RESEARCH ARTICLE

Doxorubicin Hydrochloride-loaded Nanoparticles for Oral Delivery: Optimization using Design of Experiments

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ABSTRACT

Doxorubicin hydrochloride (DOX) has low oral bioavailability due to the presence of active efflux from intestinal P-glycoprotein receptors. Because of the difficulties associated with the oral administration of DOX, there is not yet a commercially available oral formulation of DOX. Nanocarrier system was manufactured utilizing poly (lactic-co-glycolic acid) (PLGA) and treated with chitosan to provide a surface coated. Nanoparticles (NPs) were created using a modified emulsification solvent diffusion (nanoprecipitation) method by electrostatic conjugation of chitosan to modify nanoparticle surfaces. The model was built using a Box-Behnken design with three independent components: X1 (PLGA), X2 (poloxamer 188), and X3 (chitosan concentration). The optimized chitosan-PLGA NPs had a mean particle size of 153.6 nm and a positive zeta potential of 21.51 mV. More than 85% DOX permeated through the everted gut by DOX-loaded chitosan-NPs as compared to NPs prepared with PLGA alone. Chitosan nanoparticles caused a 6-fold increase in DOX intestinal permeability. The findings pointed to the possibility of using chitosan-based nanoparticles of doxorubicin for oral treatment for cancer.

Keywords: Chitosan, Design of Experiments, Doxorubicin, Nanoparticles, Oral delivery, PLGA.

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INTRODUCTION

The administration of cancer medicines through the digestive system is an attractive option. The chemical, physical, and biological barriers in the gastrointestinal tract (GIT) limit the bioavailability of substances.[1] This decreases the likelihood that the medication will be effective in treating a particular disease. Recent nanomedicine and material science developments provided several delivery platforms to overcome multiple hurdles associated with oral administration. These platforms have been generated in recent decades. Because to the existence of active efflux from intestinal P-glycoprotein receptors, and doxorubicin hydrochloride (DOX-HCl) have poor oral bioavailability.[2] DOX is a member of the anthracycline glycoside class of drugs. DOX is the anticancer medication of choice for use during the first phase of treatment for a wide variety of malignancies. Researchers have been motivated to develop novel and cutting-edge strategies for entrapping this medication in various nanocarriers due to the nephrotoxicity and cardiotoxicity associated with doxorubicin. This medication is used to treat a number of different cancers.[3] It is possible that oral chemotherapy will allow the cancer cells to be exposed to lower concentrations of anticancer medications. This release over a longer period allows for better efficacy and less adverse effects. In addition, oral chemotherapy can give cancer cells a longer, more continuous exposure to anticancer medicines at a relatively lower concentration and, therefore, safer.

Additionally, oral chemotherapy can give the cancer cells a longer period of exposure to a comparatively lower concentration of anticancer drugs, which is a safer concentration due to the longer exposure period. Patients choose oral chemotherapy not only because it has the potential to be more successful and have a lower level of toxicity but also because it is cost-effective, flexible in its dose schedule, and easy. People undergoing cancer treatment may experience an improvement in their "quality of life" due to the development of oral chemotherapy, which has made the possibility of "chemotherapy at home" possible. This might improve the

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patient's "quality of life" and promote compliance. Due to DOX's extremely limited availability *via* GIT, oral medication administration is challenging. Because DOX has a pKa of 8.3, which places it in the category of a weak amphipathic base, it will be necessary to load it into the aqueous inner part of a delivery system. This will be the case in order to ensure that the gradients can be achieved.

Molecularly tailored therapies are needed to improve traditional chemotherapeutic drugs' efficacy and toxicity.[4] Drug-loaded nanoparticles (NPs), and controlled nanosystems can manipulate drug release allowing for focused therapy when the microenvironment is changed.[5]

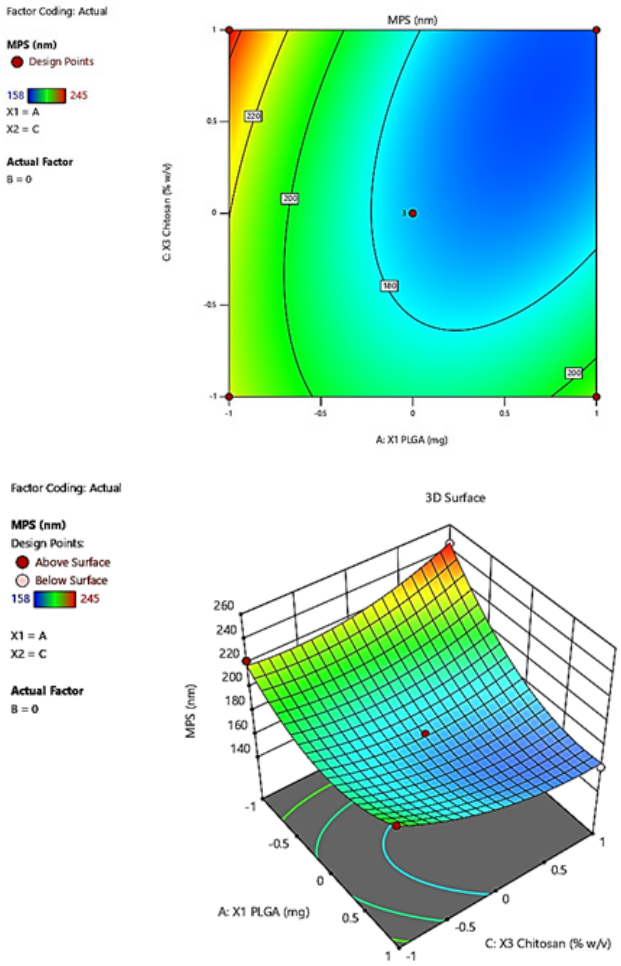

Figure 1: Contour plot (above) and 3D RSM plot (below) for BB Designed output of MPS

NPs are biocompatible and soft due to their high-water content. Natural polymer-based NPs are employed to deliver bioactive compounds. Due to its cationic nature and capacity to sustain drug release, the biodegradable, biocompatible, and non-toxic polymer chitosan is frequently used in drug delivery systems.[6] NPs made from chitosan are pH- and temperature-sensitive.

NPs are becoming increasingly popular as a targeted drug delivery system due to their enhanced bioactivity and successful therapy. As a result, the nanoparticles reduce the systemic toxicity associated with oral cancer treatment. Cancer cells can be killed by loading chemotherapeutic drugs onto these carriers, which are typically formed of polymeric and inorganic nanoparticles, stabilizing them during transport and releasing them at the appropriate times.

Regarding the use of PLGA based NPs, previously reported studies have shown an increased bioavailability for docetaxel.[7]

Due to intestinal P-glycoprotein receptor activity, causing efflux of DOX-HCl has a limited oral bioavailability. There is currently no oral formulation for DOX on the market since oral administration of DOX is still difficult. In this study, an attempt is being made to use cationic nanoparticles to have the possibility of oral delivery of NPs.

MATERIALS AND METHODS

Materials

DOX was given by RPG life-sciences Pvt. Ltd., Mumbai, India. We bought chitosan from the CIFT, Cochin, India, which has a 100K molecular weight and 79% deacetylation. Glacial acetic acid, methanol, acetone, ethyl acetate, and glutaraldehyde were supplied by S. D. Fine-Chem Ltd., Mumbai, India, while Poloxamer 188 was acquired from BASF.

Preparation of DOX-loaded PLGA Nanoparticles

A modified emulsification solvent diffusion (nanoprecipitation) approach was used to produce NPs.[8] The NPs were made by adding 10 mg of DOX to 3 mL of acetone. To this mixture was added 50 mg of PLGA (dissolved in 10 mL of ethyl acetate). This organic phase was continuously stirred at room temperature using a magnetic stirrer as the 0.75% w/v Pluronic F68-containing aqueous phase was introduced and then

Table 1: Box Behnken Design: Factors, Levels and Transformed Values of Factors

Factor	*-1 (Lower Level)*	*0 (Middle Level)*	*+1 (High Level)*
X1: PLGA (mg)	50	75	100
X2: Poloxamer 188 (% w/v)	1	1.5	2.0
X3: Chitosan (% w/v)	0.75	1.0	1.25

Table 2: Formulation of DOX loaded chitosan-PLGA Nanoparticle surface modified with poloxamer

Formulation	*Particle Size (d.nm)*	*Polydispersity index (PDI)*	*Zeta Potential (mV)*
DOX-PLGA-CH NPs	160.21	0.315 ± 0.017	23.34
Chitosan-PLGA-NPs	153.6 ± 8.0	0.345 ± 0.032	21.51
Blank PLGA-NPs	130.7 ± 9.7	0.202 ± 0.093	−38.29

Table 3: Output of BB Design for the Critical Quality Attribute of MPS

Batch No.	*X1*	*X2*	*X3*	*MPS (nm)*	*MPS ± SD*
DPCN1	-1	-1	0	206	2.3
DPCN2	-1	0	-1	223	4.6
DPCN3	-1	0	1	245	2.8
DPCN4	-1	1	0	241	1.9
DPCN5	0	-1	-1	215	4.5
DPCN6	0	-1	1	171	2.7
DPCN7	0	0	0	173	4.7
DPCN8	0	0	0	176	5.5
DPCN9	0	0	0	172	3.7
DPCN10	0	1	-1	170	4.3
DPCN11	0	1	1	206	2.9
DPCN12	1	-1	0	205	2.5
DPCN13	1	0	-1	210	6.1
DPCN14	1	0	1	162	4.4
DPCN15	1	1	0	158	4.8

DPCN: DOX-PLGA-Chitosan NP

Table 4A: Regression Statistics

Multiple R	*0.997027*
R Square	0.994062
Adjusted R Square	0.983374
Standard Error	3.678768
Observations	15

Table 4B: ANOVA of the BB Design

	SS	*df*	*MS*	*F*	*Significance F*
Regression	11328.07	9	1258.674	93.00547345	0.00005
Residual	67.66667	5	13.53333		
Total	11395.73	14			

Table 4C: Output of Regression Equation.

	Coefficients	*Standard Error*	*Stat for t test*	*p-value*
Intercept	173.66	2.12	81.766	0.005
X1	-22.5	1.30	-17.299	0.001
X2	-2.75	1.30	-2.114	0.088
X3	-4.25	1.30	-3.267	0.022
X1X2	-20.5	1.84	-11.145	0.001
X1X3	-17.5	1.84	-9.514	0.002
X2X3	20	1.84	10.873	0.001
$X1^2$	24.16	1.92	12.623	0.005
$X2^2$	4.67	1.92	2.4375	0.058
$X\ 3^2$	12.17	1.92	6.3550	0.001

homogenized for 5 minutes at 15,000 rpm on Ultra Turrax. The organic solvent was allowed to evaporate completely before stirring stopped.

Nanoparticles Surface Modification with Chitosan

Acetic acid was used to prepare a 1% w/v chitosan solution and added to 5 mL of nanoparticle solution,[9] and then the

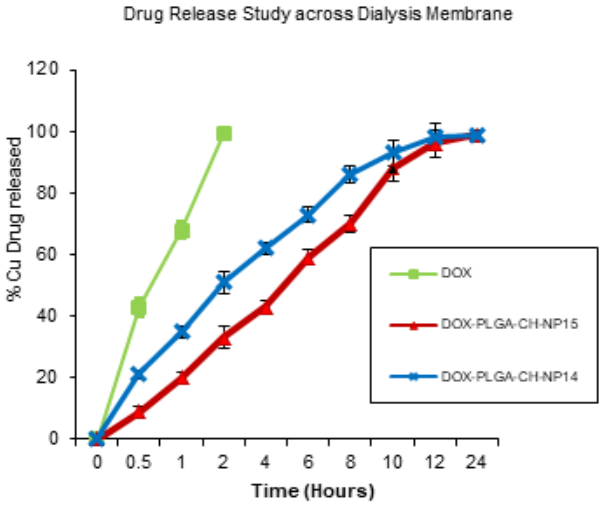

Figure 3: Drug release study at pH 5.0.

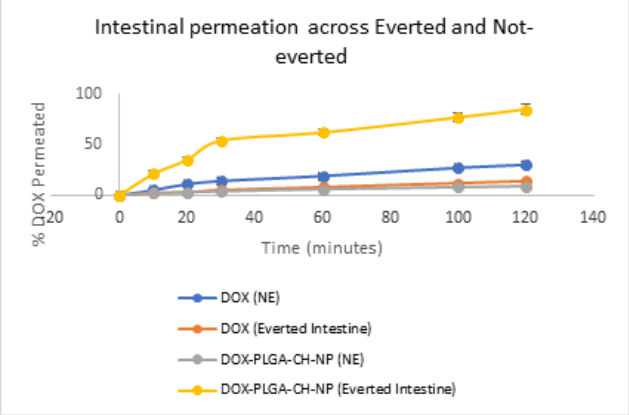

Figure 4: Intestinal permeation of Plain DOX and DOX NPs across Everted and Not- everted (NE) Chick Intestine.

Table 5. Residual Output

Observation	*Predicted Y*	*Residuals*
206	207.25	-1.25
223	219.25	3.75
245	245.75	-0.75
241	242.75	-1.75
215	217.5	-2.5
171	169	2
173	173.6667	-0.66667
176	173.6667	2.333333
172	173.6667	-1.66667
170	172	-2
206	203.5	2.5
205	203.25	1.75
210	209.25	0.75
162	165.75	-3.75
158	156.75	1.25

Table 6: Drug release kinetics

Release Kinetic Parameter	*DOX-PLGA-CH-NP15*	*DOX-PLGA-CH-NP14*
Regression coefficient value for Zero order	0.994	0.973
Regression coefficient value for First order	0.852	0.898
Regression coefficient value for Higuchi fit	0.988	0.966
Regression coefficient value for Korsemeyer fit	0.998	0.955
Korsemeyer n value	0.52	0.82

mixture was stirred at 400 rpm (Moderate speed to avoid collisions) at room temperature for a period of 2 hours. This process promoted surface modification of NPs with chitosan through electrostatic conjugation.[10] The unconjugated chitosan was separated using centrifugation as the separation method.

Box-behnken Design to Optimize Mean Particle Size of NPs

The design of experiments (DoE) as an optimization tool was used to produce the larger outcome from the smallest input in the allotted time.[11,12] The independent and dependent variables are understood and provided with optimum correlations in such experimental designs using the response surface methodology (RSM). Three independent parameters were considered at three levels and fitted into Box-Behnken (BB) design. The three levels used were high, middle and lower with the designation + 1, 0 and -1, respectively.[13] Three center point duplicates were used, resulting in a total of 15 runs. Table 1 shows the three factors taken as X1 (concentration of PLGA), X2 (concentration of Pluronic F68) and, X3 (concentration of Chitosan), and the transformed values of these factors (Figure 1).

Equation 1 was created using the Design Expert software and reflects the polynomial equation (Design Expert ® v10 (DX13), USA).

$Y=\beta_O+\beta_1X1+\beta_2X2+\beta_3X3+\beta_{12}X1X2++\beta_{13}X1X3+\beta_{23}X2X3+\beta_{11}X12+\beta_{22}X22+\beta_{33}X32$ [Eq.1]

Where Y stands for the response, X for the three elements that make up the independent variable, and stands for the model coefficients.

Mean Particle Size and Surface Charge

The Malvern Zetasizer 3000 was utilized to investigate the particle size and zeta potential of nanoparticles suspended in distilled water (Malvern Instruments, UK). The particle size was determined using the photon correlation spectroscopy technique, and the zeta potential was investigated using electrophoretic light scattering.

In-vitro Release of DOX from Nanoparticles

NPs equivalent to 100 mg of DOX were suspended in PBS, kept in a dialysis bag, and submerged in a 100 mL media. The multimedia drug release using saline phosphate buffer of pH 7.4 and acetate buffer of pH 5.0 was performed at 485 nm in UV-vis spectrophotometer. DOX concentration was monitored at predefined intervals. Data obtained from drug release was analyzed using kinetics models

Intestinal Permeation Studies by Intestinal Sac Model

The everted intestinal sac was used to investigate and compare the permeation of free DOX and DOX-loaded chitosan NPs. Standard techniques were used to prepare everted and normal intestinal sacs using freshly excised chick intestine.[14] The amount of DOX permeated across the intestine (receptor compartment pH 6.8) was evaluated spectrophotometrically.

RESULTS AND DISCUSSION

Preparation of DOX-loaded PLGA NPs

With the help of emulsification solvent diffusion methods blank PLGA NPs were successfully prepared. The results of preliminary lab trials of chitosan-PLGA-NPs and DOX-loaded chitosan-PLGA-NPs is shown in Table 2.

Increasing the concentration of chitosan was responsible for increasing the mean particle size because the polymer was deposited as a coating material on the nanoparticles. This deposition was also evident by a change in surface charge form a negative to positive. PLGA-based NPs have a net zeta potential of −38.29 mV and size 130.7 ± 9.7 nm. This was changed in chitosan-PLGA NPs to a positive zeta of 21.51 mV and an increase in size to 153.6 ± 8.0 nm. (Table 3)

The polydispersity index was low for all three lab trials within 0.202 to 0.345, indicating uniformity of prepared NPs by the method used in the preparation of NPs.

Optimization of MPS by BB Design

ANOVA scrutinizes the regression model to produce the best results and employs the F-tests to statistically test the equality of the means. To determine the overall importance of model's conclusions, *p-value* is added to the data from the F statistics.

The fact that the model has an f-*value* of 93.01 indicates that it is significant. A noise level of this magnitude would only have a 0.01% chance of producing an *f*-value of this magnitude. The prepared 15 batches of NPs using the BB design had MPS in the range of 158 and 245 nm (Table 3).

The numerical digit known as R^2 gives information about how well the statistical data fits the regression line. This information can be found by looking at the R^2. A high R^2 value of 0.9940 is indicative of the significance of the model (Table 4B)

The significance of the model can be determined by observing the *p-value* which is less than 0.05. In this case X1, X3, X1X2, X1X3, X2X3, $X1^2$ and $X3^2$ are significant model terms (Table 4C). This clearly eliminated the role of both the linear impact and quadratic impact of poloxamer concentration on mean particle size.

The low residual values (between 3.75) in Table 5 demonstrate agreement between the projected Y values of MPS and the observed values obtained during the investigation.

The 4.54 *f-value* for the lack of fit is not significant when compared to the pure error. 18.58% of the significant *f*-values from lack of fit are attributable to noise. A significant model fit is what we aim for Less than 0.2 separates the expected R^2 from the amended R^2. The SNR is measured with reasonable accuracy, with values greater than 4 being preferred. The value obtained is 29.630 and is a good ratio. This model is able to guide the design well.

The final regression equation for Y1 (MPS) in terms of actual factors is represented in Eq.2:

$$Y1(MPS)=173.7-22.5X1-2.75X2-4.25X3-20.5X1X2-17.5X1X3+20X2X3+24.2\ X12+4.7X22+12.2X32 \quad [Eq.2]$$

The correlations between the three numerical variables are graphically represented by response surface plots (Figure 1) in three dimensions and contour plots in two dimensions.

The suggested coded values by software predicting the desirability solution was X1 to be kept at 0.89, X2 at 0.97 and X3 at 0.16. In the response surface plot the desirable area is blue-colored and using the desirable values, an MPS of 157 nm can be obtained.

The present study's perturbation plot reflects no effect of factor B (X2: concentration of poloxamer) on MPS indicated by the near straight line. In contrast, both factor X1 and X2 have significant effect on MPS.

Drug Release Studies

The plain drug being hydrophilic, was completely released within 2 hours, whereas NPs were able to sustain it for 24 hours at pH 5.0 (Figure 2).

The two optimized formulations DOX-PLGA-CH-NP15 and DOX-PLGA-CH-NP14 were selected for studying the drug release studies. It was seen that both these formulations could sustain the drug release till 24 hours. Batch DOX-PLGA-CH-NP14 showed a bit faster drug release in comparison. In terms of kinetics (Table 6) DOX-PLGA-CH-NP15 reflected zero order kinetics and the diffusion kinetics was fickian (n value 0.5).

Intestinal Permeation Studies by Intestinal Sac Model

Several theories have been put out to explain how orally delivered nanocarriers are absorbed. While regular enterocytes may be able to endocytosize NPs smaller than 220 nm.[15] The hydrophobicity of nanocarriers is said to substantially impact drug release. Due to their extreme hydrophobicity, NPs might be more readily absorbed by Peyer's patches. Cancer treatment places a considerable deal of emphasis on directing orally delivered nanocarriers to the lymphatics because tumor cells typically spread through the lymphatic system to secondary sites or organs.

The NPs transported DOX much better than free DOX (approximately 6.07 folds). Only 14% of DOX was absorbed when DOX solution was used (Figure 3). The drug has a log P=1.3 for intestinal diffusion but the disparity in the results could be due to P-glycoprotein pumps (P-gp pumps) in enterocyte luminal parts. DOX can migrate from intestinal serosa to mucosa.

Permeation of DOX-loaded Cs-NPs was 85 % as it could have bypassed the P-gp efflux pumps in everted gut (Figure 4). DOX intestinal permeability increased 6-fold with chitosan NPs. When DOX is encapsulated in chitosan NPs, it is inaccessible to P-gp pumps and can pass the intestinal wall. This considerable increase in intestinal permeability may be due to chitosan's effect on tight junctions and paracellular DOX transport. Otherwise, in earlier studies, the NPs prevented simple drug diffusion.

Nanocarriers of DOX that are delivered orally may offer important clinical benefits. Several ideas have been put forward to explain how nanocarriers that are taken by mouth are taken in.

Normal enterocytes could endocytose NPs smaller than 220 nm, and 100–200 nm polyalkylcyanoacrylate nanocapsules filled with iodinated oil could move from one cell to another outside of the cell.[2]

Chitosan polymer has been proven in the past in the production of DOX-HCl NPs and to be effectively responsible for improving the intestinal absorption of DOX throughout the whole of the small intestine.[15]

CONCLUSION

It was determined that the developed chitosan-poloxamer NPs of DOX had the ability to decrease and sustain drug release for up to 24 hours. The NPs demonstrated pH-dependent release and zero-order diffusion by Fickian mechanism. These pH-sensitive NPs would surely help in the delivery of therapeutic agents by oral route for cancer treatment.

The intestinal penetration of DOX was approximately brought up to 85% by the utilization of the chitosan NPs. Further *in-vivo* studies will be needed to confirm the intestinal absorption of DOX. However, a promising platform opened up by these chitosan NPs of DOX in oral delivery for poorly absorbable drugs.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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