INTRODUCTION

Nanoparticles are one of the promising drug delivery systems for controlling particle size, surface properties and release of therapeutic ingredients in order to reach the target at the therapeutic desirable proportion and rate regimen (Mohanraj, Chen, 2006). Biocompatible, biodegradable and non-biodegradable polymers as Chitosan derivatives, PLA, PLGA, EC, are used for the preparation of polymeric nanoparticles by dissolution, entrapment, encapsulation or attachment of a drug to a nanoparticle matrix (Nagavarma et al., 2012). NP matrix carriers can improve the encapsulation efficiency and stability of the drugs inside the NPs and provide effective drug levels over longer periods of time compared to traditional therapy (Cooper, Harirforoosh, 2014). Different methods are used for the preparation of nanoparticles. One of the most used is the solvent evaporation method. Uniform concentration of drug at the site of absorption, maintaining of stable plasma concentration and reducing toxic effects can be achieved by developing controlled-release drug delivery systems (Barzegar-Jalaliet al., 2012).

Diclofenac sodium (DC) is a non-steroidal anti-inflammatory drug used for treatment of inflammatory diseases. DC has a short half-life of 1-2h and should be administered frequently at a high dose, which leads to severe undesirable effects and rises the possibility for missing a dose (Arias et al., 2009). The development of sustained dosage release forms was needed to avoid
theses inconveniences (Krishna Sailaja, Nandini, 2016). The design of experiment (DoE) is a valuable tool used for optimization. It allows the finding of the optimal conditions for the best responses of experiments and understand the relationship between the dependent and independent variables in the formulation or process development (Vera Candioti et al, 2014). The response surface methodology (RSM) is the combination of statistical and mathematical techniques based on the recapitulation of experimental data from experimental design (Trivedi et al, 2015). One of the promising RSM used in DoE is central composite design (Yang et al, 2014).

The objective of this study was to formulate and characterize DC-loaded EC-NP with the aim to evaluate the effect of the process variables on the characteristics involved using Central Composite Design.

MATERIAL AND METHODS

Materials

Diclofenac sodium was purchased from CAYMAN chemical company. Ethylcellulose (viscosity 22cP, 48% ethoxyl), Polyvinylalcohol (87-90% hydrolyzed, average mol wt. 30.000-70.000) and dialysis bags (cut-off 12 kDa) were procured from Sigma Aldrich USA. All other solvents and ingredients used were of analytical grade.

Methods

Preparation of nanoparticles

DC loaded EC nanoparticles were prepared by the W/O/W emulsion solvent evaporation method. First, 1mL of a DC aqueous solution (the internal aqueous phase) was emulsified by vigorous magnetic stirring into a 5mL of EC organic solution (ethyl acetate). Then this primary emulsion (W/O) was diluted in 10 mL of PVA aqueous solution (the external aqueous phase) while stirring using a homogenizer (KINEMATICA, Polytron PT 2500 E) in order to create the W/O/W emulsion. The NP suspension was obtained after solvent evaporation under magnetic stirring at room temperature. NP were separated by centrifugation (Sigma 3-30 KS, Germany) at 20.000 rpm for 20 min. The supernatant was kept for drug assay as described later.

Characterization of nanoparticles

Entrapment efficiency (EE)

For measuring drug entrapment efficiency in the NPs, the supernatant part of the centrifuged NPs sample was carefully removed and examined to determine the amount of non-encapsulated drug after dilution with purified water and analysis by UV-visible spectroscopy (Shimadzu, UV 1800, Japan) at 276 nm. Entrapment efficiency (EE) was calculated as follows:

\[
EE = \frac{\text{Initial weight of feeding drug} - \text{Weight of not encapsulated drug in supernatant}}{\text{Initial weight of feeding drug}} \times 100
\]

Average particle size

The particle size of nanoparticles was determined using dynamic light scattering technique at 25ºC using a Zetasizer (Horiba scientific, nano partica SZ-100). All measurements were performed in triplicate.

Fourier Transform Infrared spectroscopy (FTIR)

FT-IR spectra for DC, EC and the optimized nanoparticles were generated by means of FT-IR spectrophotometer (JASCO, FT/IR-6300, United States). This was used to investigate whether there was any degradation or chemical interaction between the polymer and the active component after formulation. Spectra were recorded from the powder in the range of 400–4000 cm\(^{-1}\), at room temperature.

Differential scanning calorimetry (DSC)

DSC thermograms of DC, EC, and the optimized NPs were determined by a differential scanning calorimeter (DSC 131, SETARAM instrumentation, France). Each sample, 2 to 3 mg, was accurately weighed into a close aluminum solid pan. The scanning rate was...
run at 10°C/min from 20 to 300°C under argon purge. DSC analysis of pure DC and EC was performed to identify the drug melting point peak and polymer glass transition temperature (Tg), respectively. The optimized NPs of DC–EC were also analyzed to observe the change of the melting endotherm of DC.

**SEM**

Morphological observation of optimized NPs was carried out using Scanning Electron Microscopy (JSM-7100F). NPs powder was mounted onto metal stubs using double-sided adhesive tape. The stubs were then coated with conductive carbon black. The morphology of the particles was then examined.

**In vitro dissolution**

*In vitro* dissolution studies were performed using USP Type II dissolution test apparatus (Paddle) (Distek 2500, Inc., USA) at 50 rpm and a temperature of 37 °C ± 0.5. In a dialysis bag, 18.7 mg of the optimized NPs containing 4.6 mg of DC was diluted by Phosphate buffered saline solution (PBS, pH=7.4), then was immersed into a Pyrex flask that contains 500 mL of PBS (pH=7.4). At predetermined intervals, 3mL of aliquots were withdrawn and replaced by the same volume of PBS (pH=7.4). Then the aliquots were filtered using a 0.45 μm membrane filter, diluted suitably, and analyzed by a UV spectrophotometer at 276 nm. The dissolution study was carried out in triplicate and their average was used for determining the release kinetics.

**Kinetics of drug release**

The *in vitro* drug release data was analyzed according to zero order, first order, Higuchi and Korsemeyer-peppas model. The selection of the most suitable model was based on the regression coefficient.

**Experimental design**

The effects of formulation factors on the NPs characteristics and the optimization procedure were examined by employing a CCD. The design and statistical analysis were performed by Minitab 18® Software for design of experiments (DOE).

| TABLE I - Variables in CCD | Level used, real and coded values | Constraints |
|-----------------------------|----------------------------------|-------------|
| **Independent variables (Factors)** | Low (-1) | Intermediate (0) | High (+1) | Maximize (20-100) | Minimize (100-400) |
| X₁: Mass Ratio of DC / EC (%) | 66.66 | 73.33 | 80 | | |
| X₂: PVA concentration (%) | 0.5 | 0.7 | 0.9 | | |
| X₃: Homogenization speed (rpm) | 8000 | 9000 | 10000 | | |

Experimental factors and their levels were determined in preliminary studies using full factorial design (data not shown). The factors evaluated in this investigation, were the mass ratio of DC/ EC (X₁: $\frac{DC}{EC}$ x 100), the PVA concentration (X₂: w/v %) and the homogenization speed (X₃: rpm) with different levels for each factor as described in Table I (coded and real values). The evaluated responses were the entrapment efficiency (Y₁) and the average particle size (Y₂).
The CCD design and the data obtained are summarized in Table II. The quadratic non-linear model generated by the design is in the following form:

$$Y = A_0 + A_1X_1 + A_2X_2 + A_3X_3 + A_{12}X_1X_2 + A_{13}X_1X_3 + A_{23}X_2X_3 + A_{11}X_1^2 + A_{22}X_2^2 + A_{33}X_3^2$$

(2)

Where Y is the measured response associated with each factor level combination; $A_0$ is an intercept, $A_1$, $A_2$, $A_3$ are the linear regression coefficients, $A_{12}$, $A_{13}$, $A_{23}$ are the interactive regression coefficients, and $A_{11}$, $A_{22}$, $A_{33}$ are the quadratic regression coefficients; $X_1$, $X_2$, and $X_3$ are the studied factors; $X_1^2$, $X_2^2$, $X_3^2$ are the quadratic effects, $X_1X_2$, $X_2X_3$, $X_1X_3$ represent the interaction between the variables (Vera Candioti et al, 2014). An analysis of variance (ANOVA) was performed to establish the optimum conditions.

The desirable optimum region was selected by the desirability function method, wherein the constraints for choosing an optimum formulation were further narrowed as shown in Table I. First, the responses $Y_1$ and $Y_2$ are transformed into individual desirability function $d_1$ and $d_2$ that vary over the range $0 \leq (d_1, d_2) \leq 1$ (Fitrianto, Midi, 2012). For the first response, the desirability function $d_1$ should be maximized as follow:

$$d_1 = \frac{Y_1 - L}{T - L}$$

(3)
For the second response, the desirability function $d_2$ should be minimized as follow

$$d_2 = \frac{(Y_2-U)}{(T-U)}$$

(4)

Where, $T$ is the target value desired, $U$, $L$ are the upper and lower acceptable values of response. The overall desirability value ($D$) is calculated by the following equation

$$D = \sqrt{d_1 \times d_2}$$

(5)

The validation of the derived polynomial equations and the optimized formulation selected was carried out by the preparation of four optimum checkpoint formulations based on their predicted values for the response variables. The error prediction was calculated by comparing experimental values of the responses with the predicted values (Motwani et al., 2008).

### RESULTS AND DISCUSSION

#### Experimental design

The results were assessed for $R^2$, adjusted $R^2$, $p$-values (model and lack of fit) as quality indicators for the model. Comparing the second order model and full quadratic model, the second order model had the highest $R^2$ values, highly significant model $p$-value ($p<0.001$), and insignificant lack of fit $p$-value ($p>0.10$) as listed in Table III.

For the fitted model, the experimental response might be represented by the following regression equation for both responses $Y_1$ and $Y_2$:

$$Y = A_0 + A_1X_1 + A_2X_2 + A_3X_3 + A_{11}X_1^2 + A_{22}X_2^2 + A_{33}X_3^2$$

(6)

#### TABLE III - Regression analysis of the studied responses

| model                | Adjusted $R^2$ | $R^2$ | $p$-value | Lack of fit | Observation |
|----------------------|----------------|-------|-----------|-------------|-------------|
|                      |                |       | model     |             |             |
| Y1                   |                |       |           |             |             |
| Second order         | 0.859          | 0.904 | $<0.001$*** | 0.892       | Suggested   |
| Full Quadratic       | 0.828          | 0.906 | $<0.001$*** | 0.723       | -           |
| Y2                   |                |       |           |             |             |
| Second order         | 0.792          | 0.857 | $<0.001$*** | 0.528       | Suggested   |
| Full Quadratic       | 0.737          | 0.861 | $<0.001$*** | 0.329       | -           |

The followings are the regression equations of the fitted model with statistically significant terms:

$$Y_1 = 51.07 + 1.03X_1 + 0.02X_2 - 0.93X_3 - 2.7X_1^2 - 2.62X_2^2 - 2.41X_3^2$$

$$Y_2 = 245.99 + 4.02X_1 + 25X_2 - 11.55X_3 + 2.15X_1^2 - 0.51X_2^2 - 0.51X_3^2$$

ANOVA was carried out to evaluate the significance of the fitted models on the responses and their quantitative effects. The effects of the model terms (intercept and coefficient) and associated $p$-values for the two responses are listed in Table IV. The value and sign of the quantitative effect correspond to the extent and the trend of the terms influence on the response, respectively. The term with a positive value in the regression equation show synergistic effect, whereas a negative value shows an antagonistic effect between the factor and the response.
TABLE IV - The coefficients of responses in the CCD design

| Term  | Coefficient | Responses | \( \text{Y1} \) | \( \text{Y2} \) |
|-------|-------------|-----------|----------------|----------------|
| \( X_1 \) |             | 1.034     | 4.022          |                |
|       |             | 0.025*    | 0.225          |                |
| \( X_2 \) |             | 0.823     | 25.004         |                |
|       |             | 0.064     | < 0.001***     |                |
| \( X_3 \) |             | -0.934    | -11.555        |                |
|       |             | < 0.001***| 0.003**        |                |
| \( X_1^2 \) |             | -2.707    | 2.152          |                |
|       |             | < 0.001***| 0.496          |                |
| \( X_2^2 \) |             | -2.627    | -0.518         |                |
|       |             | < 0.001***| 0.869          |                |
| \( X_3^2 \) |             | -2.415    | -0.518         |                |
|       |             | < 0.001***| 0.869          |                |
| Intercept |             | 51.074    | 245.990        |                |
|       |             | < 0.001***| < 0.001***     |                |

For the optimized formulation, the observed value of drug loading (DL) is \( \text{DL} = 24.59\% \), the predicted maximum values of the responses are \( \text{EE} = 49.09\% \) and particle size = 226.83 nm along with an individual desirability of 0.36 and 0.57, respectively. The overall desirability has a value of 0.458 with factors setting at 73.66\% for the mass ratio, 0.56\% for the PVA concentration and 9220.84 rpm for homogenization speed (Figure 1).

For the four checkpoint formulations, the results of the evaluation for EE and particle size were found to be within acceptable limits as listed in Table V. The validity of the generated regression equations was evaluated by determination of the error prediction.
Fourier Transform Infrared

The FTIR spectra revealed that there was no interaction between DC and the polymer. The characteristic absorption peaks of Diclofenac Sodium were obtained at wave numbers of 3384.46 cm⁻¹ (NH stretching of the secondary amine), 1570.74 cm⁻¹ (–C=O stretching of the carboxyl ion), 1554.34 cm⁻¹ (C=C ring stretching) and at 741.49 cm⁻¹ (C-Cl stretching) (Kebebe, Belete, Gebre-Mariam, 2010). The absorption peaks of EC were obtained at 1051.01 cm⁻¹ (C–O–C stretching), 2969.84 cm⁻¹ (C–H stretching) (Madni et al., 2014). In the IR spectrum of NPs, peaks corresponding to DC, NH stretching of the secondary amine (3387.35 cm⁻¹), C-Cl stretching (743.42 cm⁻¹) still present, in contrary to C=O stretching of the carboxyl ion and C = C ring stretching which disappear or are buried in the peaks of EC indicating drug entrapment and the absence of chemical interaction between polymer and drug in nanoparticles as shown in Figure 2.
**DSC**

Thermal analysis is a supportive tool for determining the dispersion of the drug in polymeric materials. DSC thermograms of the DC, EC and optimized NPs are represented in Figure 3. The pure drug showed a high endothermic peak indicating its melting point at around 280°C which was absent in NPs.
FIGURE 3 - DSC thermograms of DC (A), EC (B), optimized NPs (C)

SEM

The SEM micrographs showed that uniform NPs were successfully prepared using the solvent evaporation method. The optimized nanoparticles have a spherical shape and a smooth surface as shown in Figure 4.

FIGURE 4 - SEM micrographs of the optimized Diclofenac Sodium nanoparticles
In vitro release kinetic evaluation

In vitro drug release studies were performed to determine the sustained release nature of the formulation. In EC Formulation the drug release was slow and the spread extended. In fact, over a time period of 24 hours only 53.98% of the drug has been released from the EC Formulation (Figure 5).

FIGURE 5- In vitro release profile of optimized formulation

The dissolution data were fitted to various kinetic equations and mechanism of drug release investigated. Equations (7-10) below are Zero order, First order, Higuchi and Korsmeyer-Peppas model, respectively.

\[ Q_t = K_0 t Q_t = K_0 t \]  \hspace{1cm} (7)

\[ \ln Q_t = \ln Q_0 - K_1 t \ln Q_t = \ln Q_0 - K_1 t \]  \hspace{1cm} (8)

\[ Q_t = K_h t^{1/2} Q_t = K_h t^{1/2} \]  \hspace{1cm} (9)

\[ \frac{Q_t}{Q_\infty} = K_p t^n \frac{Q_t}{Q_\infty} = K_p t^n \]  \hspace{1cm} (10)

Where, \( Q_t \) is the percentage of drug released at time \( t \), \( Q_0 \) is the initial amount of drug present in the formulation, \( K_0, K_1, K_h, \) and \( K_p \) are the constants, \( Q_t/Q_\infty \) is the fractional drug release at time \( t \) and \( n \) is the diffusional exponent characterizing the transport mechanism. The criteria for selecting the most appropriate model were based on the regression coefficient (R²) which was determined from the slope of the following plots: Cumulative percent drug release vs. Time (Zero order kinetic model), Log cumulative of percent drug remaining vs. Time (First order kinetic model), Cumulative percent of drug release vs Square root of Time (Higuchi model), Log cumulative percent drug release vs. Log time (Korsmeyer-Peppas model). In Korsmeyer-Peppas model, first 60% of drug release was fitted and the release exponent “n” was calculated which is indicative of drug release mechanism. According to Korsmeyer theory, if \( n < 0.45 \) then the drug release follows Fickian diffusion mechanism, for \( 0.45 < n < 0.89 \) it follows Anomalous (non-Fickian) diffusion and \( n >0.89 \) for Super Case II release mechanism (Lokhande et al., 2013).
The results of Table VI show that the optimized nanoparticles follow a Korsmeyer-Peppas release model ($R^2=0.97$). The $n$ value (0.42) is lower than 0.45 indicating that the release follows a fickian diffusion mechanism.

**CONCLUSIONS**

The application of CCD is a useful tool for optimizing DC-loaded EC nanoparticles prepared by the emulsion solvent evaporation technique. The optimized nanoparticles obtained displayed an average particle size of 226.83 nm with a narrow polydispersity index (0.271), an EE of 49.09 % and a slow and prolonged drug release over a period of 24 hours by fickian diffusion mechanism governed by a Korsmeyer-Peppas release kinetics type. Ethylcellulose nanoparticles of Diclofenac sodium can be of significant practical use for a sustaining drug release and decreasing side effects.

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