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ORIGINAL ARTICLE

QbD-based Formulation Optimization and Characterization of Polymeric Nanoparticles of Cinacalcet Hydrochloride with Improved Biopharmaceutical Attributes

Geliştirilmiş Biyofarmasötik Özelliklere Sahip Sinakalset Hidroklorürün Polimerik Nanopartiküllerinin QbD Tabanlı Formülasyon Optimizasyonu ve Karakterizasyonu

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ABSTRACT

Objectives: The aim of the present work was to prepare QbD enabled optimization, and to improve the oral bioavailability of freeze–dried polymeric nanoparticles of cinacalcet hydrochloride manufactured by nanoprecipitation and ultrasonication methods using polymers PLGA, and poloxamer-188.

Materials and Methods: The initial screening and optimization were carried out for the formulations by employing Taguchi and Box-Behnken Designs. The FT-IR and DSC revealed no interactions and had no incompatibility among the selected drug and polymers. The nanoparticles were characterized for % drug release, particle size analysis, zeta potential, PDI, SEM, TEM, P-XRD, TGA, DTA, *in vitro*, and *in vivo* drug release study.

Results: *In vitro* drug release study showed sustained release of the drug from the optimized batch by diffusion mechanism. The optimized nanoparticle formulation was recognized by numerical and graphical methods using validation of the experimental model. The optimized batch was stable as per the ICH stability guidelines for 6 months with no considerable alternation noticed in particle size, entrapment efficiency, and *in vitro* drug release. The pharmacokinetic parameters of AUC and Cmax data for the optimized formulation increased 3- and 2.9-folds compared to the pure-drug suspension.

Conclusion: The prepared polymeric nanoparticles formulation is an alternative delivery system for enhanced therapeutic efficacy and bioavailability potential of a model drug to manage long-term normocalcemia in patients with preliminary hyperparathyroidism.

Key words: PLGA, polymeric nanoparticles, Taguchi, P-XRD, optimization, bioavailability potential

ÖZ

Amaç: Mevcut çalışmanın amacı, QbD özellikli optimizasyon hazırlamak ve polimerler PLGA ve poloksamer-188 kullanılarak nano-prepikipitasyon ve ultrasonikasyon yöntemleri ile üretilen cinacalcet hidroklorürün dondurularak kurutulmuş polimerik nanopartiküllerinin oral biyoyararlanımını geliştirmekti.

Gereç ve Yöntemler: Formülasyonlar için taguchi ve Box-Behnken Tasarımları kullanılarak ilk tarama ve optimizasyon gerçekleştirildi. FT-IR ve DSC hiçbir etkileşim ortaya koydu ve seçilen ilaç ve polimerler arasında uyumsuzluk yoktu. Nanopartiküller % ilaç salınımı, partikül boyutu analizi, zeta potansiyeli, PDI, SEM, TEM, P-XRD, TGA, DTA, *in vitro* ve *in vivo* ilaç salınım çalışması ile karakterize edildi.

Bulgular: *In vitro* ilaç salınım çalışması, ilacın difüzyon mekanizması ile optimize edilmiş partiden sürekli olarak salındı. Optimize edilmiş nanopartikül formülasyonu, deneysel modelin doğrulanması kullanılarak sayısal ve grafiksel yöntemlerle tanındı. Optimize edilmiş parti, parçacık boyutu, tuzak

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verimliliği ve *in vitro* ilaç salınımında fark edilen önemli bir değişiklik olmadan 6 ay boyunca ICH stabilite yönergelerine göre kararlıydı. Optimize edilmiş formülasyon için AUC ve Cmax verilerinin farmakokinetik parametreleri, saf ilaç süspansiyonu ile karşılaştırıldığında 3 ve 2,9 kat arttı.

Sonuç: Hazırlanan polimerik nanopartiküller formülasyonu, ön hiperparatiroidisi olan hastalarda uzun süreli normokalsemiyi yönetmek için model bir ilacın gelişmiş terapötik etkinliği ve biyoyararlanım potansiyeli için alternatif bir doğum sistemidir.

Anahtar kelimeler: PLGA, polimerik nanopartiküller, Taguchi, P-XRD, optimizasyon, biyoyararlanım potansiyeli

INTRODUCTION

Nanodrug delivery systems in medicine have evolved as a dependable and reliable technological boon as site-specific drug targeting in nanodrug development in the previous two decades.[1,2] Polymeric-based nanoparticles are colloidal nature systems measuring around 10-100 nm. The experimental findings achieved are mostly within proximity of 100-500 nm. The polymeric-based nanoparticulate systems have been considered an area of extensive research in novel drug delivery systems because of their all-encompassing biocompatibility and ease of altering properties.[3]

Numerous scientists worldwide have discovered abundant approaches, such as nanoprecipitation, solvent evaporation, salting out, emulsification-diffusion, and supercritical fluid technology.[4] The polymeric nanoparticulate (PN) systems offer high applicability as an active delivery system. These PN versions comprehensively avail the drug near the intended site with augmented therapeutic activity and minimal adverse effects.[5] These biopolymeric systems offer pivotal effectiveness with diminished toxicity and a potentiated therapeutic index.[6,7]

The polymeric micelle systems offer supplementary properties for safe and effective drug targeting at the tissue site, consistent biocompatibility, and potentiated stability during effective drug release.[8-10] The current methodology of the drug cinacalcet hydrochloride (CIH) encompasses the investigations streamlined to enhance the adequate oral bioavailability by nanoprecipitation-sonication techniques.[11-15] These findings may lead to achieve enhanced stability, effective dissolution rate, and less toxicity.[16] Nowadays, a heavy focus of attention has evolved in applying such materials for drug delivery implementation. These polymeric micelles are usually biodegradable and biocompatible hydrophobic polymer blocks, such as PCL, PLA, PEG, etc.[17,18] From the varied range of biopolymers, poly-lactic-co-glycolic acid (PLGA) is considered an efficient and suitable class of copolymers that can be utilized for different Food and Drug Administration-approved therapeutic devices.[19-23]

CIH, a modern-day, first-line, well-known calcimimetic drug, is indicated for the safer management of tertiary hyperparathyroidism in people with chronic renal disorder, dialysis, and hypercalcemia in patients with parathyroid carcinoma.[24,25] The oral form of CIH is considered the frontline medication in the generation of agents (i.e., the calcimimetics), with an innovative mechanism of action with absolute bioavailability of 20-25% and a log p value of 6.8.[26,27]

The overall productiveness of the concept of QbD in optimizing the appropriate experimental design space is boosting vast acceptance in the development of pharmaceuticals.[28,29] Box-Behnken Design (BBD) optimization is a notable response surface method, which is predictable in determining the specific interactions of the parameters opted in optimization.[30-32]

Few research findings on freeze-dried PNs of CIH, prepared by the nanoprecipitation with the ultrasonication method, except for few commercially marketed tablets of the strength of 30, 60, and 90 mg, are available in the literature. An alternative system of drug delivery can be developed for an enhanced therapeutic efficacy and bioavailability potential of CIH.

MATERIALS AND METHODS

Materials

CIH was obtained as a gift sample from Cadila Healthcare Pvt. Ltd., Mumbai, India. PLGA was obtained as a gift sample from Dr. Reddy's lab, Hyderabad, India. Poloxamer-188 was received from Himedia, Mumbai, India. Mannitol was obtained from Himedia Chemicals Pvt. Ltd., Mumbai, India. Further, the necessary reagents, chemicals, and solvents used in this study were of analytical grade and utmost quality. The most authentic ARRIVE guidelines recommended that all animal studies or experiments be conducted in an alliance including Scientific Procedures Act, 1986 in the UK and insisted on allied, EU Directive 2010/63/EU guidelines for experiments on animals were authorized by the Animal Care Committee, RIPS, Berhampur, Institutional Animals Ethics (926/PO/Re/5/06/CPCSEA, approval no: 87).

Methods

Target product profile (TPP)

TPP was predefined for the PN drug delivery system formulation of CIH to improve the oral bioavailability of the drug. The imperative principles of quality TTP (QTPP), such as the strength, administration route of the formulations, and their related pharmacokinetics-based process determining variables and factors, packaging stability attributes, drug release, and pharmacokinetic profiles of the drug.[17,28]

Critical quality attributes (CQAs)

Among the entire TPPs, several crucial and promising QAs are designated as CQAs on the basis of the criticality of effect upon patients' benefit. From the prepared polymeric formulations, CQAs such as time of stirring, mean particle size distribution, and zeta potential (ZP) were selected as per the TPPs.[17,27]

Screening of formulation excipients

Intrinsic solubility analysis

Drug substance intrinsic solubility was estimated in various solvents, such as water, acetonitrile, phosphate buffer pH of

6.8 and 7.4, 0.1N HCl, methanol, ethanol, dimethyl sulfoxide (DMSO), acetone, PEG200, PEG 400, and n-octanol. The drug's adequate capacity was included in each solvent and set aside on a mechanical shaker (Rivotek, Rivieria Glass Pvt. Ltd., Mumbai, India) along with a water bath regulated at 37±0.5°C for 72 h. The vials were subsequently observed from distinct intervals for absolute solubilization of the drug substance, and afterward, the drug was further included if essential. All notable excipients were permitted to move into the centrifuged tubes (Spinwin MC02, TarsonsPvt Ltd.) Kolkata, India). The distinct quantity of the solubilized drug was detected and analyzed by ultraviolet-visible (UV-vis) spectroscopy (UV-vis spectrophotometer, Labindia Ltd., Mumbai, India) for isolation of the undissolved or immiscible drug at a wavelength-maximum of 279 nm (i.e., λ max of the drug) from the supernatant fraction.[20]

Development of analytical method by ultrafast liquid chromatography (UFLC)

A simple, rapid reverse phase RP-UFLC method was used for the quantification of CIH. Drug separation was performed on a 250×4.6 mm ID, ODS C_{18} column. The mixture of (50:50, %v/v) acetonitrile:phosphate buffer i.e., TBHS solution of 25 mL as a mobile phase and filtered through a 0.45 µm millipore filter at a flow rate of 0.5 mL/min. Chromatographic detection was performed at a λmax of 223 nm, and an analytical column was maintained at a constant temperature (25±1°C).[33]

Identification of QTPPs and CQAs in product development

The QTPPs and CQAs are the major QbD elements to achieve product development and objectives for CIH nanoparticles, and the QTPP elements were set up based on drug sustained release, ZP, poly dispersibility index, and particle size. The QTPPs and CQAs parameters are depicted in Table 1.

Preliminary screening of influential factors using Taguchi design

The fundamental screening was attempted by exercising a combination of the 7-factors 2-levels Taguchi design to establish the significant vital factor(s) affecting the CQAs. For the Taguchi design, a combination of eight formulations was suggested and prepared.

Preparation of PN formulation

The development of PNs was attempted by adopting nanoprecipitation, followed by the ultrasonication method. The required quantity of PLGA was dissolved in the organic phase (acetone) at 50°C and added to the acetone solution of drug CIH. The organic phase was included drop-wise into the additive (stabilizer) solution of poloxamer-188 (aqueous phase) with the glass syringe outfitted with a needle (gauge size, 26) at 3 mL/min and a stirring speed of 5000-15000 rpm at 25°C (sample homogenizer T18 DIGITAL IKA RV, Germany). The ultrasound state parameter was set to 3 s with an interval of 2 s at 40 W for 5 min. The residual amount of acetone was evaporated at 40°C beneath condensed pressure, using a Rotary evaporator (IKA RV 10 digital, Germany) for 2 min. The obtained nanosuspension was centrifuged (RC 4815F, Eltek India) at 9000 rpm for 30 min and lyophilized for 36 h at -54°C.

Systematic formulation optimization studies

The BBD response surface design with 3-factors and 3-levels of mixture components was taken into account for optimizing the PN formulations. Design expert ver. 12.1.1 software (Stat-Ease, Minneapolis, MN, USA) was used for generating the experimental trials, where the ratio of Drug:PLGA (mg) (X1), poloxamer-188 concentration % w/v (X2), and stirring speed (X3) was utilized as the independent variables or factors and with 3-levels (-1, 0, and 1) was built to estimate the significant effect of these assorted variables or responses, namely cumulative % drug release QT24% (Y1), particle size in nm (Y2), ZP in mV (Y3), and polydispersity index (Y4). A sum of 17 trial formulations was organized together with five consecutive cumulative replicates of the center point trial, and further CQAs were formulated for evaluation.

Lyophilization of optimized PNs

Extensive research has proven that the samples obtained by lyophilization exhibit a porous structure with increased redispersibility and long-term steadiness.[34] The lyophilized process was performed by using lyophilizer using ALPHA 1- 2 LO Plus CHRIST in order to produce the powdered freeze-dried state at a pressurized vacuum of 0.01 KPa for about 48 h at -50°C to obtain a drug loaded lyophilized PNs in case of run

Table 1. Quality target product profile and critical quality attributes for developing polymeric nanoparticles of cinacalcet hydrochloride

QTTPs	Target	CQAs	Pre-determined target	Justification
Dosage type	Extended-release dosage forms	Cumulative drug release at 24 h (QT24%)	75-85%	Sustained release of drug is the objective of the study and is important for better absorption
Dosage form	Polymeric nanoparticles	Zeta potential	≥±20 mV	Highly critical factor as per the stability perspective of the nano suspensions
Drug release and absorption	C_{max} and AUC higher compared to pure drug	Mean particle size (nm)	100-200 nm	Particle size in these ranges is highly critical and important for better absorption of drug
Dispersity	High dispersity	PDI	0-0.4	Uniformity in the particle distribution by size is essential for therapeutic activity and, hence, is highly critical

QTTPs: Quality target product profiles, CQAs: Critical quality attributes, C_{max}: Maximum plasma concentration, AUC: Area under curve, PDI: Polydispersity index

no: 16. The selected optimized formulation was further freeze-dried to the powder form by applying a suitable cryoprotectant (i.e., mannitol (2%) and then subjected to micrometric characterization.

Characterization of freeze-dried PNs

Fourier-transform infrared spectroscopy (FT-IR)

The FT-IR spectroscopy was performed effectively for estimating the possible physical interactions of drug CIH. FT-IR spectra of selected CIH and physical mixture (PM) with PLGA and poloxamer-188 were recorded on IR using KBr around the 4 cm^{-1} resolution. The compatibility studies of the drug-excipients were undertaken by computing the range of transmittance from 4000 to 400 cm^{-1}. Peak matching was done to identify and determine any significant interactions among the other additives with CIH.[35]

Differential scanning calorimetry (DSC)

DSC studies were done to assess the interaction between the drug and the polymer. All the required samples (10 mg) were subjected to heat in aluminum pans through effluent gas containing dry nitrogen. The DSC thermograms of pure drug of CIH, excipients, and their respective PMs with CIH were determined.[36]

Entrapment efficiency (EE)

The percentage EE of CIH in the formulated or prepared PNs was anticipated directly by collecting the CIH content in the PNs. Samples of 10 mL of CIH PNs were allowed to centrifuge at 9000 rpm for 30 min at -4°C using a cooling centrifuge. The unencapsulated free drug can be removed using centrifugation dialysis.[37] The supernatant free drug was calculated and validated, employing the UV-spectrophotometric method at wavelength 279 nm. The drug EE (DEE) or (DEE %) of nanoparticles was determined and calculated as indicated below by equation (1).

% Entrapment Efficiency=(Total amount of drug-Free Drug)/(Amount of total drug content)×100.....(Eq.1)

Particle size and ZP measurement

Particle size, polydispersity index, and ZP were effectively determined by Photon Correlation Spectroscopy using the Zeta-sizer Nano-ZS Make-Malvern instrument. ZP implies that its value can be associated with the steadiness of colloidal dispersions. A high ZP will present the immovability or steadiness intended for molecules and particles that are small enough.[38]

In vitro diffusion studies

In this *in vitro* drug release study, the dialysis bag diffusion technique was implemented for pure-drug CIH. Formulations (5 mL) were placed in the dialysis bag, hermetically sealed, and dropped into 150 mL of 0.1 N HCl under sink conditions for the first 2 h. Then it was transferred into phosphate buffer solution of pH 6.8 for 24 h. The whole system was kept at 37°C with continuous magnetic stirring at 200 rpm. A sample (2 mL) was used by pipetting it from the compartment of the receptor at prefixed time intervals and replaced by a fresh and accurate quantity of 0.1 N HCl and phosphate buffer of pH 6.8. Then, this 1 mL sample was taken, and 1 mL of ethyl acetate was added. The sample was vortexed in a cyclomixer, and 0.5 mL of this solution's supernatant layer was made in a test tube, kept for drying, and the mobile phase was added to this test tube and analyzed under RP-UFLC.[38-41] Using a non-Fickian diffusion mechanism, kinetic studies were analyzed, allied with a concentration gradient, diffusion mechanics, and the extent of swelling.[42,43]

Solid-state characterization

Powder X-ray diffraction (P-XRD)

P-XRD (Rigaku, Japan, Smart Lab 9 kW) was implemented for diffraction studies. P-XRD studies were performed on the samples by exposure to nickel-filtered CuKa radiation (40 kV, 30 mA) and allowed for the scan. Samples required for P-XRD related investigation were pure drug and optimized lyophilized PNs of CIH. The results were then recorded as peak height (intensity) versus time (h).

Scanning electron microscopy (SEM)

SEM studies the texture or exact appearance of nanoparticles. A high resolution SEM (Jeol, Japan, JSM-6390LV) at 30 kV was used. The formulation bearing to be tested sticks to the metallic stub, which is carbon-coated. SEM is useful for a detailed study of surface morphology. A high-energy electron helps to scan across the surface of a specimen, having an Au and Pt coating, which assists in improving contrast and the signal-to-noise ratio.[44] The pure drug and optimized lyophilized PNs of CIH were studied and appropriately examined for determining surface morphology.

Transmission electron microscopy (TEM)

The exterior appearance or outline of the PNs was determined by TEM (100s, JEOL Ltd, Japan) and the PNs of CIH, which was lyophilized and diluted with 2 mL of distilled water and consistently mixed by ultrasonication for 3 min. The samples were arranged by inserting a drop of PNs of CIH upon a coated copper grid and air-dried.[45]

Thermogravimetric analysis (TGA)

TGA studies were implemented to justify the moisture content associated with weight loss in isothermal or non-isothermal stability studies. TGA denotes a vital aspect to identify and measure the amount of moisture content in pharmaceutical preparations.[46] During the stages of preformulation investigations, it is considered as an accurate method for the distinctness of polymorphs from hydrates or identification of monohydrates from among other hydrates, which may not be possible by DSC alone.[34,47]

Differential thermal analysis (DTA)

It is well understood that thermal analytical techniques are highly requisite to study the polymorphisms and predict drug stability, solvation, degradation, drug compatibility with excipients, and impurity studies. Moreover, as compared to all, DTA is a well-established thermal method intended for an improvement to the melting point determination.[48]

In vivo pharmacokinetic study

A single dosage bioavailability technique was intended in animals under an unfed state. The estimation of the oral bioavailability for the optimized CIH formulation with respect to an aqueous suspension of CIH pure drug was determined in rabbits.[49] The male rabbits of a healthy breed were selected for the present investigation. Then 1 mL of blood was collected carefully from the ear vein of the animal as a blank sample. Then 6.3 mg of pure drug dissolved in 12.6 mL of distilled water was given to the animal orally, and 2.6 mL of formulation was given to another animal. The blood sample from the ear vein of both rabbits was drawn periodically of 2 h interval at a range of time points (0, 2, 4, 6, 12, 18, and 24 h). The collected samples of blood were subjected to a centrifuge for 20 min, at 5000 rpm, after 20 min. The supernatant layer of serum was carefully collected with the aid of a micropipette. The bioanalysis of the collected samples was done by using the analytical UFLC technique. A collection of pharmacokinetic criteria like half-life (t1/2), maximum plasma concentration (C_{max}), elimination rate constant (K), maximum time to attain peak plasma concentration (T_{max}), and area under the curve (AUC) were calculated. The Animal Care Committee permitted the pharmacokinetic study, RIPS, Berhampur, Institutional Animals Ethics (926/PO/Re/5/06/CPCSEA, approval no: 87). All the animal experimentation congregated in accordance with the guidelines of ARRIVE and performed in association with the UK Animals (Scientific Procedures) Act, 1986 and connected guidelines, EU Directive 2010/63/EU for animal experiments.

Accelerated stability study

The accelerated stability studies were performed as per the ICH guidelines of optimized nanoparticles filled with hard gelatin capsules dosages form was subjected to accelerated stability at temperature 40°C and RH of 75% (i.e., relative humidity) for a 6-month period applying stability chamber (TH-200G, Thermolab, Thane, India). The samples were removed carefully from stability at 0,1,2,3, and 6-month time intervals and subjected for evaluation of particle analysis, ZP, and drug release.

RESULTS

Excipients selection on the basis of solubility studies

CIH showed a mean saturation solubility in selected solvents of 3660 µg/mL, 3256 µg/mL, and 2.471 µg/mL in acetone, DMSO, and ethanol. Among different solvents, acetone showed the highest quantitative solubility and, hence, was selected. Most negligible solubilities were observed in methanol 0.000345 µg/mL.

Taguchi screening design for identifying critical factors

The preliminary screening (Taguchi OA design) was applied to filter out the most influential factors with several trials for each element; two levels opted for low and high (1 and 2). Table 2 shows the respective coded and actual values for the formulations based on the CQAs. The influence of multiple factors, like A-PLGA concentration, B-poloxamer-188 concentration, C-Stirring speed, D-Stirring time, E-Ultrasonication time, F-Temperature, and G-Stirring type were studied. The p values of the regression coefficients (R^2) were determined to evaluate the relevance of each factor on each response. The model factors A, B, and C are significant since the p value is less than the standard α value (0.05), and other factors having p values higher than 0.1000 indicate the model terms are not significant. Thus, from the factor screening study, the factors A-Drug:PLGA concentration, B-poloxamer-188 concentration, and C-Stirring speed were finally selected as influential factors for further optimization.

Experimental design, optimization, and analysis

By keeping the other factors constant at a low level, the concentrations of PLGA, poloxamer-188, and stirring speed were changed. On the basis of preliminary data from Pareto-chart analysis, three levels were selected (-1, 0, and 1) for each of the factors. Table 3 represents a total of 17 runs on applying a three-factor at three-level 3^3 BBD. The characterization studies of each formulation were done to investigate the effect of different factors, like A-Drug:PLGA concentration, B-poloxamer-188 concentration, and C-Stirring speed on individual CQAs.

Response surface analysis of 2D and 3D plot

Effect of the factor on CQA QT24%

Figure 1a, it portrays the 2D (contour) and 3D plots of the CQA QT24%. Thorough understanding, it is anticipated that at a low level (-1) of Drug:PLGA concentration and high level (1) level of poloxamer-188 concentration, the red region is prevalent, more than 75% of drug release in 24 hr. run no: 16 has a maximum percentage of drug release, i.e., 76.945 %. In contrast, run no: 14 has a minimum QT24% value, i.e., 29.411 %, due to the high level (1) of Drug:PLGA concentration. Increased polymer concentration increases the level of particle size distribution and aggregation, which retards the release behavior. The result suggests an optimum drug concentration: Polymer ratio is required for better dissolution of the drug. It can also be inferred that the concentration of stabilizer (poloxamer-188) has a noticeable impact on improved drug dissolution.

Effect of the factor on CQA PS

Figure 1b, portrays the 2D (contour) and 3D plot of the CQA PS. The particle size ranges from 147.898 nm for run 16 to 450.211 nm for run 8. It has been noted that at low level (-1) of factors A-Drug:PLGA concentration and high level (1) of B-poloxamer-188 concentration indicated by the blue zone, where the lower range of particle size is achieved.[50] It can be assumed that at the lower level of A-Drug:PLGA concentration efficiently assists in getting a reduction in the particle size, and this characteristic increases significantly concerning a higher level, depicted by the dark yellowish zone. An increase in the stirring rate also influences the particle size, i.e., size reduction.[51]

Effect of the factor on CQA ZP

Figure 1c, portrays the 2D (contour) and 3D plots of the CQA polydispersity index (PDI). Both A-Drug:PLGA concentration and

B-poloxamer-188 concentration seem to influence the CQA ZP. It ranges from -6.321 mV for run 12-22.7 mV for run 16 at a low level 0.5 of A-Drug:PLGA concentration and B-poloxamer-188 concentration at more than level 1 show higher value, which predicts to have a substantial impact on the CQA.[52]

Effect of the factor on CQA PDI

Figure 1d, portrays the contour plot and the 3D plot of the CQA PDI. Both A-DRUG: PLGA concentration and B-poloxamer-188 concentration seem to equally influence the PDI. It ranges from 0.12 to run 14 to 0.65 for run 8. The results showed that, PDI's value remains below 0.2 only when both A-Drug:PLGA concentration and B-poloxamer-188 concentration have a value above the level 0.5. Uniform-size distribution is a vital requirement for getting drug absorbed at GI membrane. The stabilizer system is responsible for maintaining uniform-size distribution.

Analysis of variance (ANOVA) of BBD design

The summary of ANOVA for different factors and their significance with respect to the quadratic model was determined. After conducting the design matrix, the resultant model F value for QT24%, PS, ZP, and PDI is calculated as 21.76, 11.80, 5.06, and 5.72, respectively. P values of the model for various CQAs was less than 0.05 (α=0.05), which justifies that the quadratic model is significant. The lack-of-fit p values for QT24%, PS, ZP, and PDI were calculated as 0.565, 0.157, 0.001, and 0.455. It is not significant, relative to pure error (i.e., p value $>$ α), which is desirable for a fit model. For the CQA QT24%, the model terms, such as A, B, and C^2 are significant. For the CQA PS, the model term B is substantial. A, AB, A^2 are significant model terms regarding of ZP. In PDI as CQA, the model terms such as B, AB, and B^2 are significant. P values less than 0.05 indicate the model terms are significant.

DISCUSSION

Summary of BBD quadratic model

The BBD quadratic model summary in the optimization process is applied to optimize the PNs of CIH. In CQA QT24%, the predicted R^2 of 0.7627 is acceptable, with the adjusted R^2 of 0.9211. The precision ratio of 17.021 estimates good signal-to-noise ratio. In PS's case, the predicted R^2 of 0.2844 is not close to the adjusted R^2 of 0.8586 because it may indicate a significant block effect, with the precision ratio of 12.012 indicating an adequate signal. For ZP, the predicted R^2 of -1.0737 implies that the overall mean may be a better predictor with the adjusted R^2

Table 2. Design matrix for factor screening as per Taguchi design along with the experimental results of various CQAs and factors with their respective low and high levels

Runs	A	B	C	D	E	F	G	QT24%	Particle size (nm)	ZP (mV)	PDI
1	2	1	2	1	2	1	2	47.983	252.4	-27.8	0.299
2	2	2	1	1	2	2	1	67.342	181.0	22.32	0.175
3	1	2	2	2	2	1	1	32.341	276.3	-22.3	0.356
4	2	2	1	2	1	1	2	72.903	191.5	18.231	0.127
5	1	1	1	2	2	2	2	47.785	266.7	-22.3	0.368
6	2	1	2	2	1	2	1	70.234	224.1	-17.312	0.221
7	1	1	1	1	1	1	1	59.234	266.7	-23.6	0.368
8	1	2	2	1	1	2	2	63.456	286.7	-29.1	0.653
Factors	**Codes**							**Low level (-1)**		**High level (+1)**	
PLGA concentration (mg)	A							20		60	
Poloxamer-188 concentration (gm%)	B							0.5%		1.5%	
Stirring speed (rpm)	C							5000		10000	
Stirring time (h)	D							1		2	
Ultrasonication time (min)	E							5		10	
Temperature °C	F							25		40	
Stirring type	G							Magnetic		Mechanical	

CQAs: Critical quality attributes, PLGA: Poly-lactic-co-glycolic acid, PDI: Polydispersity index, ZP: Zeta potential

of 0.6956. For PDI, the predicted R^2 of 0.0433 is not as close to the adjusted R^2 of 0.7264, with a precision ratio of 7.696, indicating an adequate signal.

Analysis for identification of overlay plot and design space

In the case of optimization, the preferable target was allotted for various responses regarding QT24%, PS, ZP, and PDI as per the target identified in finding various QTPPs and CQAs. Based on the required quality target product profiles (QTTP), limits for different CQAs were set and processed for optimization. Run 16 was the optimized PNs of CIH, where BBD achieved comprising 30 mg of CIH: 30 mg of PLGA, poloxamer-188 (1.5% w/v) concentration, and stirring speed of 10000 rpm. Evaluation of the proposed optimized formulation showed QT24% of 76.945%, PS of 147.898 nm, ZP of 22.7 mV, and PDI of 0.398. The optimized PNs of CIH exhibited to achieve the QTTP in an optimum composition.

Characterization of PNs

FT-IR

CIH-polymer interactions were assessed for CIH and physical PM with PLGA and poloxamer-188. The observations were recorded on IR using KBr with a resolution of 4 cm^{-1} over the region 4000-400 cm^{-1}, FT-IR analysis for pure CIH exhibited absorption spectral bands as shown in Figure 2, at 1517 cm^{-1} designated to methyl (-CH3), 1338 cm^{-1} assigned to (-CH2), 2909 cm^{-1} selected to amide (-NH), 796 cm^{-1} fixed to the trifluoromethyl (-CF3), and absorption bands at 805 cm^{-1} assigned to be designated to benzene ($-C_6H_6$). The corresponding peaks obtained for the PM from the spectral analysis showed no alterations. The outcome showed the compatibility between CIH and other excipients.

DSC

DSC curve of CIH exhibited an endothermic peak at a temperature of 181.9°C, the onset temperature of 178.3°C, and the end set temperature of 184.9°C, matching its melting point. The DSC thermograms of CIH and PMs of CIH with excipients were observed. The thermogram of CIH showed an intense endothermic sharp peak at fusion temperature of 181.90°C with onset temperature at 178.33°C, and latent heat of fusion was observed to be -28.26 mJ, predicted crystalline drug nature whereas that of PMs also depicted the same, as shown in Figure 3. Studies indicated no change in peak characteristics for pure drug and formulation; thus, no interactions between drug and excipients were inferred in the present study.

Micromeritic studies

Table 4 enlists micrometric properties of lyophilized PNs, where the angle of repose is 27.96±1.5 degrees and % moisture content is 2.8±0.4, respectively. Based on these micrometric properties, Run-16 was selected to be the best formulation with better flow properties.

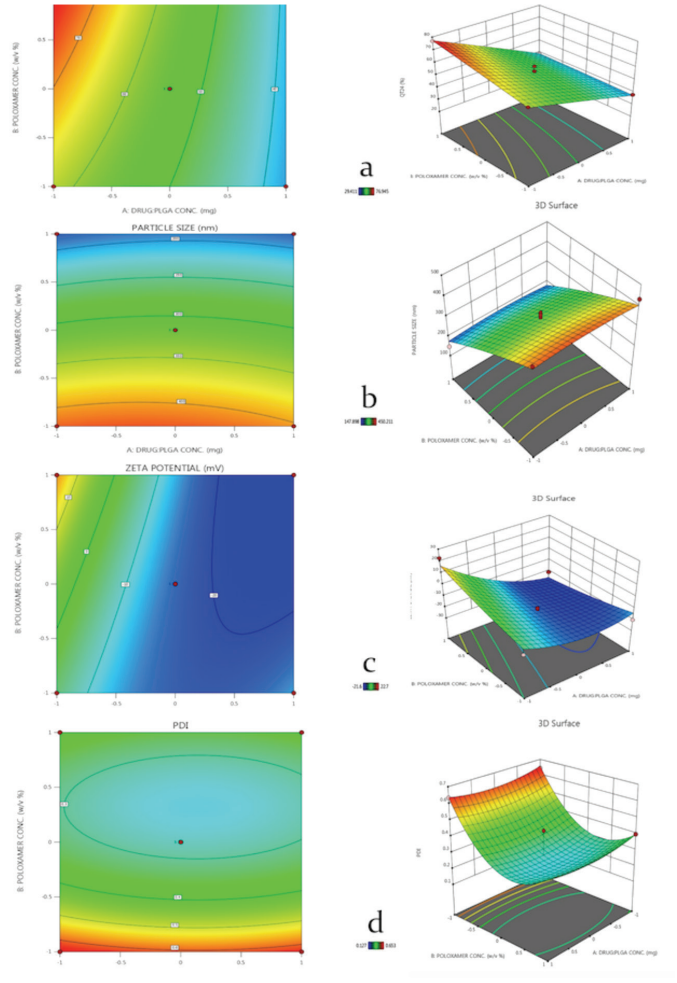

Figure 1. Contour plots (2D) and response surface plots (3D) of selected independent factors on selected dependant factors: QT24% (a); particle size (b); zeta potential (c), and polydispersibility index (d)

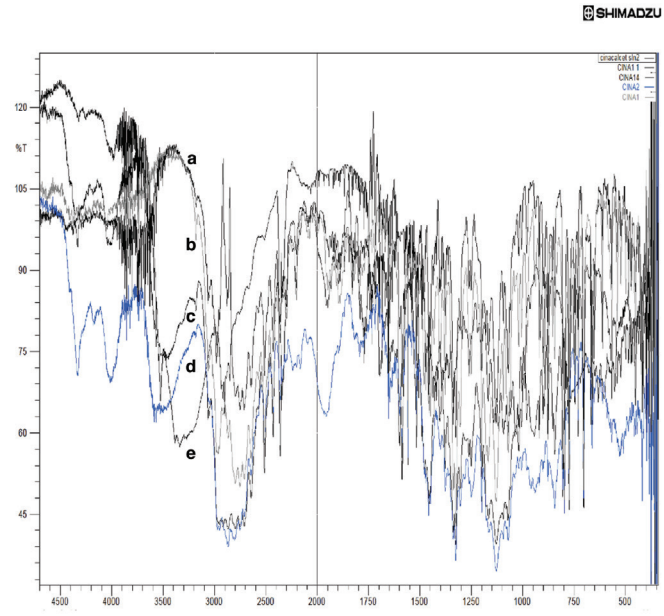

Figure 2. FT-IR spectra of pure drug (a); physical mixture of drug with PLGA polymer (b); physical mixture of drug with polaxomer-188 (c); polaxomer-188 (d) and, PLGA polymer (e)

FT-IR: Fourier-transform infrared spectroscopy, PLGA: Poly-lactic-co-glycolic acid

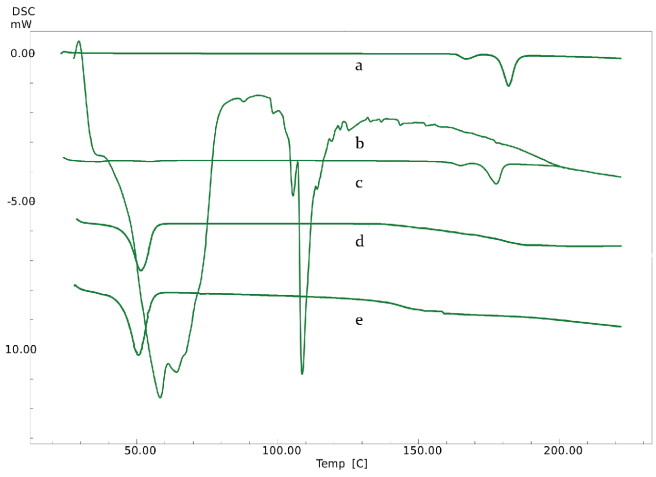

Figure 3. DSC thermogram of pure drug (a); PLGA polymer (b); drug with PLGA polymer (c); drug with polaxomer-188 (d), and polaxomer-188 (e)
DSC: Differential scanning calorimetry, PLGA: Poly-lactic-co-glycolic acid

EE

Among all the trials Run-16 (i.e., Drug:PLGA concentration 30:30 mg), poloxamer-188 1.5% w/v at stirring speed of 10000 rpm was found to have higher EE (69.56%).

Particle size and ZP determination

The zeta-sizer instrument analyzed the particle size of all formulations. The optimized size range for the PNs of CIH was 147.8 nm [i.e., for Run-16 (Figure 4a)]. The developed CIH loaded PLGA-NPs formulation showed spherical surface morphology and uniform particle size distribution of <200 nm. The increase in PLGA concentration had a potential behavior on the particle size, which produced a hazy appearance (i.e., due to the increased aggregation). The ZP results for the respective formulation were 22.7 mV for Run-16. (Figure 4b).

P-XRD

The X-RD patterns of optimized PNs of CIH and pure-drug CIH are depicted in Figure 5a and Figure 5b. Pure-drug CIH showed

Table 3. Composition of various PNs of CIH as per BBD along with the obtained CQAs responses and their coded levels QT24% cumulative % drug release at 24h

Runs	Factor 1 A:X1 PLGA:Drug ratio (mg)	Factor 2 B:X2 polaxomer-188 concentration (%w/v)	Factor 3 C:X3 stirring speed (rpm)	Response Y1 QT24%	Response Y2 Particle size (nm)	Response Y3 Zeta potential (mV)	Response Y4 PDI
1	-1	0	-1	60.345	390.311	13.23	0.453
2	1	0	-1	35.692	348.781	-21.21	0.432
3	0	0	0	48.824	345.453	-18.28	0.428
4	1	1	0	38.567	168.312	-21.6	0.389
5	-1	-1	0	62.542	432.367	-10.324	0.642
6	0	1	1	60.321	232.345	-18.674	0.299
7	0	1	-1	45.432	236.544	-19.421	0.349
8	0	-1	-1	42.871	450.211	-11.984	0.653
9	0	0	0	53.567	334.021	-15.27	0.257
10	0	0	0	54.987	323.237	-15.311	0.234
11	0	0	0	55.342	293.245	-16.322	0.231
12	-1	0	1	67.311	290.312	-6.321	0.171
13	1	-1	0	37.985	432.211	-20.3	0.634
14	1	0	1	29.411	286.768	-21.24	0.127
15	0	0	0	59.093	290.578	-18.431	0.231
16	-1	1	0	76.945	147.898	22.7	0.398
17	0	-1	1	44.252	403.231	-12.234	0.543

Independent variables	Levels		
	Low level (-1)	Middle level (0)	High level (+1)
X1: DRUG:PLGA ratio (mg)	1:1(30 mg)	1: 1.5 (45 mg)	1:2 (60 mg)
X2: Polaxomer-188 concentration (%)	0.5%	1%	1.5%
X3: Stirring speed (rpm)	5000	10000	15000

PNs: Polymeric nanoparticles, CIH: Cinacalcet hydrochloride, BBD: Box-Behnken Design, CQAs: Critical quality attributes, PDI: Polydispersity index, PLGA: Poly-lactic-co-glycolic acid

sharp peaks at the diffraction angles, such as 11.9°, 15.3°, 16.9°, 19.3°, 22.4°, 23.6°, and 25.2°, indicating a typical crystalline pattern. Optimized PNs of CIH showed a reduction (i.e., the minimal peak intensity at those angles), indicating amorphous form and confinement of the drug at the molecular level in the freeze-dried form.

SEM and TEM

Figure 6a and Figure 6b illustrate the scanning electron microscopic pictures of pure-drug CIH and optimized PNs of CIH. The SEM of pure-drug CIH appears to be a rough surface with crystalline structures. However, the TEM studies of the optimized PNs of CIH predict the amorphous structure with spherical smooth-surfaced particles (Figure 7).

TGA

The TGA curve of pure-drug CIH exhibits that at an initial temperature of 23°C, the weight loss is found to be 0.045 mg, with % weight loss of 2.76, which observed a straight line up to a temperature of 280°C with 1.42 mg with % weight loss of 93.42. The TGA curve of optimized PNs of CIH exhibited at 24°C temperature, weight loss was found to be 0.24 mg (% weight loss of 8.22), followed by a sharp decrease of curve observed at 180°C with weight loss of 1.70 mg (57.58%), as depicted in Figure 8a and 8b, and its decline up to 480°C; however, later on, it showed a straight line up to 800°C. This curve indicates that the optimized formulation seems to be significant and thermo-stable concerning % weight loss compared to CIH's pure drug.

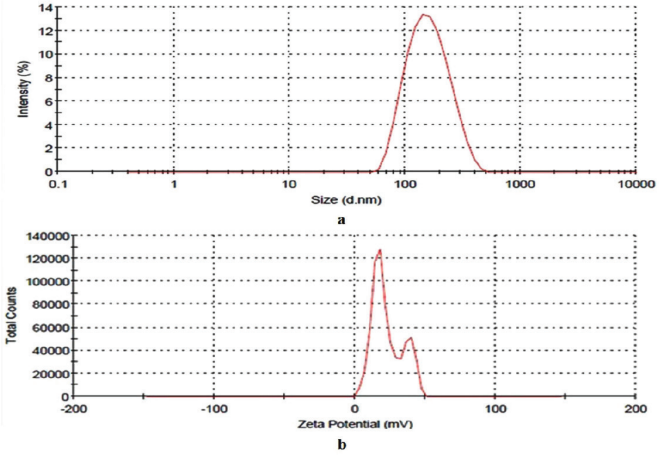

Figure 4. Particle size distribution and zeta potential curves of optimized formulation batch

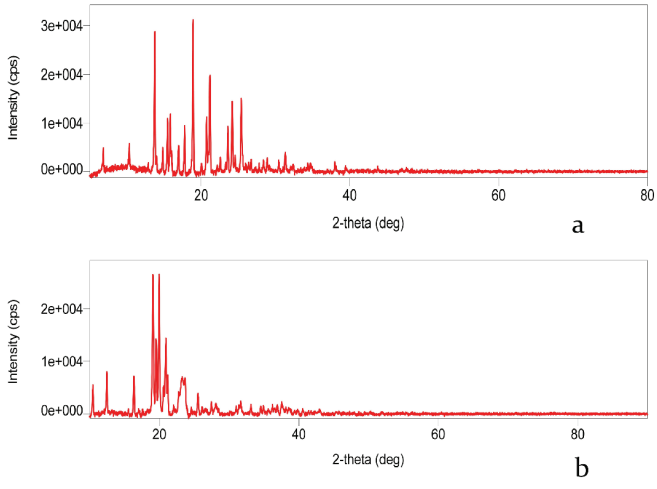

Figure 5. X-RD curves of pure drug (a) and optimized formulation batch (b)
X-RD: X-ray diffraction

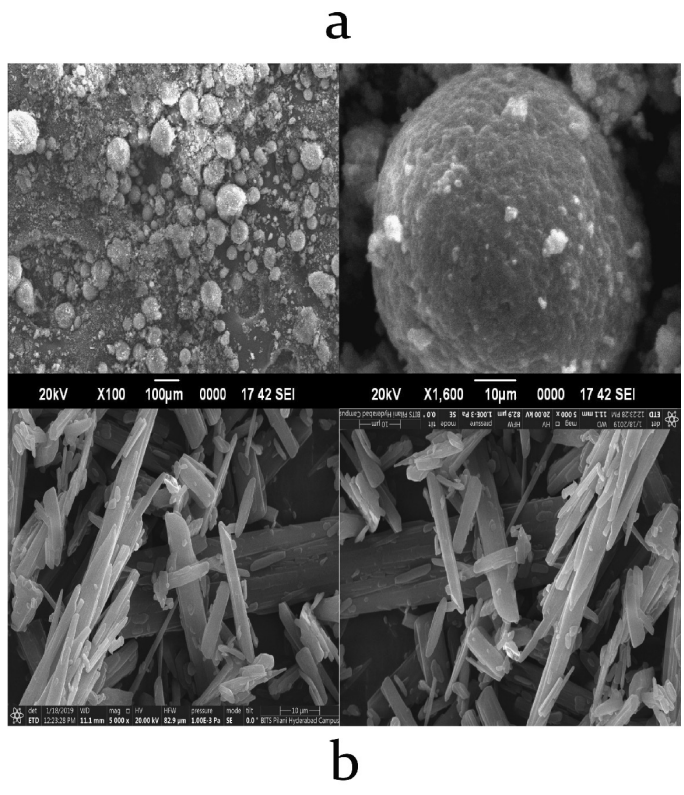

Figure 6. SEM images of optimized formulation batch (a) and pure drug (b)
SEM: Scanning electron microscopy

Table 4. Carr's index, angle of repose, moisture content, and *in vivo* pharmacokinetic parameters of pure drug and optimized formulation batch

Formulations	Carr's index	Angle of repose (Θ)	Moisture content (%)	C_{max} (µg/mL)	T_{max} (h)	K_e	AUC^{∞} 0 (µg/h)/m L	$t_{1/2}$
Pure drug (CIH)	16.66±1.08	35.5±1.98	3.2±0.31	0.671	4	190.773	10.457	0.0036
Optimized formulation	12.20±0.98	27.96±1.5	2.8±0.4	1.945	6	192.737	31.558	0.0035

CIH: Cinacalcet hydrochloride, AUC: Area under curve

DTA

The DTA curve of CIH exhibited melting point at 181°C, a significant decrease in intensity of peak, which signifies an endothermic reaction with respect to the change in the melting point. Similarly, in the case of optimized PNs showed melting point at 226°C, that means sharp change in peak of curvature in case of optimized batch due to the change in melting point at enthalpy of 348.6 mJ. Finally the onset of peaks for the optimized batch showed at 21°C and 67.31°C with the endset enthalpy at 67.31 and 13.5 J respectively. The details of DTA thermograms of optimized PNs formulations and its pure drug of CIH are depicted in Figure 9a and 9b.

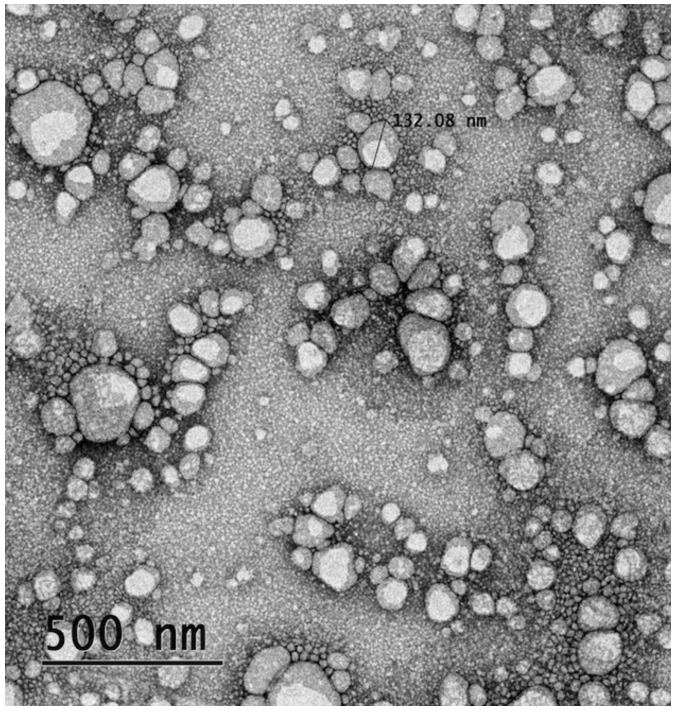

Figure 7. TEM image of optimized formulation batch
TEM: Transmission electron microscopy

In vitro diffusion studies

The behavior pattern of drug release for the optimized PNs of CIH and pure-drug of CIH is illustrated in Figure 10. The pattern of drug release was observed from the *in vitro* diffusion studies for the optimized drug loaded PNs and pure drug as shown in Figure 10. The graph indicated the optimized batch showed a substantial improvement drug release or nearly two times drug release than compared to pure drug after performing 6 h study. Hence, an optimum combination of Drug:PLGA and poloxamer-188 provides a better dissolution profile than the pure drug. For the better understanding about the drug release mechanism and fitting kinetic models, different kinetic model equations are applied such as zero-order, first order and Higuich models respectively. After applying such models, the R^2 values for each kinetic model were calculated separately for pure drug as well as for drug loaded PNs. The obtained R^2 values for pure drug, 0.921 in case of zero-order, 0.934 for first-order and 0.940 Higuchi model, and similarly the optimized drug loaded PNs R^2 values was found to be 0.835, 0.868, and 0.944, respectively. The R^2 obtained for different kinetic models suggested that Higuchi model for pure-drug CIH and optimized PNs of CIH were highest fit. The value of release exponent (n) for the pure-drug CH and optimized PNs of CIH formulation

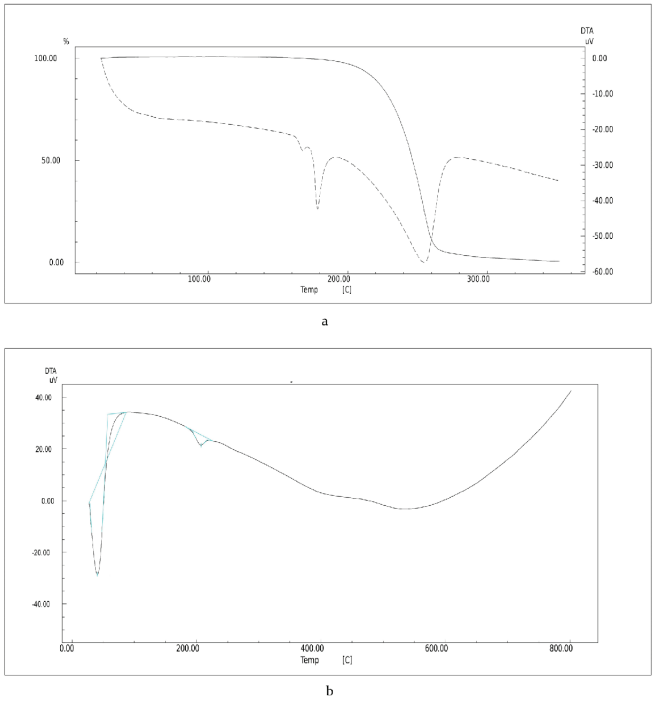

Figure 8. TGA plot of pure drug (a) and optimized formulation batch (b)
TGA: Thermogravimetric analysis

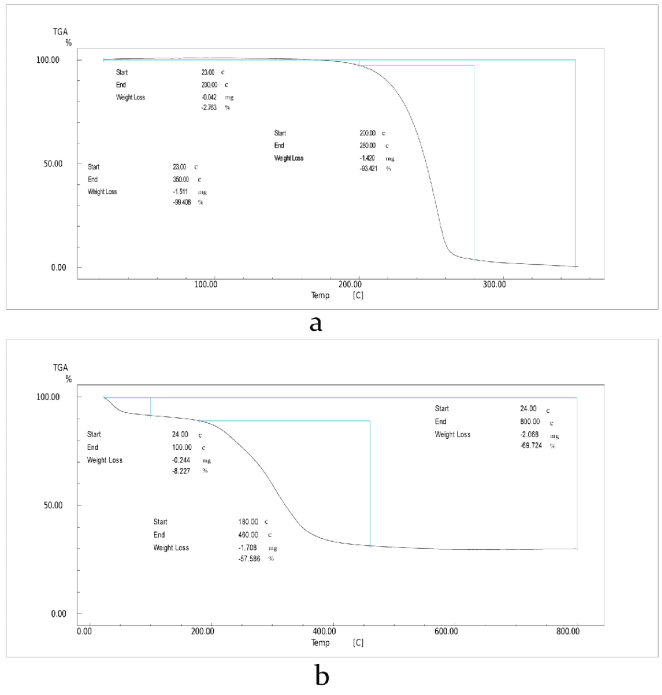

Figure 9. DTA plot of pure drug (a) and optimized formulation batch (b)
DTA: Differential thermal analysis

was 0.689 and 0.478. Hence, the drug release from pure drug follows Fickian diffusion kinetics, whereas optimized PNs of CIH follow non-Fickian diffusion kinetics.

In vivo pharmacokinetic study

From the *in vivo* pharmacokinetic study data table (Table 4) and mean plasma concentrations vs. the time curve (Figure 11) showed that T_{max} was achieved at 6 h in case of optimized PNs and at 4 h in case of pure drug, which indicated sustained release time of the drug. C_{max} of optimized PNs of CIH was 1.945 mcg/mL, whereas compared to the pure drug of CIH, it is 0.671 µg/mL. AUC of optimized PNs of CIH 31.558 (µg/h/mL) was revealed as a more than three-fold increase as compared

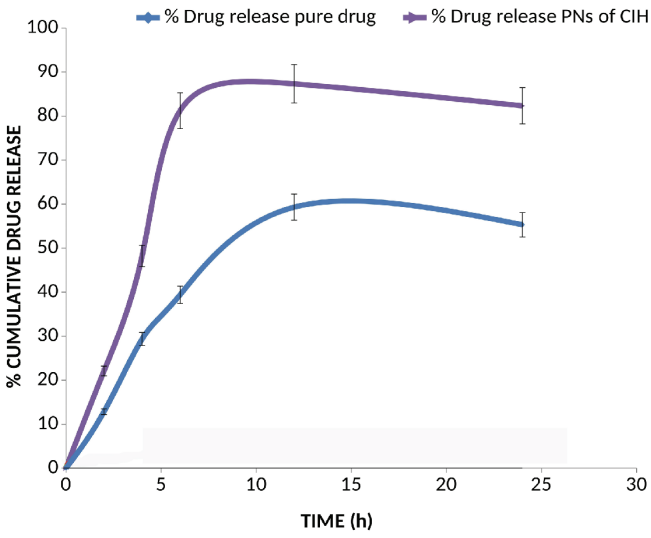

Figure 10. *In vitro* drug release curve of pure drug vs. optimized formulation batch

PNs: Polymeric nanoparticles, CIH: Cinacalcet hydrochloride

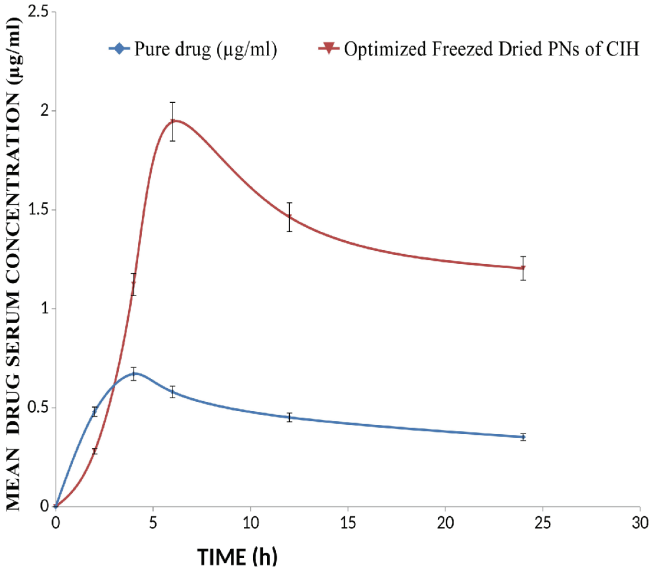

Figure 11. Serum concentration (µg/mL) vs. time (h) curve of optimized formulation batch vs. pure-drug suspension

PNs: Polymeric nanoparticles, CIH: Cinacalcet hydrochloride

to the AUC of the pure CIH 10.457 (µg/h/mL). The rationale for the boosting up in bioavailability is its improvement in the dissolution and absorption profile of drugs through the gastrointestinal membrane. *In vivo* studies proved a significant elevation in the drug CIH absorption and permeation profile concerning optimized PNs of CIH, which is evident from the distinctly superior pharmacokinetic parameter in contrast to pure drug.

Accelerated stability outcomes

The p values of the design obtained during accelerated stability studies are stated in Supplement 1. The p value was more than 0.05 for all the CQAs, indicating no significant change. Hence, the optimized freeze-dried PNs of CIH were found to satisfy the stability criteria as minimal substantial alterations in CQAs throughout the stability period.

CONCLUSION

This research instigates a systematized elaboration of PNs of a novel therapeutic for hyperparathyroidism-CIH-using a quality-by-design approach to improve drug bioavailability and sustained drug release. In the process of QbD, the first QTTPs and CQAs were identified with proper justification. Taguchi screening resulted in primary screening, followed by orderly optimization using the BBD. The regression equation and response surface were analyzed. ANOVA model was applied in the identification of the specific appreciable model term. Optimization of freeze-dried PNs of CIH was taken by coding the high and low-value range for various CQAs. The design space identification was confirmed from the overlay plot. The optimized single dose of freeze-dried PNs of drug obtained using BBD consisted of 30 mg of CIH, 30 mg of PLGA, and 1.5% w/v of poloxamer-188. The optimized freeze-dried PN formulation showed an optimum particle size of 147.89 nm, ZP at 22.7 mV, EE of 69.56%, and *in vitro* drug release of more than 75% after 24h. *In vivo* studies showed 3-folds enhancement in oral bioavailability with increased C_{max} for optimized formulation compared to a pure drug in an aqueous suspension. Accelerated stability study of optimized PNs validates the insubstantial changes in the CQAs during a stored period of 6 months, which was distinct by p values for all CQAs. The conclusive justification of the present study is that an optimum combination of 30 mg of CIH:30 mg of PLGA and 1.5% w/v of poloxamer-188 for the PLGA-based PNs of the drug may effectively be implemented to achieve the desired objective of sustained drug release and enhanced bioavailability.

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ETHICAL ISSUES

All the animal studies performed in the present work were carried out before the approval of the study protocol. The pharmacokinetic study was permitted by the Animal Care Committee, Roland Institute of Pharmaceutical Sciences, Berhampur, Institutional Animals Ethics (926/PO/Re/5/06/CPCSEA, approval no. 87). All the animal experimentation complied with the ARRIVE guidelines and were performed in association with the UK Animals (Scientific Procedures) Act, 1986 and connected guidelines, EU Directive 2010/63/EU for animal experiments.

Conflict of interest: No conflict of interest was declared by the authors. The authors are solely responsible for the content and writing of this paper.

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Supplement 1. Drug release, particle size, zeta potential, and PDI of optimized PN formulations at accelerated stability conditions

Time (months)	QT24% (cumulative drug release)	Particle size (nm)	Zeta potential (mV)	PDI
0	76.945	147.898	22.7	0.398
1	73.394	168.493	18.342	0.232
2	71.452	182.312	-13.41	0.421
3	68.341	168.301	-12.311	0.311
6	66.311	190.451	-22.212	0.390
P value $\alpha \leq 0.05$=significant	0.072	0.263	0.217	0.481

PDI: Polydispersity index, PN: Polymeric nanoparticle