Dual agents loaded polymeric nanoparticle: Effect of process variables

Deepak Sharma¹*, Gilphy Philip¹*, Reema Gabrani¹, Javed Ali², Shweta Dang¹

¹Department of Biotechnology, Jaypee Institute of Information Technology, Noida, Uttar Pradesh, ²Faculty of Pharmacy, Jamia Hamdard, Hamdard Nagar, New Delhi, India

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

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]

Studies have established the heterogeneity of GABA, 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. 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. \( X_1 \) and \( X_2 \) (\( a = 1, 2 \)) 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.[12,15,17] 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:

\[ \text{Entrapment efficiency} (\%) = \frac{\text{Total amount of drug} - \text{Amount of free drug}}{\text{Total amount of drug}} \times 100 \]

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          |
| \( X_1 = \) Polymer concentration (w/v)                    | Low Medium High |
| \( X_2 = \) Surfactant concentration (w/v)                  | 25 37.5 50      |
| 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 predetermined 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). 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 3.8-8.8% and 1.5-3.5%, respectively.

**Effect on z-average**

Polynomial equation was constructed for the measured response:

\[
Y_1 = 234 + 27.33 X_1 - 19.67 X_2 + 11.25 X_1 X_2 + 27.36 X_1^2 - 11.64 X_2^2 
\]

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:

\[
Y_2 = 91.3 + 9.83 X_1 + 0.17 X_2 - 0.5 X_1 X_2 - 12.07 X_1^2 - 0.071 X_2^2
\]

**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) |
|-----|-----------|----------------|------------------------|-----------------------|------------------------|----------------------------------|
|     | 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±1.6 |
| 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:

\[ Y_2 = 88.21 + 10.5X_1 - 0.17X_2 - 2X_1X_2 - 13.4X_1^2 - 0.43X_2^2 \]

As indicated in the above polynomial equation for hesperidin, the 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 hesperdin-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...
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.

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