RESEARCH ARTICLE

Box-Behnken Design for Formulation, Characterization and *In-vivo* Antidiabetic Activity of Pioglitazone Loaded Nanostructured Lipid Carriers

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ABSTRACT

This study employs a 2^3 full factorial design in the design of experiment (DOE) technique to assess the impact of critical quality attributes (CQAs) on critical process parameters (CPPs) in the formulation of pioglitazone (PGZ) loaded nanostructured lipid carriers (NLCs). The three chosen CPPs are the quantity of liquid lipid (Captex 300), the proportion of solid lipid (Glyceryl Palmitostearate), and the amount of surfactant. The CQAs evaluated are entrapment efficiency (%EE) and particle size. The PGZ-loaded NLCs are prepared through a solvent injection method. The lipid phase, consisting of dissolved medication and lipids in ethanol, is rapidly injected into an aqueous phase containing the surfactant. The resulting dispersion undergoes analysis for entrapment effectiveness and particle size. Additionally, the *in-vivo* antidiabetic activity of the optimal formulation is examined using diabetic rat models induced by streptozotocin (STZ), and relevant biochemical parameters are assessed. The results demonstrate that the proposed PGZ-loaded NLCs exhibit superior antidiabetic activity and favorable physicochemical characteristics compared to free PGZ. This study provides valuable insights into the formulation and characterization of NLCs for enhanced diabetes management, emphasizing their potential as a promising delivery system to improve the therapeutic efficacy of antidiabetic drugs like PGZ.

Keywords: Formulation, Characterization, Pioglitazone, Nanostructured Lipid Careers, Diabetic treatment

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INTRODUCTION

Diabetes mellitus remains a significant global health concern, characterized by elevated blood glucose levels requiring effective management strategies. Pioglitazone (PGZ), an oral antidiabetic drug known for its insulin-sensitizing properties, plays a crucial role in diabetes treatment plans. However, the low bioavailability of PGZ, common among weakly water-soluble medications, poses challenges to achieving optimal therapeutic outcomes. Inadequate bioavailability may necessitate higher drug dosages, potentially leading to adverse effects.[1]

Nanotechnology-based drug delivery systems have emerged as promising solutions to address the bioavailability challenges associated with poorly water-soluble drugs. Nanostructured lipid carriers (NLCs), colloidal systems composed of liquid and solid lipids, offer advantages such as improved drug stability, controlled release, and enhanced solubility.[2,3]

This study employs a novel approach using a Box-Behnken design to prepare and characterize PGZ-loaded NLCs, aiming to optimize drug delivery systems for efficient diabetes treatment. The Box-Behnken design, a statistical experimental methodology, allows for the simultaneous investigation of multiple critical process parameters (CPPs) and their impact on the critical quality attributes (CQAs) of the drug delivery system.[4,5]

The primary objective is to overcome the challenges associated with PGZ's low bioavailability by utilizing NLCs as a carrier system. The study aims to maximize drug encapsulation efficiency, control particle size, and enhance drug release kinetics to improve therapeutic efficacy while minimizing potential side effects. This research contributes to the advancement of drug delivery systems, providing insights into the formulation and optimization of PGZ-loaded NLCs for more effective diabetes management.

MATERIAL AND METHODS

Application of Full Factorial Design for Optimization of NLCs

Three critical process parameters (CPPs) have been studied in relation to the prepared NLC critical quality attributes (CQAs), namely, %EE (R1), particle size (R2), and their main effects and

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interactions with respect to the amount of solid lipid (Glyceryl palmitostearate) (A), amount of liquid lipid (Captex 300) (B), and concentration of surfactant (C). A design of experiment (DOE) (2^3 full factorial design) was used for this.

Formulation Development of PGZ loaded Nanostructured Lipid Careers

Solvent injection was used to prepare lipid careers loaded with pioglitazone. According to Table 1, a precisely weighed quantity of the medication and the lipid (Glyceryl Palmitostearate (GPS) and Captex 300) were dissolved in 1-mL of ethanol at 40°C. With a glass syringe of 1-mL, the entire lipid phase was quickly injected into the 30 mL aqueous phase, which contained a determined Pluronic F127 levels (surfactant), which had been pre-stirred on a magnetic stirrer (REMI, India) at a particular speed. To get the organic solvent to evaporate, the churning was kept up for two hours. Filtered at a ratio of 0.45, the resultant dispersion was free of excess lipids. After removing the samples for particle size analysis, NLCs were separated using centrifugation at 50,000 rpm for one hour. The recovered NLCs were once more suspended in two milliliters of deionized water following two washings. Following that, they were freeze-dried in a Dry/Shell Freeze System at -10°C with 5% mannitol acting as a cryoprotectant. They were then cryopreserved at -80°C for 48 hours. The resultant freeze-dried particles were stored in the refrigerator while more research was conducted.[6]

Freeze-drying of NLC Dispersion

The dispersions of PGZ-NLCs were lyophilized to produce a dry formulation. The resulting PGZ-NLC was placed in a freezer and frozen at -20ºC for the entire night. After that, it was placed in a lyophilizer and left there for 48 hours at -70ºC. Following lyophilization, the lyophilized form of PGZ-NLC was removed from the lyophilizer and examined in vitro and for physicochemical properties.[7]

Evaluation of Prepared PGZ-NLC

Entrapment efficiency

By evaluating the amount of drug that isn't entrapped in an aqueous medium using the technique of centrifugation, the entrapment efficiency (EE) of PGZ-NLC was ascertained. The nanoparticles were centrifuged at 5,000 rpm for 15 minutes at 4°C in a high speed cooling centrifuge (C-24, Remi) using Nanosep centrifuge tubes with ultrafilters with molecular weight cutoffs of 100 KD (Pall life sciences, India). The supernatant was then separated. A UV-vis spectrophotometer was used to measure the amount of medication in the supernatant at 234 nm following the appropriate dilution (Labindia 3000+).[8] The net percentage entrapment efficiency was then ascertained using a formula & result of entrapment efficiency as shown in Table 2.

$$\%EE = \frac{\text{Total drug content} - \text{Free drug Total drug content}}{\text{Total drug content}} x\ 100$$

Table 1: Different preparations in accordance with 2^3 factorial designs

Std	*Run*	*Factor 1: Glyceryl Palmitostearate (mg)*	*Factor 2: Captex 300 (mg)*	*Factor 3: Pluronic F127 (%)*
3	1	150	150	0.75
8	2	300	100	1
1	3	150	50	0.75
5	4	150	100	0.5
16	5	225	100	0.75
10	6	225	150	0.5
14	7	225	100	0.75
2	8	300	50	0.75
11	9	225	50	1
12	10	225	150	1
13	11	225	100	0.75
17	12	225	100	0.75
7	13	150	100	1
4	14	300	150	0.75
6	15	300	100	0.5
9	16	225	50	0.5
15	17	225	100	0.75

Table 2: Results of entrapment efficiency and particle size

F. code	*Response 1: % EE*	*Response 2: Particle Size (nm)*	*F. code*	*Response 1: % EE*
F1	66.65	165.58	F1	66.65
F2	76.65	125.45	F2	76.65
F3	68.78	173.32	F3	68.78
F4	65.98	160.54	F4	65.98
F5	69.98	145.87	F5	69.98
F6	64.47	155.95	F6	64.47
F7	70.12	145.65	F7	70.12
F8	75.65	115.47	F8	75.65
F9	69.74	178.98	F9	69.74
F10	65.32	145.74	F10	65.32
F11	66.85	146.65	F11	66.85
F12	68.78	147.33	F12	68.78
F13	68.78	175.45	F13	68.78
F14	71.47	110.25	F14	71.47
F15	72.23	115.85	F15	72.23
F16	73.32	136.85	F16	73.32
F17	69.11	153.32	F17	69.11

Determination of particle size

The average particle size of PGZ-NLC was calculated using dynamic light scattering (DLS) and a Malvern zetasizer (Malvern zetasizer, Worcestershire, UK) from SAIF RGPV, Bhopal. Keep the sample in a polystyrene cuvette; observations were made at a fixed 90-degree angle. In order to make sure that

Table 3: Experimental results with predicted responses

Formulation	*Run order*	*Composition, glyceryl palmitostearate (mg)/Captex 300 (mg)/ Pluronic F127 (%)*	*Response*	*Predicted value*	*Experimental value*
OPF1	8	300/50/0.75	% EE	76.82	75.65
			Particle Size	117.93	115.47
OPF2	14	300/150/0.75	% EE	70.90	71.47
			Particle Size	112.41	110.25
OPF3	15	300/100/0.5	% EE	73.17	72.23
			Particle Size	112.61	115.85

Table 4: Outcome of Zeta Potential and amount of drug of optimized formulation OPF1, OPF2 and OPF3

S. No.	*Formulation code*	*Zeta potential*	*% Drug content**
1.	OPF1	-39.65	98.85
2.	OPF2	-43.25	99.48
3.	OPF3	-32.15	97.65

Table 5: Results of *In-vitro* drug Release Study of formulation OPF1, OPF2, and OPF3

Time	*Formulation code*		
	OF1	*OF2*	*OF3*
0.5	26.65	24.45	20.21
1	34.58	30.32	23.32
2	52.23	42.32	29.98
4	69.98	51.14	35.45
6	73.32	63.32	49.95
8	85.45	78.98	56.65
10	96.65	89.95	63.32
12	98.85	95.65	73.37

the Its light scattering intensity fell between the the sensitivity of the instrument, the dispersion sample was diluted to a ratio of 1:9 v/v using distilled and de-ionized water. A range of measurements for the sizes of the particles in the sample being studied is called the polydispersity index. The polydispersity index is calculated by dividing the average weight by the number of average molecular weights. It is employed to show the distribution range of vesicles' diameter.[9]

Experimental results with predicted responses

Comparing expected responses to genuine outcomes from a portion of the research is one technique to assess the quality of the predicted responses. Usually, to accomplish this, a subset of the experimental runs is chosen at random to act as a set for validation, and the subsequent runs are then used to construct a prediction model. The analysis can be done both with and without the projected answers to assess how incorporating them might affect the experimental results. This makes it possible to identify and measure the effects of the anticipated responses.

Based on the Design of Experiments (DoE) formulation, four ideal formulations have been selected in order to create nanostructured lipid carriers as shown in Table 3. The compositional experimental values were found to be both within the allowed limit and reasonably similar to the projected values, which is why these formulations were chosen. This suggests that the predicted model was dependable and accurate when it came to the DoE formulation. Consequently, it is expected that using these improved formulations will produce nanostructured lipid carriers with the desired characteristics. It is important to keep in mind that any report on the application of these formulations must acknowledge the use of DoE and explicitly disclose the reasoning behind their selection. To aid in decision-making, it should also include information on the experimental values and how they compare to the projected values.[10]

Zeta potential

An electrophoretic light-scattering (ELS) spectrophotometer (Malvern zetasizer, Worcestershire, UK) was used to measure

Table 6: *In-vitro* drug release data for OPF2

Time (h)	*Square root of time(h)$^{1/2}$*	*Log time*	*Cumulative %drug release*	*Log cumulative %drug release*	*Cumulative %drug remaining*	*Log cumulative %drug remaining*
0.5	0.707	-0.301	24.45	1.388	75.55	1.878
1	1	0	30.32	1.482	69.68	1.843
2	1.414	0.301	42.32	1.627	57.68	1.761
4	2	0.602	51.14	1.709	48.86	1.689
6	2.449	0.778	63.32	1.802	36.68	1.564
8	2.828	0.903	78.98	1.898	21.02	1.323
10	3.162	1	89.95	1.954	10.05	1.002
12	3.464	1.079	95.65	1.981	4.35	0.638
24	4.899	1.38	99.05	1.996	0.95	-0.022

the zeta potential of the NLC. The samples were directly added into a quartz cuvette, and all measurements were conducted at 25°C Zeta potential of optimism batch shown in Table 4.

To extract the medication from the lipid matrix, the 10 mg NLC sample should be appropriately prepared by dispersing it in 10 mL of methanol. Take 0.1 mL of the medicine that was extracted, and dilute it with methanol to make 10 mL. Utilizing a UV-vis spectrophotometer (Labindia 3000+), measure the absorbance of this solution and use the celebration curve approach to determine the drug content.[11]

In-vitro drug release study

In order to conduct the NLC dissolving study, a dialysis bag was filled with an suitable NLC suspension volume (equivalent to 10 mg), and the bag was then submerged in the release medium (100 mL). Vibrations of the releasing media were made using a magnetic stirrer at 100 rpm. The NLC were exposed to artificial gastric juice with a pH of 1.2 for the first two hours. Two hours later, pH 6.8 phosphate buffer was added in place of the release medium. Three milliliters of the dispersion were taken out of the release medium at predetermined intervals, and to keep the volume constant, a new volume of release medium was quickly added. When utilizing the UV technique to evaluate the samples, the same analytical conditions were applied as previously described table no. 5 shows results of in vitro drug release study of formulation OPF1,OPF2 and OPF3. Table 6 shows results of in vitro drug release data for OPF2 optimized batch, theses shows 99.05 cumulative % drug release at 24 Hr. Table no. 7 shows regression analysis of the OPF3 optimized formulation and from this observation, OPF3 formulation follows korsmeyers peppas equation.[12]

Table 7: Data from a regression analysis of the OPF3 optimized formulation

Batch	*Zero order*	*First order*	*Higuchi's model*	*Korsmeyers peppas equation*
	R^2	R^2	R^2	R^2
OF3	0.752	0.963	0.911	0.972

In-vivo Antidiabetic Activity of Nanostructured Lipid Carriers

Animals

Wistar rats weighing between 150 and 200 grams were kept in groups of six in controlled temperatures and humidity levels of 25 ± 2°C and 55 to 65%, respectively, with a regular 12-hour light/dark cycle. Prior to doing the trials, the rats were given seven days to become used to the lab environment. Every experiment was run in a quiet room from 8:00 to 15:00. A distinct group of six rats participated in each series of trials. The Institutional Animal Ethics Committee was founded by the Ministry of Environment and Forests, Government of India, New Delhi, India (IAEC) to oversee and regulate the use of experimental animals. The IAEC granted approval for the animal experiments.[13]

Induction of experimental diabetes in rats

The injection of a 60 mg/kg streptozotocin solution intraperitoneally (pH 4.5) was given to rats that had fasted the previous night. After 48 hours, rats' blood glucose levels were measured; those exceeding 250 mg/dl were utilized in the tests and were considered diabetic.

Experimental Protocol

The animals were split up into groups of six rats apiece.

Group I: Rats were given the vehicle (0.5 mL of distilled water per day/rat) and served as the normal-control group.

Group II: Rats were used to control diabetes and were given the vehicle (0.5 mL per day of distilled water).

Group III: STZ+ Pioglitazone (5 mg/mL p.o.) for 21 days

Group IV: STZ+ nanostructured lipid carriers of Pioglitazone (5 mg/ml p.o.) for 21 days as shown in Table 8

Table 8: The anti-diabetic impact of pioglitazone's nanostructured lipid carriers on blood glucose levels (mg/dl) in rats with STZ-induced diabetes

Groups	*Treatment*	*Day 0*	*Day 7*	*Day21*
1	Normal	71.6 ± 3.2	73.5 ± 3.5	74.6 ± 3.8
2	Diabetic control	268.9 ± 7.5#	288.5 ± 7.8#	313.2 ± 8.8#
3	STZ+ Pioglitazone (5 mg/mL p.o.)	259.7 ± 6.5	172.5 ± 6.6**	149.3 ± 5.2***
4	STZ+ nanostructured lipid carriers of Pioglitazone (5 mg/mL p.o.)	260.7 ± 6.7	188.4 ± 6.9*	137.5 ± 5.4***

The values (n = 6) are expressed as mean +/- S.E.M.According to Tukey's post hoc test after one-way ANOVA, values are statistically significant at # p<0.01 vs. normal group, ***p<0.0001, **p<0.01, and *p<0.05 vs. diabetes control group, respectively.

Table 9: Antidiabetic effect of nanostructured lipid carriers of pioglitazone on serum lipid profile i.e. TG, Total protein, Insulin in STZ-induced diabetic rats

Group	*Treatment*	*TG (mg/dL)*	*Total protein(g/dl)*	*Insulin (mIU/L)*
1	Normal	81.5±3.5	7.7 ± 2.5	40.3 ± 2.8
2	Diabetic control	151.9 ± 4.9#	6.0 ± 2.9#	24.6 ± 2.1#
3	STZ+ Pioglitazone (5 mg/mL p.o.)	99.1 ± 3.8***	11.1 ± 3.2***	31.8 ± 2.8***
4	STZ+ nanostructured lipid carriers of Pioglitazone (5 mg/mL p.o.)	92.3 ± 3.7***	13.3 ± 3.7***	37.9 ± 2.8***

The values (n = 6) are expressed as mean±S.E.M. According to Tukey's post hoc test after a one-way ANOVA, the values are statistically significant at # p<0.01 vs. the normal group and *** p<0.0001 vs. the diabetes control group, respectively.

Blood sampling and glucose estimation

The ocular venous plexus was perfused with blood using retro-orbital hemorrhage in order to estimate the biochemical parameters and determine the glucose level via tail snipping as shown in Table 9.

Blood sampling and glucose estimation

Using the tail snipping method, blood was collected in order to measure blood glucose. Blood was extracted using the retro-orbital bleeding technique from the ocular venous plexus in order to estimate different lipid profiles and biochemical markers A shown in Table 8.[14-17]

Statistical Analysis

With GraphPad Instant 8.0.1, all data were analyzed and variables of interest were entered. The standard error of the mean (SEM) is represented as the mean ± in all statistical analyses. When appropriate, a one-way ANOVA was used to analyze the data. When compared to the vehicle, *p<0.05, **p <0.01, and ***p <0.001 were deemed statistically significant.[18-21]

RESULTS AND DISCUSSION

The study evaluated various formulations (F1 to F17) of Pioglitazone (PGZ)-loaded nanostructured lipid carriers (NLCs) using a Box Behnken design. Formulations F2, F8, F14, and F15 demonstrated the highest percentage of drug encapsulation efficiency (%EE) at around 71 to 73%, indicating effective PGZ encapsulation. Particle sizes were smallest in F8, F15, and F16 (115–137 nm), showcasing potential for nanoscale drug carriers. Further investigations are needed to determine the best formulation for specific pharmaceutical applications.

The Design of Experiments (DoE) results for chosen formulations (OPF1, OPF2 and OPF3) showed close alignment between experimental and expected values for %EE, suggesting accurate encapsulation efficiency prediction. Although OPF2 and OPF3 exhibited smaller-than-anticipated particle sizes, discrepancies warrant further examination. Despite minor differences, all three formulations demonstrated high % EE, beneficial for drug delivery systems. Zeta potential measurements indicated good stability, with OPF2 showing the highest electrostatic repulsion. The drug content percentage was highest in OPF2 (99.48%), emphasizing successful drug integration.

In an anti-diabetic study on streptozotocin-induced diabetic rats, both pioglitazone and its nanostructured lipid carriers significantly reduced blood glucose levels. Nanostructured lipid carriers showcased enhanced anti-diabetic effects. Additionally, the formulations positively influenced the blood lipid profile, insulin levels, and triglycerides, suggesting potential for improving lipid metabolism. These findings highlight the potential of nanostructured lipid carriers as a promising delivery strategy for enhancing the therapeutic benefits of pioglitazone in diabetes care, warranting further research for a comprehensive understanding.

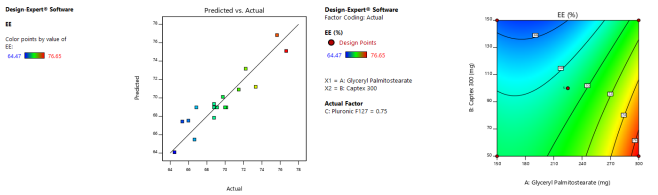

Figure 1: Response surface plots for Entrapment Efficiency

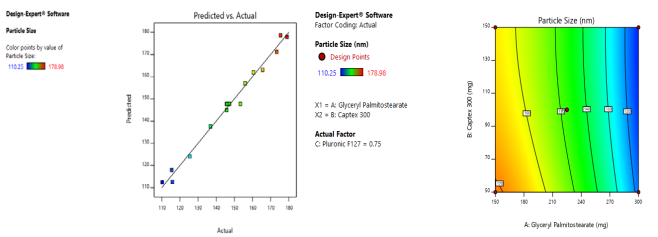

Figure 2: Response surface plots for particle size

CONCLUSION

In conclusion, the study effectively utilized a Box-Behnken design to optimize and characterize PGZ-loaded NLCs. The optimized formulation exhibited favorable physicochemical properties and demonstrated significantly improved in vivo antidiabetic activity compared to free PGZ in diabetic rat models. The findings suggest that the enhanced therapeutic effects could be attributed to increased drug solubility and bioavailability facilitated by the nanostructured lipid carriers. This study highlights the potential of utilizing NLCs for the delivery of PGZ and other antidiabetic drugs, showcasing improved medication solubility, absorption, and administration. Further research and clinical trials are warranted to fully explore the therapeutic potential of PGZ-loaded NLCs for diabetes management.

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