets (purity ≥98%), orthophosphoric acid (88%), and potassium dihydrogen phosphate (purity >98%) was Merck Ltd. located in Mumbai, India. Finar supplied the HPLC-grade organic solvent (acetonitrile and methanol). The Milli-Q (Type 1 water) was produced in a laboratory. Finar Ltd. (Ahmedabad, India) supplied 35% pure AR hydrochloric acid. We purchased a membrane filter of 0.45 μm from Riviera Glass Pvt. Ltd (Mumbai, India). 120 Å, 250 mm × 4.6 mm, 5 μm particle size Spherisorb ODS C18 column. Cetyltrimethylammonium bromide (CTAB) was procured from TCI Pharmaceuticals, Tokyo Chemical Industry (India) Pvt. Ltd (Japan), Tetraethylorthosilicate (TEOS) was acquired from Sisco Research Laboratories Pvt. Ltd. (SRL) (Mumbai, India), Triethanolamine was acquired from Loba Chemie Pvt. Ltd., Mumbai, India,

### Methods

#### Instrumentation and apparatus

The Shimadzu HPLC system LC20-AD was attached with a PDA detector (SPD-20A & SPD-M10A) and the software used for the analysis was LabSolution. All the chemicals used in the analysis were weighed carefully on the calibrated weighing balance (Sartorius Mechatronics CP225D, India). The prepared buffer solution was filtered through a glass vacuum filtration assembly unit, the 0.45 μm membrane filter was

used. Ultrasonicator (servewell instruments, India) was used to degas the solvents for 10 minutes. A calibrated pH meter from Systronics India Ltd (Ahmedabad, India) was used to check the pH of the mobile phase.

#### *Analytical methods for estimation of LND*

To find the correct wavelength for the estimation of LND, to achieve a concentration of 10 µg/ml, the produced working stock solution was utilized and further diluted. Using water as a blank, the final solution was produced and examined in a double-beam UV-Vis spectrophotometer (UV-1,800) operating in the range of 190–800 nm to identify absorption maxima ( $\lambda_{max}$ ).

#### *Screening of mobile phase and stationary phase*

Considering the pKa value of LND (2.31), buffers with pH +/- of the pKa value such as 10 mM Ammonium acetate pH 5.5 were tried. Stationary phases such as the Spherisorb ODS C<sub>18</sub> column were tried as the stationary phase. Isocratic mode was used for all indicated experiments, and the autosampler temperature was set to 10°C.

#### *Preparation of standard solution for the drug*

Accurately, 10 mg of LND dissolved in 10 ml of methanol obtained a 1 mg/l (1,000 µg/ml) concentration for the primary stock solutions. Then, for 2 minutes, the drug solution was vortexed. As a further step, we created working stock solutions of LND by diluting the primary stock solutions to concentrations between 0.6 and 12 µg/ml with the suitable diluent.

#### *Selection of critical independent variables*

According to the literature review, preliminary trials were used to examine the effect of varying processing parameters on outcomes, including peak area, retention time, and resolution (15–17). These parameters included ratio, organic phase ratio (methanol/ACN), flow rate, column temperature, and injection volume. Also, a working range was established for each variable, and a one-factor-at-a-time (OFAT) analysis was accomplished to select these independent variables and find those significantly affecting the method outcomes [18].

#### *Chromatographic conditions optimized with the help of CCD*

An ideal chromatographic approach for estimating analytes requires determining and analyzing the crucial parameters that cause effective elution of LND. Several time-consuming experiments could be performed without an experimental design. One must refine the identified critical variables to get reliable experimental parameters as part of an optimized method. Optimizing values for these essential parameters in HPLC requires knowledge of the chemistry of individual components and their relationships. DoE is a mathematical method that may help you make sense of these varied complexity levels. Several types of response surfaces are used in DoE to help find the sweet spot of parameter space where a reliable response may be obtained. Two potential methods are CCD and Box Behnken design (BBD) [13,19]. The design was quadratic to evaluate the effect of both independent and interactive variables. A more accurate picture

emerges from this analysis since it ignores the outlier values of the variables. Therefore, Design of Experiments software was utilized to fine-tune the CCD model strategy. Four variables were used as independent in this optimization process: buffer phase ratio (X1), buffer pH (X2), flow rate (X3) and injection volume (X4). The retention time (Rt) of LND (Y1), peak area of LND (Y2), and theoretical plate (Tp) (X3) were obtained at the same time.

#### *Method validation as per ICH guidelines ICH Q2(R1)*

According to ICH Q2(R1) guidelines, the developed and optimized RP-HPLC analytical method of LND was validated [1].

#### *System suitability*

This helps to check whether the system is suitable for the method development. In this validation parameter, six samples of the LND were injected with a concentration of 1 µg /m. The tailing factors (T), theoretical plate (T), and retention time (R) were calculated for a standard solution of LND.

#### *Specificity and selectivity*

Analyzing the blank interference helped determine the analytical method specificity. Three duplicate injections of blank diluent were analyzed at LND retention time.

#### *Linearity*

Five sets of injections were performed with varying doses of standard drug solution (0.5–16 µg/ml) to determine linearity. A linear calibration curve covered the whole concentration range. Peak area (y) was analyzed in relation to concentration (x) using a regression model.

#### *Accuracy*

Three injections of the known concentration (1 µg/ml) at concentrations of 80%, 100%, and 120% were used to assess the accuracy of the procedure. RSD and recovery percentages were determined.

#### *Precision (Interday and Intraday)*

The precision of the method was determined by injecting the standard sample of 1 µg/ml intra-day and inter-day. Six-time samples were injected at three different concentrations (80%, 100%, and 120%).

#### *LOD and LOQ*

To determine the limits of detection (LOD) (Equation 1) and limit of quantification (LOQ) (Equation 2), the slope and standard deviation of the responses were utilized.

$$LOD = \frac{3.3\sigma}{s} \quad \text{Equation (1)}$$

$$LOQ = \frac{10\sigma}{s} \quad \text{Equation (2)}$$

#### *Robustness*

The current method's sensitivity was investigated by changing chromatographic parameters slightly at a time, and it has been shown to be reliable under typical situations (20).

- 1) Change in the pH 6.35 and 6.45
- 2) Change in the temperature 24 and 26
- 3) Change in the flow rate 0.8 and 1
- 4) Change in the injection volume 14 and 16
- 5) Change in the wavelength +1 and -1.

#### Stability studies

##### Bench-top stability

To evaluate the LND stability in a reference solution on a laboratory bench. The sample (1 µg/ml) was kept at room temperature. The freshly (1 µg/ml) prepared and bench-top samples were injected into HPLC in triplicates after 24 hours [21].

$$\text{Similarity index} = \frac{\text{peak area of old standard} \times \text{Amount of new standard}}{\text{Average peak area of new standard} \times \text{amount of standard}} \quad \text{Equation (3)}$$

The peak area observed after 24 hours corresponds to the old standards, while the freshly prepared sample exhibits an average peak area of the newly prepared sample. The amount of drug represents the concentration of the drug in old and newly prepared samples.

##### Stress-induced degradation studies

The established analytical method performance that indicates stability was assessed by subjecting a standard drug solution containing 1 µg/ml of LND to different stress conditions in accordance with ICH recommendations. The stress-induced degradation studies conducted under acidic degradation (0.1 N HCl and 1 N HCl) [21], basic degradation (0.1 N NaOH and 1 N NaOH) [22,23], oxidative hydrolysis (3% H<sub>2</sub>O<sub>2</sub>) [24,25], photolytic degradation [26], and thermal degradation 40°C. In each degradation study, a 1 ml standard stock solution of LND with a concentration of 1 µg/ml was employed. Following the addition of 1 ml of each stressor to the drug solution, it was heated to 60°C for approximately 24 hours [27]. The resulting solution was subsequently neutralized and diluted, and the samples were injected into the HPLC for the stability analysis [28,29].

##### Application of the developed RP-HPLC analytical method

###### Fabrication of MSNs

MSNs were fabricated using a sol-gel technique. This procedure involved mixing deionized water with CTAB and NaOH, gradually adding TEOS, and stirring at 800 rpm for 2 hours at 78°C. The formulation was centrifuged at 10,000 rpm for 10 minutes, washed 3 times, and dried using a hot air oven at 60°C for 24 hours. Later, the formulation was kept for calcination in a muffle furnace at 550°C for 3 hours. Dependent factors such as particle size, zeta potential, and polydispersity index were used to fabricate the placebo MSNs.

###### Preparation for LND loaded MSNs

The drug, LND loading into MSNs was done using the incubation method. Briefly, 3 mg of LND was dissolved in 1 ml

of ethanol. Furthermore, this solution was incubated in 2 mg of MSNs ethanol solution. The step was followed by sonication of MSNs for 5 minutes and stirring of MSNs dispersion at 500 rpm for 24 hours at room temperature. The resultant dispersion was centrifuged at 15,000 rpm for 10 minutes. The step was followed by washing the precipitate with ethanol three times. Finally, the fabricated LND-loaded MSNs (LND@MSNs) were characterized and optimized using dependent variables such as particle size, zeta potential, and PDI [30–32].

###### Determination of % entrapment efficiency (% EE) and % Drug loading (% DL)

After the loading procedure, the dispersion was centrifuged at 15,000 rpm for 10 minutes, and the LND remaining in the supernatant (untrapped LND) was determined using the developed HPLC method. The % EE (Equation 4) and % DL (Equation 5) of LND in MSNs were determined using the following formula [33,34]:

$$\text{Entrapment efficiency (\%EE)} = \frac{\text{initial amount of drug} - \text{amount of drug in supernatant}}{\text{initial amount of drug}} \times 100\% \quad \text{Equation (4)}$$

$$\% \text{ Drug Loading (\%DL)} = \frac{\text{initial amount of drug} - \text{Amount of drug in supernatant}}{\text{Total weight of formulation}} \times 100\% \quad \text{Equation (5)}$$

###### Scanning electron microscopy (SEM) analysis

SEM was used to evaluate the surface characteristics of the fabricated MSNs. The samples were prepared for SEM by applying MSNs on an aluminum counterfoil. The sample was examined using a scanning electron microscope (SEM) (EVO MA18, Zeiss) after a thin layer of gold was deposited using a gold sputtering coater [35,36].

## RESULTS AND DISCUSSION

### Method development

The LND absorbance maxima were determined to be 219 nm using a UV-Vis spectrophotometer.

### Selection of mobile phase and stationary phase

During the development of the analytical method for LND, we explored various alternative mobile phases. The first attempt involved utilizing a phosphate buffer and acetonitrile/methanol at different ratios, but this resulted in LND eluting in the dead volume with a high tailing factor.

Subsequently, we tested an alternative approach using an ammonium acetate buffer and acetonitrile/methanol as the mobile phase. However, this combination caused the buffer and acetonitrile to elute LND in the dead volume, resulting in poor symmetry and a plate count of less than 2,000. In a final trial, we experimented with different ratios of ammonium acetate

and methanol as the mobile phase. This approach successfully yielded elution of LND after only 3 minutes, with a tailing factor of less than 2 and a theoretical plate count exceeding 2,000, meeting the acceptance criteria. Initially, we employed a generally used C<sub>18</sub> stationary phase (250 mm × 4.6 mm i.d., 5 µm particle size, Waters), which produced peak LND properties that met the required criteria. Further refinement and optimization of the chromatographic conditions were carried out using DoE methodology.

#### Method optimization by CCD

The optimization technique employed in this method development directly impacts the independent parameters influenced by the chromatographical results. Previously reported analytical methods for the LND quantification did not mention the effect of independent variables such as buffer ratio and flow rate. These factors can significantly affect the drug peak properties such as peak area and retention time. The absence of a comprehensive understanding of these pivotal method parameters compromises the method's robustness, elevating the risk of method failure when applied in different settings. Therefore, it is imperative to identify and thoroughly

investigate these influential factors to establish a reliable and resilient analytical method for precise LND estimation.

In this present study, we used a three-level CCD to optimize specific independent factors: buffer ratio (X1), buffer pH (X2), flow rate (X3), and injection volume (X4). The upper and lower limit values for these pivotal parameters were determined based on the results obtained from preliminary OFAT trials. We established a lower limit of 60% in the mobile phase for the buffer ratio and an upper limit of 70%. Buffer pH spanned from 5.3 (lower limit) to 5.8 (upper limit), while the flow rate ranged from 0.8 ml/minute (lower limit) to 1 ml/minute (upper limit). Injection volume covered 5 µl (lower limit) to 10 µl (upper limit). However, relying exclusively on OFAT-based approaches can be laborious and challenging. Therefore, the incorporation of CCD and other response surface designs expedites the optimization process and enhances the development of a robust analytical technique.

There were 25 trial runs carried out in all, with one center point. Table 2 presents the specifics of these trial runs along with the replies that went along with them. Significant interactions between the independent variables were found using the Analysis of variance (ANOVA) analysis, and the observations are shown in Table 3.

**Table 2.** Independent variables recommended by the DoE and the associated responses.

Std	Run	X1	X2	X3	Y1	Y2	Y3	Y4
17	1	55	5.55	0.9	7.5	3.7195	459,354	2155.95
14	2	70	5.3	1	10	5.0885	498,718	2657.81
18	3	75	5.55	0.9	7.5	7.0275	434,375	3364.34
23	4	65	5.55	0.9	2.5	4.64	163,854	2602.19
4	5	70	5.8	0.8	5	6.2575	304,547	3172.41
11	6	60	5.8	0.8	10	4.5635	592,250	2544.57
24	7	65	5.55	0.9	12.5	4.6515	720,892	2510.75
2	8	70	5.3	0.8	5	6.2695	608,523	3081.39
16	9	70	5.8	1	10	4.9865	493,681	2823.09
15	10	60	5.8	1	10	3.6625	480,460	2375.78
20	11	65	6.05	0.9	7.5	4.6045	442,554	2651.53
10	12	70	5.3	0.8	10	6.334	614,009	2994.08
1	13	60	5.3	0.8	5	4.5995	302,559	2289.17
19	14	65	5.05	0.9	7.5	4.6245	389,419	2425.75
22	15	65	5.55	1.1	7.5	3.7555	366,429	2311.05
6	16	70	5.3	1	5	5.077	249,011	2775.68
5	17	60	5.3	1	5	3.6815	241,638	2210.57
3	18	60	5.8	0.8	5	4.5525	298,469	2551.95
8	19	70	5.8	1	5	4.9855	247,784	2896.4
12	20	70	5.8	0.8	10	6.26	615,116	3082.5
25	21	65	5.55	0.9	7.5	4.649	441,332	2552.59
9	22	60	5.3	0.8	10	4.602	605,344	2281.29
21	23	65	5.55	0.7	7.5	5.9865	568,637	2741.74
13	24	60	5.3	1	10	3.6895	483,145	2172.92
7	25	60	5.8	1	5	3.6535	240,893	2362.22

Higher levels of factors (75% buffer phase, 6 pH, 1 ml/minute flow rate, and 12.5 µl injection volume) are indicated by +1; intermediate point of 0.5 ml/minute, 0.9 ml/minute flow rate, and 7.5 µl injection volume is indicated by a value of 0; while an lower level of factors (55% buffer phase, 5 pH, 0.7 ml/minute flow rate, and 5 µl injection volume) is indicated by a value of -1.

**Table 3.** ANOVA results of central composite design.

Response	Rt of LND (Y1)		Peak area of LND (Y2)		Theoretical plate (Y3)	
F-value	37.72		39.75		19.42	
p-value	Model	<0.0001	Model	<0.0001	Model	<0.0001
	A	<0.0001	A	0.2525	A	<0.0001
	B	0.7038	B	0.4428	B	0.0007
	C	<0.0001	C	0.0002	C	<0.0001
	D	0.9123	D	<0.0001	D	0.1757
	AB	0.8879	AB	0.2309	AB	0.2398
	AC	0.1839	AC	0.2131	AC	0.0827
	AD	0.9603	AD	0.2675	AD	0.3470
	BC	0.9377	BC	0.1993	BC	0.8504
	BD	0.9490	BD	0.2281	BD	0.7859
	CD	0.9587	CD	0.7850	CD	0.9473
R2	0.964		0.913		0.962	
Adjusted R2	0.938		0.852		0.936	

The symbols A, B, C, and D stand for buffer ratio, pH, flow rate, and injection volume; AB, AC, AD, BC, BD, and CD denote combinations of these variables.

**Impact of independent factors on Rt of LND (Y1)**

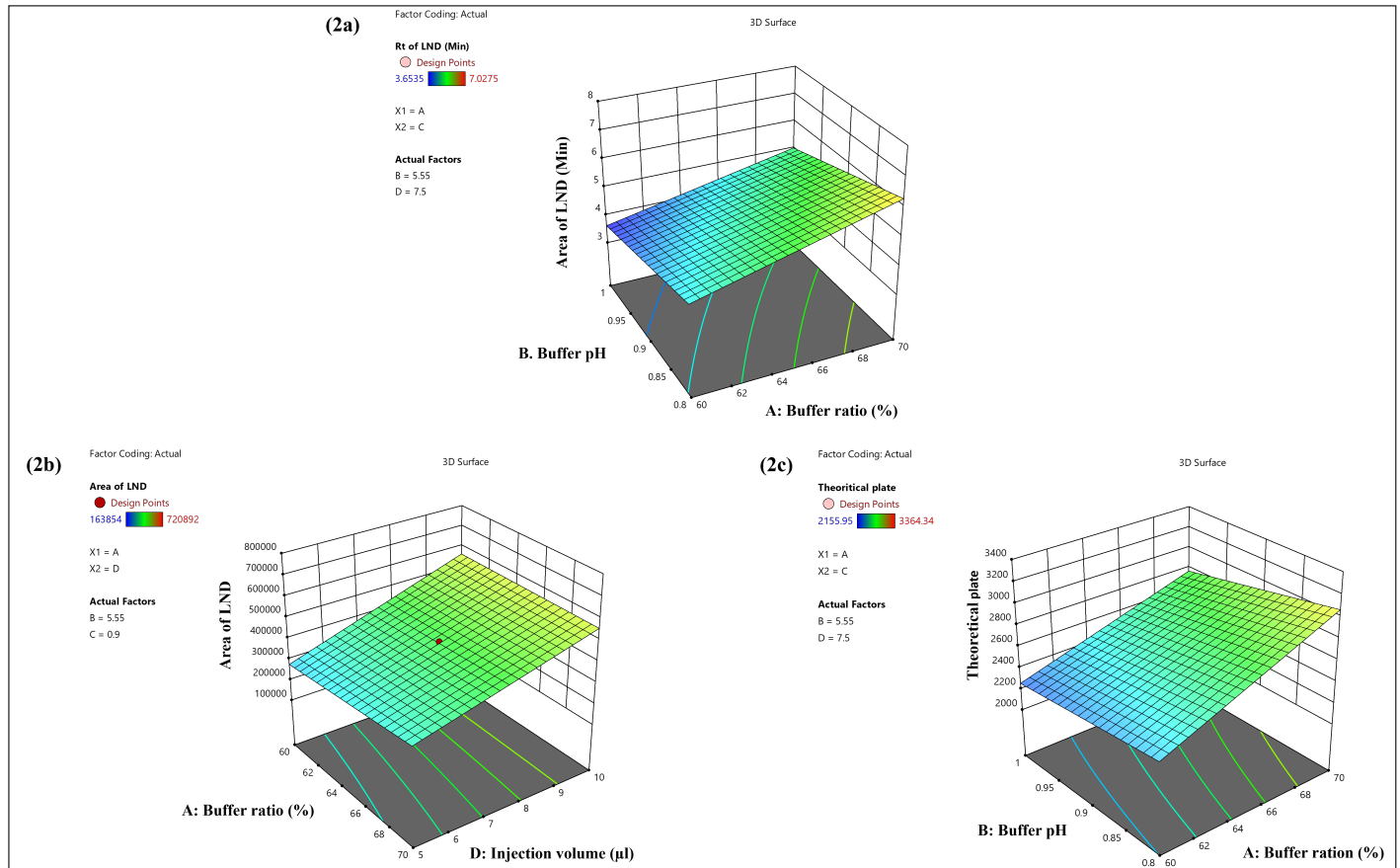
The quadratic equation (Equation 6) and ANOVA analysis revealed the independent factors, buffer ratio (X1), and flow rate (X3), demonstrating an effect on the  $R_t$  of LND. Conversely, buffer pH (X2) and injection volume (X4) had a lesser impact on retention time. Specifically, an increase in buffer ratio and a decrease in flow rate led to an extended retention time for LND. In this study, we altered X1 and X3 values from 55% to 75% and from 0.8 to 1 ml/minute, resulting in a shift in  $R_t$  from 3.25 to 7.02 minutes. The effects of these independent variables, X1 and X3, are depicted in the 3D and perturbation plots shown in Figures 2a and 3a.

$$Y1 = +4.88 + 0.7863 \times A - 0.0192 \times B - 0.5448 \times C + 0.0055 \times D - 0.0087 \times AB - 0.0846 \times AC + 0.0031 \times AD - 0.0048 \times BC - 0.0039 \times BD + 0.0032 \times CD$$

Equation (6)

**Impact of independent factors on the peak area of LND (Y1)**

The quadratic equation (Equation 7) ANOVA analysis revealed that independent variables, flow rate (X3) and injection volume (X4), exhibited a significant impact on



**Figure 2.** 3D surface response graphic illustrating how independent factors have an impact: (2a) on the retention time of LND, (2b) on the peak area of LND, and (2c) on the theoretical plate of LND.

the peak area of LND. Conversely, buffer ratio (X1) and buffer pH (X2) had a relatively minor effect on the LND peak area. Specifically, a decrease in flow rate (X3) and an increase in injection volume (X4) led to an augmentation in the peak area of LND. In this study, we adjusted X3 and X4 values from 0.8 to 1 ml/minute and 5 to 10  $\mu$ l, increasing the peak area from 163,854 to 720,892. The effects of these independent variables, X3 and X4, are visually depicted in the 3D and perturbation plots featured in Figures 2b and 3b.

$$Y2 = +4.34 + 14028.13 \times A - 9311.58 \times B - 58745.96 \times C + 1.251 \times D - 18782.13 \times AB - 1878213 \times AC - 16623.81 \times AD + 19397.31 \times BC + 18145.50 \times BD + 4003.56 \times CD \quad \text{Equation (7)}$$

### Impact of independent factors on the theoretical plates (Y3)

The quadratic equation (Equation 8) ANOVA analysis revealed that independent variables, buffer ratio (X1), buffer pH (X2), and flow rate (X3), exerted an influence on the  $T_p$  of LND. In contrast, injection volume (X4) had a relatively minor impact of the  $T_p$  for LND. Notably, an increase in buffer ratio (X1), an increase in buffer pH (X2), and a decrease in flow

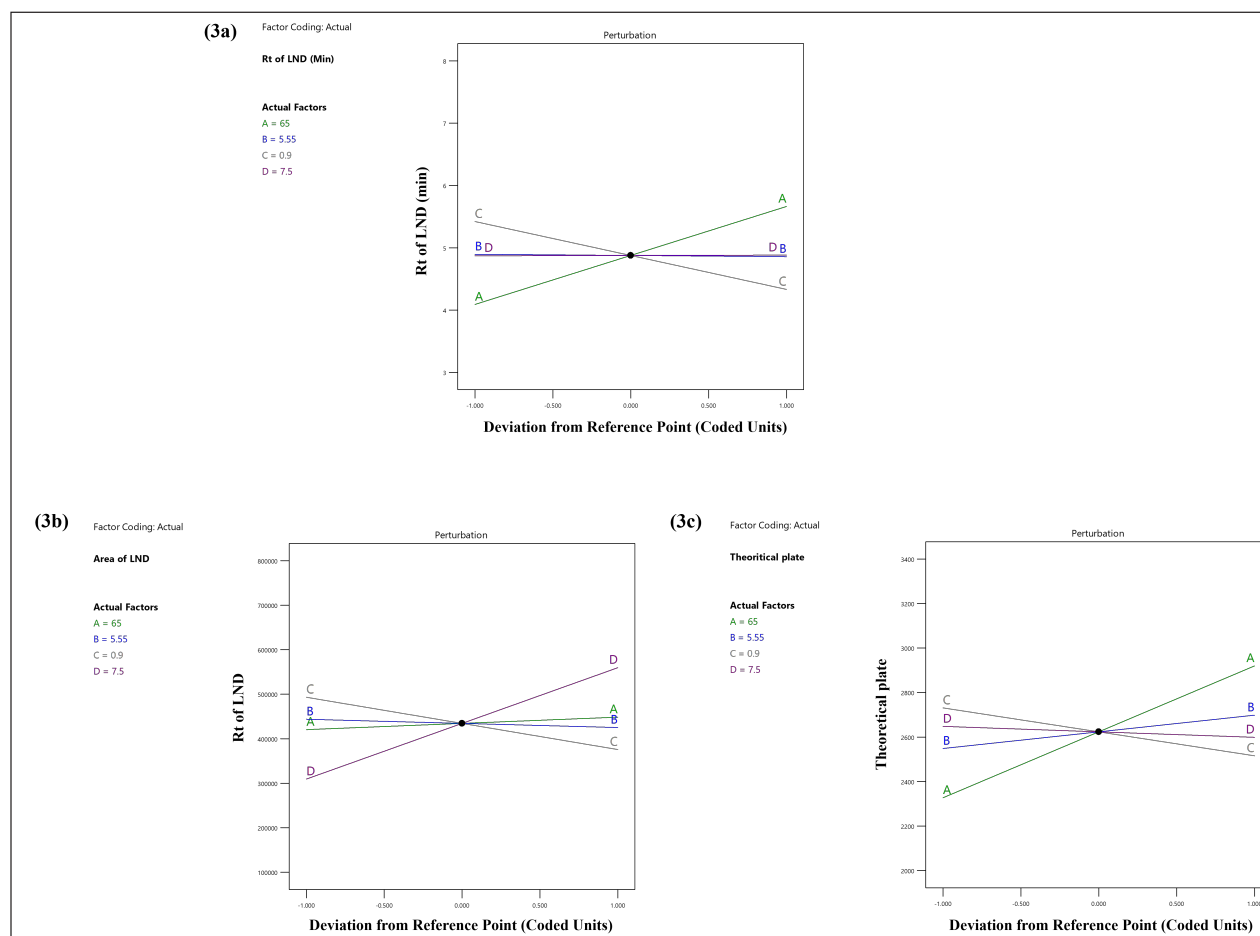
rate (X3) resulted in an elevation of the theoretical plate for LND.

In this study, we raised X1, X2, and X3 values from 55% to 75%, from 5 to 6, and from 0.8 to 1 ml/minute, respectively, leading to an increase in the  $T_p$  from 2155.95 to 3364.34. The effects of these independent factors, X1, X2, and X3, are graphically illustrated in the 3D and perturbation plot featured in the Figures 2c and 3c.

$$Y3 = 2623.51 + 296.32 \times A + 74.90 \times B - 107.68 \times C - 24.61 \times D - 25.95 \times AB - 39.49 \times AC - 20.57 \times AD - 4.06 \times BC + 5.85 \times BD - 1.42 \times CD \quad \text{Equation (8)}$$

### Desirability

The preceding analysis yielded an optimized RP-HPLC analytical method with a desirability value of 0.933. The resulting method specified a buffer ratio of 70%, a buffer pH of 5.5, and an injection volume of 10  $\mu$ l. To validate the suggested method, it was executed in six replicates, and the obtained outcomes for each response were compared with the values indicated by the software. This comparison revealed



**Figure 3.** Perturbation diagram that illustrates how independent variables interact (3a) on the retention time of LND, (3b) on the peak area of LND, and (3c) on the theoretical plate of LND.

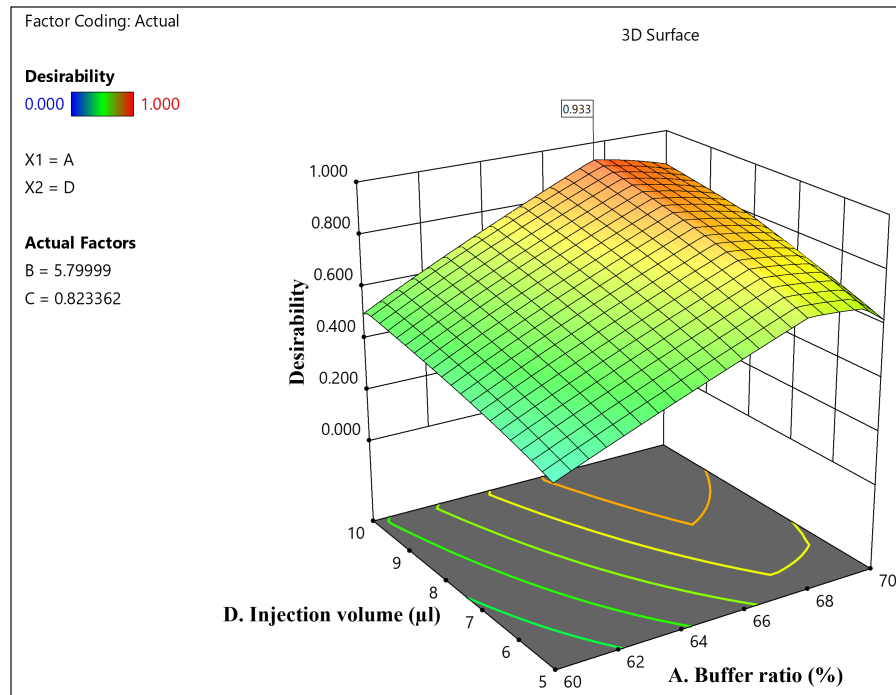


Figure 4. Desirability plot from the ANOVA analysis.

that the % relative error for the responses remained below 10%. Subsequently, the suggested method underwent further validation in accordance with the ICH Q2 (R1) guidelines. Figure 4 displays the chromatogram that was achieved under optimal circumstances.

#### Method validation

Parameters including retention duration, tailing factor, resolution, and theoretical plates were calculated to assess the system's applicability (Table 4).

#### System suitability

The optimized RP-HPLC method was run for system suitability, so parameters such as tailing factor,  $R_t$  and  $T_p$  were calculated. All the outcomes for the system suitability were satisfactory and came under the acceptance criteria.

#### Linearity

Linearity was obtained for the LND with the calibration range of 0.06–12 µg/ml. The  $R^2$  value for LND was calculated from the calibration curve 0.999, respectively. The linearity equation for the LND was  $y = 78569x - 3710.7$ .

#### Sensitivity

The LOD and LOQ were found to be 11.79 ng/ml and 35.74 ng/ml, respectively.

#### Accuracy

Three separate tests were conducted to determine the accuracy of the established method: 80%, 100%, and 120%. As per the ICH recommendations, the percent

Table 4. Validation data of the optimized analytical method.

Parameters	Lenalidomide
Retention time (minute)	5.84 ± 0.03
Peak area	520558.3 ± 1120.764
Tailing factor	1.27 ± 0.016
Theoretical plate	3204.57 ± 77.25
Resolution	-
Linearity range (µg/ml)	0.062–12
Regression equation	$y = 78569x - 3710.7$
Correlation coefficient ( $r^2$ )	0.9993
LOD (ng/ml)	11.79
LOQ (ng/ml)	35.74
Accuracy	% Recovery
80%	99.15
100%	98.38
120%	100.62
Precision	Repeatability Inter-day
%RSD	0.46 1.15

recovery of LND fell within an acceptable range between 90% and 110%. The approach that was devised was precise and accurate.

#### Precision

The LND's intra- and inter-day precision was examined. It was observed that the intra-day and inter-day %CVs were less than 2.0%.



### Robustness

Small adjustments to the temperature, injection volume, flow rate, pH, and wavelength of the buffer were assessed. These chromatographic condition adjustments had no discernible impact on the theoretical plate, peak area, retention factor, or tailing factor. [Table S1](#). This suggests that the established approach for the LND estimate is reliable.

### Bench-top stability

After 24 hours, the recovery from the working solution was found to be 100%–105% and %CV was less than 2%.

### Stress-induced degradation studies

The forced degradation study was conducted to assess the stability of LND under various stress conditions, simulating the conditions encountered during formulation development. As depicted in [Figure 6](#), LND demonstrated susceptibility to certain stress conditions. Specifically, it exhibited significant degradation in acidic and alkaline environments. In a 0.1M HCl solution, degradation exceeded 65%, while in a 1M HCl solution, degradation was over 85%. In alkaline conditions, degradation was substantial, with more than 75% in a 0.1M NaOH solution and over 90% in a 1M NaOH solution. In addition, exposure to an H<sub>2</sub>O<sub>2</sub> solution resulted in degradation

exceeding 35%. However, LND showed better stability under other stress conditions. At elevated temperatures, degradation was less than 35%, and exposure to sunlight (UV) caused less than 13% degradation.

### Application of the developed RP-HPLC analytical method

#### Characterization of fabricated placebo MSNs and LND@MSNs

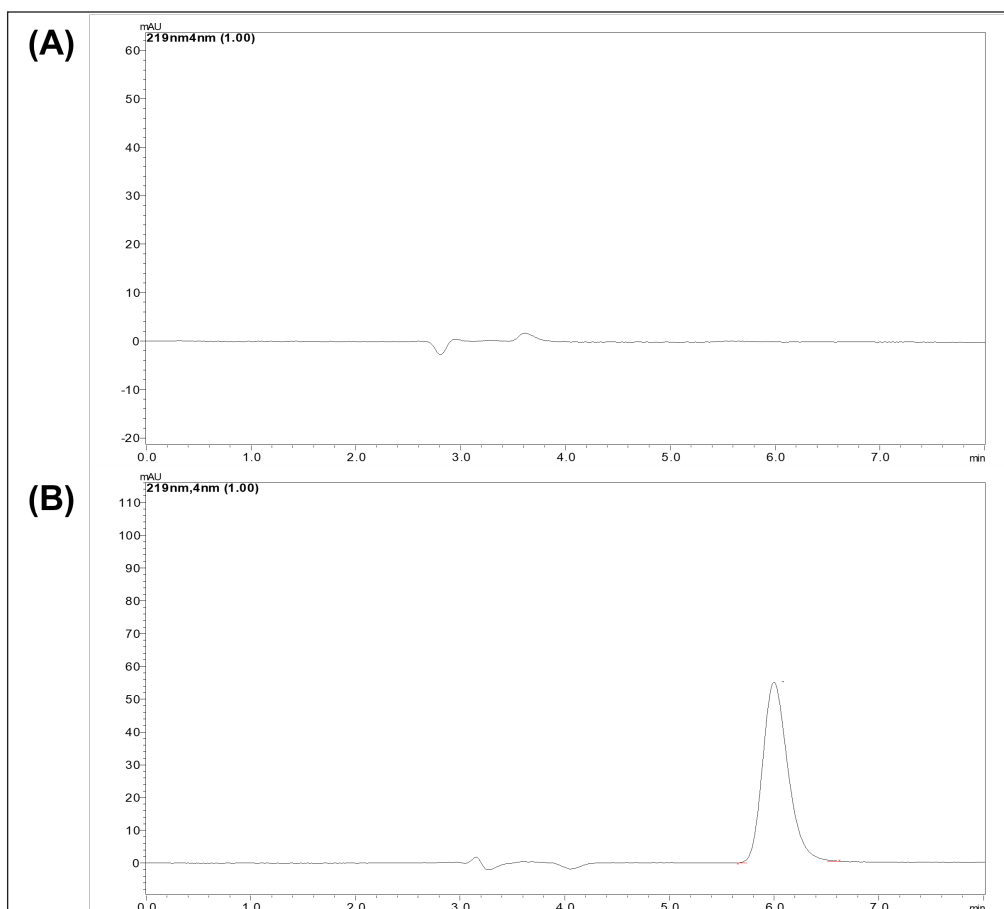
The average particle size of placebo MSNs and LND@MSNs was found to be 246 nm and 280 nm, respectively. Notably, the placebo MSNs and LND@MSNs showcased a polydispersity index of 0.149 and 0.088, indicating uniform particle size distribution within the formulation as shown in the figures. The Zeta potential of placebo MSNs and LND@MSNs was found to be -28.9 mV and -32.7 mV ([Figure 7](#)).

#### The specificity of the validated method

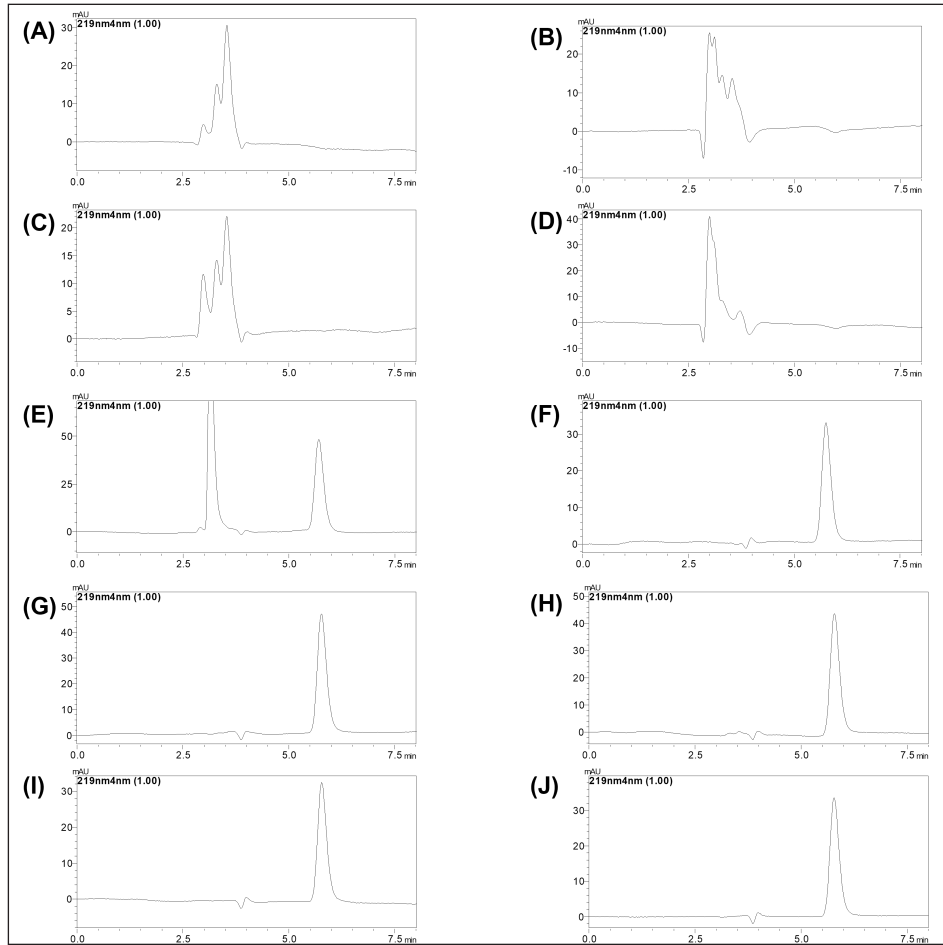
The absence of interference from the excipients at the R<sub>t</sub> of LND confirms the method's specificity for accurately estimating the drug within the MSNs. The chromatogram of placebo and LND-loaded MSNs is shown in [Figure 5](#).

#### % Entrapment efficiency and % drug loading

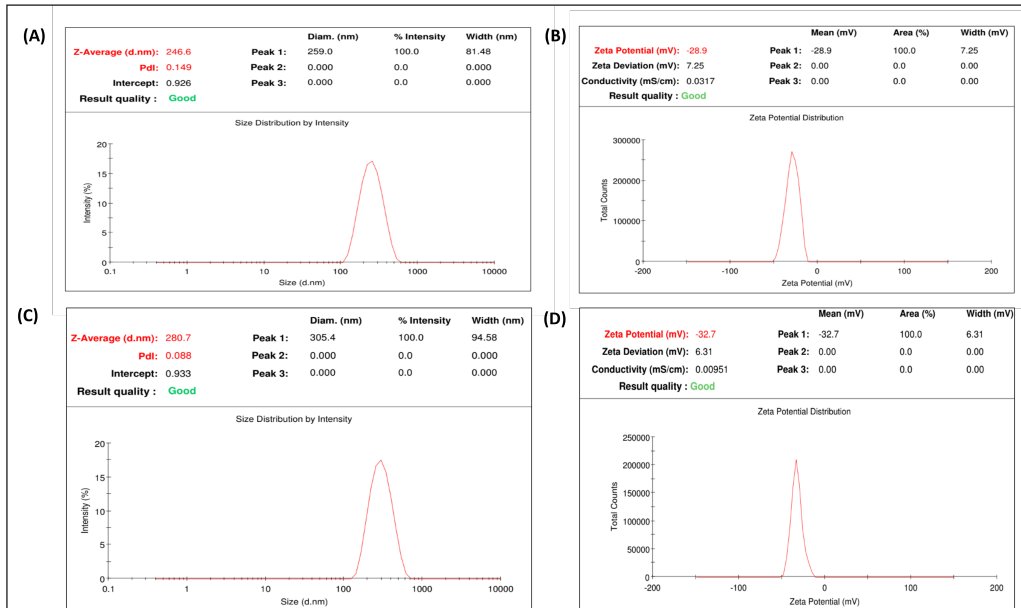
The % EE for LND in MSNs was found to be 76.66% and % DL for LND in MSNs was found to be 14%, respectively.



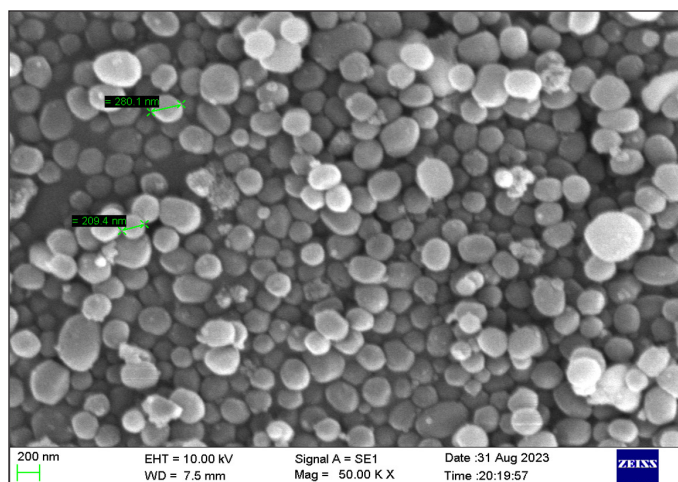
**Figure 5.** Chromatogram obtained at optimized conditions (A) Chromatogram of blank MSNs, (B) Chromatogram of LND loaded MSNs.



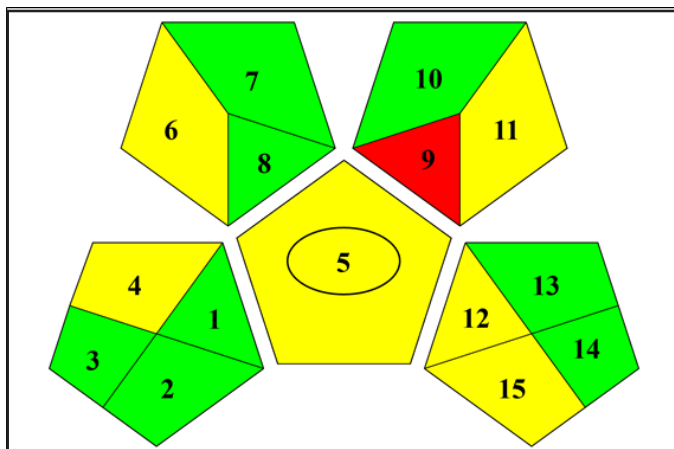
**Figure 6.** Chromatogram of (A) 0.1N HCl, (B) 1N HCl, (C) 0.1N NaOH, (D) 1N NaOH, (E) H<sub>2</sub>O<sub>2</sub>, (F) photothermal, (G) Temperature 40°C, (H) Temperature 60°C, (I) Bench-top stability, and (J) standard.



**Figure 7.** Characterization of fabricated formulation: (A) Particle size of placebo MSNs, (B) Zeta potential of placebo MSNs, (C) Particle size of LND@MSNs, and (D) Zeta potential of LND@MSNs.



**Figure 8.** SEM result of loaded MSNs in drug LND therefore in figure showing the average value of loaded LND with 209 nm and actual size of drug-loaded MSNs with 280 nm.



**Figure 9.** Diagram shows the suggested HPLC procedure for estimating LND from MSNs using the Green Analytical Procedure Index pictogram. The color red represents a high danger to the environment, while yellow and green symbolize lower danger and better greenness. Eight parameters relating to the sample (collection, preservation, transport, storage, types of processing method, scale of extraction, solvent/reagent used, additional reagent, and so on), three parameters relating to reagents and compounds (amount to solvent/reagent, health hazard, and safety hazard) and four parameters relating to instrumentation (energy, occupational hazard, waste, and waste treatment).

#### Scanning electron microscopy

The SEM image of LND@MSNs revealed the formulation morphology and each particle nanostructure (Figure 8). The morphology of the synthesized MSNs can be modified by varying the CTAB and TEOS concentrations. Adjusting the CTAB and TEOS concentration in MSNs average size was found to be 209 nm and surface morphology was spherical and aggregated.

#### GREENNESS ASSESSMENT OF DEVELOPED HPLC METHOD

The recommended analytical method of environmental friendliness was evaluated by analyzing twelve GAPI criteria

pertaining to the equipment, reagents and compounds, and sample. Independent variables include things like extraction volume, solvent or reagent used, storage, transportation, processing, and additional reagents. The risks associated with chemicals and reagents were the amount of solvent or reagent required and possible threats to the health and safety of humans. Energy, workplace risk, waste, and waste treatment were taken into account [37]. We generated an image (Figure 9) that illustrates the method of environmental friendliness using the GAPI program. Within a pictogram, a circle at the center symbolizes the capacity to serve both qualitative and quantitative goals. Red denoted a severe environmental concern, whereas yellow and green denoted less of a threat and greater greenery, respectively. The strategy received a total score of six green, six yellow, and three red, making it ecologically friendly.

#### CONCLUSION

An ecofriendly RP-HPLC method for the determination of LND was successfully developed. The three-level CCD was used to optimize the analytical method. The desirability from the DoE analysis was found to be 0.933. The optimized method was validated as per ICH guidelines, the parameters were linearity, sensitivity, accuracy, precision, and robustness. Furthermore, stress-induced degradation study confirmed the sensitivity of the validated method. Using the validated method, the calculated %EE and %DL were 76.66% and 14.00% for the MSNs formulation. The excipients of MSNs did not show any interference with the retention time of LND. This method could be employed for the estimation for the successful formulation development and analysis of these drugs.

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#### LIST OF ABBREVIATIONS

ANOVA, Analysis of variance; CCD, Central composite design; DoE, Design of expert; LND, Lenalidomide; MSNs, Mesoporous silica nanoparticles; OFAT, One-factor at a time; ICH, International Conference on Harmonization

#### CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

#### AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit

to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

### ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

### DATA AVAILABILITY

All the data is available with the authors and shall be provided upon request.

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### USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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**SUPPLEMENTARY MATERIAL**

Supplementary data can be downloaded from the journal's website link here: [[https://japsonline.com/admin/php/uploads/4424\\_pdf.pdf](https://japsonline.com/admin/php/uploads/4424_pdf.pdf)]