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Inhaled dry powder of bedaquiline loaded nano-carrier for the treatment of multi-drug resistant tuberculosis

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ABSTRACT: According to ICH Q8(R2) guideline, this research presents development of inhaled dry powder of bedaquiline loaded nano-carrier for the treatment of MDR-tuberculosis to mitigate the side effects of marketed oral dosage form. Circumscribed Central composite design was utilised to optimize the bedaquiline fumarate (BDQ) loaded NLCs formulated by solvent injection technique and examine the impact of independent variables such as SP Crodamol ML-MBAL-LQ-(RB), Lipoid S 100 and MyrjTM S 40 on dependent variables such as %drug loading, zeta potential, vesicle size, %entrapment efficiency & %In-vitro drug release (Q12). The optimized formulation gave a sustained drug release up to 12 hrs (97.12% ± 0.89%). It formed a stable emulsion with desired zeta potential (-34.98 mV), high entrapment efficiency (65.42% ± 0.49%), smaller vesicle size (175.51 nm) and sufficient drug loading (18.01% ± 0.14%). Further the BDQ loaded NLCs emulsion was lyophilized using mannitol as lyoprotectant and ethylene glycol as cryoprotectant. The lyophilized cake was sifted from #100 and then to #120 and filled in Red/Transparent coloured size "3" hard gelatin capsule. It had good flow characteristics; maximum drug content & the formulation provided no impedance to BDQ release. In-vitro lung deposition study showed that the inhaled dry powder of BDQ loaded NLCs could be deposited in the deep lung tissue & GSD=1 showed that all the particles were of same size. The minimum inhibitory concentration (MIC) of inhaled dry powder of BDQ loaded NLCs capsule 50 mg was found to be 2 µg/ml which was less than the available marketed dosage form. According to ICH Q1C guideline, the formulation was found stable for 6 month accelerated & long term stability conditions. The inhaled dry powder of BDQ loaded NLCs reduced the peripheral tissue exposure and dosing frequency, gave a target specific action, improved the bioavailability and patient compliance.

KEYWORDS: Bedaquiline fumarate; NLCs; DPIs; ICH Q8(R2) guideline; ICH Q1C guideline.

1. INTRODUCTION

After 40 years, FDA approved bedaquiline fumarate (BDQ) for the management of pulmonary multi drug resistant tuberculosis (MDR-TB) [1,2,3,4]. It inhibits proton pump of mycobacterial ATP synthase, an enzyme necessary for production of energy in mycobacterium tuberculosis bacteria [1,2,3,4]. The effective half-life of BDQ in plasma is approximately 24 hours, but its metabolite M2 has a long term elimination half-life of approximately 5.5 months due to slow release from peripheral tissues due to characteristics of cationic amphiphilic drug (CAD) [1,2,3,4]. BDQ accumulation in tissues is caused by binding of CAD to intracellular phospholipids. This results in undesirable side effects of BDQ such as hemoptysis, arthralgia, QT prolongation, and hepatic related side effects. When the BDQ consumption is discontinued, the rate at which the BDQ is eliminated from the tissues depends on both; the rate at which the CAD dissociates from the phospholipid and the rate at which the CAD is eliminated from the tissue [1,2,3,4].

Regulatory authorities approved the use of nanostructured lipid carriers (NLCs) which is composed of biocompatible, physiological and biodegradable emulsifier, liquid lipid & solid lipid in a number of drug delivery systems [5,6,7]. Administering lyophilized BDQ loaded NLCs via inhalation enhances the absorption and retention of BDQ in the deep lung tissue [5,6,7].

Inhaled dry powder of BDQ loaded NLCs has the potential to achieve relatively uniform distribution of BDQ dose among the alveoli. It reduces the peripheral tissue exposure, reduces the dosing frequency, enhances the bioavailability, improves the patient compliance and overcomes the side effects associated with available marketed dosage form [8,9,10].

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2. RESULTS AND DISCUSSION

2.1 Results of optimization of BDQ loaded NLCs

The results of all the central composite design batches listed in Table 1. Figure 1 and Figure 2 displayed the vesicle size of batch F15 and the zeta potential of batch F8, respectively.

Table 1. Evaluation of central composite design batches

Sr. No.	Batches	Vesicle Size (nm)	Zeta Potential (mV)	%Entrapment Efficiency ± S.D (n=3)	% Drug Loading ± S.D (n=3)	%*In-Vitro* Drug Release (Q12) ± S.D (n=3)
1	F1	144.9	-12.3	41.98 ± 0.52	38.64 ± 0.19	91.22 ± 0.16
2	F2	402.1	-10.5	46.19 ± 1.39	27.71 ± 0.63	88.36 ± 0.39
3	F3	167.7	-11.8	56.63 ± 0.29	18.03 ± 0.26	90.24 ± 0.15
4	F4	399.5	-10.3	63.85 ± 0.59	15.17 ± 1.08	86.61 ± 0.21
5	F5	113.3	-35.6	49.69 ± 0.35	34.08 ± 0.55	93.69 ± 0.32
6	F6	323.6	-32.3	54.99 ± 1.25	25.22 ± 0.41	90.17 ± 0.23
7	F7	198.1	-25.2	59.87 ± 0.96	16.99 ± 0.98	92.28 ± 0.18
8	F8	323.9	-22.8	64.42 ± 0.28	14.67 ± 0.78	90.15 ± 0.59
9	F9	192.4	-26.5	52.16 ± 1.68	24.6 ± 0.61	93.37 ± 0.11
10	F10	503.3	-22.5	60.22 ± 0.55	17.6 ± 0.17	89.16 ± 0.01
11	F11	191.6	-28.7	41.55 ± 0.29	44.38 ± 0.36	93.28 ± 0.32
12	F12	220.4	-15.2	68.47 ± 0.58	14.31 ± 0.82	91.32 ± 0.02
13	F13	206.8	-9.5	50.68 ± 1.39	21.65 ± 0.64	89.49 ± 0.03
14	F14	187.7	-35.1	60.45 ± 0.32	19.33 ± 0.47	93.19 ± 0.02
15	F15	179.8	-34.7	64.12 ± 1.21	20.35 ± 0.59	97.08 ± 0.16

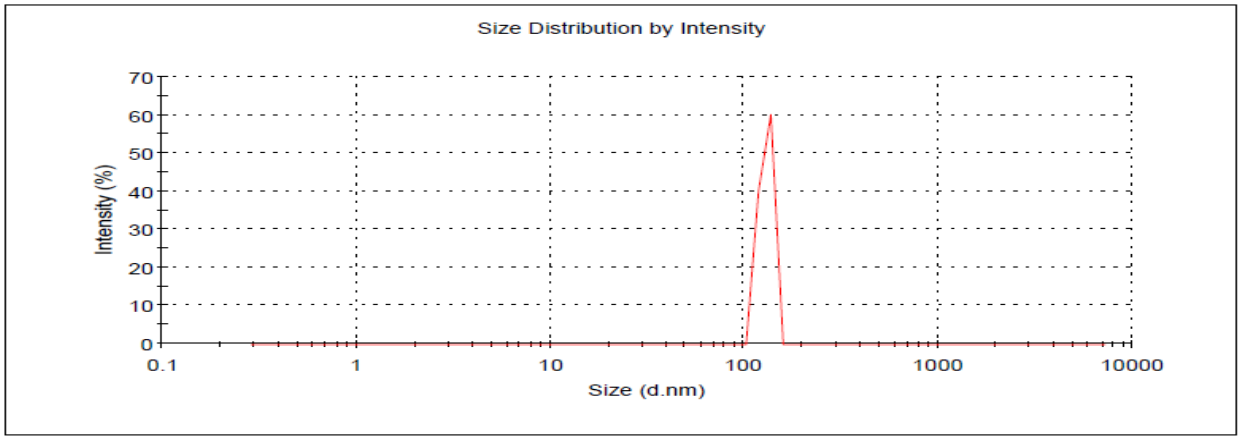

Figure 1: Vesicle Size of Batch F15

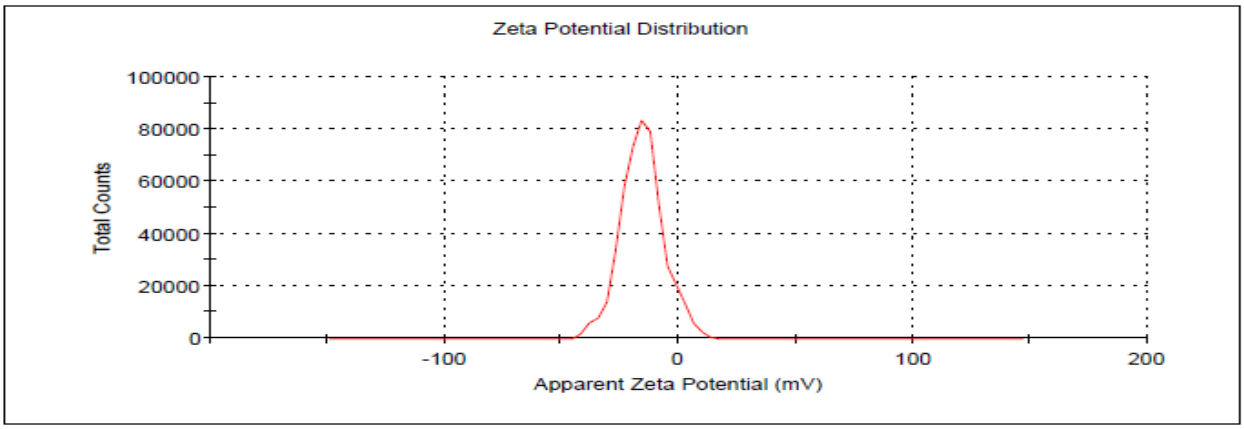

Figure 2: Zeta Potential of Batch F8

2.1.1 Results of %In-vitro drug release (Q12)

Figure 3 showed a plot of the % cumulative drug release (Q12) vs time based on the results of the %In-vitro drug release (Q12) of all the central composite design batches stated in Table 2.

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Table 2. Results of %*In-vitro* drug release (Q12)

Time (Hrs)	(*In-vitro* drug release (Q12) ± S.D., (n=3)) Batch No.														
	F1	**F2**	**F3**	**F4**	**F5**	**F6**	**F7**	**F8**	**F9**	**F10**	**F11**	**F12**	**F13**	**F14**	**F15**
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1	12.62 ± 1.21	15.21 ± 1.12	16.24 ± 1.16	7.26 ± 1.06	19 ± 1.09	11.63 ± 1.15	16.21 ± 1.22	18.22 ± 1.21	13.29 ± 1.15	15.23 ± 1.14	18.61 ± 1.17	7.23 ± 1.11	6.25 ± 1.09	19.57 ± 1.08	16.91 ± 1.24
2	21.2 ± 1.09	24.63 ± 1.19	25.29 ± 1.14	19.63 ± 1.12	27.2 ± 1.07	20.15 ± 1.23	25.62 ± 1.16	27.96 ± 1.19	22.39 ± 1.22	26.3 ± 1.11	26.33 ± 1.13	18.26 ± 1.21	15.69 ± 1.12	26.48 ± 1.16	28 ± 1.06
3	29.32 ± 1.11	31.27 ± 1.52	32.67 ± 1.32	28.45 ± 1.33	36.3 ± 1.11	28.32 ± 1.24	31.36 ± 1.11	35.61 ± 1.21	37.45 ± 1.22	32.38 ± 0.56	39.47 ± 1.19	26.89 ± 1.45	21.8 ± 1.40	37.62 ± 1.26	35.59 ± 1.21
4	38.69 ± 1.15	42.63 ± 1.18	44.21 ± 1.21	35.63 ± 1.14	43.4 ± 1.19	36.45 ± 1.27	39.63 ± 1.29	43.27 ± 1.33	43.69 ± 1.08	42.36 ± 1.36	45.88 ± 1.38	30.67 ± 1.36	29.5 ± 1.41	45.73 ± 1.05	49.51 ± 1.22
5	47.43 ± 1.12	49.36 ± 1.19	52.89 ± 1.23	43.21 ± 1.34	53.6 ± 1.29	47.75 ± 1.09	48.59 ± 1.16	52.93 ± 1.12	53.68 ± 1.22	49.78 ± 1.26	59.61 ± 1.41	38.36 ± 1.46	38.3 ± 1.34	59.32 ± 1.15	57.23 ± 1.21
6	59.21± 1.17	54.89 ± 1.23	58.12 ± 1.05	56.53 ± 1.13	65.9 ± 1.33	58.9 ± 1.16	56.91 ± 1.28	65.29 ± 1.09	59.82 ± 1.18	58.89 ± 1.12	68.21 ± 1.19	46.6 ± 0.51	47.8 ± 1.55	67.85 ± 1.29	65.59 ± 1.16
7	63.87 ± 1.13	65.23 ± 0.45	68.02 ± 1.41	62.39 ± 1.43	72.2 ± 1.34	65.12 ± 1.12	69.75 ± 1.05	71.56 ± 1.17	66.69 ± 1.26	65.68 ± 1.18	76.96 ± 1.16	59.2 ± 1.13	55.1 ± 1.41	73.73 ± 0.49	74.47 ± 1.14
8	70.22 ± 1.22	73.36 ± 1.25	76.24 ± 1.29	71.69 ± 1.33	76.3 ± 1.27	71.78 ± 1.23	78.63 ± 1.09	75.21 ± 1.18	73.59 ± 0.55	72.63 ± 1.19	81.36 ± 1.22	65.6 ± 1.13	66.7 ± 1.36	77.28 ± 1.27	82.69 ± 1.18
9	75.69 ± 1.24	75.29 ± 1.14	81.54 ± 1.43	73.65 ± 1.37	82.0 ± 1.08	76.47 ± 1.11	82.15 ± 1.28	81.63 ± 1.13	79.55 ± 0.50	76.28 ± 0.40	85.89 ± 1.26	72.5 ± 1.35	72.3 ± 1.28	82.05 ± 1.34	87.54 ± 1.23
10	81.22 ± 1.18	80.63 ± 1.19	83.45 ± 1.51	78.17 ± 1.41	86.2 ± 1.21	81.78 ± 1.28	86.45 ± 1.32	84.26 ± 0.46	85.17 ± 1.29	81.03 ± 1.10	88.45 ± 1.38	80.4 ± 1.46	78.2 ± 1.09	86.73 ± 1.39	90.23 ± 1.12
11	86.39 ± 1.14	85.17 ± 1.26	88.24 ± 0.56	82.27 ± 0.46	90.2 ± 1.24	85.24 ± 1.41	90.01 ± 1.30	87.59 ± 1.39	90.04 ± 1.18	85.48 ± 1.16	90.64 ± 0.47	85.1 ± 1.17	83.0 ± 1.16	90.35 ± 1.43	93.89 ± 1.28
12	91.22 ± 1.16	88.36 ± 1.39	90.24 ± 1.15	86.61 ± 1.21	93.6 ± 1.32	90.17 ± 1.23	92.28 ± 1.18	90.15 ± 0.59	93.37 ± 1.11	89.16 ± 1.01	93.28 ± 1.32	91.32 ± 1.02	89.43 ± 1.03	93.19 ± 1.02	97.08 ± 1.16

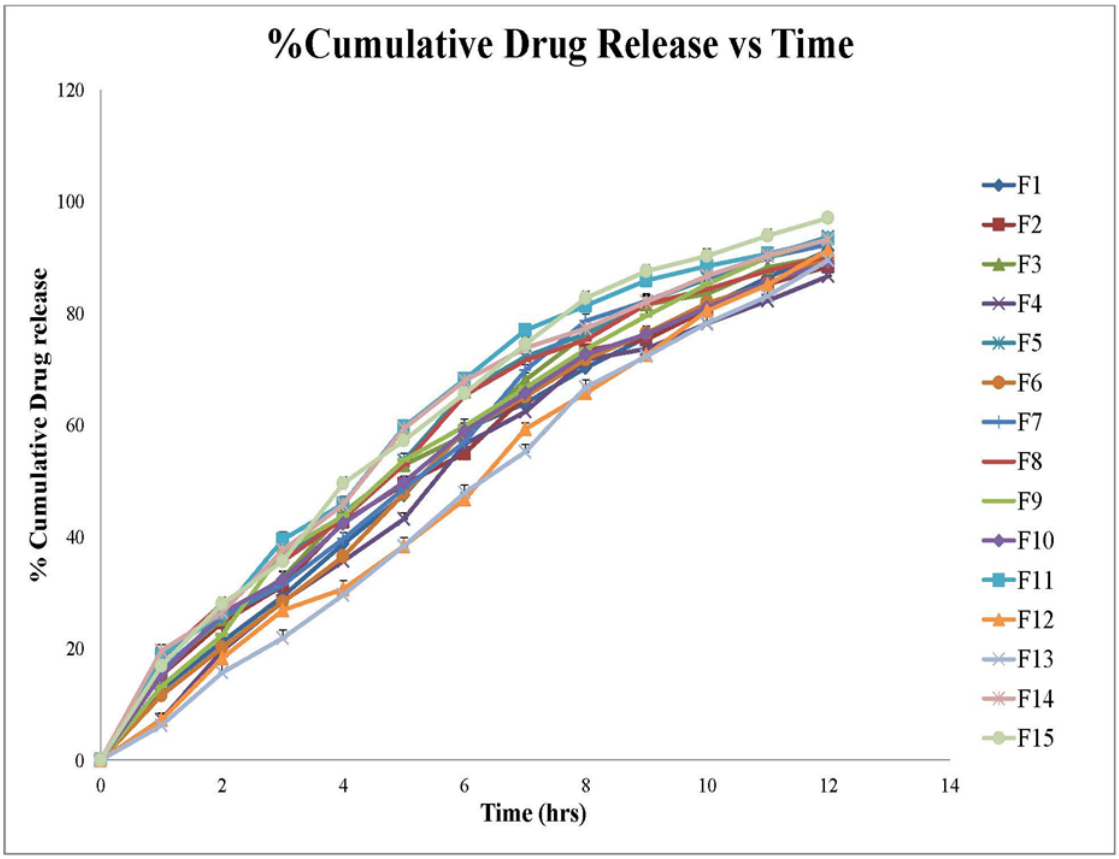

Figure 3. Plot of %cumulative drug release vs. time of %*in-vitro* drug release (Q12)

Figure 3 shows the plot of %cumulative drug release in 12 hours as a function of time for all formulations is displayed in Figure 3. The formulation provided no impedance to BDQ release. BDQ was released gradually and steadily over the period.

2.1.2 Results of statistical parameter obtained from ANOVA study

In the result of ANOVA for vesicle size stated in Table 3, the model F-value of 137.54 implied that the model was significant. P-value less than 0.0500 indicated that model terms were significant. In this case X1, X2, X3, X1X2, X1X3, $X1^2$, $X2^2$ were significant model terms.

Table 3: Result of ANOVA for vesicle size

Source	Sum of square	d.f.	Mean Square	F- Value	P- value
Model	1.951E+05	9	21676.16	137.54	<0.0001
X1	1..330E+05	1	1.330E+05	844.21	<0.0001
X2	1730.61	1	1730.61	10.98	0.0078
X3	2572.12	1	2572.12	16.32	0.0024
X1X2	1509.75	1	1509.75	9.58	0.0113
X1X3	2922.30	1	2922.30	18.54	0.0015
X2X3	526.50	1	526.50	3.34	0.0975
$X1^2$	52508.90	1	52508.90	333.18	<0.0001
$X2^2$	1502.48	1	1502.48	9.53	0.0115
$X3^2$	729.97	1	729.97	4.63	0.0569
Residual	1576.00	10	157.60		
Cor Total	1.967E+05	19			

In the result of ANOVA for zeta potential stated in Table 4, the model F-value of 81.43 implied that the model was significant. P-value less than 0.0500 indicated that model terms were significant. In this case X1, X2, X3, X2X3, $X1^2$, $X2^2$, $X3^2$ were significant model terms.

Table 4: Result of ANOVA for zeta potential

Source	Sum of square	d.f.	Mean Square	F- Value	P- value
Model	1929.00	9	214.33	81.43	<0.0001
X1	18.11	1	18.11	6.88	0.0255
X2	137.31	1	137.31	52.17	<0.0001
X3	952.51	1	952.51	361.88	<0.0001
X1X2	0.1800	1	0.1800	0.0684	0.7990
X1X3	0.7200	1	0.7200	0.2735	0.6124
X2X3	46.08	1	46.08	17.51	0.0019
$X1^2$	236.36	1	236.36	89.80	<0.0001
$X2^2$	353.31	1	353.31	134.23	<0.0001
$X3^2$	335.87	1	335.87	127.60	<0.0001
Residual	26.32	10	2.63		
Cor Total	1955.32	19			

In the result of ANOVA for %entrapment efficiency stated in Table 5, the model F-value of 132.48 implied that the model was significant. P-value less than 0.0500 indicated that model terms were significant. In this case X1, X2, X3, X2X3, $X1^2$, $X2^2$, $X3^2$ were significant model terms.

Table 5: Result of ANOVA for %entrapment efficiency

Source	Sum of square	d.f.	Mean Square	F- Value	P- value
Model	1247.30	9	138.59	132.48	<0.0001
X1	88.86	1	88.86	84.94	<0.0001
X2	691.71	1	691.71	661.24	<0.0001
X3	98.90	1	98.90	94.54	<0.0001
X1X2	0.6384	1	0.6384	0.6103	0.4528
X1X3	0.3120	1	0.3120	0.2983	0.5969
X2X3	20.16	1	20.16	19.27	0.0014
$X1^2$	119.65	1	119.65	114.38	<0.0001
$X2^2$	156.81	1	156.81	149.90	<0.0001
$X3^2$	138.70	1	138.70	132.59	<0.0001
Residual	10.46	10	1.05		
Cor Total	1257.76	19			

In the result of ANOVA for %drug loading stated in Table 6, the model F-value of 128.64 implied that the model was significant. P-value less than 0.0500 indicated that model terms were significant. In this case X1, X2, X3, X1X2, $X2^2$ were significant model terms.

Table 6: Result of ANOVA for %drug loading

Source	Sum of square	d.f.	Mean Square	F- Value	P- value
Model	1195.92	9	132.88	128.64	<0.0001
X1	98.85	1	98.85	95.70	<0.0001
X2	908.07	1	908.07	879.11	<0.0001
X3	11.43	1	11.43	11.06	0.0077
X1X2	26.68	1	26.68	25.83	0.0005
X1X3	0.8515	1	0.8515	0.8244	0.3853
X2X3	3.80	1	3.80	3.67	0.0843
$X1^2$	0.9637	1	0.9637	0.9330	0.3569
$X2^2$	145.15	1	145.15	140.52	<0.0001
$X3^2$	0.0266	1	0.0266	0.0257	0.8758
Residual	10.33	10	1.03		
Cor Total	1206.25	19			

In the result of ANOVA for %*In-vitro* drug release (Q12) stated in Table 7, the model F-value of 61.93 implied that the model was significant. P-value less than 0.0500 indicated that model terms were significant. In this case X1, X2, X3, $X1^2$, $X2^2$, $X3^2$ were significant model terms.

Table 7: Result of ANOVA for %*In-vitro* drug release (Q12)

Source	Sum of square	d.f.	Mean Square	F- Value	P- value
Model	215.19	9	23.91	61.93	<0.0001
X1	27.05	1	27.05	70.06	<0.0001
X2	4.07	1	4.07	10.54	0.0088
X3	18.94	1	18.94	49.05	<0.0001
X1X2	0.0480	1	0.0480	0.1245	0.7316
X1X3	0.0882	1	0.0882	0.2284	0.6430
X2X3	0.2113	1	0.2113	0.5472	0.4765
$X1^2$	73.57	1	73.57	190.56	<0.0001
$X2^2$	51.67	1	51.67	133.84	<0.0001
$X3^2$	71.86	1	71.86	186.12	<0.0001
Residual	3.86	10	0.3861		
Cor Total	219.05	19			

Based on results of statistical parameters obtained from ANOVA study stated in Table 8, the "Predicted R^2" value was in reasonable agreement with the "Adjusted R^2" value. The difference between "Predicted R^2" & "Adjusted R^2" was less than 0.2. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable.

Table 8: Results of statistical parameters obtained from ANOVA study

Response	Adjusted R^2	Predicted R^2	Adequate precision	C.V. (%)
Vesicle Size	0.9848	0.9356	43.4225	5.39
Zeta Potential	0.9744	0.8981	25.6234	6.41
%Entrapment Efficiency	0.9842	0.9346	35.6502	1.77
%Drug Loading	0.9837	0.9346	39.4259	4.47
%*In-Vitro* Drug Release (Q12)	0.9665	0.8626	22.0931	0.6699

2.2.Validation of experimental model and optimization by numerical method

It is possible to predict optimum levels for the independent variables by using the desirability function.

Table 9: Validation of experimental model and optimization by numerical method

Dependent Variables	Goal	Limits
Vesicle Size	In range	160 nm - 200 nm
Zeta Potential	In range	(-35 mV) - (-30 mV)
%Entrapment Efficiency	Maximize	62% - 66%
%Drug Loading	In range	18% - 22%
%*In-Vitro* Drug Release (Q12)	Maximize	95% - 97.08%

2.2.1 Composition of Solution 1

The desirability values of solution 1 is displayed in Figure 4 and the composition is mentioned in Table 10.

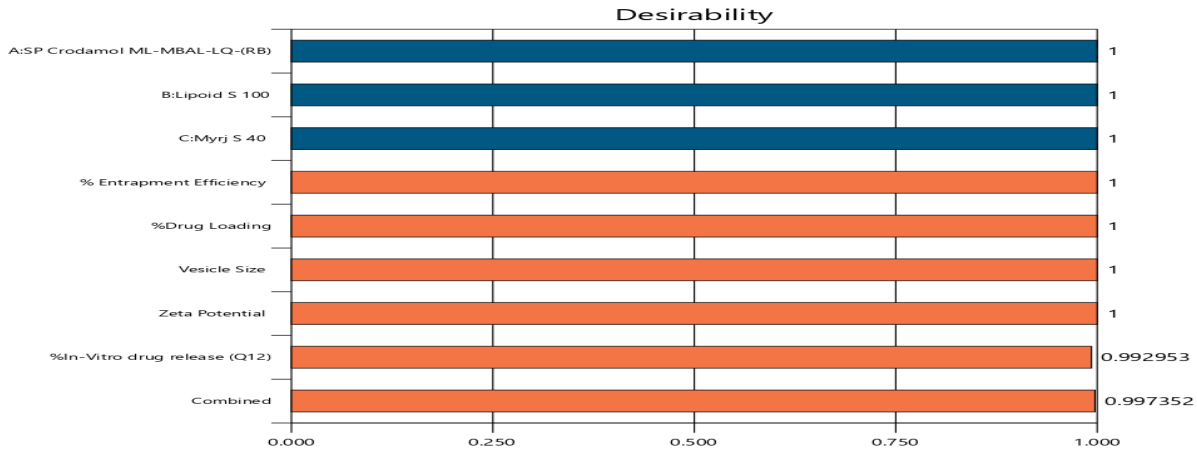

Figure 4. Desirability values of solution 1

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Table 10: Composition of Solution 1

Ingredient	Quantity (mg)
Drug	60.45
SP Crodamol ML-MBAL-LQ-(RB) (X1)	56.65
Lipoid S 100 (X2)	207.04
Myrj™ S 40 (mg) (X3)	22.40

2.2.2 Results of optimized batch (Solution 1)

The results of optimized batch (solution 1) listed in Table 11. Figure 5 and Figure 6 displayed the vesicle size and the zeta potential of optimized batch (solution 1), respectively.

Table 11: Results of optimized batch (Solution 1)

Property	Predicted Response	Observed Result ± S.D
Vesicle Size (nm)	172.342	175.51
Zeta Potential (mV)	-35.0016	-34.98
%Entrapment Efficiency	65.998	65.42 ± 0.49
%Drug Loading	18.256	18.01 ± 0.14
%*In-Vitro* Drug Release (Q12)	97.066	97.12 ± 0.89

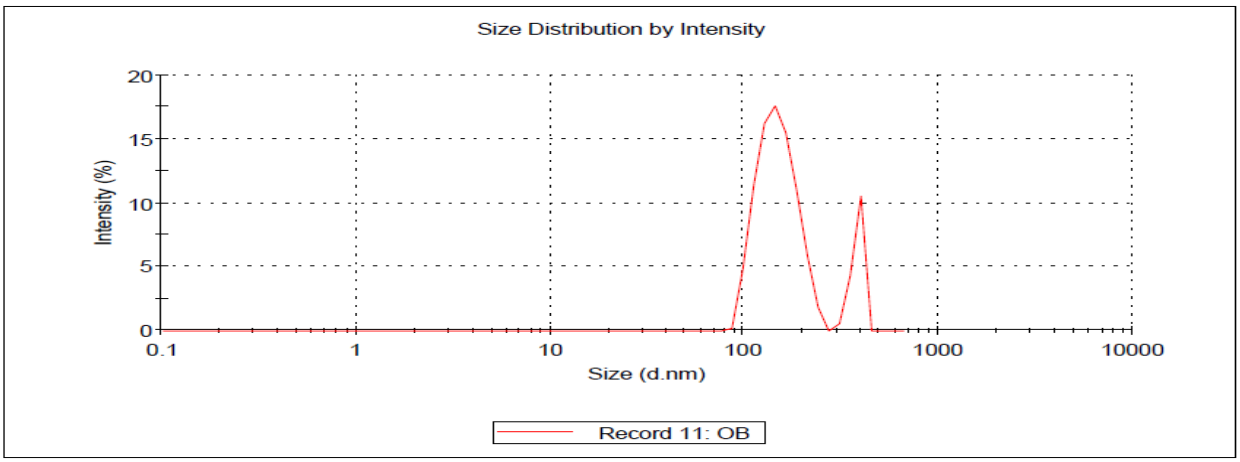

Figure 5: Vesicle size of optimized batch (Solution 1)

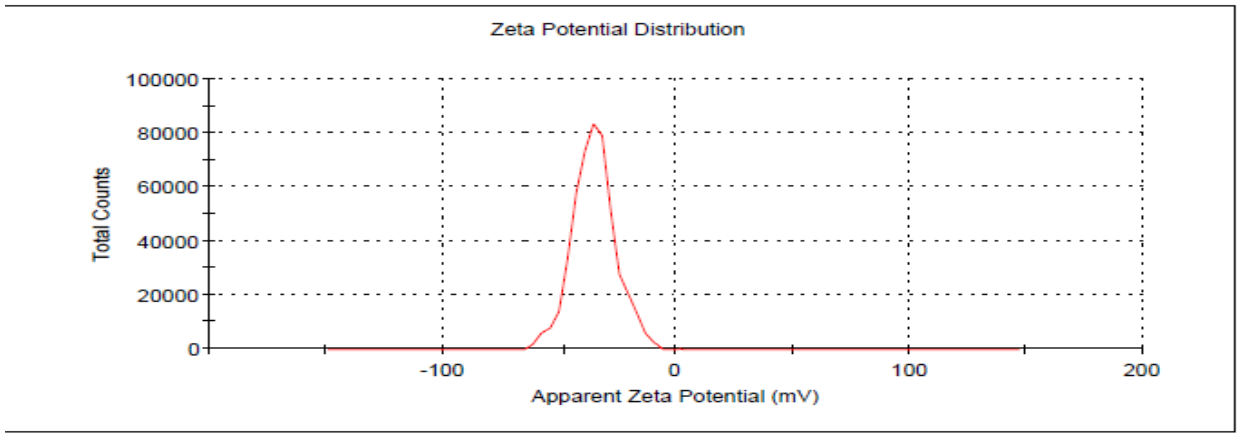

Figure 6: Zeta potential of optimized batch (Solution 1)

Table 12: Results of %*In-vitro* drug release (Q12) of optimized batch (Solution 1)

Time (hr)	%Cumulative Drug Release ± S.D (n=3)
0	0
1	18.89 ± 1.95
2	30.15 ± 0.98
3	37.85 ± 1.12
4	46.76 ± 1.45
5	55.19 ± 0.84
6	64.58 ± 1.22
7	70.15 ± 1.39
8	75.65 ± 1.56
9	82.76 ± 1.45
10	89.64 ± 1.99
11	93.87 ± 2.05
12	97.12 ± 0.89

Figure 7 and Table 12 shows the plot of Cumulative % drug release in 12 hours as a function of time for optimized formulation. The formulation provided no hindrance to BDQ release. BDQ was released slowly and steadily over the period.

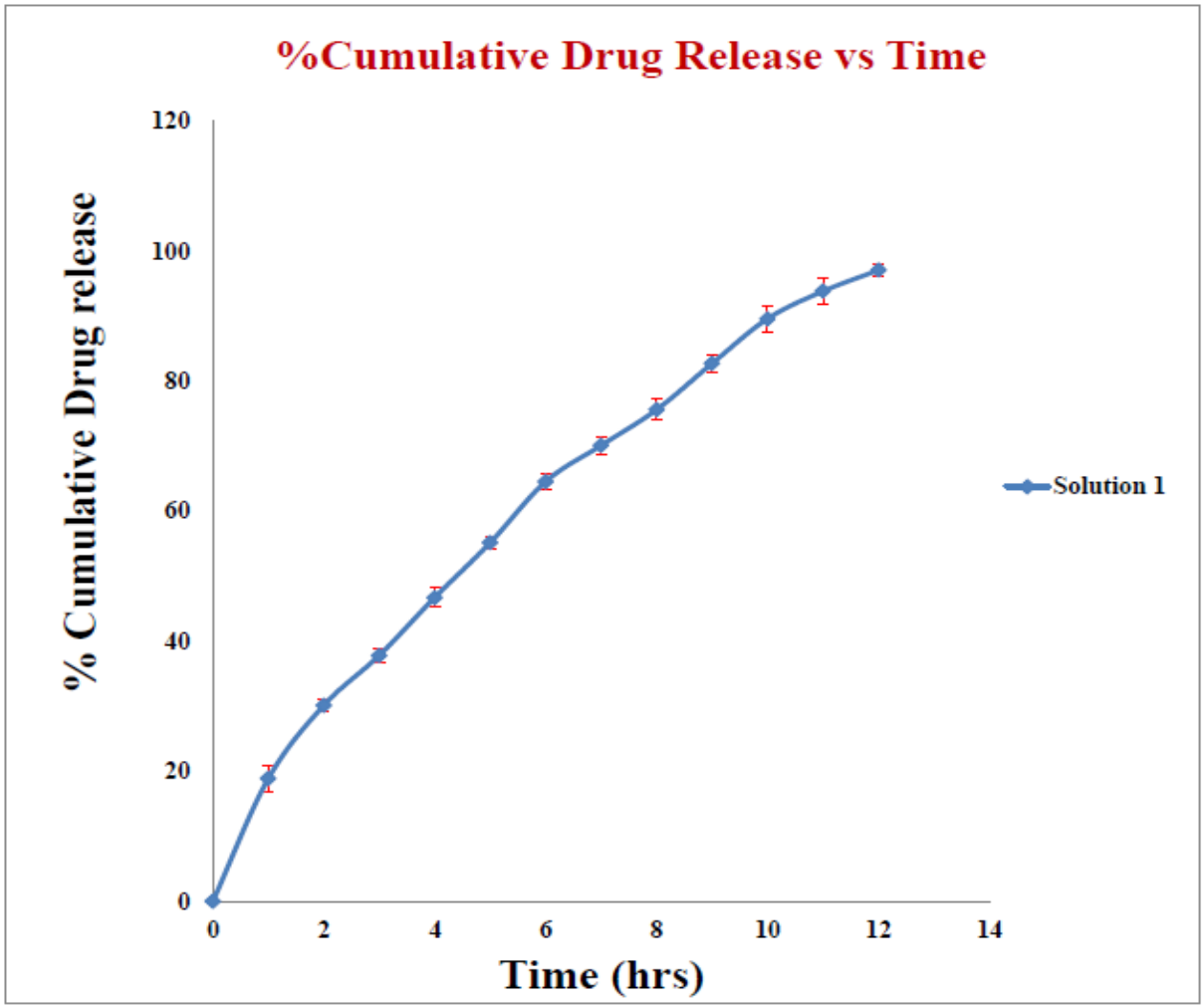

Figure 7: Plot of %cumulative drug release vs. time of %*In-vitro* drug release (Q12) of optimized batch (Solution 1)

2.2.3 Drug release kinetic models of optimized batch (Solution 1)

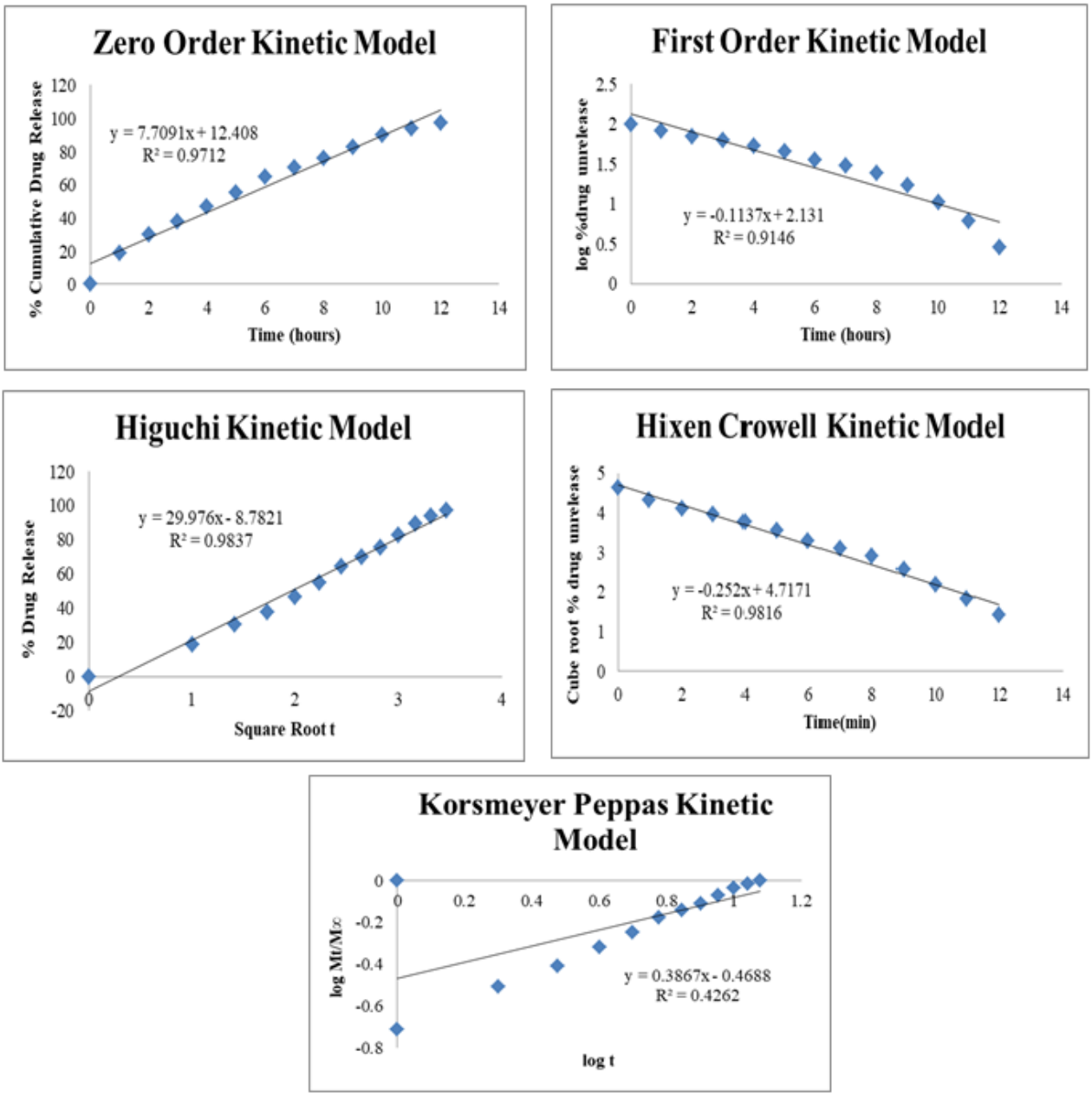

Figure 8: Drug release kinetic models of optimized batch (Solution 1)

Table 13: Result of drug release kinetic models of optimized batch (Solution 1)

Zero Order		First Order		Higuchi		Hixen Crowell		Korsmeyer Peppas	
R^2	K	R^2	K	R^2	K	R^2	K	R^2	K
0.9712	7.7091	0.9146	-0.1137	0.9837	29.976	0.9816	-0.252	0.4262	0.3867

Figure 8 and Table 13 showed the drug release was best fitted to the Higuchi kinetic model. So the drug release follows the diffusion mechanism.

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2.2.4 Response surface plot of optimized batch (Solution 1)

Response surface plot of optimized batch (solution 1) displayed in Figure 9 to Figure 13.

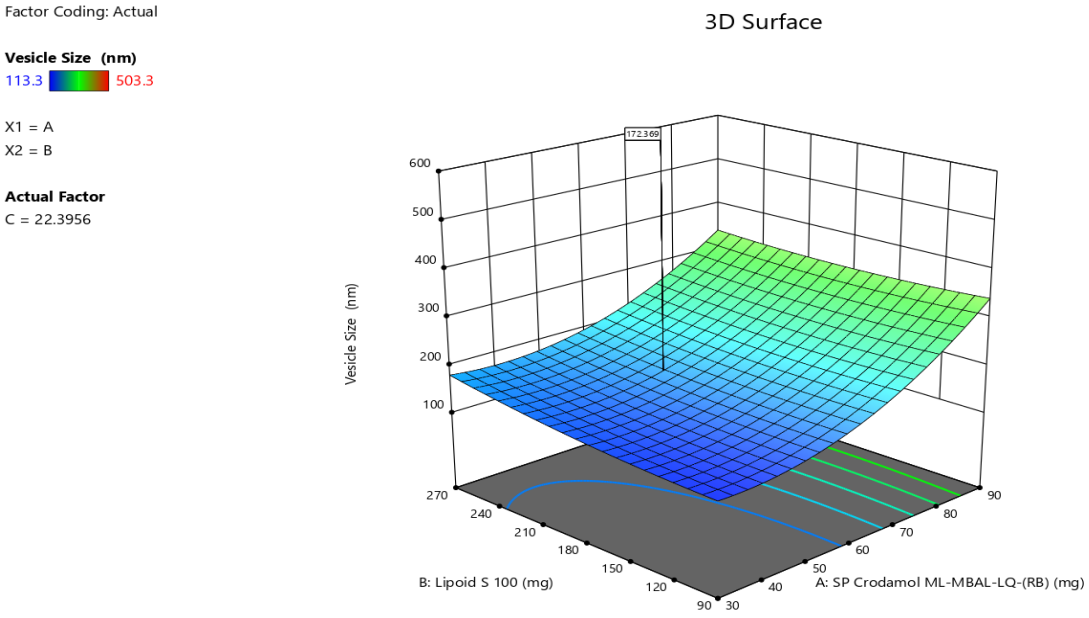

Figure 9. Response surface plot of vesicle size

Vesicle Size = +179.66 + 98.70 X1 + 11.26 X2 – 13.72 X3 – 13.74 X1X2 – 19.11 X1X3 + 8.11 X2X3 + 60.36 $X1^2$ + 10.21 $X2^2$ + 7.12 $X3^2$

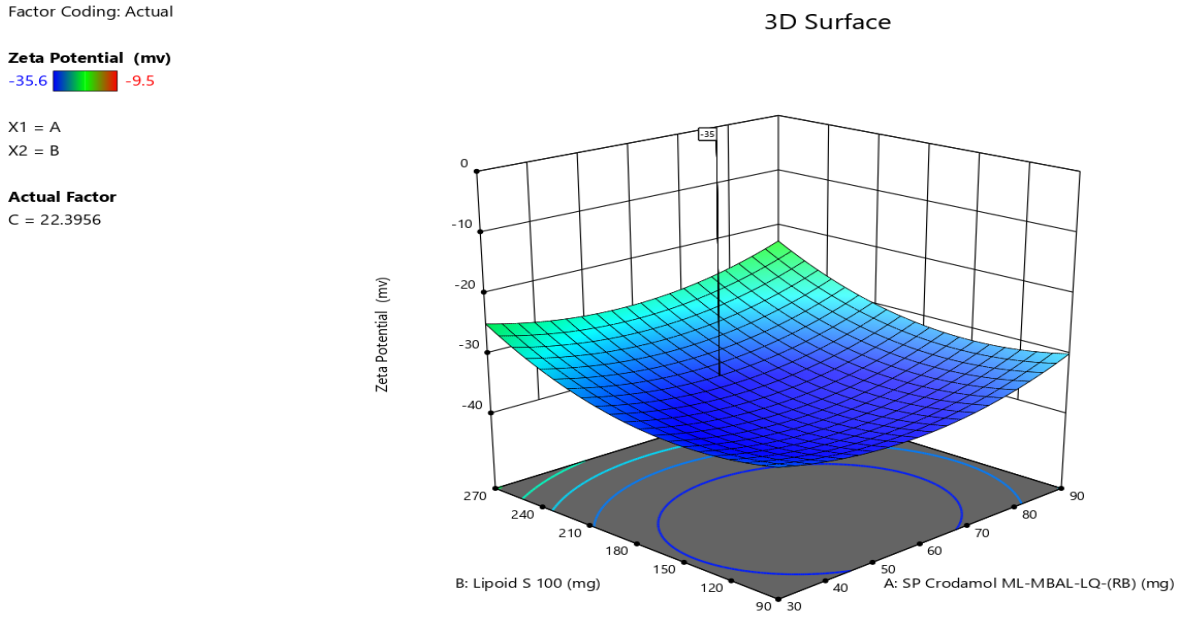

Figure 10. Response surface plot of zeta potential

Zeta potential = -34.77 + 1.15 X1 + 3.17 X2 – 8.35 X3 – 0.1500 X1X2 + 0.3000 X1X3 + 2.40 X2X3 + 4.05 $X1^2$ + 4.95 $X2^2$ + 4.83 $X3^2$

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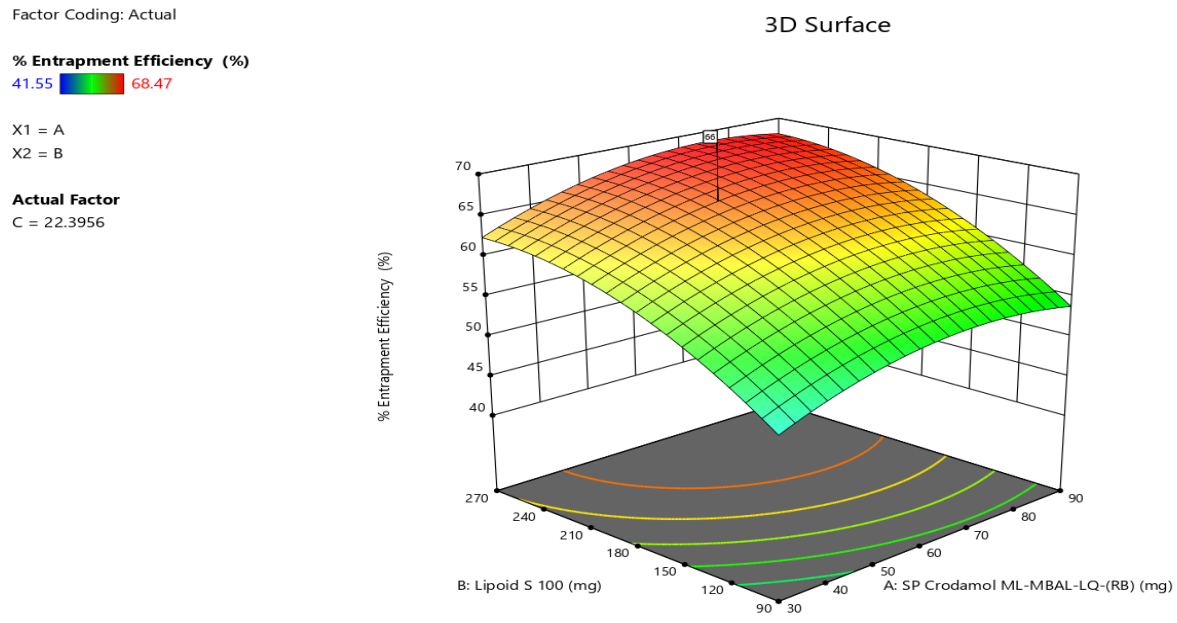

Figure 11. Response surface plot of %entrapment efficiency

%Entrapment efficiency = +64.13 + 2.55 X1 + 7.12 X2 + 2.69 X3 + 0.2825 X1X2 – 0.1975 X1X3 – 1.59 X2X3 – 2.88 $X1^2$ – 3.30 $X2^2$ – 3.10 $X3^2$

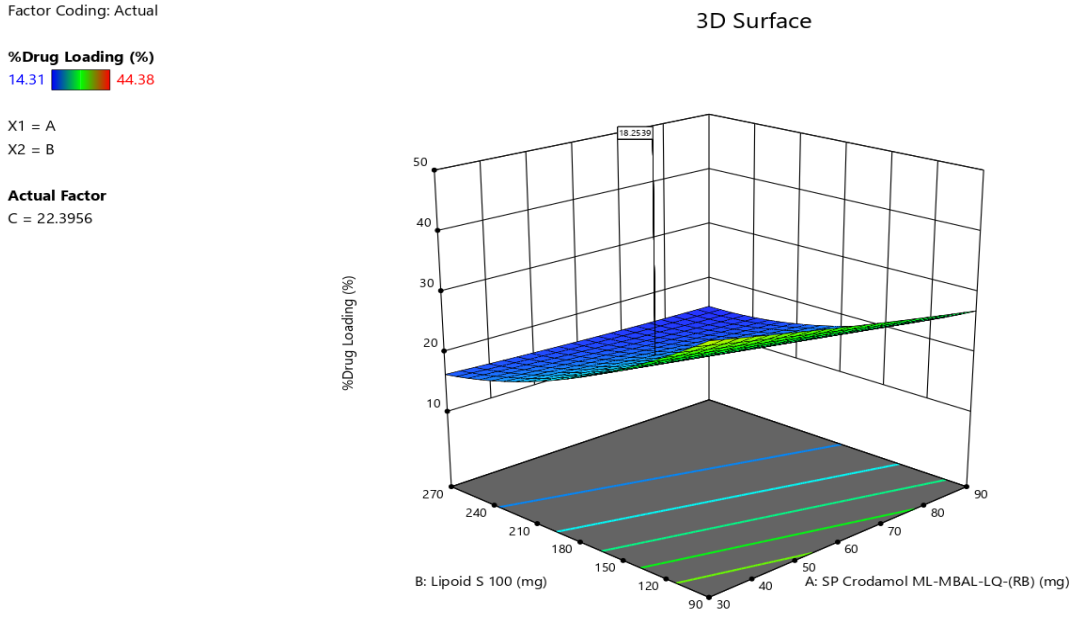

Figure 12. Response surface plot of %drug loading

%Drug loading = +20.35 – 2.69 X1 – 8.15 X2 – 0.9147 X3 + 1.83 X1X2 + 0.3263 X1X3 + 0.6888 X2X3 + 0.2586 $X1^2$ + 3.17 $X2^2$ + 0.0429 $X3^2$

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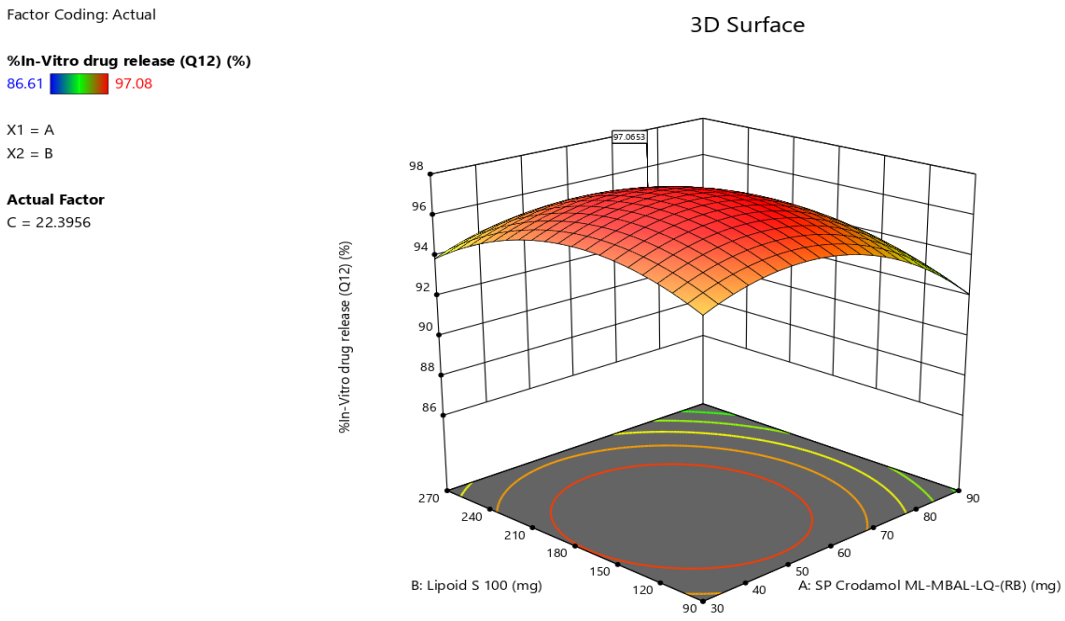

Figure 13. Response surface plot of %*in-vitro* drug release (Q12)

%*In-vitro* drug release (Q12) = +97.11 - 1.41 X1 - 0.5460 X2 + 1.18 X3 + 0.0775 X1X2 + 0.1050 X1X3 + 0.1625 X2X3 - 2.26 $X1^2$ - 1.89 $X2^2$ - 2.23 $X3^2$

The Optimized formulation (Solution 1) gave a sustained drug release up to 12 hrs. It formed a stable emulsion with a desired zeta potential. It had a high entrapment efficiency and smaller vesicle size. 18.01% of the NLCs were composed of BDQ, which indicated sufficient BDQ loading.

2.3 Lyophilization of BDQ Loaded NLCs

2.3.1 Results of Lyophilized BDQ Loaded NLCs of Batch 1

Results of Lyophilized BDQ Loaded NLCs of Batch 1 is listed in Table 14.

Table 14: Results of Lyophilized BDQ Loaded NLCs of Batch 1

Test	Specifications	Result
Angle of Repose(°)	≤30°	25.22°
Bulk density (g/cc)	-	0.0453
Tapped density (g/cc)	-	0.051
Carr's index (%)	≤15	11.18
Hausner's ratio	≤1.18	1.126
Moisture content (%w/w)	≤1.0	0.07
Empty Capsule weight (Size '3') (mg)	48.28 ± 5%	48.26
Average filled weight (mg)	71.33 mg ≅ 60.45 mg of BDQ ± 5%	71.29
Average weight of filled capsule (mg)	119.61 ± 5%	119.55
%Drug Content ± S.D	90.00%-110.00%	99.93% ± 0.52
%*In-Vitro* drug release (Q12) ± S.D	NLT 95.00%	98.12% ± 0.97

2.3.2 Results of %in-vitro drug release (Q12) of Lyophilized BDQ Loaded NLCs of Batch 1

Figure 14 and Table 15 shows the results of %cumulative drug release vs. time of %*in-vitro* drug release (Q12) of of Lyophilized BDQ Loaded NLCs of Batch 1.

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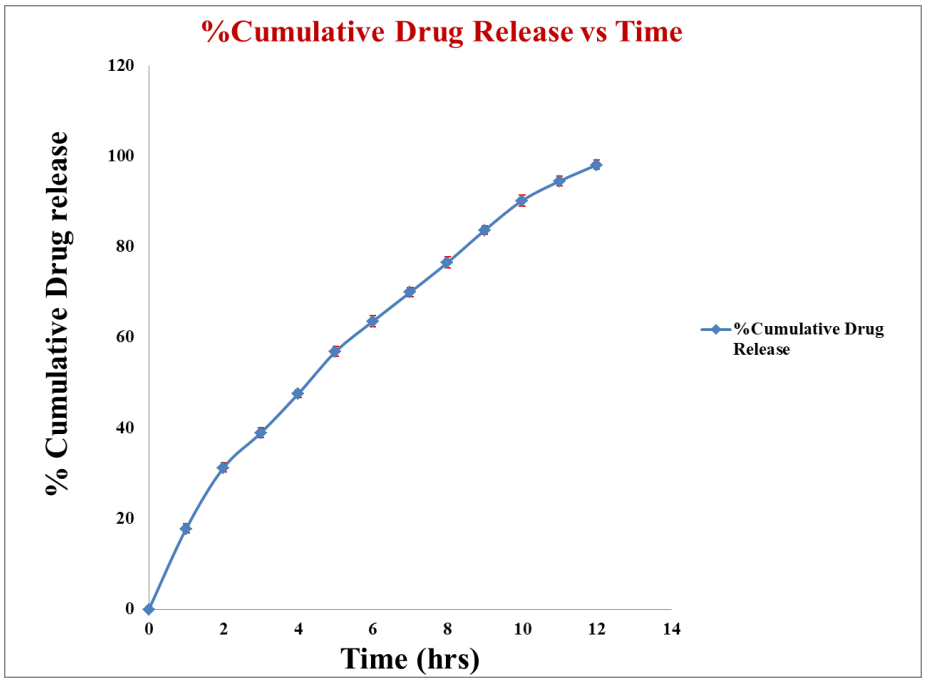

Figure 14: Plot of %cumulative drug release vs. time of %*in-vitro* drug release (Q12) of of Lyophilized BDQ Loaded NLCs of Batch 1

Table 15: Results of %*In-vitro* drug release (Q12) of lyophilized BDQ loaded NLCs of Batch 1

Time (hr)	%Cumulative Drug Release ± S.D (n=3)
0	0
1	17.79 ± 0.99
2	31.25 ± 1.05
3	38.95 ± 1.14
4	47.53 ± 0.90
5	56.87 ± 1.10
6	63.57 ± 1.26
7	69.98 ± 1.01
8	76.53 ± 1.21
9	83.69 ± 0.93
10	90.21 ± 1.24
11	94.52 ± 1.04
12	98.12 ± 0.97

The plot of %cumulative drug release in 12 hours as a function of time for lyophilized BDQ loaded NLCs of batch 1 is displayed in Figure 14. The formulation provided no impedance to BDQ release. BDQ was released gradually and steadily over the period.

2.4. In-vitro lung deposition study by andersen cascade impactor

2.4.1 Distribution of drug at various stages of andersen cascade impactor of Batch 1

Table 16 and Figure 15 shows the Distribution of drug at various stages of andersen cascade impactor of Batch 1.

Table 16: Distribution of drug at various stages of andersen cascade impactor of Batch 1

Stages of ACI	No. of Capsule										Avg.	±SD
	1	2	3	4	5	6	7	8	9	10		
Device (D) (mg)	0.11	0.10	0.09	0.10	0.11	0.12	0.11	0.13	0.09	0.11	0.11	0.01
Capsule shells (mg)	0.13	0.11	0.12	0.11	0.10	0.09	0.11	0.09	0.01	0.09	0.10	0.03
Induction port (IP) (mg)	0.26	0.29	0.39	0.38	0.22	0.12	0.32	0.32	0.13	0.11	0.25	0.11
Pre-separator (PS) (mg)	0.11	0.08	0.11	0.25	0.15	0.19	0.11	0.10	0.08	0.05	0.12	0.06
Stage 0 (mg)	0.11	0.10	0.08	0.09	0.19	0.08	0.12	0.11	0.13	0.12	0.11	0.03
Stage 1 (mg)	1.12	1.23	1.05	1.32	1.01	1.08	1.11	1.08	1.21	1.02	1.12	0.10
Stage 2 (mg)	1.58	1.75	1.42	1.64	1.63	1.45	1.57	1.59	1.42	1.58	1.56	0.11

Stage 3 (mg)	2.02	2.11	1.99	1.98	2.33	2.01	2.11	2.12	2.32	2.09	2.11	0.13
Stage 4 (mg)	10.58	10.89	10.93	10.59	10.42	10.65	10.14	10.11	10.93	10.59	10.58	0.30
Stage 5 (mg)	22.78	22.65	22.69	22.54	22.79	22.58	22.72	22.45	22.48	22.78	22.65	0.13
Stage 6 (mg)	22.04	22.01	22.02	22.05	22.34	22.13	22.06	22.07	22.04	22.01	22.08	0.10
Stage 7 (mg)	7.76	7.91	7.74	7.65	7.54	7.76	7.93	7.79	7.85	7.65	7.76	0.12
Stage 8 (mg)	1.28	1.29	1.29	1.30	1.26	1.29	1.28	1.25	1.25	1.26	1.28	0.02
Filter (F) (mg)	0.98	0.99	0.97	0.97	0.98	0.97	0.96	0.97	0.98	0.99	0.98	0.01
Total recovery (mg)	70.86	71.51	70.89	70.97	71.07	70.52	70.65	70.18	70.92	70.45	70.80	0.37

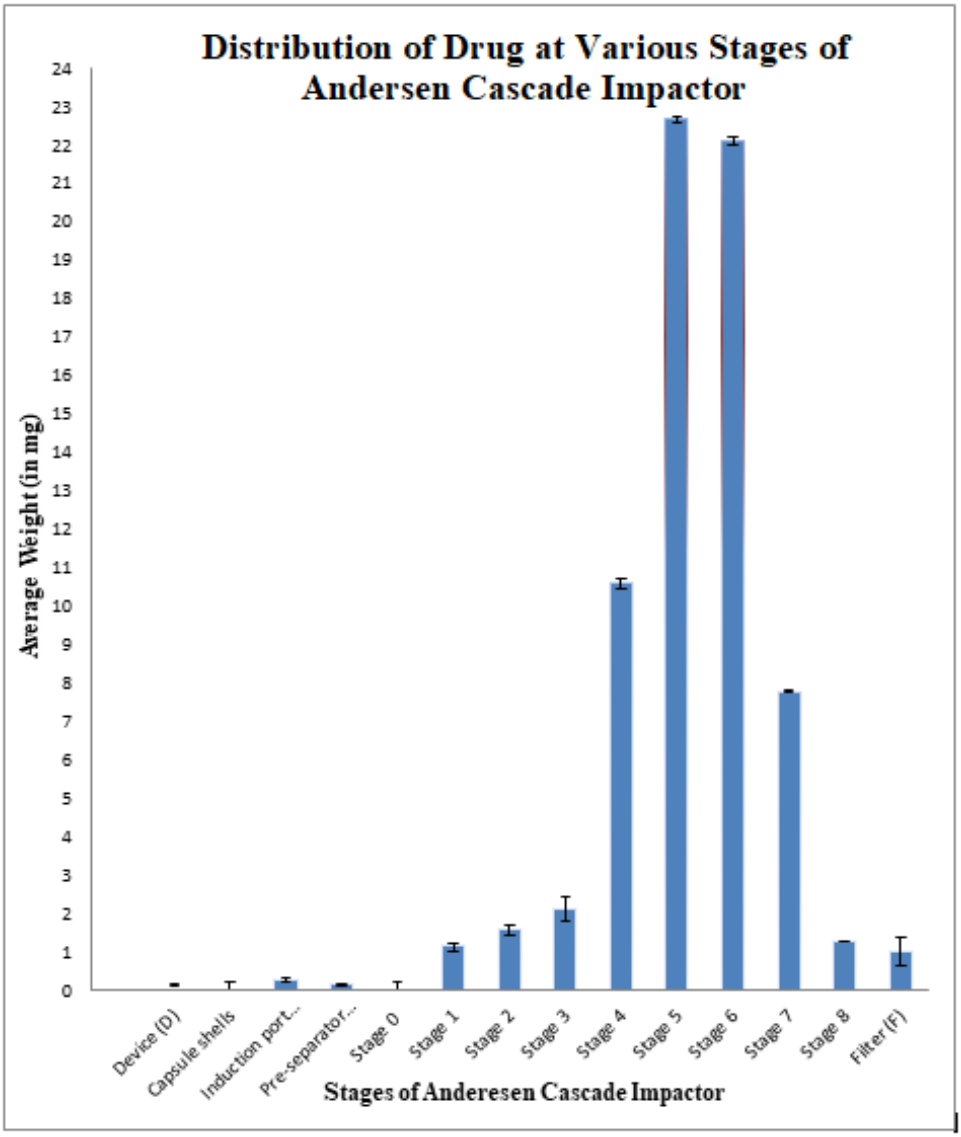

Figure 15. Distribution of drug at various stages of andersen cascade impactor of Batch 1

2.4.2 Calculation for mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD) of Batch 1

In-vitro lung deposition study showed that the DPIs of BDQ loaded NLCs could be deposited in the deep lung tissue. GSD=1 showed that all the particles were of same size. This formulation has great potential to treat the MDR-Tuberculosis and it can reduce the side effects associated with available marketed dosage form. Calculation for MMAD (µm) and GSD of Batch 1 and results are listed in Table 17 and Table 18 respectively.

Table 17: Calculation for MMAD (µm) and GSD of Batch 1

Parameters	Criteria
Sample flow rate Q (l/min)	27.000
Sampling volume (m^3)	0.027000
Time	1 min.

Stage No.	Cut-Point Dp (µm)	Weight gain W3 (mg)	Conc ΔC (mg/m^3)	log10Dp	$\Delta log_{10}Dp$	ΔC/Δlog10Dp ($mg.m^3 log_{10}\mu m$)	GMD (µm)	W/W_{tot} (%)	Cumulative mass % <Dp
1	21.30	1.12	41.48	1.33	0.37	111.93	32.63	1.60	98.40
2	14.80	1.56	57.78	1.17	0.16	365.41	17.75	2.22	96.18
3	9.80	2.11	78.15	0.99	0.18	436.49	12.04	3.01	93.17
4	6.00	10.58	391.85	0.78	0.21	1839.03	7.67	15.09	78.08
5	3.50	22.65	838.89	0.54	0.23	3583.72	4.58	32.30	45.78
6	1.55	22.08	817.78	0.19	0.35	2311.83	2.33	31.49	14.29
7	0.93	7.76	287.41	-0.03	0.22	1295.51	1.20	11.07	3.22
8	0.52	1.28	47.41	-0.28	0.25	187.77	0.70	1.83	1.40
F	0.26	0.98	36.30	-0.59	0.30	120.57	0.37	1.40	0.00
Total		**70.12**	**2597.04**						

	Cut-Point Dp (µm)	Cumulative Mass % <Dp
Value above the 50% cummulative mass<Dp	6.00	78.08
Value below the 50% cummulative mass<Dp	3.5	45.78
Value above the 16% cummulative mass<Dp	3.5	45.78
Value below the 16% cummulative mass<Dp	1.6	14.29

Table 18: Summary Results of Batch 1

Parameters	Results
Inhalable concentration (C_{tot}) mg/m^3	2597
%mass in the thoracic fraction (<11.64µm)	94%
%mass in the respirable fraction (<4.25µm)	55%
MMAD (µm)	4
GSD	1

2.5. *In-vitro* antimicrobial activity

2.5.1 Observation of bacterial growth after 8 weeks

MIC (Minimum inhibitory concentration) is defined as the highest dilution that exhibits at least 99% inhibition. The result of this is much affected by the size of the inoculum. Results were reported as visual growth found on L.J media and lowest dilution of drug had no growth on media while positive control showed significant growth.

Table 19: Observation of bacterial growth after 8 weeks of *in-vitro* antimicrobial activity

Inhaled dry powder of bedaquiline loaded NLCs capsule 50 mg (Batch 1)	
Sample	**Colonies Observed or not**
Negative Control (Drug-100 µg/ml)	No Growth
Positive Control (H_{37}RV Strain)	4+
100 µg/ml, 50 µg/ml, 12.5 µg/ml, 10 µg/ml, 8 µg/ml, 6.25 µg/ml, 5 µg/ml, 4 µg/ml, 3.125 µg/ml, 2.5 µg/ml, 2 µg/ml	No Growth
1.25 µg/ml, 1 µg/ml	1+
0.5 µg/ml, 0.25 µg/ml	2+
Bedaquiline Tablets 50 mg	
Sample	**Colonies Observed or not**
Negative Control (Drug-100 µg/ml)	No Growth
Positive Control (H_{37}RV Strain)	4+
100 µg/ml, 50 µg/ml, 12.5 µg/ml, 10 µg/ml, 8 µg/ml, 6.25 µg/ml, 5 µg/ml, 4 µg/ml, 3.125 µg/ml, 2.5 µg/ml	No Growth
2 µg/ml, 1.25 µg/ml	1+
1 µg/ml, 0.5 µg/ml, 0.25 µg/ml	2+

Table 20: Results of *in-vitro* antimicrobial activity

	Method	L.J. Medium (Conventional Method)
	Bacteria	$H_{37}RV$ Culture
Sr. No.	**Product Name**	**MIC (µg/ml)**
1	Inhaled Dry powder of bedaquiline loaded NLCs capsule 50 mg (Batch 1)	2
2	Bedaquiline Tablets 50 mg	2.5

In-vitro antimicrobial activity of developed inhaled dry powder of bedaquiline loaded NLCs capsule 50 mg was compared with the bedaquiline tablets 50 mg. From the Table 19 and Table 20, the minimum inhibitory concentration (MIC) of inhaled dry powder of bedaquiline loaded NLCs capsule 50 mg was found to be 2 µg/ml which is less than the available marketed dosage form.

2.6. Stability study

2.6.1 Results of accelerated stability data

The results of accelerated stability studies of Batch 1 is listed in Table 21.

Table 21: Accelerated stability studies (40°C±2°C & 75%±5%RH)

Product Name	Inhaled Dry Powder of Bedaquiline loaded NLCs			**Stability start date**	18/02/2023
Strength	60.45 mg eq. to 50 mg	**Batch No.**	Batch 1	**Packing**	60 DPIs Capsule packed in HDPE Bottle.
Mfg. Date	01/2023	**Exp. Date**	12/2024		
Test	**Specification**			**Initial**	**After 180 days**
Appearance	White coloured free flowing powder filled in Red/Transperent coloured size "3" hard gelatin capsule.			Complies	Complies
Average filled weight (mg)	71.33 ± 5%			71.29	71.32
Angle of Repose (°)	≤30°			25.22°	24.96°
Hausner's ratio	≤1.18			1.126	1.136
Carr's index (%)	≤15			11.18%	11.96%
Moisture Content (% w/w)	≤1.0			0.07	0.05
%Drug Content ± S.D	90.00-110.00			99.93 ± 0.52	99.89 ± 0.89
%*In-Vitro* Drug Release (Q12) ± S.D	NLT 95.00			98.12 ± 0.97	97.91 ± 0.26

2.6.2 Results of long term stability data

The results of Long term stability studies of Batch 1 is listed in Table 22.

Table 22: Long term stability studies (30°C±2°C & 75%±5%RH)

Product Name	Inhaled Dry Powder of Bedaquiline loaded NLCs			**Stability start date**	18/02/2023
Strength	60.45 mg eq. to 50 mg	**Batch No.**	Batch 1	**Packing**	60 DPIs Capsule packed in HDPE Bottle.
Mfg. Date	01/2023	**Exp. Date**	12/2024		
Test	**Specification**			**Initial**	**After 180 days**

Appearance	White coloured free flowing powder filled in Red/Transperent coloured size "3" hard gelatin capsule.	Complies	Complies
Average filled weight (mg)	71.33 ± 5%	71.29	71.30
Angle of Repose (º)	≤30º	25.22º	25.05º
Hausner's ratio	≤1.18	1.126	1.129
Carr's index (%)	≤15	11.18%	11.52
Moisture Content (% w/w)	≤1.0	0.07	0.09
%Drug Content ± S.D	90.00-110.00	99.93 ± 0.52	99.78 ±1.06
%*In-Vitro* Drug Release (Q12) ± S.D	NLT 95.00	98.12 ± 0.97	0.56

2.6.3 Results of %in-vitro drug release (Q12) of DPIs capsule after accelerated and long term stability study of Batch 1

Table 23 and Figure 16 showed the results of *%in-vitro* drug release (Q12) of DPIs capsule after accelerated and long term stability study of Batch 1.

Table 23: Results of %*in-vitro* drug release (Q12) of Batch 1

Time (hr)	%Cumulative Drug Release ± S.D (n=3)		
	Initial	**Accelerated Stability**	**Long Term Stability**
0	0	0	0
1	17.79 ± 0.99	16.96 ± 1.14	17.95 ± 0.63
2	31.25 ± 1.05	30.19 ± 0.98	30.96 ± 1.19
3	38.95 ± 1.14	37.21 ± 0.66	37.67 ± 0.57
4	47.53 ± 0.90	45.96 ± 0.32	46.22 ± 0.92
5	56.87 ± 1.10	54.23 ± 0.41	55.37 ± 1.03
6	63.57 ± 1.26	62.45 ± 0.87	62.74 ± 0.96
7	69.98 ± 1.01	68.02 ± 1.27	70.21 ± 0.81
8	76.53 ± 1.21	75.41 ± 0.74	77.02 ± 0.37
9	83.69 ± 0.93	82.98 ± 1.04	84.39 ± 0.45
10	90.21 ± 1.24	89.54 ± 0.86	90.61 ± 0.99
11	94.52 ± 1.04	94.16 ± 0.87	95.10 ± 0.68
12	98.12 ± 0.97	97.91 ± 0.48	98.35 ± 0.39

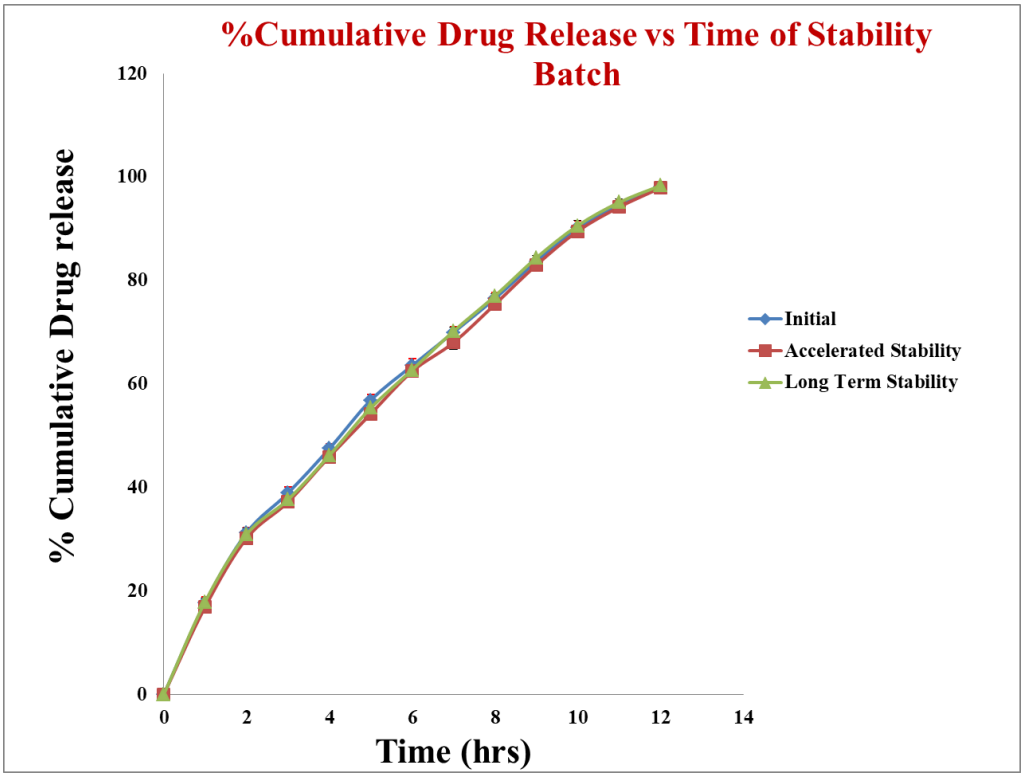

Figure 16. Plot of %cumulative drug release vs. time of %*in-vitro* drug release (Q12) of DPIs capsule after accelerated and long term stability study of Batch 1

Results of stability studies showed that there was no significant change in Appearance, Characteristics of Flow Properties, Moisture Content (%), %Drug Content, %*In-Vitro* Drug Release (Q12) when stored at accelerated and long term stability conditions for 180 days.

3. CONCLUSION

The optimized formulation of BDQ loaded NLCs emulsion gave a sustained drug release up to 12 hrs. It formed a stable emulsion with a desired zeta potential. It had a high entrapment efficiency and smaller vesicle size and sufficient drug loading. The BDQ loaded NLCs emulsion was lyophilized with addition of equivalent proportion of cryoprotectants & lyoprotectants which protected the BDQ from physical and chemical change during freezing and drying stage and it formed a perfect cake. The prepared capsules of inhaled dry powder of bedaquiline loaded NLCs had good flow characteristics and maximum drug content. The formulation provided no impedance to BDQ release. *In-vitro* lung deposition study showed that the inhaled dry powder of BDQ loaded NLCs could be deposited in the deep lung tissue. The minimum inhibitory concentration (MIC) of inhaled dry powder of bedaquiline loaded NLCs capsule 50 mg was less than the available marketed dosage form. There was no appreciable difference in appearance, characteristics of flow properties, %drug content, moisture content (%), %*in-vitro* drug release (Q12) of inhaled dry powder of BDQ loaded NLCs when stored at accelerated and long term stability conditions for 180 days. The developed inhaled dry powder of BDQ loaded NLCs formulation delivered directly to the lungs and had great potential to treat the MDR-Tuberculosis. İt gave a target specific action, reduced the dosing frequency, improved the bioavailability, reduced the peripheral tissue exposure and overcome the side effect associated with marketed solid oral dosage form.

4. MATERIALS AND METHODS

4.1 Sample, Chemical and Reagent

BDQ was received as a gift sample from Dishman Carbogen Amcis Ltd., India. Analytical-grade reagents were utilized throughout the study. Di-sodium hydrogen phosphate heptahydrate ($Na_2HPO_4.7H_2O$), sodium phosphate monobasic monohydrate ($NaH_2PO_4.H_2O$), hydrochloric acid, sodium hydroxide, ethylene glycol and mannitol were procured from Sigma-Aldrich Chemicals Pvt. Ltd., India. SP

crodamol ML-MBAL-LQ-(RB) & Myrj™ S 40 was received as a gift sample from Croda, India. Lipoid S 100 was received as a gift sample from Lipoid, Germany. Ethanol was procured from Suvidnath Laboratories, India.

4.2 Preparation of BDQ loaded NLCs [6,7,11]

BDQ loaded NLCs was prepared by solvent injection technique. Lipid phase was prepared by dissolving SP crodamol ML-MBAL-LQ-(RB), lipoid S 100 and BDQ in ethanol above 5°C of their melting point. Aqueous phase was prepared by mixing of Myrj™ S 40 in purified water and heated to the same temperature as lipid phase. Lipid Phase was injected into aqueous phase through inject needle under continuous stirring at 2000 RPM & 70°C-75°C for 45 minute on magnetic stirrer to create transparent or translucent O/W emulsion. Probe sonicated the O/W emulsion and cooled it at room temperature to create NLCs [5-7].

4.2.1 Optimization of BDQ loaded NLCs

A Central Composite design was employed to optimize the formulation of BDQ loaded NLCs. The design was employed to study the effect of independent variables, i.e. Amount of SP Crodamol ML-MBAL-LQ-(RB) (X1), amount of Lipoid S 100 (X2), amount of MyrjTM S 40 (X3) on dependent variables Vesicle Size (Y1), Zeta Potential (Y2), % Entrapment Efficiency (Y3), %Drug Loading (Y4), %*In-Vitro* Drug Release (Q12) (Y5).

A statistical model incorporating interactive and polynomial terms was utilized to evaluate responses.

$$Y= b_0 + b_1X1 + b_2X2 + b_3X3 + b_{11}X1^2 + b_{22}X2^2 + b_{33}X3^2 + b_{12}X1X2 + b_{13}X1X3 + b_{23}X2X3$$

Where Y is the dependent variable, b0 is the arithmetic mean response of the fifteen runs, b1, b2 and b3 are the estimated coefficients for the factor X1, X2 and X3 respectively. The main effects (X1, X2, X3) and Quadratic effect (X12, X22, X32) represent the average result of changing one factor at a time from its low to high value. The interaction terms X1X2, X1X3 and X2X3 shows how the response changes when two factors are simultaneously changed.

List of independent and dependent variable and Central composite design layout for formulation of BDQ loaded NLCs is mentioned in Table 24 and Table 25 respectively.

Table 24: List of independent and dependent variable

Independent Variable	Dependent Variable
SP Crodamol ML-MBAL-LQ-(RB) (X1)	Vesicle Size (Y1)
Lipoid S 100 (X2)	Zeta Potential (Y2)
Myrj™ S 40 (X3)	% Entrapment Efficiency (Y3)
	%Drug Loading (Y4)
	%In-Vitro Drug Release (Q12) (Y5)

Table 25: Central composite design layout for formulation of BDQ loaded NLCs

Batch No.	Drug BDQ (mg)	Independent Variable Levels in actual unit (mg)		
		X1	X2	X3
F1	60.45	30	90	10
F2	60.45	90	90	10
F3	60.45	30	270	10
F4	60.45	90	270	10
F5	60.45	30	90	30
F6	60.45	90	90	30
F7	60.45	30	270	30
F8	60.45	90	270	30
F9	60.45	9.55	180	20

F10	60.45	110.45	180	20
F11	60.45	60	28.64	20
F12	60.45	60	331.36	20
F13	60.45	60	180	3.18
F14	60.45	60	180	36.82
F15	60.45	60	180	20

Where,
X1 = Amount of SP Crodamol ML-MBAL-LQ-(RB)
X2 = Amount of Lipoid S 100
X3 = Amount of Myrj™ S 40

4.2.2 Validation of experimental model and optimization by numerical method

In order to assess the reliability of the equations that described the impact of the factors on Vesicle Size (Y1), Zeta Potential (Y2), % Entrapment Efficiency (Y3), %Drug Loading (Y4), %In-Vitro Drug Release (Q12) (Y5), an additional checkpoint batch experiment was concluded. The following formula was used to determine the %relative error between the predicted and observed values.

$$\% \text{ Relative Error} = \frac{\text{Predicted Value} - \text{Observed Value}}{\text{Predicted Value}} \times 100$$

The primary goal of the formulation development was to determine the optimum level of variables to ensure that the finished product has the intended or targeted qualities. Thus, the desirability function was used to carry out the optimization. After establishing the criteria for various dependent variables, the optimized formulation—which had the Desirability Function (D) value closest to 1 was selected from the potential formulations.

4.3 Evaluation parameters of BDQ loaded NLCs

4.3.1 Vesicle Size and Zeta Potential [12]

The zeta potential (ZP) and vesicle size of NLCs was examined using a zeta sizer. The mean vesicle size was determined using photon correlation spectroscopy, which examines variations in dynamic light scattering brought on by the brownian motion of the particles. At 25°C, the mean diameter of 10 mm diameter cells was measured at a 90° angle. When examining the physical stability of any colloidal system, the ZP reflecting the electric charge on the vesicle surface is a valuable measurement. An electrophoretic light scattering method is used to determine it. After an appropriate dilution of all samples with the original dispersion media, all size and ZP measurements were performed at 25°C using disposable polystyrene cells and disposable plain folded capillary zeta cells, respectively.

4.3.2 % Entrapment Efficiency (EE) & % Drug Loading [12]

The %EE of BDQ loaded NLCs was determined after separation of the non-entrapped BDQ. The centrifugation method was used to measure the %EE of BDQ in NLC's emulsion. The BDQ loaded NLCs emulsion was frozen in eppendorff tubes at -20°C for a duration of 24 hours. After getting the frozen sample out of the freezer, it was allowed to defrost at room temperature before being centrifuged for 50 minutes at 4°C at 14000 rpm. The NLC pellets were reconstituted in a 90:10 ratio of acetonitrile:DMF and centrifuged again. To make sure the unentrapped BDQ was no longer present, this washing process was carried out twice. Every time, the supernatant was extracted from the pellets to be used for the free BDQ analysis. Acetonitrile:DMF (90:10) was used as a blank in the spectrophotometric analysis of the BDQ content, which was done using the following formula:

$$\% \text{ Drug loading} = \frac{\text{Weight of BDQ in NLCs}}{\text{Total weight of tested NLCs}} \times 100$$

$$\% \text{ Entrapment efficiency} = \frac{\text{Total amount of BDQ in NLCs} - \text{Amount of BDQ in supernent}}{\text{Total amount of BDQ in NLCs}} \times 100$$

4.3.3 %In-Vitro Drug Release (Q12) [13]

The *in-vitro* release upto 12 hours of BDQ loaded NLCs through an artificial cellophane membrane was determined by a simple dialysis method in Franz diffusion cell. The receptor medium consisted of 25 ml of pH 7.4 phosphate buffer, which was kept at 37±0.5 ^{o}C and continuously stirred at 100 rpm using a magnetic stirrer with thermostat control. The donor compartment contained a sample of BDQ loaded NLCs emulsion (10 ml ≅ 43.18 mg BDQ). 2 ml samples were removed from the receptor compartment and instantly replaced with an equivalent volume of fresh phosphate buffer pH 7.4 at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 hours of intervals. For every investigation, triplicate experiments were carried out, and the sink condition was constantly maintained. All samples were examined spectrophotometrically for BDQ content at a wavelength of 285.88 nm against phosphate buffer pH 7.4 as a blank and BDQ release in 12 hours (Q12) was computed.

4.3.4 Kinetic Modelling of Drug Release

To find out the drug release from the system followed which kinetic model, In-Vitro drug release (Q12) of emulsion of Bedaquiline loaded NLCs were subjected to various models such as Zero order, First order, Higuchi kinetics, Korsmeyer Peppas and Hixson Crowell equations.

The zero order (Equation 1) drug release described that the release rate was independent of its concentration.

$$C = k_0 t \quad \text{..... (1)}$$

Where, k_0 is zero order rate constant expressed in unit of concentration/time and t is time. A plot of amount of drug release versus time will be linear for zero order kinetics.

First order equation (Equation 2 & 3) describe that the release rate was concentration dependent.

$$\text{Log } C_t = \text{Log } C_0 - k_1 t/2.303 \quad \text{..... (2)}$$

$$\text{Log } C_0 - \text{Log } C_t = k_1 t/2.303 \quad \text{..... (3)}$$

Where, C_t is the amount of drug release in time t, C_0 is the initial concentration of drug and k_1 is the first order rate constant. Here, the graphical representation of log of % cumulative drug remaining versus time will be linear with a negative slope.

In general way, Higuchi model (Equation 4) can be simplifies as:

$$Q = k_H . t^{1/2} \quad \text{..... (4)}$$

Where, k_H is the Higuchi dissolution constant. Higuchi describes drug release as a diffusion process based on the Fick's Law. For diffusion controlled process a plot of Q versus square root of time is linear.

The Hixson-Crowell cube root law (Equation 5) describes the release from systems where there is a change in surface area and diameter of particles or tablets.

$$Q_0^{1/3} - Q_t^{1/3} = K_{HC} . t \quad \text{..... (5)}$$

Where, Q_t is the remaining amount of drug in the dosage form at time t, Q_0 is the initial amount of the drug in tablet and K_{HC} is the rate constant for Hixson-Crowell rate equation.

Korsmeyer derived a simple relationship which describes drug release from a polymeric system (Equation 6). To find out the mechanism of drug release, first 60% drug release data is fit into Korsmeyer-peppas Model.

$$M_t/M_\infty = K.t^n \quad \text{..... (6)}$$

Where, M_t/M_∞ is fraction of drug released at time t, n is diffusion exponent indicative of the mechanism of transport of drug through the polymer. The n value is used to characterize different release mechanisms as given in table 26.

Table 26: Interpretation of Diffusion Release Mechanism

Release Exponent (n)	Drug Transport mechanism
0.45	Fickian Diffusion
0.45<n = 0.89	Anomalous (Non Fickian) Diffusion
0.89	Case-II Transport
n>0.89	Super Case-II Transport

4.4 Lyophilization of BDQ Loaded NLCs [14]

The process in which water is removed from a product after it is frozen and placed under a vacuum, allowing the ice to change directly from solid to vapor without passing through a liquid phase is called freeze drying or lyophilization.

Mannitol as lyoprotectant and ethylene glycol as cryoprotectant was used to lyophilize the optimized batch (Solution 1) of BDQ loaded NLCs. The lyophilized cake was passed through #100 and after that through #120. It was evaluated for the flow properties and filled in Red/Transparent coloured size "3" hard gelatin capsule.

4.4.1 Lyophilization process parameter & formulation consideration

The process parameter of lyophilization process is mentioned in Table 27. The Composition and evaluation parameer of lyophilized formulation is mentioned in Table 28 and Table 29 respectively.

Table 27: Lyophilization Process Parameter

Parameter	Criteria
Time	48 hours
Freezing Temperature	-70°C to -40°C
Vacuum	
Primary Drying	180µbar – 200µbar
Secondary Drying	50µbar – 80µbar

Table 28: Composition of lyophilized formulation (Batch 1)

Ingredient	Quantity/Dose
BDQ Loaded NLCs (optimized batch (Solution 1))	14 ml Emulsion contain 60.45 mg of BDQ
Mannitol	5.44 mg
Ethylene Glycol	5.44 mg

Table 29: Evaluation parameters of inhaled dry powders (DPIs) of BDQ Loaded NLCs capsule

Test	Specifications
Angle of Repose(°)	≤30°
Bulk density (g/cc)	-
Tapped density (g/cc)	-
Carr's index (%)	≤15
Hausner's ratio	≤1.18
Moisture content (%w/w)	≤1.0
Empty Capsule weight (Size '3') (mg)	48.28 ± 5%
Average filled weight (mg)	71.33 mg ≅ 60.45 mg of Drug ± 5%
Average weight of filled capsule (mg)	119.61 ± 5%
%Drug Content ± S.D	90.00%-110.00%
%*In-Vitro* drug release (Q12) ± S.D	NLT 95.00%

4.5 *In-vitro* lung deposition study by andersen cascade impactor [15,16]

The aerosolization behaviour of the inhaled dry powder of BDQ loaded NLCs (Batch 1) was examined by Andersen Cascade Impactor (ACI) having 8 stage, induction port and mouthpiece adaptor (Copley Scientific Ltd., Nottingham, UK).

Before aerosolization, silicone oil was applied to every ACI stage to prevent particle bounce. The capsule of inhaled dry powder of BDQ loaded NLCs was placed into the aerolizer device. Powder was inhaled from the DPI device into the ACI at a flow rate of 27 L/min for 1 min. A digital flow meter was used to regulate the air flow rate through the ACI. The aerosolization performance of formulation was performed for 10 samples. Powder retained on each stages was collected, dissolved in ACN: DMF (90:10) and measured for the absorbance at λmax: 284.99 nm by the UV spectrophotometry method. The %mass in the thoracic fraction, %mass in the respirable fraction, mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD) were calculated.

4.6 *In-vitro* antimicrobial activity [17]

In-vitro antimicrobial activity of inhaled dry powder of BDQ loaded NLCs (60.45 mg of BDQ equivalent to 50 mg of bedaquiline) (Batch 1) was performed by measuring the Minimum Inhibitory Concentration (MIC) technique and compared with and BDQ tablets (60.45 mg of BDQ equivalent to 50 mg of bedaquiline) conducted at Microcare Laboratory & Tuberculosis Research Centre, Surat.

The lowest concentration of an antimicrobial agent that inhibits the growth of more than 99% of microorganisms in a solid medium or broth dilution susceptibility test is known as the minimum inhibitory concentration (MIC).

An isolate $H_{37}RV$ strain was tested for susceptibility to BDQ formulations using Lowenstein-Jensen (LJ) medium. The drug concentration of 100 µg/ml, 50 µg/ml, 12.5 µg/ml, 6.25 µg/ml. 3.125 µg/ml, 10 µg/ml, 5 µg/ml, 2.5 µg/ml, 1.25 µg/ml, 8 µg/ml, 4 µg/ml, 2 µg/ml, 1 µg/ml, 0.5 µg/ml, 0.25 µg/ml was prepared with serial dilution in DMSO for inhaled dry powder of BDQ loaded NLCs and BDQ tablets and it was added in different sterile tubes containing 5ml of LJ medium.

The standard strain of mycobacterium tuberculosis $H_{37}RV$ was used as culture. The test mixture was containing 3 x 10^8 organism/ml and it was compared with McFarland 1 standard. 0.1ml of the $H_{37}RV$ culture was inoculated in the each sterile tube containing 5 ml of LJ medium with different concentrations of the drug. The tubes were placed at an angle and incubated at 37°C ± 1°C for 8 weeks after the inoculation.

Table 30: Criteria of quantifying and reporting the growth of bacteria in L.J. Medium

Number of Colonies	Report
0-50	Actual count
50-100	1+ (use the actual count)
100-200	2+ (use the approximate count)
200-500	3+
Confluent growth	4+

4.7 Stability study [18]

6 month accelerated and long term stability were conducted according to ICH Q1C guidelines. The prepared inhaled dry powder of BDQ loaded NLCs 50 mg (Batch 1) were subjected to stability studies in HDPE container at two different temperature at accelerated Condition (40°C ± 2°C & 75% ± 5%RH) and at long term Condition (30°C ± 2°C & 75% ± 5%RH) and evaluated for appearance, average filled weight (mg), hausner's ratio, carr's index (%), angle of repose, %drug Content, moisture Content & %In-vitro drug release (Q12) after a period of 180 days.

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