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Research Article

FORMULATION OPTIMIZATION AND CHARACTERIZATION OF GANCICLOVIR LOADED DRY CHITOSAN NANOPARTICLES

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ABSTRACT

Objective: The objective of this work was to formulate, optimize, and characterize ganciclovir (GCV) loaded dry chitosan nanoparticles (CSNPs).

Methods: The GCV loaded CSNPs was prepared by ionic gelation method. Box–Behnken design was employed to optimize the influence of independent process and formulation variables like drug to polymer ratio, concentration of sodium tripolyphosphate, and stirring time (min) on the dependent variables such as particle size (PS) and drug encapsulation efficiency (% EE). The optimum conditions were determined by regression analysis of the output data.

Results: The independent variables had interactive effects and they affected both the responses. The optimum formulation had PS within the range of 100-120 nm and % EE between 85% and 86%. The prepared GCV loaded CSNPs were dried by fluidized bed drying method. Fourier transform infrared spectra showed there was no physicochemical interaction between GCV and CS. Powder X-ray diffraction study showed less intense crystalline peaks indicated that GCV may exist in the formulation as amorphous nanodispersion or molecular dispersion form. Differential scanning calorimetry study was performed which indicated that the drug was molecularly dispersed inside the matrix of CS. Higuchi model was the best to fit the *in vitro* release data for the GCV loaded CSNPs.

Conclusion: From the results, it can be concluded that the GCV loaded dry CSNPs were formulated, optimized, and characterized using desired pharmacotechnical properties.

Keywords: Chitosan nanoparticles, Box–Behnken design, Sodium tripolyphosphate, Ionic gelation.

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INTRODUCTION

Polymeric nanoparticles (NPs) have been studied as promising drug delivery systems. Different NPs have been formulated and characterized with the use of natural as well as synthetic polymers. Based on the physicochemical and biological properties, chitosan (CS) a natural polymer attracts the researchers to work with Hamidi *et al.* [1].

CS has been proved to have many other intrinsic properties, such as low toxicity (lethal dose 50% in mice is 16 g/kg body weight) [2], biocompatibility and biodegradability (Cordova *et al.*, 2008). It is mucoadhesive polymer and can increase the residence time at the site of absorption and has favorable controlled drug-release abilities [3]. It has been demonstrated that CS (when protonated) affects cell permeability and enhances paracellular permeability of drugs across the mucosal epithelia by opening the intercellular tight junctions.

Ganciclovir (GCV) 9-(1,3-dihydroxy-2-propoxymethyl) guanine, is a Biopharmaceutics Classification System Class III drug. GCV (log p = −1.7) having molecular weight 255.2 g/mol. It is the first antiviral drug that proved to be efficacious in the treatment of *Cytomegalovirus* (CMV) disease in humans [4]. GCV is also used for maintenance therapy and prophylaxis of CMV. Oral bioavailability of GCV is ~5%. Possible reasons for the poor bioavailability are poor permeability through the gastrointestinal tract (GIT) and P-glycoprotein substrate activity [5]. Therefore, it is necessary to develop a drug delivery system for GCV which can enhance the absorption of GCV. Different drug delivery systems for GCV have been developed these days, such as GCV loaded albumin NPs [6], solid lipid NPs [7], Long-circulating liposome-encapsulated GCV [8].

This study focus on development of GCV loaded dry CSNPs with an ionic gelation method. The 2^{5-2} fractional factorial design was employed to screen the process and formulation parameters. Response surface methodology with the utilization of Box–Behnken experimental design (BBD) was employed for optimization of the particle size (PS) and entrapment efficiency (% EE) of GCV loaded dry CSNPs, as they are properties of great influence on the pharmacokinetic (i.e., biodistribution) and the pharmacodynamic (i.e., therapeutic efficacy) of the drug loaded NPs [9]. The optimized formulation was investigated for physicochemical characterizations such as scanning electron microscopy (SEM), Fourier transform infrared spectroscopy (FTIR), and differential scanning calorimetry (DSC) study were employed for detection of particle morphology and any physicochemical interaction with the CS. *In vitro* release profile was investigated.

MATERIALS AND METHODS

Materials

GCV was kindly gifted by Bakul Fine Chem Research Center, Mumbai. CS (molecular weight=110 kDa, 80.0% degree of deacetylation) was gratis sample from Chitopharm S, Norway (USA). Sodium tripolyphosphate (TPP) (Cross-linking agent) was purchased from Sigma-Aldrich, Mumbai (India). The water used was pretreated with the Milli-Q plus system (Millipore, Q-5 UVS, India). All other materials of analytical grade were used.

Methods

Preparation of GCV loaded CSNPs

The GCV loaded CSNPs were prepared by ionic gelation method [10]. The possible mechanism for ionic gelation is that the ionic interaction

between positively charged CS with negatively charged sodium TPP [11]. Briefly, CS solutions were prepared by dissolving CS into Milli-Q® water at different concentrations containing glacial acetic acid (2%). GCV was dissolved in Milli-Q® water containing different concentrations of TPP. NPs were formed by adding TPP solution drop wise onto a CS solution under mechanical stirring (Remi, India) at room temperature. Thereafter, the CS-TPP particle suspension was processed under ultrasonication by Probe sonicator (VCX-500, Vibra cell, USA) for 5 minutes, producing CSNPs with controlled PS. The prepared GCV loaded CSNPs were dried by fluidized bed drying (FBD) method [12]. The GCV loaded CSNPs dispersion was centrifuged at 10,000 rpm for 30 minutes. The sediment was mixed with microcrystalline cellulose and Aeroperl R 300 in the ratio of 3:1. The prepared wet mass was subjected to FBD at 60-70°C for 4-6 minutes. The dried particles were allowed to pass from sieve 40# and further characterized.

Experimental design

From the prior experience and literature survey, the process and formulation parameters were identified and screened through 2^{5-2} fractional factorial design. Eight batches were prepared and evaluated for PS, % EE and morphology. Drug:polymer ratio (w/w), concentration of TPP (%w/v), stirring time (min), sonication time (min), and stirring speed (rpm) were taken as the independent variables while PS (nm), EE (%), and morphology were taken as dependent variables for 2^{5-2} fractional factorial design.

A BBD was used with, three-factors, three-level, and fifteen-runs to optimize the preparation conditions. The polynomial equations relating factors and responses were obtained by design of experiment software® (9.0.6). Table 1 shows the three variables (Drug:polymer ratio, concentration of TPP, and stirring time) were represented by A, B, and C, respectively. The responses (PS and %EE) were represented by Y_1 and Y_2, respectively [13]. A number of check-point experiments were carried out in the optimal area of the BBD to verify the optimization procedure validation, as predicted by desirability analysis and by comparing the experimental and predicted response.

Characterization

PS determination

The size of the particles was determined by dynamic laser scattering technique (Malvern nano S90, Malvern Instruments, UK) PS analyzer at temperature of 25°C with an angle of 900.

Entrapment efficiency (% EE)

% EE of the drug was determined by high-performance liquid chromatography (HPLC) (LC-2010C HT, Shimadzu, Japan). Formulations were centrifuged at 10,000 RPM for 30 minutes. Supernant was collected and analyzed for drug content at 254 nm by HPLC (LC-2010C HT, Shimadzu, Japan) [14]. The % EE was calculated as follows:

$$\%EE=(Sa-Sb)/Sa*100 \quad (1)$$

Where, Sa is the total amount of drug in system, Sb is the amount of drug in supernant after centrifugation.

Table 1: Variables with their coded and actual values for BBD

Independent variables	Low	Medium	High
Coded values	(−1)	(0)	(1)
A=Drug: Polymer ratio (w:w)	1:2	1:3	1:4
B=Concentration of TPP (%w/v)	0.3	0.4	0.5
C=Stirring time (min)	50	100	150
Dependent variables constrains			
Y_1=Particle size (nm) 100-200 nm			
Y_2=EE (%) Maximum			

EE: Entrapment efficiency, BBD: Box–Behnken experimental design

Morphology observation

SEM (JSM 6010 LA, JEOL, USA) was used to access the morphology/shape of the dried CSNPs. A sample of dried CSNPs was placed on a double stick tape over aluminum stubs to get a uniform layer of particles. Sample was platinum coated for 20 seconds. Then the sample was observed by SEM at 10 kV [15].

FTIR spectroscopy

FTIR spectrum was recorded using NICOLET-6700 (Thermo Scientific, USA) to confirm the cross-linking reaction between the phosphoric group of the TPP and amino group of CS. The pellet was prepared by homogenously dried formulation in dried potassium bromide in a mortar and pestle and then powder was compressed under vacuum using round flat face punch to produce pellet compact. The sample was placed in IR light path and spectra were scanned over the wavelength number range of 4000-400 cm^{-1} [16].

DSC study

DSC study was performed by DSC (DSC-60, Shimadzu Corporation, Japan) to characterize the physical state of GCV in CSNPs [17]. The heat flow as a function of temperature was measured over a temperature range of 50-400°C at heating rate of 10°C/min [18].

In vitro drug release study

In vitro drug release study of GCV loaded CSNPs dispersion, marketed formulation (Natclovir: 250 mg Capsule) and drug solution was carried out in diffusion cell apparatus (J-FDC-07, Orchid Scientifics and Innovations India Pvt., Ltd.) in phosphate buffer pH 6.8. At predetermined time intervals, the samples were withdrawn and replenish with fresh medium and the absorbance was measured by HPLC at 254 nm. Data obtained from the *in vitro* drug release for formulation were fitted to various kinetic models. Each experiment was performed in triplicate. The drug release mechanism and linearization were determined by finding the goodness of fit (R^2) and sum squared of residuals (SSR) for each kinetic model [19].

RESULTS AND DISCUSSIONS

The 2^{5-2} fractional factorial design revealed that the drug to polymer ratio, concentration of TPP, and stirring time was affected more significantly on the PS and % EE. The PS was found to be in the range of 112.6±1.1 to 364.9±4.759 nm and % EE in the range of 10±5.4 to 83.57±1.4% with spherical shape.

Model fitting by BBD

The screening design suggested that drug to polymer ratio, concentration of TPP, and stirring time were most significant factors. These significant factors were further optimized through the BBD. The measured significant variables range between 121.20±2.7 and 294.5±15.4 nm for PS and between 59.95±0.5 and 85.15±1.1 for % EE. These indicated that PS and % EE of the prepared CSNPs were largely influenced by the selected variables. The polynomial equations for both responses were as follows:

$$Y_1=+121.53-17.54A\ (p<0.05)-0.13B\ (p<0.05)-4.39C\ (p>0.05)-11.79\ AB\ (p<0.05)\ -9AC\ (p<0.05)+19.93BC\ (p<0.05)+86.16A2\ (p>0.05)+40.18B2\ (p>0.05)\ -66.06C2\ (p>0.05) \quad (2)$$

$$Y_2=+84.02-0.47A\ (p<0.05)+2.55B\ (p<0.05)\ -0.53\ C\ (p<0.05)\ +3.58AB\ (p<0.05)-\ 3.23AC\ (p<0.05)-2.78BC\ (p>0.05)-12.11A2\ (p>0.05)-5.98B2\ (p>0.05)+0.085C2\ (p>0.05) \quad (3)$$

Where, Y_1 is the PS, Y_2 is the % EE. A, B, and C is drug:polymer ratio, concentration of TPP and stirring time, respectively. AB, AC, and BC are the interaction terms which show how the responses change when two variables are concurrently changed, while the effect of changes in each single variable on the response is reflected by A, B, and C the main effect terms and A^2, B^2, and C^2 the quadratic terms. Table 2 presents the results of which the analysis of variance (ANOVA) was conducted to test the

quadratic models significance and the lack of fit for the experimental data.

Equations (2) and (3) show the effect of the independent variables on the PS and % EE. As A, B, and C the three independent variables changed simultaneously, the coefficient of interaction of Y_1 and Y_2 also changed. There is increase in the PS due to increase in the concentration of TPP would be due to cross-linkage between TPP and CS. As there is increase in the concentration of TPP, there would be more tripolyphosphoric ions to cross-link with amino groups on CS chains. As stirring time increases, there is increase in the PS. This may be due to aggregation of particles [20].

There is decrease in the % EE with increase in the drug:polymer ratio. This may be due to the fact that at higher concentration CS molecules in the dispersion are present very close to each other during precipitation by polyanion. Hence, they got precipitated to form a large particle and this larger particle is having low entrapment of drug [20].

To check the significance of the quadratic models and the lack of fit for the experimental data the ANOVA was conducted (Table 2). Fischer's ratio (F) confirms the models significances, where FY_1=9.71 (p=0.011<0.05) and FY_2=5.93 (p=0.032<0.05). The value of correlation coefficient (R^2) for equation was found to be R_1^2=0.961 and R_2^2=0.934, indicating a good fit.

The Student's t-test was applied to determine the significant difference between actual and predicted value of dependent parameters. The p-value for the PS and % EE was found to be 0.372 and 0.468, respectively, which showed that at 5% level of significance, there is no significant difference between actual and predicted values of PS and % EE.

Fig. 1 shows the three-dimensional response surface plots of independent variables interactive effects on the PS and % EE. Optimum conditions for preparation of CSNPs can be achieved for different values of independent variables within the experimental range, followed by desirability function. At optimized conditions, GCV loaded CSNPs had the PS 121.20±2.7 nm and % EE of 85.15±1.1% with the desirability of 0.978 (Fig. 2). There was no significant difference between the predicted and actual values of the measured responses as the p>0.05. Fig. 3 shows overlay contour plot of the location of the desirable region for selection of optimized NP formulation of GCV.

Table 2: ANOVA analysis for measured responses

Response	Source	df[a]	SS[b]	MS[c]	F ratio[d]	p value
Y_1	Model	9	48816.51	5424.06	9.71	0.111
	Error	5	2794.25	558.85	-	-
	Total	14	51610.76	-	-	-
Y_2	Model	9	823.00	91.44	5.93	0.0321
	Error	5	77.10	15.42	-	-
	Total	14	900.10	-	-	-

[a]Degree of freedom, [b]Sum of square, [c]Mean sum of square, [d]Model MS/error MS, ANOVA: Analysis of variance

Morphology observation

SEM micrographs are shown in Fig. 4 which revealed that prepared particles were spherical in shape which is favorable for the uptake through the GIT.

FTIR study

In the FTIR spectra of cross-linked CS (Fig. 5), the peak of 1655 cm^{-1} disappeared and two new peaks at 1645 cm^{-1} and 1554 cm^{-1} appeared. The disappearance of the band could be attributed to the linkage between the phosphoric and ammonium ions. The cross-linked CS also showed a peak for P=O at 1155 cm^{-1} [21]. Cross-linked CS-TPP in the NPs also shows broader peak at 2934-3421 cm^{-1} indicates hydrogen bonding between OH and NH_2 groups [22]. Hence, this confirms the cross-linking between CS and TPP.

DSC study

DSC studies (Fig. 6) were performed to understand the behavior of the cross-linked CS on application of thermal energy. Polysaccharides usually have a strong affinity for water, and in solid state, these macromolecules may have disordered structures that can be easily hydrated. The endotherm related to evaporation of water is expected to reflect the molecular changes brought in after cross-linking.

The thermogram of CS showed endotherm at 102.36°C with the enthalpy of fusion (ΔH) 81.93 J/g. The water-holding capacity of cross-linked CS with TPP was found to be more (ΔH 244.24 J/g) compared with that of plain CS. The cross-linking of CS with TPP at different pH modifies the crystalline nature of CS. The hydrophilicity of cross-linked CS is higher at pH 3, which might be responsible for its increase in the water holding capacity. On the basis of these results, it can be stated that increase in the polar groups and reduction in crystalline domains caused an increase in the water-holding capacity of cross-linked CS. These results are in agreement with the work done by Kittur [23]. An endothermic melting peak of GCV was observed at 253.88°C. The absence of this peak in the thermogram of formulation indicates that the drug was molecularly dispersed inside the matrix of CS as a solid solution.

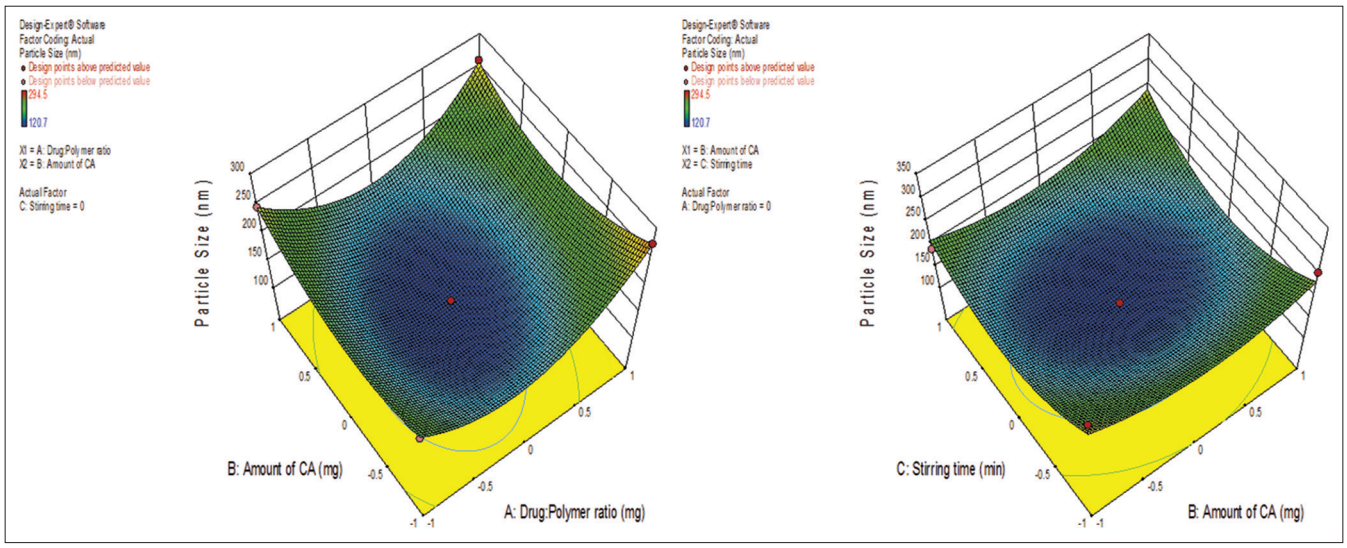

Fig. 1: Three-dimensional response surface curves for particle size

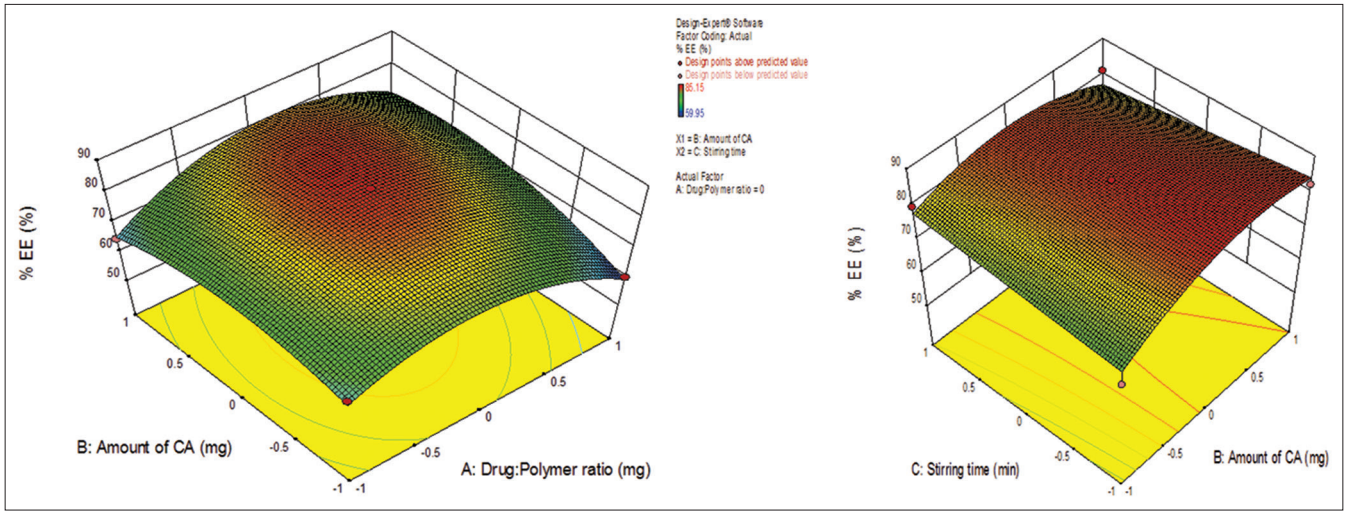

Fig. 2: Three-dimensional response surface curves for % entrapment efficiency

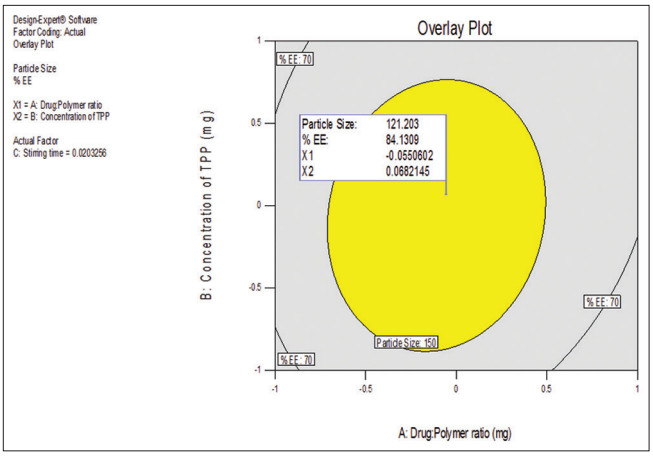

Fig. 3: Overlay contour plot showing the location of the desirable region for selection of optimized nanoparticle formulation of ganciclovir

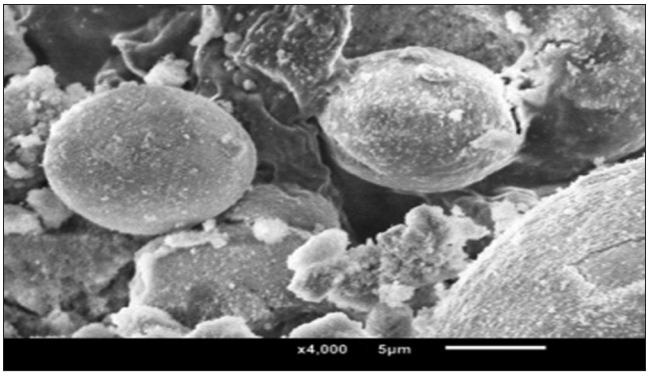

Fig. 4: Scanning electron microscopy micrograph of ganciclovir loaded chitosan nanoparticles

In vitro drug release study

The *in vitro* drug release study of GCV loaded CSNPs, marketed formulation and drug solution was carried out in pH 6.8 phosphate buffer. Drug exhibited sustained release behavior up to the 24 hrs. Drug release was found up to 71.63±0.36%, 45.36±1.96%, and 27.23±1.19% in GCV loaded CSNPs, marketed formulation and drug solution, respectively, at the end of 24 hrs. It was noteworthy that

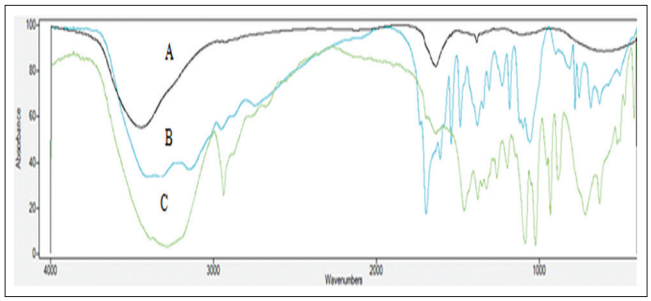

Fig. 5: Fourier transform infrared spectra of (a) Chitosan, (b) ganciclovir (GCV), (c) GCV loaded chitosan nanoparticles

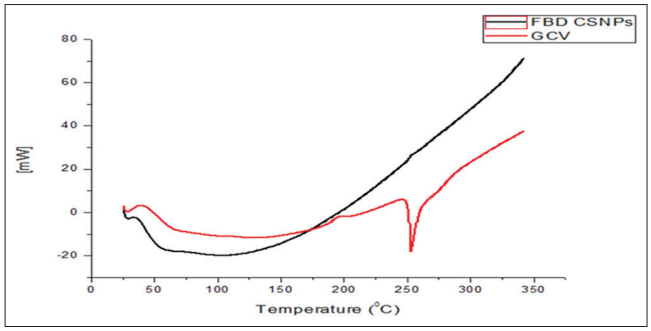

Fig. 6: The overlay differential scanning calorimetry thermogram of ganciclovir (GCV) and GCV loaded chitosan nanoparticles (CSNPs) (fluidized bed drying CSNPs)

formulation showed an initial burst release possibly due to the small size of the NPs. As the particle diameter was reduced, the specific surface area increased, while the path length to the surface of the drug decreased [24]. In the comparison with the marketed formulation the prepared GCV loaded CSNPs released drug in higher amount. The model having highest value of R^2 and lowest value of SSR is considered as the best model to fit [25]. From the results, it was found that drug release from CSNPs follows Higuchi model (R^2=0.9078; SSR=0.3796) indicating that drug release as a diffusion process based on the Fick's law [18].

CONCLUSION

In the presented research, the process and formulation parameters were optimized through the BBD. The variables drug to polymer

ratio, concentration of TPP, and stirring time affected the PS and % EE more significantly. The GCV loaded dry CSNPs showed diffusion controlled release mechanism up to 24 hrs along with ease of formulation.

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