In-vitro and *In-vivo* Characterization of Entacapone Loaded Nanostructured Lipid Carriers Developed by Quality-By-Design Approach

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Abstract

Entacapone, a reversible catechol-o-methyl transferase inhibitor, is used to enhance the action of dopamine agonists by reducing their metabolism and the "Wearing-off" effects associated with long-term use in the treatment of Parkinson's disease. It is used as an adjunct to levodopa/Carbidopa therapy. Due to limited dissolution and first-pass clearance, it suffers from low and variable bioavailability. The present study aims to explore the potential of nanostructured lipid carriers (NLCs) for the delivery of Entacapone. The Quality by Design (QbD) approach was used for the systematic development of NLCs. The 2^3 full factorial design was investigated using Design-Expert®11 software. The three independent variables viz. content of total lipid (X1), surfactant (X2), and sonication time (X3) were optimized against two responses viz. particle size and entrapment efficiency. The optimized NLCs were characterized for their size, surface morphology, entrapment efficiency, drug release, thermal and crystallographic studies. The prepared Entacapone-loaded NLCs were capable of sustaining the release of the drug within therapeutic concentration for a prolonged time. In-vivo pharmacokinetic studies in Entacapone-loaded NLCs showed an increase in $t_{1/2}$, AUC $_{0-\infty}$, MRT compared to free drug, which confirms the enhancement of bioavailability while the reduction in elimination (K_{el}) depicts prolonged action of Entacapone by loading in NLCs. The results displayed Entacapone loaded NLCs have promising potential for oral delivery and may enhance the therapeutic effect and thereby reduce the dosing frequency which is a major issue with the present drug.

Introduction

Parkinson's disease (PD) is a neurodegenerative disorder affecting 7 to 10 million people worldwide. It is caused due to the impairment of small areas in the brain that control posture, balance, and movement [1, 2]. There is no perfect treatment available for PD; however, therapy aims to minimize the impact of symptoms. The symptomatic treatment involves dopamine agonists like levodopa and MAO-B inhibitors. However, conventional treatment becomes less effective as the disease worsens and produces end-of-dose adverse effects known as "wear off" symptoms. These symptoms are characterized by the reappearance of both motor and non-motor movements of PD as a result of previous carbidopa-levodopa therapy [3].

Entacapone, a nitro catechol compound (Fig. 1) has been approved for clinical use in patients with PD. It inhibits the degradation of dopamine and levodopa by blocking the enzyme Catechol-O-Methyl Transferase (COMT) [4]. It enhances the action of dopamine and reduces the onset of motor complications to a certain extent. It is used along with carbidopa-levodopa therapy to overcome the "wear-off" symptoms [5, 6]. But Entacapone is a BCS class IV drug with low aqueous solubility and low permeability [7]. Moreover, the bioavailability may also be affected by high lipophilicity, pre-systemic clearance in the gastrointestinal mucosa, and P-gp efflux mechanism [8]. Therefore, the major challenge is to formulate the drug delivery system that possibly tackles all these problems and increases the bioavailability and residence of the drug.

A number of technologies can be used to enhance the dissolution characteristic of such low solubility and low permeability drugs. A lipid-based formulation is an alternative approach for developing a product from laboratory scale to commercial level successfully [9]. Lipid-based formulations are potential drug carriers due to their propensity to improve the solubility of lipophilic drugs and ultimately augment oral bioavailability [10].

Nanostructured lipid carriers (NLCs) are novel lipid-based drug-delivery carriers comprised of solid and liquid lipids as a core matrix. These are second-generation lipoidal carriers that can overcome the limitations of first-generation solid lipid nanoparticles (SLNs), such as inefficient drug loading, drug leakage, polymorphism, and irrelevant gelation. The oil phase (liquid lipids) produces imperfections in the solid lipid, which increases carriers' structural fluidity and results in high drug loading [10, 11]. Moreover, the use of affordable, biodegradable and biocompatible lipids and ease of formulation with high stability made this delivery system an attractive promising alternative to first-generation nano-metric systems. NLCs have much justified to overcome the basic shortcomings of other Nano-carriers and are chosen for their known benefits like easy scale-up, high stability, high drug loading and minimum drug leakage, and prolonged drug release. Additionally, it has been reported that NLCs, in the intestinal lumen is exposed to lipases and co-lipase enzymes and are transformed into mixed micelles which are readily absorbed. Moreover, the drug is transported through the lymphatic route or payer's patches, which eventually enhances oral absorption [11].

The possibility of formulation and development of Entacapone NLCs via optimization and analysis by in-vitro and in-vivo studies has not been explored yet, leading us to current research work. So the primary aim was to explore the potential of NLCs for the delivery of Entacapone in the management of Parkinson's disease using the Quality by Design (QbD) approach. The secondary objective was to characterize it by Fourier transform infrared spectroscopy (FTIR), differential scanning calorimetry (DSC), powder X-ray diffraction (PXRD), scanning electron microscopy (SEM), drug particle size, zeta potential, entrapment efficiency, stability study. And finally, to evaluate it by in-vivo pharmacokinetic studies in Wistar rats.

Material And Methods

Materials

Entacapone was obtained as a generous gift from Macloeds Pharmaceuticals Ltd. Mumbai, India. Glycerol monostearate (GMS), Oleic acid, and Tween 80 were received as a gift sample from S.D. Finechem. Ltd. Mumbai. All the chemicals such as Acetonitrile, Ethanol, Methanol, Trimethylamine were of analytical grade and purchased from Merck Specialtie Pvt. Ltd., Mumbai, India.

Pre-formulation study

Pre-formulation studies are an essential prerequisite in the development of any drug delivery system. It gives the information needed to define the nature of the drug substance and provides a framework for drug combination with the dosage form. Therefore, there is a need for pre-formulation studies on the obtained sample of the drug. The most important prerequisite of any formulation development is to identify and check the purity of the drug, which otherwise affects the product's quality. These can be done by melting point, UV spectroscopy, infrared spectroscopy (IR), and differential scanning calorimetry (DSC) determination.

Fourier transform infrared spectroscopy

The Fourier Transform Infrared Spectrum of the drug was measured in the solid-state as a potassium bromide (KBr) mixture. Initially, ground Pure Entacapone was mixed thoroughly with KBr, an infrared transparent matrix at a 1:100 (sample: KBr) ratio. KBr pellets were produced by pressurizing 10-12 MT in a motorized pellet press

(Kimaya Engineers, India). The pellet was then scanned over a wave range of 4000 - 400 cm^{-1} and spectra were obtained using an FTIR spectrometer (Mettler-Toledo India Private Limited).

Differential scanning calorimetry

The thermal behaviour of pure drug was determined by using differential scanning calorimetric analysis. Thermogram for Entacapone was obtained using DSC (DSC star system, Mettler-Toledo India Private Limited). The drug was sealed in perforated aluminium pan and heated at a constant rate of $10^{\circ}\text{C}/\text{min}$ over $40\text{-}400^{\circ}\text{C}$.

Solubility in liquid lipids

To investigate the solubility of Entacapone in liquid lipids, a weight of 15 mg Entacapone was mixed with 2 mL of the tested oil within a test tube, and 3 mL of ethanol was added until a homogeneous mixture was obtained. The aqueous phase of the above mixture was separated from the lipids by centrifugation at 25000 rpm for 20 min in high-speed centrifugation. The clear supernatant obtained after centrifugation was suitably diluted and analyzed for Entacapone content at 306 nm max by UV spectroscopy to study its partition behavior with various lipids. The liquid oil that dissolved the highest amount of Entacapone was selected for use only in the "inaccuracy" test.

Solubility in solid lipids

For studying the solubility in solid lipids, accurately weighed 30 mg of the Entacapone was taken in a test tube; the solid lipid was added in increments of 0.5 g, and the test tube was heated in a controlled temperature water bath kept at 80°C till the clear melt was achieved. In this way, the quantity of lipid required to solubilize 30 mg of Entacapone was estimated.

Preparation of Entacapone loaded NLCs

Entacapone -NLCs were prepared using the probe sonication method described by Ajiboye et al. [12]. Briefly, a 70:30 GMS (solid lipid) and oleic acid (liquid lipid) concentration were kept constant. In the lipidic phase, the solid lipid such as GMS was melted, and 100 mg Entacapone (drug) was utilized. The drug was dissolved in the melted lipid phases which were kept in a heating water bath at 80°C . The aqueous phase is comprised of surfactant (tween 80) dissolved in water. Both the phases were maintained at a temperature above the melting point of the lipid (80°C). At this temperature, the melted hot lipid phase was then dispersed in the hot surfactant phase, obtaining a pre-emulsion under mechanical stirring (Remi Instruments Ltd., Mumbai, India) at 1800 rpm for 10 mins. Then this warm pre-emulsion was introduced into a probe sonicator (Sonics & Materials, Inc. United States) at an amplitude of 60% to form the NLC's dispersion. Then the NLC's so formulated were allowed to be cooled at room temperature, which was further used for characterization.

Optimization of NLCs by Quality by Design

Quality by Design (QbD) approach was used to optimize the developed NLCs. The 23 full factorial design was investigated using Design-Expert®11 software (Stat-Ease, Inc, US) [13]. The three independent variables viz. content of total lipid (X1), surfactant (X2) and sonication time (X3) were optimized against two responses viz. particle size and entrapment efficiency. Each response coefficient was studied for statistical significance at the 95% confidence level. A $p > 0.05$ value depicts that the model was significantly fit for the study. All the independent variables, their levels as well as the actual and coded values of these variables are given in (Table 1). The effect of the variables on the responses was statistically interpreted by the Fishers ANOVA test. The

model suitability was interpreted by comparing adjusted and regular correlation coefficient (r^2). Moreover, all variables' simultaneous interaction on the responses was interpreted graphically with the 2D counter plot and 3D surface response plots. For optimization, the constraint was applied as minimum particle size, maximum entrapment efficiency (%), and minimum polydispersity index.

Table 1
2³ Factorial design for Entacapone loaded NLCs

Run	X1 (gm)	X2 (%)	X3
F1	0.5	1	13
F2	1	2	15
F3	0.5	2	15
F4	0.5	1	13
F5	1	2	13
F6	1	1	15
F7	1	1	13
F8	0.50	1	13

Lyophilisation of Entacapone NLCs

Trehalose was added to the aqueous dispersion of NLCs and frozen at -75°C in deep freeze for 76 hours. The frozen NLCs are then lyophilized by a freeze dryer (VirTis Benchtop K, SP scientific, Warminster USA) and stored in sealed vials for further studies [14].

Characterization of Nanoparticles

Determination of particle size and PDI of prepared NLCs

The average particle size, polydispersity index (PDI), and surface charge of the NLCs was measured using a dynamic light-scattering method (Zetasizer Nano ZS, Malvern, USA) [15]. It measures statistical fluctuations in the intensity of light scattered by particles caused by random Brownian motion. Dilute NLCs solution were screened for particle size at varying scattering intensity.

Surface morphology

The surface morphology and the particle size of the formed NLCs were depicted using scanning electron microscopy (SEM, Zeiss, Germany). The gold coating of an aqueous dispersion of the NLCs was done by dual ion beam sputtering system. The gold-coated droplets were then placed through accelerating voltage of 5.0kV and magnification of 10-20 KX at $25 \pm 2^{\circ}\text{C}$ temperature. The image of the NLCs was then captured at various magnifications as required [16].

Encapsulation efficiency

The percent encapsulation efficiency (%EE) of Entacapone in NLCs was estimated using the process described by Patil et al. [16] The suspension of entacapone-NLCs were centrifuged at 20,000 rpm and the concentration of untrapped drug in the supernatant was estimated using UV–visible spectrophotometer at 306 nm λ_{max} . Values of EE% were calculated using following Equation.

$$\%EE = \frac{\text{Initial drug in NLCs} - \text{Amount of unbound drug}}{\text{Initial drug in NLCs}} \times 100$$

Differential scanning calorimetry

DSC is a basic way to investigate the crystallinity or amorphous state of drugs, polymers, and formulations by determining the temperature and energy divergence at the phase transition. The thermal behavior of the Entacapone, bulk GMS, physical mixture of Entacapone with GMS in 1:1 ratio and lyophilized Entacapone -NLCs were evaluated by DSC calorimeter (DSC STAR system, Mettler-Toledo, India Pvt. Ltd.). This study is helpful in interpreting the physiochemical changes in the samples with respect to temperature. Moreover, it also provides vital information regarding drug and excipient compatibility. The samples were kept under constant heating rate with an increment of 10°C/min for a temperature range of 25–300 °C under nitrogen purge, and the resultant thermograms were recorded.

X-ray diffraction

The crystalline nature of the NLCs was evaluated by X-ray diffractometer (Bruker AXS D8 Advance, Germany). The samples of pure Entacapone, a physical mixture in (1:1) ratio, and lyophilized NLCs were screened for XRD patterns in the 3 to 80-degree range with a chart speed of 5 per minute.

Accelerated stability studies

Entacapone –NLCs was subjected to accelerated stability studies [17]. The optimized batch of lyophilized drug-loaded NLCs were kept for accelerated stability study according to ICH Q1A (R2) guidelines [18]. The guideline suggests long term stability at 5°C ± 3°C and accelerated stability at 25°C ± 2°C/60% RH ± 5% RH for 3 Months for products to be stored in refrigerators. The stability of the NLCs was checked with respect to particle size, PDI, zeta potential and entrapment efficiency.

In-vitro release studies of Entacapone NLCs

Entacapone -NLCs were placed in dialysis bag with a pore size of 2.4 nm and molecular weight cut off between 100 kDa. The dialysis bags were tied at both ends and were placed in the beaker containing 250 mL hydrochloric acid (0.1 N) for an initial 2 h. Then the media was replaced with phosphate buffer saline pH 6.8. The beaker was maintained at 37 ± 2°C and stirred at a constant speed of 100 rpm [19]. At regular intervals of 0, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 18 and 24 h, 500 µL of dissolution medium was removed and was replaced with the fresh buffer at the same temperature to maintain sink condition. The amount of Entacapone in the aliquots was analyzed by UV-Visible spectrophotometer at max 306 nm [20].

Pharmacokinetic study

To study the in-vivo fate of Entacapone, in-vivo pharmacokinetic study was carried out for optimized formulation and plain drug suspension as per the below discussed protocol. The experimental protocol in the present study

was approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and the Institutional Animal Ethics Committee (IAEC) of R.C. Patel Institute of Pharmaceutical Education and Research, India (Protocol No. RCPIPER/IAEC/201).

Experimental design

The experiment was carried out on healthy adult Sprague-Dawley rats with weight range from 200 to 250 g. The animals were housed in acrylic cages (45×24×20 cm), maintained at constant room temperature (25±2°C), relative humidity (50±5%) and 12-h dark/light cycle with ad libitum access to pelleted chow food and drinking water [21, 22]. Before dosing, the animals were fasted for the period of 12 h prior and 4 h post with free access to water. Animals were divided into two groups (n = 4 per group). Control group received a suspension of free Entacapone suspended in 0.5% w/v sodium CMC [23] and the test group received the drug loaded optimized formulation at a dose of 3 mg/kg body weight [24, 25].

Pharmacokinetic data analysis

The blood samples were collected in an aliquot by the retro-orbital plexus of each animal at the scheduled time intervals (viz., 0.5, 1, 2, 4, 8, 12, and 24 h), and later animals were sacrificed. The serum concentration vs. time profile for the drug and formulations were estimated from the non-compartmental pharmacokinetic model, and the parameters were evaluated by Kinetica software, 5.0 (Thermo Fisher Scientific, USA). The PK parameters such as peak plasma concentration (C_{max}), time to reach C_{max} (T_{max}), AUC_{0-∞}, Mean residence time (MRT) and half-life were calculated using the concentration-time plot of the drug.

Sample treatment procedure

The Eppendorf tube consisting of an aforementioned mixture was meticulously vortex-mixed (Macro Scientific Work Pvt Ltd, Delhi, India) for 30 s followed by centrifugation at 15,000 rpm for 10 min at 6 °C to separate denatured protein. After centrifugation, 20 µL of the filtered supernatant (0.45 µm membrane filter) was injected into HPLC system and analyzed at 306 nm [20, 26].

Results And Discussion

Pre-formulation studies

The melting point of Entacapone was found to be 143°C and 144°C by glass capillary method and DSC, respectively. The observed melting point was then confirmed with the standard melting point of Entacapone i.e. 142-146°C. The UV spectrum of the drug showed λ_{max} of 306 nm. The IR spectrum confirmed the major functional groups present in the drug were in concordance with the reference IR spectrum of Entacapone.

Solubility Determination of Entacapone

Solubility of Entacapone in different solid lipid and liquid lipids were estimated. The results indicate that the relative distribution of Entacapone in GMS was higher 42.01±0.05mg than the other solid lipids. Whereas, Oleic acid has higher solubility 47.71±0.77mg of the drug compared to the other liquid lipids. Therefore, GMS and Oleic acid were chosen as the solid and liquid lipid for formulation of Entacapone loaded nanostructured lipid carriers (NLCs) respectively.

Preparation of Entacapone loaded NLCs

The probe sonication technique was used to prepare Entacapone loaded NLCs. This technique produces smaller NLCs with minimum poly-dispersity and high drug loading. Moreover, it is reproducible, robust and easy for large scale production. The GMS and Oleic acid were chosen as solid lipid: liquid lipid, respectively. Based on the results it was suggested that Tween 80 was sufficient to effectively cover 1% of the nanoparticle surface and prevent agglomeration during the homogenization process. Lipid nanoparticles stabilized with the Tween 80 have low particle size and high storage stability.

Optimization of NLCs by Quality by Design

After selecting the most important factors affecting Entacapone NLCs, 2³ simple full factorial design was used to determine optimal levels of these variables. Table 2 depicted the experimental results for the responses such as the particle size (Y1) and drug entrapment efficiency (Y2) of the NLCs. The particle size of all batches were between 161 and 259 nm. The entrapment efficiency of all the batches was found to be in the range of 80-92%. A mathematical relationship between factors and parameters was generated by factorial design analysis using Design-Expert software.

Table 2
Formulations batches of Entacapone- NLCs

Batch	X1	X2	X3	Y1	Y2
F1	0.50	1.00	13	240	89
F2	1.00	2.00	15	161.2	92.5
F3	0.50	2.00	15	213.4	85
F4	0.50	1.00	13	225.2	90
F5	1.00	2.00	13	230.5	88
F6	1.00	1.00	15	206.4	83
F7	1.00	1.00	15	218.4	84
F8	0.50	1.00	13	220.8	86

The statistical significance and interaction of each variable were estimated using different polynomial models. It was observed that the quadratic polynomial model was accurate to predict and interpret the relationship between the CQAs with the given responses. This was also confirmed with the F-test ANOVA (p<0.05) value which depicts the significance of the model. Table 3 summarizes all the results of full factorial design. The comparable high correlation coefficient (R²) values of all the responses signifies the best fit of the quadratic polynomial model. The insignificant (p>0.05) lack of fit values represents the pure error which also confirms the best fit of model. Moreover, the difference between predicted R² and adjusted R² values with respect to actual R² value is less than 0.2 which signifies the accuracy of the selected model.

The coefficients of regression equations for fitted quadratic model:

$$\text{Particlessize} = + 31.15 + 153.23\text{X1} - 41.63\text{X2} - 18.85\text{X3} + 0.70\text{X1X2} - 6.93\text{X1X3} + 5.58\text{X2X3}$$

$$\text{Entrapmentefficiency} = + 90.63 + 3.37\text{X1} + 2.62\text{X2} + 1.87\text{X3} - 0.62\text{X1X2} - 0.87\text{X1X3} - 1.12\text{X2X3}$$

Where, X1, X2 and X3 are coded values for independent variables for concentration of total lipid, concentration of surfactant, and sonication time respectively.

Table 3
Statistical report of Critical quality attributes using full factorial design

Models	Model Fit		Actual	Adjusted	Predicted	S.D	% C.V.
	F value	P value	R ²	R ²	R ²		
Particle Size (Y1)	339.16	0.0415	0.9995	0.9966	0.9686	10.04	3.23
% EE (Y2)	258.33	0.0476	0.9994	0.9955	0.9587	0.35	0.39

To study the simultaneous interaction of all the CQAs, three-dimensional (3D) response surface plots (Fig. 2) were generated and studied. It was observed that total lipid concentration was directly proportional to the particle size of the NLCs and increased when the concentration of lipid increases. However, surfactant concentration was inversely proportional to the particle size of the NLCs. Moreover, sonication time has also pronounced effect on particle size and was inversely proportional to each other. This might be due to the higher molecular clouding at high lipid concentration which leads to agglomeration of NLCs. In contrast at higher concentration of surfactant sufficient charge were developed on the surface of NLCs which reduces the agglomeration and stabilizes the formed NLCs. While at higher sonication time NLCs breakdown to smaller size. Consequently, all three CQAs significantly affect the EE and it increases with increase in total lipid concentration and surfactant concentration while reduces with increase in sonication time. The better availability of lipid and surfactant increases the drug dissolution and entrapment capacity of the NLCs. While sonication tends to leached out the drug from formed NLCs. The observation was well with previously reported results of particle size and EE [27–29]. Therefore, optimal responses could be achieved when the variable amount was the total lipid concentration (X1) 0.5%, surfactant concentration (X2) 1–2% and 13–15 minutes of sonication time (X3).

Characterization of NLCs

Particle size, Poly-dispersity index and Zeta potential

The morphology of the optimized batch was studied with the help of scanning electron microscopy (SEM) and confirms the non-spherical shape and nano-metric size of the drug loaded NLCs (Fig. 3). This was also confirmed by the measurement of particle size, PDI and zeta potential using Malvern Zeta Sizer and found to be 161.2 ± 16 nm, 0.444 and -28 mV respectively. The negative zeta potentials were likely due to the negatively charged lipid (GMS) and surfactants (Tween 80).

Entrapment efficiency

The entrapment efficiency of the optimized batch of drug loaded NLCs was found to be $82.5 \pm 3\%$. The high entrapment was likely due to the increased percentage of liquid lipid (oleic acid) which having greater solubility of drug and the less ordered solid lipid matrix.

Differential scanning calorimetry (DSC)

DSC studies were conducted for Entacapone, bulk GMS, their physical mixture in 1:1 ratio and freeze dried NLCs of the optimized batch and shown in Fig. 4. The overlay of DSC thermogram shows the sharp endothermic peak of free Entacapone at 163.6°C and endothermic peak for GMS at 61.57°C. Distinct peaks of both GMS and Entacapone is observed in the graph obtained with represents no chemical interaction. The DSC thermogram of lyophilized NLCs showed one endothermic peak at 61.49 °C due to the existence of lipidic phase and very small peak at 163°C. The reduced intensity of peak was due to the molecular incorporation of Entacapone into the lipid matrix of the GMS. This suggests that the Entacapone exists in the amorphous state in the formulation and is homogeneously dispersed in the NLCs. Thus, the DSC study concluded the absence of any chemical interaction between Entacapone and the lipidic phase. A lowering of the melting point of the lipid matrix as compared to their individual stuffing was observed which may be due to the presence of tween 80.

X-Ray Diffraction

X-ray diffraction pattern (XRD) studies of the purified drug Entacapone, GMS, physical mixture and lyophilized Entacapone nanostructured lipid carrier (NLCs) formulation were performed using an X-ray diffractometer. The 2θ value of Entacapone as a pure drug showed sharp diffraction peaks which reflects the crystalline nature of pure Entacapone. However, the physical mixture and NLCs show the amorphous nature in the diffractogram indicating that the new structure has not been formed. This may be due to the molecular entrapment of the drug in NLCs. Similar studies were also reported by various formulation scientist [27, 28]. The X-ray diffraction patterns of Entacapone and freeze-dried NLCs preparation are given in Fig. 5.

In-vitro drug release

The Entacapone loaded NLCs showed dual release behavior with initial burst release of the surface bound drug and then delayed release of the matrix drug through diffusion mechanism. After 12 h approximate $98.82 \pm 3.8\%$ drug release was observed from NLCs as compared to free drug which showed complete release within 2.5 h (Fig. 6). This depicts the sustain release nature of Entacapone loaded NLCs.

Moreover, the kinetic models depict that the Entacapone released from NLCs is governed by the Korsmeyer Peppas model, which is indicative of a diffusion mediated drug release (Fig. 7). The similar results were reported by several researchers for NLCs [15, 29].

A more stringent test was used to distinguish between the mechanisms of drug release. The release data was fitted to the Peppas exponential model, $M_t/M_\infty = Kt^n$, where M_t/M_∞ is the fraction of drug released after time t ; k is the kinetic constant; and n is the release exponent which characterizes the drug transport mechanism. It was observed that the n value is 0.658 means it is $0.45 < n < 0.89$ which clearly indicates that it follows non-fickian transport as reported by Peppas et al. [30].

Accelerated Stability Studies of Entacapone-NLCs

In the stability studies before and after the lyophilisation of the formulation the effect of temperature was noted. Initial particle size, PDI, and drug content were determined at the time of preparation and then the batch was divided into two equal portions, which were stored under different temperature conditions in a refrigerator $5^{\circ}\text{C} \pm 3^{\circ}\text{C}$ for 3 Months and at $25^{\circ}\text{C} \pm 2^{\circ}\text{C}/60\% \text{ RH} \pm 5\% \text{ RH}$ for 3 Months. Continuous monitoring of the sample with respect to particle size, PDI, and zeta potential was done up to 3 months. The obtained results are presented in Table 4. It was observed that at room temperature initially the sample remained stable for around 12-15 days and slowly aggregation started which was not that significant in cold condition. From the data it can be interpreted that Optimized Entacapone NLCs were relatively stable under refrigerated condition.

Table 4
Stability study of lyophilized Entacapone loaded NLCs

Sampling Time	PS (nm)	PDI	EE %
At the time of preparation	161.2	0.444	92.51
$25^{\circ}\text{C} \pm 2^{\circ}\text{C}/60\% \text{ RH} \pm 5\% \text{ RH}$ for 3 Months			
1 Month	170.3	0.483	80.33
2 Month	192.7	0.472	74.74
3 Month	203.5	0.497	64.32
Storage Condition in refrigerator $5^{\circ}\text{C} \pm 3^{\circ}\text{C}$ for 3 Months			
1 Month	166.3	0.454	90.12
2 Month	174.6	0.482	79.89
3 Month	186.4	0.488	70.81

Pharmacokinetic study

After single-dose administration of plain Entacapone and drug-loaded NLCs, the drug was observed in plasma for 24 h above its therapeutic concentration. However, the free drug was cleared from circulation within 1-2.5 h (Fig. 8).

The plasma profile shows that C_{max} and T_{max} of drug-loaded NLCs significantly differed from the free drug. K_{el} of the free drug was higher than NLCs, indicating an increase in the $t_{1/2}$ of the latter. Moreover, the mean residence time (MRT) and $\text{AUC}_{0-\infty}$ of NLCs are significantly ($p < 0.001$) increased as compared to a free drug (Table 5).

Table 5
Pharmacokinetic parameters of free Entacapone and Entacapone loaded NLCs

Parameters	Free Entacapone	ENT-NLCs
C _{max} (ng/mL)	1592±99	2993±322**
T _{max} (h)	0.5±0.08	1.0±0.13*
t _{1/2} (h)	0.704±0.01	4.74±0.58***
k _{el}	0.847±0.02	0.14±0.01**
AUC _{0-∞} (ng.h)/mL	2227.5±121.6	21588.3±1067.6***
MRT (h)	1.18±0.12	6.77±0.23***
Notes: *** P < 0.001 as compared with the plain Entacapone group.		

Conclusion

In the current study Entacapone loaded Nanostructured lipid carrier was developed by probe sonication method and optimized with QbD approach. The 2³ full factorial design was explored to optimize and drive relationship between the screened Critical parameters and the desired responses. The quadratic polynomial model was used to derive significant interaction terms. Moreover, the 2D and 3D surface response graphs were used to interpret the simultaneous impact of all the CQAs on the selected responses. It was found that the concentration of lipoidal phase play an important role in particle size, drug entrapment and drug release from NLCs. SEM studies revealed the non-spherical shape and nano-metric size of the drug loaded NLCs, which was further confirmed by Malvern Zeta Sizer. Both DSC and XRD studies proved the compatibility of the drug with the lipids used. The in-vitro release pattern displayed a sustained release pattern of Entacapone from NLCs. In the accelerated stability study the particle size and zeta potential was stable at room temperature and comparatively more stable in refrigerated conditions. From the in-vivo pharmacokinetic results, it can be concluded that the orally administered Entacapone NLCs displayed better bioavailability by enhancing the AUC in plasma drug concentration profile. The Nanoscale particle size, enhanced solubilisation, prevention of extensive metabolism and enhanced efficiency will ultimately help to reduce the dose and improve the overall therapy. Hence we conclude that preparation of Entacapone NLCs can be appropriate approach for improving the oral bioavailability and pharmacological bioactivity of the poorly water-soluble drugs.

Abbreviations

Mean particle size, MPS; Poly-dispersity index, PDI; MRT, Mean residence time; AUC, area under the curve; Elimination constant, k_{el}.

Declarations

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Ethics approval

“The study was conducted according to the guidelines of the Committee for the Purpose of Control and Supervision of the Experiments on Animals (CPCSEA), Government of India and experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) of R. C. Patel Institute of Pharmaceutical Education and Re-search, Shirpur, District- Dhule, Maharashtra, India (**Protocol No. RCPIPER/IAEC/201**)

Consent to participate:

Not Applicable

Consent for publication:

All authors agreed with the content and that all gave explicit consent to submit the findings of research to the journal for publication.

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The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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All authors contributed to the study. project administration, conceptualization; methodology and original draft was prepared by Dr. Yogeeta Agrawal, software; validation; formal analysis, reviewing draft was carried out by Dr. Kiran Patil, investigations at laboratory were carried out by Pratiksha Joshi, Reviewing and editing was done by Mrugendra Potdar, data curation was done by Dr Hitendra Mahajan, Pharmacokinetic investigation was performed by Dr. Kartik Nakhate.

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Figures

Figure 1

Structure of Entacapone.

Figure 2

Response surface plot showing the effect of variables i.e. total lipid concentration and surfactant concentration over the particle size [A] and entrapment efficiency [B].

Figure 3

Figure 4

DSC overlay thermogram overlay of A) Entacapone NLCs; B) Physical mixture of Entacapone and Glyceryl Mono stearate; C) Entacapone, D) Glyceryl Mono stearate

Figure 5

XRD of plain Entacapone [A] and Lyophilized Entacapone -NLCs [B]

Figure 6

In-vitro drug release of free Entacapone and optimized Entacapone-NLC

Figure 7

Drug release kinetics of Entacapone loaded NLCs

Figure 8

Plasma drug profile of free Entacapone and drug loaded NLCs in Wistar Rats

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