Original Research Article

Dual agents loaded polymeric nanoparticle: Effect of process variables

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Abstract

Aim and Objectives: In the present investigation dual agents i.e., hesperidin and diazepam loaded polymeric nanoparticles (NPs) were formulated by nanoprecipitation method and optimized using three-level factorial design. **Methods:** The developed NPs were optimized keeping poly (lactic-co-glycolic) acid (PLGA), poloxamer amount as independent process variable and z-average, percentage drug entrapment as a dependent response. The optimized NP was subjected to in vitro drug release study to investigate drug release mechanism from NP. Cell viability assay was performed on Vero cell line to confirm the safety of NP. **Results:** Drug loaded NP showed z-average in the range of 189-307 d.nm with percentage drug entrapment for diazepam and hesperidin 62-89% and 68-92%, respectively. *In vitro* drug release studies showed controlled drug release behavior was observed from polymeric NP across dialysis membrane compared to aqueous drug solution. Cell viability assay showed drug dependent cytotoxicity on Vero cell line, however, polymeric NP showed less cytotoxicity compared with aqueous drug solution.

Key words: Three levels factorial design, diazepam, hesperidin, optimization, Polymeric nanoparticles

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INTRODUCTION

Benzodiazepines have been widely used for central nervous system (CNS) disorders such as strokes, ischemia, epilepsy, and psychiatric disorders. They work on specific postsynaptic sites and enhance the activity of gamma-aminobutyric acid (GABA) receptors. Diazepam is a sedative-hypnotic, anti-anxiety, and an antiepileptic drug.[1,2] Diazepam can readily cross the blood-brain barrier, but it gets rapidly redistributed out of the brain. Repeated dosing is required to achieve proper therapeutic efficiency, which can lead to problems of dependency and tolerance. Usefulness of benzodiazepines is hence compromised by the occurrence of several adverse effects such as ataxia, amnesia, alcohol intolerance, and residual sedation after chronic use.[1,2]

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Studies have established the heterogeneity of $GABA_A$ receptors and the pharmacological, and electrophysiological study of the different subtypes has allowed the search of new ligands with improved selectivity.

Natural neuroprotective compounds can be used in combination with synthetic drugs to enhance the efficacy of a treatment, lower the dosage, and thus decrease the toxic effects. Hesperidin, a natural flavonoid, and diazepam have been reported to show synergistic action.[3] Combining synthetic drugs with natural agents can modify the index of dependence and decrease toxic effects. Hesperidin has anti-inflammatory anti-oxidant activity on CNS.[4,5] Fernández *et al*. 2005, reported synergy between hesperidin and diazepam for sedation and sleep enhancing properties.[3] Integration of drugs into carrier molecules overcomes problems of poor stability and drug degradation. Nanoencapsulation can be a possible solution for short half-life and drug degradation.[6,7]

Drugs are encapsulated in biodegradable polymeric nanoparticles (NPs) for controlled release of the drug to brain.[6,7] PLGA has been widely explored for the preparation of polymeric NPs and is well reported for mucoadhesive properties, increased drug stability, and high encapsulation efficiencies.[6-9] Oral and intravenous route of drug delivery have the efficiency to some extent but offer several intrinsic limitations like increased hepatic pass metabolism resulting in the diminished bioavailability of the drug molecule.

The present investigation was aimed to formulate and develop diazepam combined with hesperidin was encapsulated into biodegradable poly (lactic-co-glycolic) acid (PLGA) NPs. Encapsulation efficiency was calculated using the validated spectrophotometric technique. Characterization of the optimized formulation was done by *In vitro* drug release studies and cell viability analysis.

MATERIALS AND METHODS

Materials

Poly (lactic-co-glycolic) acid (PLGA) 50:50 (molecular weight 30,000–60,000) and Poloxamer 407 were purchased from Sigma-Aldrich, St. Louis, USA. Diazepam was purchased from R L Fine Chem, Bangalore, Karnataka, India. Hesperidin and acetone were purchased from Fisher Scientific, Mumbai, Maharashtra, India. Dimethyl sulfoxide (DMSO) was purchased from Qualigens. All the other solvents were of high-performance liquid chromatography (HPLC) grade.

Nanoparticles preparation

Hesperidin-diazepam loaded PLGA NPs were optimized and developed using nanoprecipitation method.[10] Organic phase was prepared by dissolving hesperidin (100 mg) in acetone using DMSO as cosolvent. Diazepam and PLGA were dissolved in acetone and mixed with hesperidin solution. The organic phase was added dropwise to an aqueous phase containing poloxamer with constant stirring of 300 rpm. The colloidal suspension was left for stirring to evaporate acetone completely. The resultant nanosuspension was collected and further subjected for *in vitro* characterization.

Optimization of drug-loaded polymeric nanoparticles

Dual agents loaded PLGA NP were formulated and optimized by investigating effect of independent variables, that is, PLGA and poloxamer amount on dependent characteristic properties, that is, percentage drug entrapment and z-average using response surface methodology (RSM).[11,12] Total 10 formulation runs were developed with two center points using three-level factorial design using Design Expert software (version 8.0.0, Stat-Ease Inc., Minneapolis, Minnesota). Polynomial equation and three-dimension (3D) response surface graphs were generated by the software for analyses of results.

$$Y = b_0 + b_1 X_1 + b_2 X_2 + b_3 X_1 X_2 + b_4 X_1^2 + b_5 X_2^2$$

Where, Y is the dependent variable, b_0 is the intercept, b_1 to b_5 are the regression coefficients. X_1 and X_2 are the coded values of the independent variables. $X_a X_b$ (a, b = 1, 2) and X_i^2 (i = 1, 2) represent the interaction and quadratic terms, respectively [Table 1].

Analytical method for drug estimation

Ultraviolet (UV) spectrophotometric (UV-1800, Shimadzu Japan) method has been developed and validated as per ICH guidelines for estimation of diazepam and hesperidin. Absorbance was taken at 282 nm and 228 nm for hesperidin and diazepam, respectively.[13,14]

Measurement of z-average and zeta potential

Z-average, polydispersity index, and zeta potential of developed hesperidin-diazepam NPs was measured using a Malvern Zetasizer Nano ZS (Malvern Instruments, UK).[12,15] The principle is based on dynamic (laser) light scattering. Zetasizer measures the intensity variation (because of the Brownian motion of NPs) of scattered light and relates it to the particle size with the help of an autocorrelation function. The measurements were performed in triplicates.

Drug entrapment efficiency and drug loading

The amount of drug entrapped in NPs is quantified from the supernatant collected after centrifugation of nanosuspension through the developed analytical method.[11,12,15,16] The drug NP suspension was centrifuged at 12000 rpm, 4°C for 30 min (Remi, Mumbai, Maharashtra, India) and the supernatant was collected after washing pellet with HPLC distilled water. The amount of unentrapped drug in the supernatant was determined by the developed analytical method. Percentage drug entrapment was calculated using following formula:

$$\text{Entrapment effciency (\%)} = \frac{\text{Total amount of drug} - \text{Amount of free drug}}{\text{Total amount of drug}} \times 100$$

Percentage drug loading was calculated by using following formula:

$$\text{Drug loading (\%)} = \frac{\text{Total amount of drug} - \text{Amount of free drug}}{\text{NP weight}} \times 100$$

In vitro studies

In vitro release behavior of hesperidin and diazepam from PLGA NP was evaluated by the dialysis bag diffusion technique.[12,15,17] The studies of the release of drug from NP were performed in phosphate buffered saline (PBS) (pH 7.4, 1% Tween 20) to create a perfect sink condition. Tween 20, a surfactant was added in the dissolution media as diazepam and hesperidin have limited aqueous solubility (0.05 mg/mL and 0.08 mg/mL, respectively). NPs were prepared, centrifuged and the pellet containing NPs were washed to remove extra drug adsorbed on the polymer surface and then redispersed in 2 mL PBS buffer solution (pH 7.4). The redispersed pellet was then transferred inside the membrane tubing

Table 1: Different levels of variables in Box-Behnken design

Independent variables	Levels		
	Low	Medium	High
X_1=Polymer concentration (w/v)	25	37.5	50
X_2=Surfactant concentration (w/v)	50	75	100
Dependent variables	**Desired constraints**		
Y_1=Z-average (d.nm)	Minimize		
Y_2=Percentage drug entrapment	Maximize		

and the tubing was then put in a conical flask. A volume of 100 mL PBS was then added to the flask to check the dissolution across the membrane. Shaker incubator was operated while stirring at 180 rpm at room temperature. A volume of 2 mL sample was taken at predetermine time intervals from the buffer solution and replaced with fresh buffer to maintain sink conditions. Samples were analyzed using a spectrophotometer to calculate the drug released from the membrane into the buffer solution.

Cell viability study

Cell viability studies were carried out on Vero (kidney epithelial cells from African green monkey) cell line using MTT reagent (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a tetrazole dye).[18,19] The whole process of cytotoxicity analysis was carried out with the maintenance of Vero cell line and then followed with MTT assay.

Percentage cell viability was calculated using formula:

$$\text{Cell viability (\%)} = \frac{\text{OD of test formulation}}{\text{OD of positive control}} \times 100$$

RESULTS AND DISCUSSION

Polymer concentration is known to play an important role in percentage encapsulation inside NPs and controlling particle size along with the release of drug from the matrix. Low polymer concentration (25 mg) resulted in less entrapment efficiency with small z-average whereas; high polymer concentration (50 mg) resulted in good entrapment but large z-average. Surfactants or stabilizers are usually involved in the process to modify the surface properties and to impart stability to NPs. Polynomial equation was generated for all the response variables. 3D response surface plots were constructed using Design Expert Software (version 8.0.0, Stat-Ease Inc., Minneapolis, Minnesota). The effect of independent variables on dependent characteristic properties of NP is shown in Table 2. The percentage drug loading for hesperidin and diazepam in the developed NP were found in the range of 3.8-8.85% and 1.5-3.5%, respectively.

Effect on z-average

Polynomial equation was constructed for the measured response:

$$Y_1 = 234 + 27.33\,X_1 - 19.67\,X_2 + 11.25\,X_1X_2 + 27.36\,X_1^2 - 11.64\,X_2^2$$

The polynomial equation shows the quantitative effect of process variables and their interaction on z-average of the developed NP. From the above equation, positive coefficient for X_1 and X_1, X_2 suggested that z-average is directly proportional to X_1. Negative sign on the coefficient for X_2 is attributed to the opposite effect of the variable on the response. The overall effects of responses are shown in Figure 1. The results suggested that with an increase in the amount of polymer the z-average increase, this could be due to aggregation of polymer particles. Whereas, with an increase in surfactant concentration the z-average decreases, this could be due to the decrease in surface tension with increasing surfactant amount which results in small particle size.

Effect on percentage drug entrapment

To investigate the impact of each variable on response Y_2 (i.e., percentage drug entrapment) polynomial equation was constructed by Box-Behnken design.

Polynomial equation for hesperidin:

$$Y_2 = 91.3 + 9.83\,X_1 + 0.17\,X_2 - 0.5\,X_1X_2 - 12.07\,X_1^2 - 0.071\,X_2^2$$

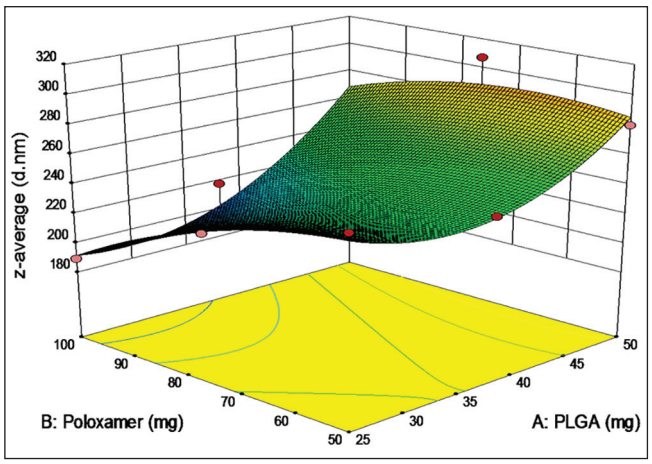

Figure 1: Effect of PLGA and poloxamer concentration on z-average

Table 2: Optimization of experimental variables and their effect on drug entrapment and z-average

Run	PLGA (mg)	Poloxamer (mg)	w/o phase volume ratio	Drug concentration mg		Z-average (d.nm) (±SD)	Percentage drug entrapment (±SD)	
				Hesperidin	Diazepam		Hesperidin	Diazepam
1	50	100	5	5	2	256±4	89±0.5	83±0.5
2	25	75	5	5	2	232±3	71±0.8	64±1
3	37.5	100	5	5	2	218± 6	92±0.5	87±1
4	37.5	75	5	5	2	229±2	91±1.2	89±0.5
5	37.5	75	5	5	2	223±4	91±0.5	88±1.3
6	50	75	5	5	2	307±5	88±0.5	85±0.8
7	25	50	5	5	2	258±2	68±1.8	62±0.5
8	37.5	50	5	5	2	243±2	91±1.5	88±1.5
9	25	100	5	5	2	189±3	69±0.5	66±0.5
10	50	50	5	5	2	280±1	90±0.5	87±1

SD: Standard deviation

Polynomial equation for diazepam:

$$Y_2 = 88.21 + 10.5\,X_1 - 0.17\,X_2 - 2\,X_1\,X_2 - 13.4\,X_1^2 - 0.43\,X_2^2$$

As indicated in the above polynomial equation for hesperidin positive sign on the coefficient for factors X_1 and X_2 show a positive impact on percentage drug entrapment. Whereas, in case of diazepam negative sign on the coefficient for factor X_2 indicates a negative impact on response Y_2. The overall effects of responses are shown in Figure 2. Polynomial equation and response surface plots suggested that with an increase in polymer amount the percentage drug encapsulation also increase, this could be due to presence polymer amount for binding of the drug. Whereas surfactant amount slightly affects percentage drug entrapment in the opposite direction, this could be due to the increase of aqueous solubility of the drugs due to the presence of surfactant that results in drug loss.

Validation of response surface methodology and optimization of drug loaded NP

Optimum formulation combination of NP was selected based on desired constraints within range for independent variables and minimized and maximized constraints for z-average and percentage drug entrapment, respectively. RSM generated various solutions and the optimized formulation (X_1 = 38.4 mg and X_2 = 100 mg with predictable response value for Y_1 = 205.7 d.nm and Y_2 = 92% for Hes and 88.17% for Dzp) were selected on the basis of desirability factor. The experimental value for response Y_1 (218 d.nm) and Y_2 (89.8% for Hes and 87.5% for Dzp) of optimized formulation were found in good agreement with the predicted values generated by RSM and the result assured the validity of RSM model.

In vitro drug release studies

In vitro release study of hesperidin-diazepam from PLGA NPs was carried out using dialysis bag diffusion technique [Figure 3]. PBS (pH 7.4 with 1% tween 20) was used as dissolution medium for evaluating the sustained release of drugs from PLGA NPs and to provide sink conditions.

Nanoparticle showed controlled drug release behavior compared to aqueous drug solution. 88.7% diazepam and 57% hesperidin were found within 6 h from aqueous drug solution, while only

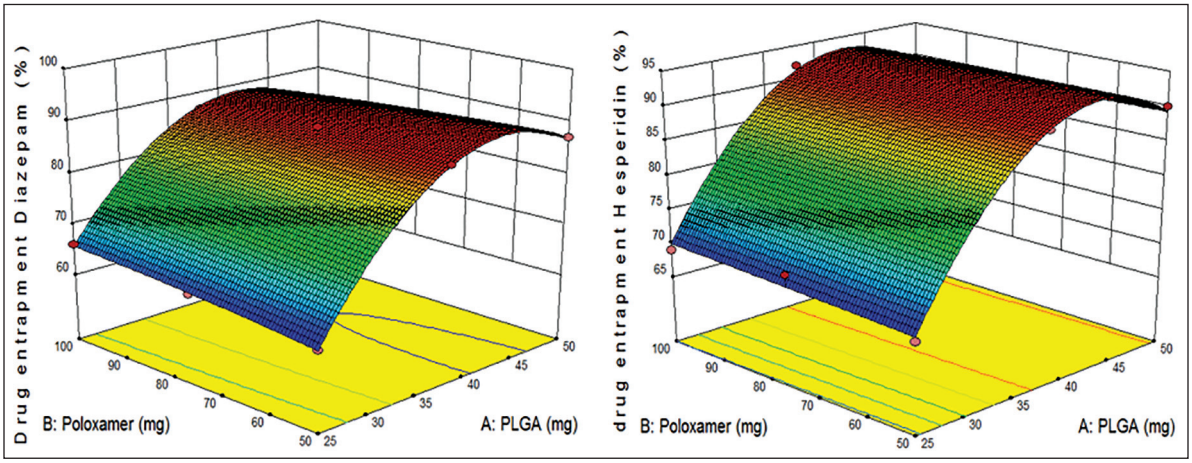

Figure 2: Effect of PLGA and poloxamer concentration on percentage drug entrapment

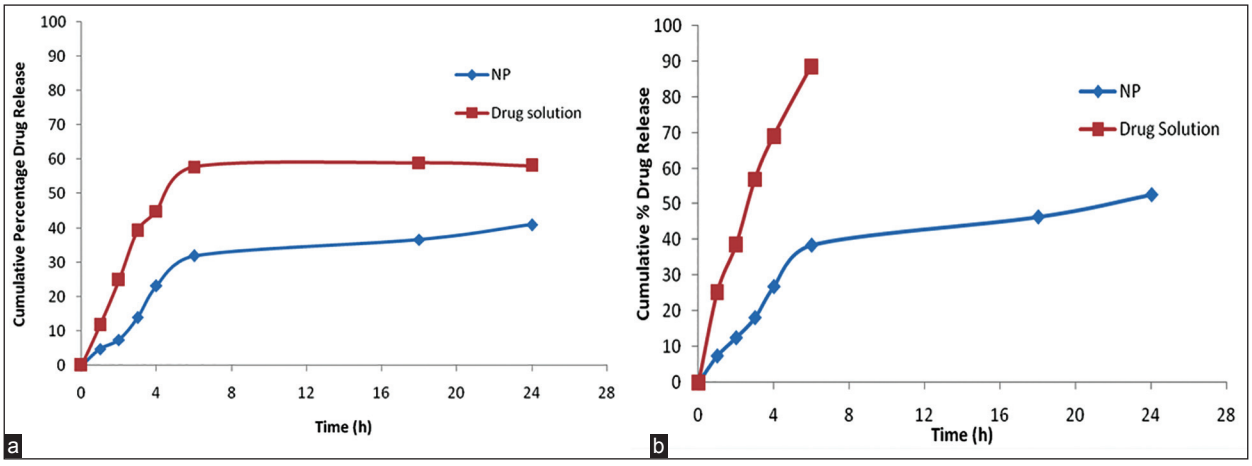

Figure 3: (a) Drug diffusion profile of hesperidin from nanoparticles (NPs) across dialysis membrane (b) Drug diffusion profile of diazepam from NPs across dialysis membrane

38.5% diazepam and 32% hesperidin were released from NPs with maximum release of 43.7% and 41% after 24 h for diazepam and hesperidin, respectively.

Diazepam and hesperidin release from NPs followed a sustained release pattern up to 24 h. With the sustained release of drugs, it is speculated that the need for repeated administrations is avoided leading to less drug accumulation and toxic effects in the body.

Cell viability assay

The cell viability analysis of hesperidin-diazepam loaded NP and placebo on Vero cell line were assessed through MTT assay.[20] The stock of NP and placebo were diluted 8 times to obtain the concentrations of hesperidin and diazepam ranging from 19.5-156.2 μg/ml to 7.8-62.5 μg/ml, respectively.

From Figure 4, it can be concluded that PLGA NPs containing hesperidin and diazepam showed lesser toxic effect to cell lines when compared with the effect of plain drug concentration and placebo. The 156 μg/ml concentration of placebo (79.2% cell viability) was showing equivalent results with 156 μg/ml concentration of plain drug (33% cell viability) and hesperidin and diazepam loaded NPs (56.8% cell viability). The results came out with the finding that the PLGA NPs can be the suitable carrier for hesperidin and diazepam as it provides a sustained release and also diminishes the toxicity.

CONCLUSION

PLGA NP containing hesperidin and diazepam were successfully prepared and optimized using three-factor design of Design Expert Software. The effect of independent variables was studied on dependent characteristic response and formulation was optimized using point prediction technique. Average particle size and size distribution of developed NPs were determined by dynamic light scattering method. Formulation with smaller particle size, monodisperse size distribution were obtained with high percentage drug entrapment was developed using RSM. *In vitro* drug release studies showed that PLGA NPs provided sustained release of the drug, which were up to 42 ± 1.2% (hesperidin) and 52.6 ± 1.35% (diazepam) after 24 h. From the cytotoxicity results, it was inferred that NPs reduces the drugs toxicity over cell line showing cell viability of 56.8%, when compared with plain drug effect over cell lines showing cell viability of 33.5% at 156.2 μg/ml and 62.5 μg/ml concentration of hesperidin and diazepam, respectively. The results from the present investigation showed that PLGA NPs could be a potential carrier option for dual agents that is, diazepam and hesperidin for controlled drug action.

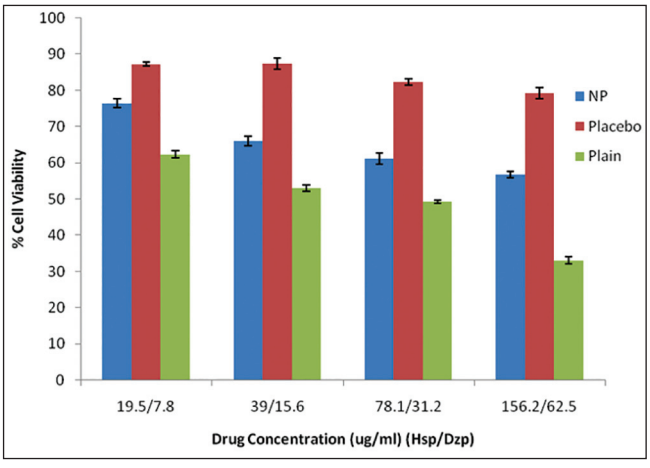

Figure 4: Cell viability analysis of hesperidin-diazepam loaded nanoparticles, placebo, and plain drug on Vero cell line

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