Novel Methotrexate-Loaded Zein Nanoparticles With Improved and Extended Biopharmaceutical Performance: QbD-Steered Development, and Extensive In Vitro and In Vivo Evaluation

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Abstract

The current work entails QbD-enabled preparation of methotrexate-loaded nanoparticles (NPs) using zein as the release-controlling natural polymer. Initially, quality risk estimation and factor screening studies using Taguchi design were undertaken to delineate “vital few” process and material attributes among “plausible so many”. Further, formulation optimization using central composite design and validation using correlation plots and percent predictive bias was carried out. Optimized NPs exhibited mean size of 159 nm, zeta potential of 14.85 mV and entrapment of 50.23%. *In vitro* dissolution kinetic modelling unearthed non-Fickian drug release extension mechanism from the proposed zein NPs. *In vitro* MTT and apoptosis assay using MCF-7 cells and cellular uptake studies using Caco-2 cells indicate remarkably superior anticancer potential of zein NPs over pure methotrexate, ascribable to their nanometric size and cationic nature. *In vivo* pharmacokinetic studies in rat construed significant enhancement by 2.15-fold in AUC_{48h} ($p < 0.001$), 1.30-fold in C_{max} ($p < 0.05$), 3.67-fold in T_{max} ($p < 0.001$), and 1.38-fold in $T_{1/2}$ ($p < 0.01$), along with notably reduced variability in biopharmaceutical performance. Establishment of significant point-to-point level A *in vitro/in vivo* correlations (IVIVC) and kinetic modeling construed the robustness and prognostic ability of drug release studies. Robustness of the nanoformulation was ratified under refrigerated storage through six months’ stability studies. Overall, the studies unequivocally indicate development of a stable nanoparticulate formulation with significantly enhanced extent, extension and consistency of biopharmaceutical performance, along with improved anticancer potential of methotrexate.

Introduction

Cancer, a proliferative syndrome, has proved to be serious threat to human health and life, since decades [1]. Breast cancer, in particular, has been the primary cause of deaths in women, with approximately 2.1 million new cases reported every year across the globe [2, 3]. Methotrexate (MTX), in this regard, plays a vital role in the therapeutic management of cancers [4]. Exhibiting poor water solubility (0.01 mg/mL) couple with poor permeability (cLog P of 0.53), it can safely be categorized as a Class IV drug as per Biopharmaceutical Classification System (BCS) [5]. Reported to follow saturable absorption through proximal intestine, mediated through specific transporters, MTX shows dose-dependent oral bioavailability [6, 7]. Accordingly, lower doses of MTX have been observed to demonstrate relatively higher oral bioavailability [8, 9]. Besides, owing to its poor solubility and permeability, coupled with saturation of transporters, MTX is documented to exhibit inconsistent pharmacokinetic behaviour and erratic bioavailability [10, 11]. As MTX is also known to observe a narrow therapeutic index, it is highly recommended to maintain and sustain plasma levels of drug at the optimal levels [12, 13].

Myriad types of nanostructured delivery systems of MTX, in this context, have been explored not only for improving its extent of bioavailability, but also in reducing the intensity and frequency of undesirable side effects like nephrotoxicity, bone marrow suppression, chronic interstitial obstructive pulmonary disease and hepatotoxicity [4, 14]. Sustained drug release characteristics has been another distinct advantage of employing such nanocarriers in drug delivery [15]. Drug delivery systems, like liposomes [16], microcapsules [17], synthetic polymer-based NPs [4, 18], lipid-based non-vesicular drug delivery systems like solid lipid nanoparticles (SLNs) [19, 20], and lipid-polymer hybrid NPs [21], have been explored to improve the biopharmaceutical potential of MTX and/or controlled release characteristics, but with limited fruition. Many of such nanocarrier systems also tend to pose serious problems of inept robustness and stability, and rapid degradation in the gastric milieu [22].

Recently, the domain of drug delivery has witnessed growing popularity of diverse polymers obtained from natural origin [23]. Zein, a natural, water-insoluble and prolamine-based amphiphilic cationic polymer, has duly been approved by the United States Federal Drug Agency as Generally Regarded as Safe (GRAS) [24]. It is a plant protein, derived usually from maize, which has found myriad uses as a wrapping material in food industries, primarily due to its capability to sustain moisture levels in prepared goods [24]. Because of its high coating capacity, coupled with low cost, biocompatibility and biodegradability, zein has also been successfully employed for developing modified release systems for the delivery of diverse drugs [25, 26], enzymes [27, 28], essential oils [29] and other substances [30, 31].

Systematic drug delivery system development to provide the required quality traits, while optimizing different possible process and material parameters, is a formidable task. This could be achieved using Quality by Design (QbD) paradigms, involving the cardinal principles of Quality Risk Management (QRM) and Design of Experiments (DoE) [32, 33]. Thus, the present work envisages organized development, characterization and evaluation of MTX-encapsulated NPs for attaining improved, sustained and consistent biopharmaceutical attributes of the drug. Zein was selected to formulate such MTX-loaded NPs for efficient and cost-effective controlled drug delivery to potentially facilitate effective drug transportation to the target cells [34].

Materials

MTX was obtained from IPCA Laboratories, Mumbai, India, as a gift drug sample. Zein was procured from Acros organics, Thermo Fisher Scientific, [New Jersey](#), USA, while 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Annexin V-FITC apoptosis kit were procured from Sigma Aldrich, Missouri, USA, and BD Bioscience, [New Jersey](#), USA, respectively. MCF-7 breast cancer and Caco-2 intestinal epithelial barrier model cells were arranged from National Centre of Cell Science, Pune, India. Different other laboratory excipients, reagents and solvent of pure analytical grade were used without any further alteration throughout the study.

Methods

3.1 Preparation of Polymeric Nanoparticles

Zein-based NPs encapsulating MTX were formulated using an already reported evaporation method, after minor modifications [4, 32, 35]. Briefly, zein was dissolved in ethanol (90 % v/v), constituting the organic phase, while phosphate buffer saline (i.e., PBS, pH 7.4), containing the requisite amounts of MTX and Pluronic F-68 (PF-68) as the stabilizer, constituted as the aqueous phase. The aforesaid organic phase was added into the aqueous phase, drop-wise, with constant stirring, employing a magnetic stirrer (REMI, Mumbai, India) to allow the diffusion and evaporation of organic phase, and eventually, the formation of colloidal dispersion of zein NPs.

3.2 QTPP and CQAs

As per QbD approach, the quality target product profile (QTPP) for the proposed polymeric nanocarriers was firstly embarked upon with an aim to improve the quality attributes of zein NPs [36]. Different critical quality attributes (CQAs), like mean particle size (PS), zeta potential (ZP) and % entrapment efficiency (EE), were identified, and plausible cause-effect relationships among the chosen CQAs and probable method or material parameters were delineated using an Ishikawa fish-bone diagram. Consequently, an initial risk estimation matrix (REM) exercise involving all the potential factors influencing the QAs (PS, ZP and % EE) was conducted by assigning these to low, medium and high levels of risk [37-39].

3.3 Factor Screening Studies

Design Expert[®] software package (Version 11, Stat-Ease Inc., [Minnesota](#), USA) was used to implement Taguchi design (TgD) with a seven-variable eight-run matrix in order to chalk out highly influential method and material parameters. Only the high- and medium-risk factors, previously identified using REM studies in Section 3.2, were considered for factor screening. **Table 1** enlists the design matrix for various variables screened, along with their high and low levels, respectively [33, 40].

3.4 Optimization Studies on Zein NPs

The CMPs, i.e., amount of polymer (X_1) and percentage of surfactant (X_2), were systematically optimized using a nonlinear second-order central composite design (CCD). **Table 2** summarises the design matrix, consisting of a total of thirteen experimental trials, along with pertinent factors and their corresponding levels.

3.4.1 Modelling data analysis, validation and optimum search

Subsequently, in order to fit the experimental data, a quadratic polynomial model was employed to optimize data analysis and model validation. Model evaluation was carried out using Pearson's correlation coefficient (R) and lack-of-fit analysis. The 2D-contour plots and 3D-response surfaces were drawn and deciphered for ostensible factor-response relationship(s). Optimal solution was selected using mathematical desirability function by "trading-off" different CQAs, and subsequently by graphical optimization, delineating the "design space" region by following the criteria of maximal values of % EE and ZP, and minimal values of PS and % cumulative drug release. Six confirmatory formulations were prepared as the validation runs (ZNP 1-6) to evaluate the predictive ability of the evolved polynomial models and DoE methodology, using percentage of prediction error (%PPE) and linear correlation plots.

3.5 Characterization of Zein NPs

3.5.1 Mean Particle size and Zeta Potential

The magnitudes of mean particle size as well as of zeta potential of the prepared NPs were measured at 25°C using a particle size analyser, (Zetasizer, ZS90; Malvern Instruments, UK). Dispersion of zein NPs was placed into a micro-electrophoretic cell, fixed at an angle of 90°.

3.5.2 Field Emission Scanning Electron Microscopy (FESEM)

Surface morphology of optimized NPs was examined using an FESEM system (SU-8010, Hitachi, Tokyo, Japan). A droplet of sample was coated with gold, and the microphotographs were captured at suitable operating conditions.

3.5.3 Transmission electron microscopy (TEM)

The prepared optimized formulation was further characterized employing TEM (Tecnai, Holland, Netherland), operating at suitable accelerating voltage. A droplet of sample was placed on a grid surface, followed by (negative) staining with 1% phosphotungstic acid. Following air-drying, the grid was exposed for imaging and the microphotographs were taken at appropriate magnification(s).

3.5.4 Encapsulation efficiency

The values of % EE of the prepared zein NPs were obtained by adopting a reported method [41, 42]. Briefly, 2 mL aliquots of the optimized prepared formulation were centrifuged (Sorvall™ Legend™ XTR, Thermo Fisher Scientific, Massachusetts, USA) at 10,000 rpm (11,200g) for 10 minutes, and free drug in the supernatant was estimated at a λ_{max} of 257 nm using a UV-Vis spectrophotometer (UV 3000+, Labindia, Mumbai, India).

3.5.5 *In vitro* drug release studies

Drug release kinetics from the optimized NPs was investigated employing the dialysis bag (MWCO 10-12, KD, Himedia) method [43, 44]. Two millilitres of suspensions, each of the drug and its optimized NPs, were placed into the dialysis bag(s) and suspended in the receptor compartment constituting phosphate buffer (pH 7.4: 50 mL) over a water bath (Rivotek, Mumbai, India), maintained at 37°C. At regular intervals, 2 mL aliquots each of medium were withdrawn and replaced with fresh solvent in equal volumes at pre-determined time intervals during the study. Quantification of MTX was carried out employing UV spectrophotometry. Drug release data were subsequently fitted into various different kinetic models to arrive at the possible mechanism(s) of MTX release from the prepared NPs.

3.6 *In Vitro* Cell Culture Studies

Cells were harvested and grown in a tissue culture flask (25 cm²) (BD, Falcon, New Jersey, USA), according to the requisite guidelines [45]. Later, the cells were passaged to an alternate flask, as per the standard protocol for *in vitro* cell culture studies [46].

3.6.1 MCF-7 Cell culture investigations

3.6.1.1 MTT cellular viability studies

Cell viability assay using free MTX and the prepared zein NPs was conducted employing an MTT assay on the MCF-7 cells, according to the procedure already reported by us [32, 47, 48]. Harvested cells (1×10^5 cells/well) were seeded in a 96-well plate and were kept for adherence to the flask surface. Free MTX and zein NPs containing equivalent amount drug amounts were added in serial concentrations, and kept aside for incubation at $37 \pm 1^\circ\text{C}$ under CO_2 (5%) environment. Subsequently, excess of zein NPs, free MTX and medium were removed, and washed with buffer (PBS; pH 7.4). Optical density (OD) was measured at 550 nm employing an ELISA plate reader (MultiscanTM FC microplate, Thermo Fisher ScientificTM, New Jersey, USA), and percentage of cell viability was quantified.

3.6.1.2 Apoptosis assay

Apoptosis assay using MCF-7 cells was carried out using Annexin V-FITC/PI kit [49]. Cells (2×10^5) were collected, incubated with MTX and zein NPs, and processed according to the standard protocol [50]. Cells were further treated with Annexin V-FITC:PI and kept aside in dark for incubation, followed by addition of binding buffer prior to its analysis using a flow cytometer (BD AccuriTMC6, California, USA).

3.6.1.3 Quantitative cell uptake analysis: Flow cytometry

A flow cytometer (BD AccuriTM C6, California, USA) was employed to study the quantitative cellular uptake of rhodamine-labelled NPs by MCF-7 cells [51]. Cells (1×10^5 cells/well) were seeded in a 6-well plate, along with complete growth medium, and were set aside for an overnight. The cells were separately incubated with free rhodamine and zein NPs-rhodamine, and kept aside for 4 h, followed by their quantification using the flow cytometer.

3.6.2 Caco-2 cells culture

3.6.2.1 Qualitative uptake using confocal microscopy

Caco-2 cells (2×10^5 cells/well) were placed in a 6-well plate (BD, Falcon, New Jersey, USA), and were set aside for 24 h for attachment of cells over the flask surface [51]. Additionally, the cells were separately incubated with free dye and dye-loaded zein NPs for 4 h. Cells were subsequently rinsed with the PBS (pH 7.4) to wipe out the excess of medium carefully and were fixed with a solution of glutaraldehyde in ethanol (2.5% v/v). After proper treatment, microphotographs of the nanocarriers entrapped within the cell(s) were captured with a confocal laser scanning microscope (Nikon C2+, Tokyo, Japan).

3.7 In Vivo Animal Studies

Animal studies were conducted as per institutional ethical committee guidelines of the Panjab University, India, after attaining the essential permission, vide number, PU/IAEC/S/15/31.

3.7.1 In vivo animal pharmacokinetic studies

Pharmacokinetic investigations were conducted on free MTX and zein NPs using Sprague Dawley female rats (weights: 180 to 225 g), supplied by the Central Animal House Facility of the Panjab University. Prior to studies, the animals were housed in regulated conditions ($25 \pm 2^\circ\text{C}/60 \pm 5\% \text{ RH}$) and fasted overnight, but were allowed *ad libitum* access to water. Further, 3 rats were randomly placed into two different groups, and were administered with free MTX (10 mg) and equivalent amount of zein NPs, employing oral gavage.

Blood samples, measuring approximately 150 μL each, were thereafter collected from rat retro-orbital plexus under mild anaesthesia at the scheduled time-points of 0.25, 0.50, 1, 2, 6, 8, 12, 24, and 48 h in HiAnticlot[®] vials (Himedia, Mumbai, India). Blood samples, thus collected, were centrifuged at 12,000 rpm (16,128 g) for 10 minutes, and supernatant plasma samples were estimated for MTX by an HPLC method, reported earlier by the authors [52]. The pharmacokinetic data analysis and modelling were conducted employing an add-in PK Solver[®] MS-Excel spread-sheet [53], adopting Wagner-Nelson technique.

Diverse pharmacokinetic parameters like area-under-curve till 48 hours (AUC_{48h}), maximal plasma drug concentration (C_{max}), time to attain C_{max} (t_{max}), biological half-life ($t_{1/2}$), and mean residence time (MRT) were computed, interpreted critically, and compared to those obtained with pure drug suspension.

3.8 *In vitro/in vivo* correlation (IVIVC)

Attempts were made to establish point-to-point linear level A IVIVC between percentages of *in vitro* drug released data with that of *in vivo* drug absorbed at the corresponding time points for MTX as well as its zein NPs, and the significance of the correlations was statistically deciphered according to the standard Fisher's ratio criterion at the appropriate degrees of freedom. The magnitudes of the total drug absorbed were calculated using Modified Wagner–Nelson technique, as MTX was found to obey one-compartment pharmacokinetic model[32].

3.9 Stability Studies

The lyophilized zein NPs were investigated for stability studies in order to predict their quality and integrity during different storage conditions over the course of time, employing an environmental chamber (Newtronic Lifecare, Mumbai, India). MTX being a photosensitive drug, amber-coloured glass vials were kept for a time period of 180 days at varied conditions of temperature and relative humidity (RH), viz., $5\pm3^{\circ}\text{C}$ and $25\pm2^{\circ}\text{C}$ (both at $60 \pm 5\%$ RH); and $40\pm2^{\circ}\text{C}$ and $75\pm5\%$ RH ($n=3$) [2, 54]. The formulations were periodically evaluated on intervals of 1, 2, 3 and 6 months for the values of their identified CQAs and compared to data obtained at the start of studies [55].

Results And Discussion

4.1 Risk Estimation Matrix (REM)

Figure 1 represents the Ishikawa fish-bone diagram, constructed to highlight the possible inter-relations among the selected method and material parameters and the requisite CQAs of zein NPs of MTX. **Figure 2** represents the REM layout of different risk levels delineated with each variable, based upon the reported scientific literature and brain storming discussions among the team members. Only the factors linked with high or medium level of risk(s), like amount of polymer, volume of aqueous phase, time of sonication, stirring time and speed, and concentration and type of surfactant, were further selected for screening studies [38]. The remaining input variables like stirring type and method of preparation were kept at constant levels throughout the studies.

4.2 Factor Screening Studies

Half-normal and Pareto plots in **Figure 3** vividly depict that the amount of polymer and percentage of surfactant were found to significantly influence the chosen CQAs, i.e., mean PS (nm), ZP (mV) and EE(%), as these remained either above Bonferroni limit and/or t-value limits. **Eq 1** displays the polynomial generated for the chosen CQAs [33].

$$Y = \beta_0 + \beta_1 + \beta_2 + \beta_3 + \beta_4 + \beta_7 \dots \text{Eq. 1}$$

where, β_0 presents the coefficient of intercept term, while β_{1-7} are the corresponding linear model coefficient terms, i.e., X_{1-7} .

4.3 Optimization studies and Response Surface Mapping (RSM)

A second-order CCD was applied to carry out the optimization studies, followed by data analysis using **Eq2**, accounting for main and interaction effects between the selected CMPs.

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_1 X_2 + \beta_4 + \beta_5 \dots \text{Eq. 2}$$

where, β_0 is the intercept term, β_1 and β_2 represent the coefficients of factors, and respectively, β_3 represents the interaction term coefficient between X_1 and X_2 , and β_4 and β_5 are the corresponding quadratic coefficients of .

Table 3 summarizes the polynomial model coefficients of varied quadratic terms and corresponding values of statistical significance of ANOVA model (p) and coefficient of correlation (R).

Figure 4(a-f) portray the effect of surfactant and polymer, on the desired CQAs explored through the 3D- and 2D-response surface curves.

Figure 4(a) portrays the 3D-response surface plot between the amount of polymer and surfactant concentration for PS. On increasing the polymer concentration, a curvilinear pattern was observed, i.e., initially a decline in the PS values, followed by a rise after the intermediate levels. A gradual decline in PS values was observed on increasing the surfactant concentration, however, this could be attributed to the tendency of surfactants to reduce the interfacial surface tension, eventually lowering the PS. The respective 2D-contour plot (**Figure 4(b)**) reinforces the aforesaid interpretations of the 3D-response surface.

Figure 4(c) shows the 3D-response surface between the amount of polymer and surfactant concentration for ZP. The values of ZP were observed to follow moderate curvilinear trends with rising levels of surfactant and/or polymer. The highest magnitude of ZP was seen at the highest levels of zein and the lowest levels of PF-68. Thus, higher levels of polymer and lower levels of surfactant were observed to be favourable for attainment of sufficiently high ZP values of the developed NPs. The parallel lines of 2D-contour in **Figure 4(d)** corroborate similar inference too.

Figure 4(e) portrays an enigmatic relationship between polymer concentration and surfactant for EE as the CQA. High EE was found to be achieved at low levels of surfactant and mid-levels of polymer. The rising ridge pattern of **Figure 4(f)** depicts that low levels of surfactant and middle levels of polymer could achieve higher entrapment of MTX.

Figure 4(g) represents a 3D-response surface for percentage of cumulative drug release plotted against the two CMAs, viz., polymer and surfactant. With increasing polymer levels, a linear declining trend was observed upto its intermediate levels, followed by a slight increase in drug release values. However, a rise in the surfactant levels was found to increase the % drug released, ostensibly owing to improved wetting and solubilizing potential of surfactant. Likewise, the respective 2D-contour plot in **Figure 4(h)** also construed analogous observations.

4.3.1 Search for Optimized Formulation and Validation Studies

The most suitable drug delivery formulation was selected by balancing variegated CQAs to obtain the preferred targets, i.e., minimal value of PS (which aids in drug absorption) and cumulative drug release till 48 h (ensures extended release profile), and maximal values of ZP (imperative for stability indication) and EE (ensures loading capacity). Following the aforesaid criteria, the optimum search was accomplished using numerical optimisation, aiming for the desirability values close to unity. **Figure 5** shows the overlay plot delineating the constitution of the optimal zein NPs as per the flag, as polymer (78 mg) and surfactant (0.35 %), attaining mean PS of 159.30 nm, ZP of 14.85 mV, EE of 50.23% and cumulative drug release of 33.90 %.

Table S 1 illustrates the composition of the different formulations, along with the observed and the anticipated values of CQAs and % PPE between these. The absolute bias or % PPE ranged between -4.55 and 4.86% with low magnitude of overall prognostic error (i.e., $0.11\% \pm 3.06$), construing a high level of predictive capability of the adopted QbD methodology.

Figure S 1 depicts the linear correlation plots, drawn between observed values of the CQAs and those predicted using RSM, and the corresponding residual curves for all the drug delivery formulations. All such correlation plots were observed to follow significant linearity, as the magnitude of R ranged between 0.9370 and 0.9787 ($p < 0.05$ to < 0.01), and the analogous residual plots were also quite controlled with narrow and indiscriminate scatter, well within the acceptable limits of 5%.

4.4 Characterization of the optimized SLNs

4.4.1 Mean Particle Size and Zeta Potential

Figure 6 (a and b) depict the mean PS of 159 nm and PDI of 0.264, indicating monodisperse homogeneous nature of the prepared polymeric dispersion. The high magnitude of ZP, i.e., 14.85 mV, construed good stability of the developed system and

high permeation potential across the mucosal membrane of the intestine [56].

4.4.2 Electron Microscopic Studies

Figure 7 (a and b) show the TEM and FESEM microphotographs of the optimised formulation, respectively, construing segregated and round-shaped particles with smooth surface and size under 200 nm. Such interpretation of size ranges was found to be in agreement with those obtained earlier during particle size measurement (Section 4.4.1).

4.4.3 Drug Release Kinetics Modelling

Studies on cumulative MTX release profiles on zein NPs revealed a sustained release pattern of MTX from the polymeric NPs, when compared to naïve drug suspension (Figure 8). It is quite evident that more than half fraction of pure MTX got released within the first 30 minutes, while it took over 8 h to release the same extent of drug from the optimized NPs. **Table 4** enlists the outcomes of fitting of release data into various mathematical models. Statistical significance of regression (i.e., F ratio) construed Korsmeyer-Peppas mathematical model ($p < 0.001$) as the most suitable strategy to unravel the mechanism of MTX release kinetics [57]. The values of diffusional release exponent (n) indicated Fickian release mechanism of MTX ($n = 0.181$), and non-Fickian or anomalous release behaviour from the optimized NPs ($n = 0.5681$) [55]. Weibull model fitting on drug release data revealed the values of dissolution curve shape parameter (β) to be ranging between 0.444 and 0.723 (i.e., < 1) for MTX as well zein NPs, thus representing an initial steep slope, followed by an exponential-type of curvature. On the other hand, the magnitude of Weibull time-scale parameter (α) for zein NPs (i.e., 4.854) were found to be superior to those of pure drug (i.e., 0.876), indicating discrete drug release extension from the NPs [58].

The values of location parameter (T_i) obtained with both pure MTX as well as with the optimized NPs indicated that the onset of dissolution process was quite instantaneous [36]. Attempts to fit the data in Baker-Lonsdale model for NPs too indicated high degree of fitness ($R = 0.9840$; $p < 0.001$) construing regulated drug release characteristics from the spherically shaped NPs [59]. On the other hand, pure drug data failed to yield statistically significant results.

4.4 Cell culture study

4.4.1.MTT assay

Figure 9 depicts concentration-dependent cell cytotoxicity of MCF-7 cells on interacting with plain MTX suspension and zein NPs, evidently indicating much higher cytotoxicity of the latter *vis-à-vis* the former. The IC_{50} values were found to be approximately 37.78 and 12.77 $\mu\text{g/mL}$ for pure drug and optimized NPs, respectively, thus justifying almost 3-folds improvement in cytotoxicity and efficacy of the latter. The enhanced cytotoxicity of the optimized formulation could be attributed to its cationic nature and minuscule particle size, which may enhance cellular internalization across the MCF-7 cells, followed by subsequent release of MTX from zein NPs [60].

3.4.2 Apoptosis assay

Figure 10 shows the quantitative estimation of programmed cell death caused by MTX and its zein NPs in the stoichiometrically equivalent concentrations (i.e., 12.77 $\mu\text{g/mL}$). Almost 12.9 % of cells were observed to be in the initial apoptotic phase on treatment with zein NPs, to that of 2.1 and 0.5 % cells for MTX pure and reference control of untreated cells, respectively. Nevertheless, the count of cells in the late apoptotic phase increased to 0.9 % (reference control) and 1.1% (MTX) to 3.4 % (zein NPs), on treatment. Superior apoptosis was observed with zein NPs, which can be ascribed to the enhanced permeation of the carrier system due to their positive charge and the subsequent internalization across the cell membrane due to its minuscule size [56].

4.4.3 Quantitative cellular uptake

Figure 11 (a-d) illustrates the cellular uptake of C_6 by MCF-7 cells, when exposed to free MTX and its optimized zein NPs. The quantum of percent C_6 values, measured using FACS analysis was found to be approximately 141-folds with the latter *vis-à-vis* the control (i.e., untreated cells). Improved fluorescent intensity of C_6 was found with zein NPs, which can again be attributed to the integrated effect of nanometric size and cationic nature of the polymer [61].

4.4.4 Cellular uptake studies

Figure 12 (a-d) depicts the qualitative cellular uptake of C_6 -loaded zein NPs (C_6 -zein NPs) and pure C_6 in Caco-2 cell lines. Negligible fluorescent characteristics were noticed, as free C_6 was not freely internalised by the cell lines [62]. Besides, high fluorescent intensity was noticed with C_6 -zein NPs (**Figure 12 (d)**), assignable again the positive charge and diminutive size of the NPs thus facilitating their endocytosis across the cellular membrane [63, 64].

4.5 In vivo pharmacokinetic studies

Figure 13 displays 48-h plasma drug level (mean $\pm$ SD) profiles of pure MTX suspension and zein NPs. The respective inset represents percentage alteration in the values of pharmacokinetic metrics following intake of MTX NPs compared pure MTX. The results showed quite enhanced biopharmaceutical attributes of zein NPs *vis-à-vis* naïve drug, with nearly 2.15-folds improvement in AUC_{48h} ($p < 0.001$), 1.30-folds in C_{max} ($p < 0.05$), 3.67-folds in T_{max} ($p < 0.001$), 1.38-folds in $T_{1/2}$ ($p < 0.01$), and 1.64-folds in MRT ($p < 0.01$). Also, the values of percent coefficient of variation (% CV) noticed with zein NPs (i.e., ranging between 5.8 and 59.8%; mean CV: 23.5%) indicated superior consistency in plasma level data over that of pure MTX suspension (i.e., ranging between 12.3 % and 81.8 %, mean CV: 41.9 %). Significant improvement in various absorption parameters and consistency in plasma level data were noticeable with NPs, than pure MTX. Overall, distinctive improvement in the magnitude of biopharmaceutical attributes demonstrates significant augmentation in the extent of bioavailability, as well as extension and consistency in of plasma MTX levels, on its incorporation in the zein NPs.

4.6 IVIVC studies

Figure 14 portrays the linear curves for level A IVIVC for MTX and its zein NPs. High values of coefficient of correlation (R) were observed for MTX zein NPs (i.e., $R = 0.9716$, $p < 0.01$) as well as for pure MTX (i.e., $R = 0.9426$, $p < 0.05$) with linear model fitted between cumulative percentages of dissolved drug *in vitro* with those of absorbed drug *in vivo* [32, 36]. Embarking upon of statistically valid IVIVC's, accordingly, corroborate the robustness of *in vitro* drug dissolution evaluation methodology, including that of drug release medium composition, dissolution apparatus type, sampling schedule and stirring conditions, as quite prognostic and simulative of *in vivo* biopharmaceutical performance of MTX. Successful establishment of such IVIVC's have already been reported in the literature for various BCS class II and IV drugs, and their consequent drug delivery systems [32, 36, 47, 65].

4.7 Stability studies

The results of stability studies are summarized in Table S2. Relatively mild to notable rise in mean size of particles may be ascribed to the reduced viscosity of the delivery system, during different storage conditions. This eventually led to the high kinetic energy and number of collisions throughout the particles ultimately resulting in aggregation of NPs at room temperature (Shah et al., 2014). Besides, modest decline was observed in the magnitude of zeta potential, cumulative drug release and efficiency of entrapment, during storage at 25 °C and 40 °C, which may majorly be accountable to the possible denaturation of the proteinaceous structure of zein, resulting in high drug leakage from the NPs [66]. The NPs, stored under the refrigerated conditions, on the other hand, were found to be stable during storage period of three months, without any significant change in the values of CQAs [67].

The NPs, stored under the refrigerated conditions, on the other hand, were found to be stable during storage period of three months, without any significant change in the values of CQAs.

Conclusion

The current studies were planned to explore the plausible enhancement and extension in biopharmaceutical performance of MTX, when formulated as zein NPs. Implementation of QbD strategy aided in holistic know-how of the nanoparticulate formulation development while achieving the QTPP requirements. NPs demonstrated significantly higher cytotoxicity and improved uptake potential than pure MTX during in vitro evaluation, ostensibly ascribable to their nanometric size and cationic surface charge. Notable extension in pharmacokinetic profile, augmentation in the extent of bioavailability, and diminution in data variability were observed with zein NPs. Stability studies ratified the robustness of zein NPs under refrigerated conditions when stored for three months. Concisely, the findings of the current research work substantiate superior formulation robustness, and extended, regulated and consistent biopharmaceutical performance of MTX NPs, resulting in its high therapeutic potential in breast tumour(s). Successful outcomes observed with polymeric NPs, employing zein as a natural, biodegradable and economical polymer, can be rationally explored and extrapolated for other biopharmaceutically enigmatic drug molecules too.

Declarations

ETHICAL STATEMENT:

- Ethics approval and consent to participate: Animal studies were conducted as per institutional ethical committee guidelines of the Panjab University, India, after attaining the essential permission, vide number, PU/IAEC/S/15/31
- **Consent for publication:** Yes
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- **Authors' contributions:**

Atul Jain: Data curation; Formal analysis; Funding acquisition; Investigation; Methodology

Teenu Sharma: Validation; Writing - original draft

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Tables

Table 1: Seven-variables eight-run Taguchi orthogonal array for screening of different material and process parameters during formulation of polymer nanoparticles of methotrexate

Run	Amount of Polymer	Type of Surfactant	Stirring Speed	Conc. of Surfactant (%)	Stirring Time	Volume of Aqueous Phase	Sonication Time
1	+1	-1	-1	+1	-1	-1	+1
2	-1	-1	+1	+1	+1	-1	-1
3	-1	+1	-1	-1	-1	-1	-1
4	-1	-1	+1	-1	-1	+1	+1
5	+1	-1	-1	-1	+1	+1	-1
6	-1	+1	-1	+1	+1	+1	+1
7	+1	+1	+1	-1	+1	-1	+1
8	+1	+1	+1	+1	-1	+1	-1
					Level of Factor studies		
Selected Parameter					Low (-1)	High (+1)	
Amount of Polymer (mg)					50	100	
Type of Surfactant (T/P)					Tween 80 (T)	Pluronic F-68 (P)	
Stirring speed (rpm)					500	1000	
Surfactant (%)					0.25	0.5	
Stirring time (min)					120	180	
Volume of aqueous phase (mL)					20	40	
Sonication time (sec)					30	60	

Table 2: Design matrix as per the CCD for the optimization of polymeric nanoparticles of MTX

Run	Coded factor levels		
	Factor 1 (X_1)	Factor 2 (X_2)	
1	+1	0	
2	-1	-1	
3	-1	+1	
4	+1	-1	
5	+1	+1	
6	0	0	
7	0	0	
8	0	-1	
9	0	0	
10	0	+1	
11	0	0	
12	0	0	
13	-1	0	
Selected parameters	Decoded factor levels		
	Low (-1)	Intermediate (0)	High (+1)
Amount of polymer (mg)	50	75	100
Surfactant (%)	0.20	0.35	0.50

Table 3: Coefficients of polynomial equation as per the second-order modified quadratic model for each of the critical quality attributes

Coefficient code	Polynomial coefficients for critical analytical attributes			
	Particle Size	Zeta Potential	EE (%)	Release (%)
β_0	+153.73	+14.58	+49.71	+33.72
β_1	+44.32	+2.62	+5.71	-0.67
β_2	-16.18	-1.45	-1.78	+5.90
β_3	-17.95	-0.4000	-6.02	+1.14
β_4	+51.65	+0.5224	-6.33	+5.07
β_5	+8.75	+1.12	+1.98	-5.35
P-value	<0.0001	<0.001	<0.010	< 0.005
R	0.9794	0.9485	0.9032	0.9109

Table 4: Results of fitting various mathematical modelling approaches to drug release kinetic data of MTX and its optimized zein nanoparticulate formulation

Pure MTX			
Mathematical Model	Correlation coefficient	Model coefficient	Statistical significance [#]
First-order	0.8485	0.086	< 0.05
Zero-order	0.7107	4.9477	< 0.05
Higuchi	0.8562	21.771	< 0.05
Weibull	0.9320	$\alpha = 0.876$ $\beta = 0.444$ $T_i = 0.00$	< 0.05
Hixson-Crowell Cube Root	0.8117	0.1832	< 0.05
Baker-Lonsdale	0.8450	0.042	< 0.05
Korsmeyer-Peppas	0.9560	0.1818	< 0.001
Zein-NPs of MTX			
Mathematical Model	Correlation coefficient	Model coefficient	Statistical significance [#]
First-order	0.9145	0.0335	< 0.05
Zero-order	0.8793	3.5539	NS
Higuchi	0.8793	3.5539	NS
Weibull	0.9579	$\alpha = 4.854$ $\beta = 0.723$ $T_i = 0.00$	< 0.05
Hixson-Crowell Cube Root	0.9242	0.0903	< 0.05
Baker-Lonsdale	0.9840	0.010	< 0.001
Korsmeyer-Peppas	0.9823	0.5681	< 0.001

[#]p computed on the basis of significance of regression; NS: not significant at 5% level (i.e., $p > 0.05$)

Figures

Figure 1

An Ishikawa-fish bone diagram depicting various factors affecting the formulation development process of zein nanoparticles

CAAs	Amount of polymer	Type of surfactant	Surfctant (%)	Sonication time	Stirring type	Stirring speed	Stirring time	Volume of aqueous phase	Method of preparation
Particle size	High	Medium	High	High	Low	High	High	Medium	Medium
Zeta potential	High	Low	Medium	Low	Low	Medium	Medium	Medium	Low
EE (%)	High	Medium	High	Medium	Low	High	Medium	Medium	Low

High-risk parameter

Medium risk parameter

Low-risk parameter

Figure 2

A risk estimation matrix (REM) for evaluating risk levels of various input parameters for zein nanoparticles

Figure 3

Pareto charts (a, c and e) and half-normal plots (b, d and f) depicting the influential variables during the screening study of zein-NPs of MTX

Figure 4

3D-response surfaces and 2D- contour plots for different CQAs, viz., particle size (a-b), zeta potential (c-d), % EE (e-f), and cumulative % drug release (g-h)

Figure 5

An overlay plot depicting the design space of the optimized formulation

Figure 6

Photo-images of (a) particle size (b) zeta potential of the optimized zein nanoparticles

Figure 7

TEM and FESEM images of optimized zein nanoparticles

Figure 8

Cumulative release profiles of MTX and zein nanoparticles in phosphate buffer pH 7.4

Figure 9

In vitro cell cytotoxicity profiles of MTX (●) and zein nanoparticles (◆), performed on MCF-7 cells lines. Data are expressed as Mean ± SD; n=3

Figure 10
Apoptotic changes in plasma membrane of MCF-7 cells with concurrent staining using Annexin V-FITC/propidium iodide; (a) Gating of cells, (b) reference control cells, (c) cells treated with MTX, and (d) cells treated with zein nanoparticles

Figure 11
Cellular uptake study using Coumarin-6 (a) Gating of cells on FSC/SSC plot, (b) fluorescence intensity of coumarin-6 treated cells, (c) fluorescence intensity in coumarin-6 loaded nanoparticles treated cells, (d) an overlay plot of (b) and (c)

Figure 12
Qualitative cellular uptake of Coumarin-6 loaded zein nanoparticles (C6-zein-NPs) using CLSM (a) TD image of MCF-7 cells representing normal cells, (b) Cells treated with coumarin-6 (c) normal MCF-7 cells (d) Overlay images (b) and (c) showing localization of C6-zein-NPs in Caco-2 cells

Figure 13

Plasma level profiles of MTX (●) and zein nanoparticles (■) at different time-points observed in rats. Data are expressed in Mean $\pm$ 1 SEM (n=3). The corresponding inset depicts percent change in different pharmacokinetic parameters

Figure 14

Establishment of point-to-point Level A correlations between the values of in vitro percent drug released and of in vivo percent drug absorbed; (a) MTX, and (b) zein nanoparticles of MTX

Supplementary Files

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