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Research Article

# Formulation and *in vitro* and *in vivo* characterization of Nifedipine stabilized nanosuspensions by nanoprecipitation method

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**ABSTRACT**

Nifedipine is a dihydropyridine calcium channel antagonist originally introduced for the treatment of angina pectoris and hypertension. Nifedipine which is a poorly water soluble drug coming under BCS class-2, however it suffers from a poor aqueous solubility, which delays its onset of action. Therefore, the purpose of the present study is to utilize the nanotechnology to formulate nanoparticles that enhance the dissolution and hence the bioavailability nifedipine. Nanosuspensions were prepared by nano precipitation method in the presence of selected stabilizers at different concentrations. The nanosuspensions were evaluated for their particle size, zeta potential, drug content and *In vitro* drug dissolution. The selected formula was freeze dried and characterized by scanning electron microscopy (SEM), fourier transforms infrared spectroscopy (FTIR), differential scanning calorimetry (DSC), and pharmacokinetic study. The *in vitro* dissolution showed higher drug release compared to the pure drug. The optimum formula has an average particle size of 225.56±4.65 nm and zeta potential of -17.84±2.17 mV. The bioavailability parameters in the rabbits were enhanced by 2 folds when compared with the marketed tablets (Calcigard®). Nanoprecipitation method was successfully employed to produce stable Nifedipine nanosuspension by using the suitable concentration of stabilizer (PVA, Tween 80, PVP & HPMC). From this study, it is concluded that formulation of Nifedipine nanosuspension may be a promising approach that improves the dissolution rate and hence oral bioavailability.

**Keywords:** Dissolution; bioavailability; nanosuspension; nifedipine; solubility

## INTRODUCTION

Nifedipine is an inadequately water soluble medication which comes under BCS Class II. This medication experiences hepatic metabolism in liver and gut wall. Poor water solubility, deficient bioavailability, fluctuating plasma levels and high sustenance reliance are the most imperative and normal issues with this medication. Significant efforts have been made for the advancement of modified medication bearers to conquer the annoying in vivo results of the medication (Barratt GM., 2003 & Amidon GL et.al., 1995). Consequently, there is a increasing requirement for a unique system that can tackle the detailing related issues associated with the delivery of hydrophobic medications keeping in mind the end goal to enhance their clinical viability and upgrade u their treatment as for pharmacoeconomics.

The rate of dissolution for ineffectively water soluble medications frequently turns into a rate-limiting step in their absorption process from GI tract (Maeda T et.al., 1979 & Chiba Y et.al., 1991). Different solubilization strategies have been utilized to enhance the medication solubility and disintegration properties, including the utilization of surfactant, water-soluble bearers, polymeric conjugates, and solid dispersions.

Nanosuspension is a proficient and brainy approach used to delivery of water insoluble medications where the medication is diminished to the submicron extend as the saturation solubility and the surface area available for dissolution enhancement subsequently increasing its dissolution rate and thus its bioavailability (White SR., 2005 & Janssen CJ., 2005). Stabilizer assumes an imperative part in the preparation of nanosuspensions. Without a suitable stabilizer, the high surface energy of nano-sized particles can initiate agglomeration of particles. The principle elements of a stabilizer are wetting the drug particles completely, and preventing Ostwald's ripening (Moschwitzer J., 2004).

Nanoprecipitation strategy displays various points of interest, in that it is a clear method, quick and simple to perform. In this methodology, the drug substance is broken down in a organic solvent, for example, acetone, acetonitrile etc. The organic solvent is vanished either by pressure reducing or by ceaseless stirring.

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Size of the drug particles was observed to be impacted by the kind of stabilizer, concentrations of stabilizer, and speed of homogenizer (Wongmekiat et.al., 2002). In the present work, nanosuspension formulation was developed by nanoprecipitation methodology, in which drug substance is broken up in a solvent, which is then added to non-solvent that causes precipitation of the fine drug molecules and the framework is balanced out by polymer and surfactant.

## MATERIALS AND METHODS

### Materials

Nifedipine drug sample was procured from J.B. Chemicals & Pharmaceuticals Ltd, Mumbai. Hydroxy Propyl Methyl Cellulose (HPMC) was procured from Loba chem. Pvt ltd, Mumbai. Poly Vinyl Alcohol (PVA) was purchased from procured from Meru Chem Private Limited., Mumbai. Tween 80 and Ethyl alcohol 95% V/V were purchased from Drugs India, Hyderabad. All other chemicals and reagents used in the study were of analytical grade.

### Methods

#### Formulation of nifedipine nanosuspensions

Nanosuspensions formulations were developed and prepared by the nanoprecipitation method (Itoh K et.al.,2003, Vikram M et.al.,2011, Kipp JE et.al.,2003 & Matteucci ME et.al.,2007). In short, nifedipine (10 mg) and stabilizers (PVA, Tween 80, PVP K44 & HPMC K4M) were made soluble in organic solvent (15 ml of 95% ethanol) to produce a series of organic solutions at room temperature (25±1°C) consisting of different concentrations of stabilizers. Distilled water holding a surfactant (1% tween 20), which serves as antisolvent system was cooled at lower temperature (below 5 °C). Consequently the organic solution was added in to aqueous solution at a slower rate (1 ml/min) with the help of syringe, under higher-speed mechanical agitation of 7000 rpm by means of Probe sonicator for 10 minutes to produce the required nanosuspension formulation. The cooling was sustained during the process by means of an ice-water bath which is controlled the rate of precipitation. The resulting coarse predispersion was comminuted using zirconium oxide beads (milling media) on a magnetic stirrer. Zirconium oxide beads were used in the preparation of nanosuspension due to their low cost and easy availability for lab scale production of nanosuspension in comparison to silver beads. Different formulation batches were prepared in respect to the formulation design. Later the prepared nanosuspensions were kept under vacuum at 25 ° C for 3 h for removal of organic solvents.

#### Characterization of nanosuspensions

#### Particle size and poly dispersity Index

The formulated nifedipine nanosuspensions were characterized by means of Photon Correlation Spectroscopy (PCS) with a Zetasizer Nano ZS apparatus. In the process of analyzing, an aliquot of the formulation was diluted before the quantification. All experiments were conducted in triplicate at 90 ° scattering angle at the temperature 25 °C.

#### Determination of zeta potential

The prepared nanosuspension formulations were subjected for zeta potential measurement by means of an additional electrode in the particle size analyzer i.e. Zetasizer, Malven. For analysis the samples were diluted with water and kept in electrophoretic cell as the electrophoretic mobility was transformed to zeta potential by means of Smoluchowski equation (Keck CM & Muller RH.,2006). Every individual sample was measured three times at room temperature and average values were estimated.

#### Determination of the total drug content

All formulations were conducted for assay test to determine the amount of pure drug present in the formulation. In brief the procedure is as follows; 0.5 ml of aliquot sample was first made soluble in 10 ml $p^H$ 7.4 phosphate buffer followed by filtration through 0.44 µm size Whatman filter paper. The drug quantity was estimated after suitable dilution with respective medium and phosphate buffer as a blank on UV Spectrophotometer (Lab India, UV 3000) at a wavelength of 238 nm. The drug content and percentage of drug content were estimated by using following equations;

$$Total\ drug\ content = \left(\frac{Total\ Volume}{Aliquot\ Volume}\right) \times Drug\ amount\ in\ aliquot \times 100$$

$$\%\ Total\ drug\ content = \left(\frac{Total\ drug\ content}{Total\ added\ drug}\right) \times 100$$

Here, Total Volume/ Aliquot Volume is the ratio of total nanosuspension quantity to the volume taken in aliquot and the total quantity of drug taken for the preparation of nanosuspension, total added drug (Chorny M et.al.,2002).

#### *In vitro* dissolution studies of the prepared formulae

The nanosuspension formulations of nifedipine were evaluated for dissolution studies in comparison with dissolution profile of pure drug. The procedure is as follows; pure drug and nanosuspension formulation (all equivalent to 10 mg of nifedipine) were weighed accurately for the study and made soluble in adequate quantity of phosphate buffer pH 7.4. The samples were placed in dialysis bag (Mw cut-off = 12,000Da). After this procedure, the formulations the formulae were released in a beaker containing phosphate buffered (500 ml) and stirred at a constant speed of 200 rpm with a magnetic stirrer at 37±0.5 °C. 5 ml sample was taken and replaced with a fresh medium at 5, 10, 15, 20, 30 and 45 min, followed by filtration and diluted suitably for the measurement of drug concentration using UV spectrophotometry at $\lambda_{max}$ 238.5 nm. The experiments were conducted for three times for each of

the selected formulation. The drug release kinetics of the formulations was performed for zero-order, first-order and Higuchi by using Microsoft Excel Add-Ins DD Solver software.

**Lyophilization of selected nanosuspensions**

To facilitate the removal of water content from the nanosuspensions, lyophilization technique is most commonly employed (Kumar MP et al., 2008). In the present work, we have subjected the formulations for the same purpose. The optimized formulations were initially exposed to lowest temperatures to make them to frozen and then lyophilized by means of a freeze dryer (Thermo Fischer Scient, Micromodul YO 230). During process formulation was placed in ampoules and pre-frozen in a deep freezer at -45$^{o}$C for a period of 24 hours, followed by ampoules were moved to glass flasks and these flasks were connected to the vacuum adapter of freeze dryer.

**Optimization of nifedipine nanoparticles**

**Process yield determination**

The percentage yield of the experimental procedure was calculated by using the following formula;

% process yield = [Mass recovered/Mass entered into the experiment] X100

Nifedipine entrapment efficiency in the dry lyophilized powder form was estimated by distributing 1 mg of powder in 10 ml of ethanol. As a result of this a suspension was obtained and this was subjected for sonication in a water bath for about 30 minutes then centrifuged at 1500 rpm, in order to remove insoluble solid particles. After this nifedipine content was estimated in supernatant layer by using UV spectrophotometry at a wavelength 238.55nm. The obtained weight was divided by the mass initially taken in the process and calculated in terms of percentage.

**Scanning electron microscopy (SEM)**

The optimized nanosuspension formulations were studied for morphological characters by means of scanning electron microscopy (SEM). This procedure was carried out at different magnification points for detailed study of morphological properties, prior to this the samples has subjected for palladium and gold coating. The images were taken at a voltage of 20 kv.

**Fourier transforms infrared spectroscopy (FTIR)**

In order to identify the incompatibilities in drug and polymers and other excipients in the formulation, the nanosuspensions were analyzed by Fourier transforms infrared (FTIR) spectroscopy method and spectras of drug sample and optimized formulations were recorded by means of FTIR spectrophotometer (Bruker Optics, Alpha). The samples were prepared by pressed pellet method; they were mixed with potassium bromide (spectroscopic grade) then compressed in to disks with a hydraulic press and then scanned in the range of 5000 to 500 $cm^{-1}$.

**Differential scanning calorimetry (DSC)**

Further incopatibility studies were confirmed by means of Differential Scanning Calorimetric method (TA Instruments, Q20). The analysis process is as follows; the drug sample and formulation in appropriate weight (4-6 mg) were sealed in the flat-bottomed aluminum pan of the differential scanning calorimeter. Then sample was analyzed at a temperature range of 0-300$^{o}$ C and heating rate was set 5 $^{o}$ C/ minute under nitrogen gas passing at a flow rate of 25 ml/min. Determination of transition temperature and melting point is done with the help of DSC device software.

***In vivo* drug absorption study**

An *in vivo* pharmacokinetic study was conducted in accordance with the ethical guidelines for investigations in laboratory animals and approved by the Institutional Animal Ethics Committee (IAEC). Twelve Rabbits (New Zealand, White) weighing 2.30 ± 0.12 kg (divided into two groups) were fasted overnight. The animals were allowed 7 days to acclimate and were given ad libitum access to standard rat chow (0.5% NaCl) and tap water until the initiation of the experiment. For dosing, each group of three rabbits was given either a 10-mg/kg subcutaneous dose of nifedipine formulated as regular suspension. Oral dosing followed the same guidelines. At the initiation of the study, the animals weighed from 297 to 329 g. Blood samples (approximately 0.2 mL per sample) were collected from each animal via jugular vein cannulae at the following time points: pre dose; 5, 15, and 30 min post dose; and 1, 2, 4, 8, and 24 h post dose. All samples were collected into tubes containing potassium ethylenediaminetetraacetic acid as an anticoagulant. Blood samples were centrifuged within 30 min of the collection, and plasma was harvested. Plasma samples were analyzed for drug concentrations by a HPLC assay method.

The plasma samples were analyzed using validated HPLC method using nitrendipine as internal standard for nifedipine. Chromatographic system consisted of model Shimadzu SPD M10 ATVP pump and rheodyne injector with 20 µL fixed volume loop and shimadzu SPD10A UV detector controlled by the software kinetica. Separation was carried out at room temperature (25$^{0}$c) phenomenax C18 (150 mm X 4.6 mm with 5 µ particle size) column. A mixture of acetonitrile: pH 6.8 phosphate (35:65) was used as mobile phase with flow rate 1ml/min and pressure was maintained at 90-150 $kg/cm^{2}$. Column temperature was maintained at 35$^{0}$C. Mobile phase was filtered through 0.45 µm membrane filter before use. The detector wavelength was set at 238 nm.

**Dose simulation**

A model based on the Wagner-Nelson equation was established in-house and was used to calculate the

**Table 1: Composition of nifedine nanosuspensions**

| Batch Code | Stabilizer | Drug (mg) | Stabilizer concentration | Surfactant |
|---|---|---|---|---|
| F1 | PVA | 20 | 0.2% w/v | 1% w/v |
| F2 | PVA | 20 | 0.4% w/v | 1% w/v |
| F3 | Tween 80 | 20 | 0.2% w/v | 1% w/v |
| F4 | Tween 80 | 20 | 0.4% w/v | 1% w/v |
| F5 | PVP K44 | 20 | 0.4% w/v | 1% w/v |
| F6 | HPMC K4M | 20 | 0.4% w/v | 1% w/v |

**Table 2: Drug content, percentage yield, particle Size (P.S.), and size distribution of nanosuspensions**

| Formulation Code | Drug Content % | % Yield | P.S. in nm | Polydispersibility Index | Zeta Potential (mV) ±*SD |
|---|---|---|---|---|---|
| F1 | 73.64 | 95.18 | 236±4.28 | 0.52 | -10.25±1.37 |
| F2 | 72.38 | 98.6 | 348±3.69 | 0.49 | -11.46±1.49 |
| F3 | 92.67 | 95.2 | 282±4.48 | 0.46 | -10.32±1.63 |
| F4 | 95.78 | 96.1 | 337±3.34 | 0.39 | -13.18±1.49 |
| F5 | 89.49 | 94.2 | 318±4.64 | 0.64 | -10.94±0.48 |
| F6 | 92.78 | 96.5 | 339±3.48 | 0.52 | -11.47±2.18 |

**Table 3: Pharmacokinetic parameters after oral administration of nifedipine formulations to rabbits**

| Parameters | Marketed formulation | Optimized formulation |
|---|---|---|
| $C_{max}$ (µg/mL) | 2.28±0.474 | 2.42±0.259 |
| $T_{max}$ (h) | 3.48±0.000 | 1.35±0.000 |
| $t_{1/2}$ (h) | 3.29±1.112 | 4.79±0.734 |
| $AUC_{0-t}$ (µg-h/mL) | 18.45±0.102 | 25.89±0.245 |
| $K_e$ (h-1) | 0.158±0.017 | 0.125±0.032 |
| MRT | 8.59±2.15 | 12.48±2.37 |

**Table 4: Correlation coefficient R2 values of various kinetic models used for analysis of the release data of nifedipine nanosuspension**

| Formulation code | Zero order | First order | Higuchi Diffusion |
|---|---|---|---|
| F1 | 0.912 | 0.985 | 0.954 |
| F2 | 0.889 | 0.988 | 0.974 |
| F3 | 0.945 | 0.979 | 0.943 |
| F4 | 0.957 | 0.988 | 0.939 |
| F5 | 0.879 | 0.979 | 0.954 |
| F6 | 0.914 | 0.994 | 0.942 |

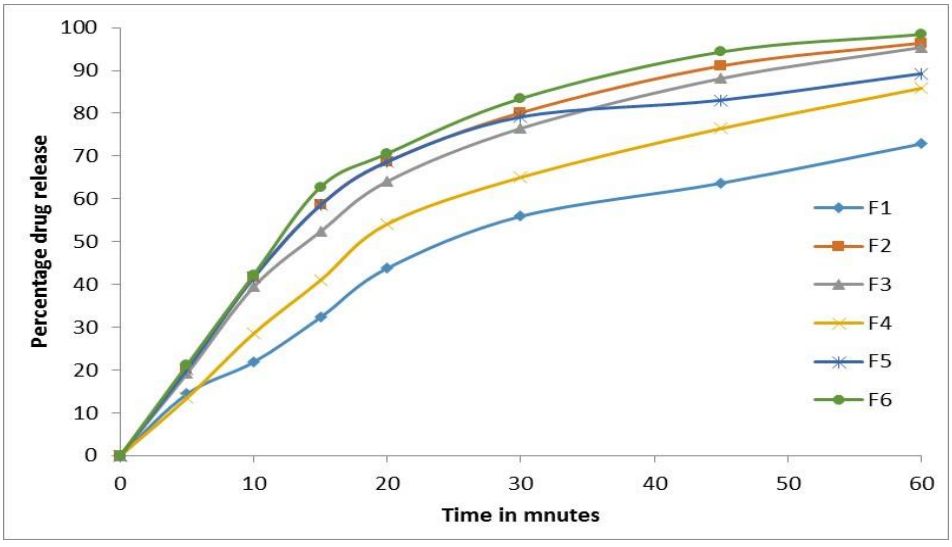


**Figure 1: In-vitro release profile of nifedipine nanosuspension formulations (F1-F6) in phosphate buffer pH 6.8**

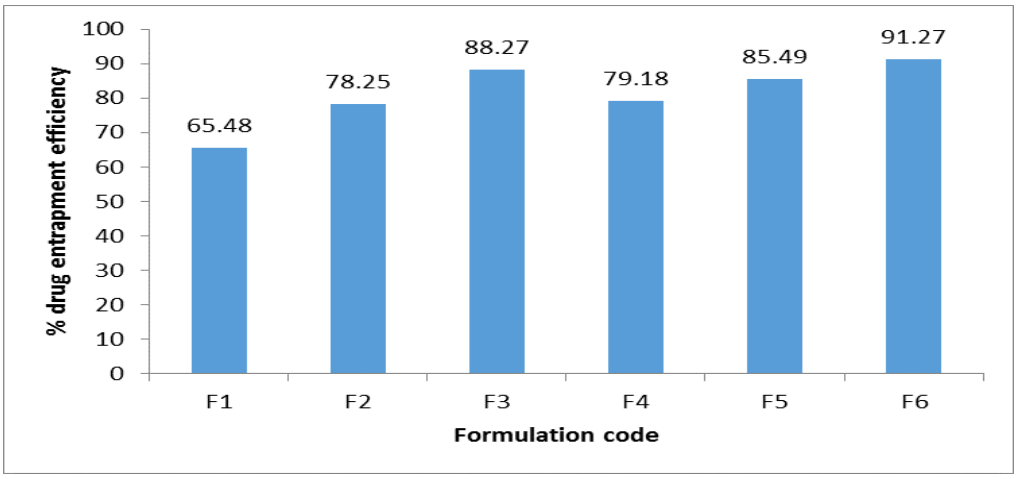


**Figure 2: Entrapment efficiency chart for various prepared nanosuspension formulations**

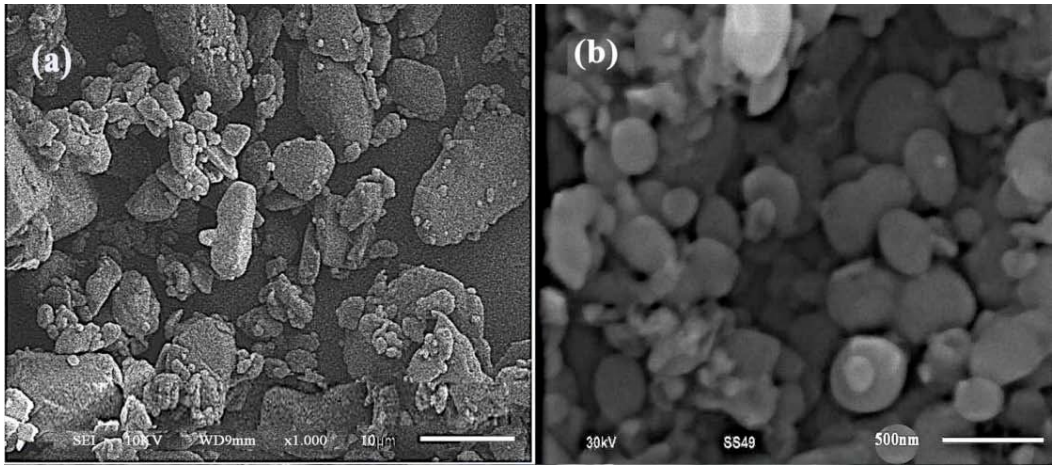


**Figure 3: Scanning electron microscopy (SEM), of (a) pure nifedipine and (b) nifedipine optimized formulation**

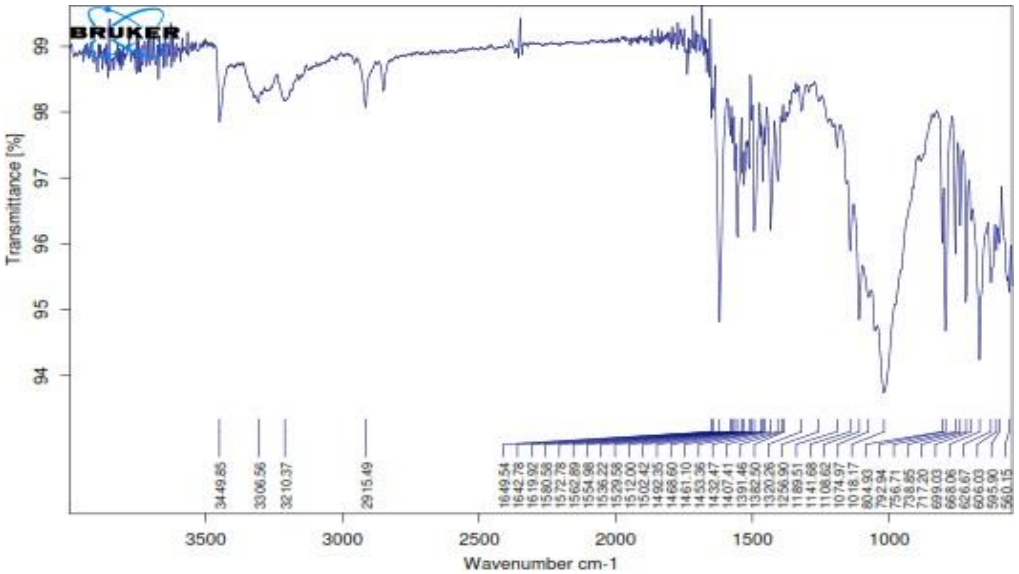


**Figure 4: Fourier transforms infrared spectroscopy of pure nifedipine and nanosuspensions (lyophilized nanosuspension)**

drug absorbed to further assess the amount of drug absorbed as a function of time (Van Eerdenbrugh et al., 2009). The utilization of the equation allows us to obtain the entire drug that is absorbed (including excreted) at different time points. This allowed us to estimate the relationship and the impact on the absorption on the surface area changes of the drug.

$$dA = V \times dCp + V \times K \times Cp \times dt$$

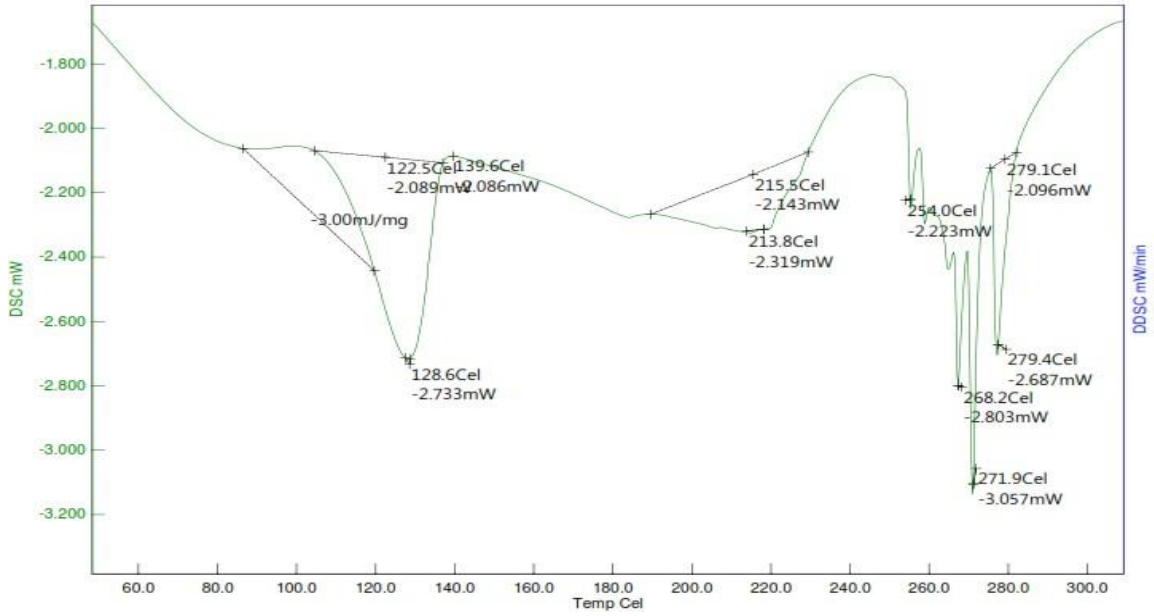


**Figure 5: Differential scanning calorimetry of pure nifedipine and nanosuspensions (lyophilized nanosuspension)**

$$A = V \times Cp + V \times K \times \int_0^t Cp \times dt$$

Where A is the drug absorbed, V is the volume of distribution, Cp is the plasma concentration, K is the elimination rate constant, and t is time. A slightly simplified gastro transit time equation was integrated in the model [27] to estimate the amount of drug entering the small intestine as a function of time.

$$M = De^{-Ke(t)}$$

Where M is the mass of the drug remaining in the stomach, D is the drug dosed, Ke is the stomach empting rate, and t is the time.

## RESULTS AND DISCUSSION

### Influence of various parameters on particle size and size distribution

The effect of stirring time on particle size was optimized by keeping 50:50 ratio of different diameter (0.5 mm to 0.8 mm and 1.4 mm to 1.8 mm) of zirconium oxide beads and keeping the drug: surfactant: milling media volume (1:3.0:50) constant. Lowest 325 nm mean particle size was achieved after 24 hrs stirring of 50:50 ratios of zirconium oxide beads. Further stirring up to 28 hrs may lead to increased particle size due to increased surface free energy.

Optimized formulation showed mean particle size of 296 nm with Polydispersity index of 0.310 (before lyophilization), with 3.0 % w/v of PVA stabilizer which was used as a stabilizer and 50 % v/v of milling media. After lyophilization a mean particle diameter was found to be 298 nm with Polydispersity index 0.321, so in lyophilization process there was no significant change in particle size and size distribution.

The nature of the stabilizer and its quantity is an important factor in controlling the size and firmness of the nanosuspensions in the process. The results were indicated decreasing the particle size with increasing the concentration of the stabilizer.

The results showed particle size reduced with the increasing of stabilizer concentration as the particle size of a batch which contains 0.1% stabilizer was 623.45±28.44 nm compared with 248.78±11.18 nm for a batch contains 0.8% stabilizer. This could be attributed to the increase in the molar substitution ratio of the polymer per drug. The increase of the hydrophilic corona surrounding the polymer to protect the nanoparticles enhances the stability and prevents particles from coalescence and preventing aggregation (Merisko-Liversidge E et.al., 1996). On the other hand, the particle size increased with the high concentration of PVP K30 which might be due to the higher viscosity of the resulting solution that might hinder particle attrition at the same milling energy. Moreover Ostwald ripening might cause agglomeration, and consequently, higher particle size values resulted (Merisko E et.al., 2003). On the other side, the poly dispersity index (PI) values were ranged from 0.08-0.517 which indicates acceptable uniformity level for most of the preparations (Patravale VB., 2004).

### Zeta potential analysis

Zeta potential of the prepared formulations was observed in the range -9.45 to -18.34. Zeta potential of nifedipine nanosuspensions was relatively low due to the shielding effect of the hydrophilic chains of the polymers used. These chains formed what is called

hydrophilic corona that is surrounding the particles and prevent the true measure for the zeta potential (Saindane., 2013). On the other hand, the importance of the colloidal stability of the nanosuspension is reduced because these formulae will be kept in dry state which is reducing the importance of zeta potential as a controlling factor.

**The percent of the total drug content**

The drug content for the prepared formulae was calculated from the experimental observations. The drug content for all formulae was calculated as a percent of the initially added drug. The amount of the drug within the formulations was more than 88 % in all samples.

***In vitro* dissolution**

The most important feature of nanosuspensions is the increase of the dissolution rate not only because of increase in surface area but also because the use of hydrophilic surfactant. The *In vitro* dissolution of nifedipine was carried out for all of the prepared nanosuspensions formulations and then compared to that of the pure drug powder. The cumulative percentage of the drug dissolved was 97.85 % at 35 min for selected nanosuspension, while the cumulative percentage of the pure drug was 36.46 at 35 min. The difference was significance at $p<0.05$ when t-test for unpaired data was applied, and the release kinetics was found to obey first-order kinetics with $R > 0.98$.

**Process Yield**

The yield of the mass recovered for processed nanosuspensions was determined after lyophilization process and was considerably high (96±3.25%) which indicated efficient processing with minimum batch variability, thus representing a negligible loss of drug during preparation.

**Morphology evaluation**

The morphological characteristics were investigated using scanning electron micrographs (SEM). The SEM image of the drug and nanosuspension showed a significant difference in the morphology of these particles. Nanosuspension sample was appeared to be spherical with the mean particle size of 226 nm. They are having narrow distribution index, while SEM of the drug showed coarse, irregular, more elongated, and within a micro range. The results showed the formation of uniform non-aggregated particles that adsorb the hydrophilic corona around them. Two distinct layers are shown; where the hydrophobic part of the polymer is directed inward the particles and the hydrophilic part of the polymers are directed outward.

**Fourier transform infrared spectroscopy (FTIR)**

FTIR spectra of nifedipine revealed that characteristic peaks of the NH stretching is at 3331 cm-1 and a band with main peak at 1688 cm-1 indicative of the C=O stretch of the esteric group. The above characteristic peaks appear in the spectra of both physical mixtures and formulations of drug with stabilizers. From these results it was confirmed that there was no interaction between the drug and stabilizers used in the nanosuspension formulations. Further characterization was done by DSC.

**Differential scanning calorimetry (DSC)**

The DSC thermogram of pure nifedipine shows a characteristic sharp endothermic melting peak at about 176.7 °C with peak onset at 171.91 °C and peak end at 175.17 °C and the heat of transition was (171 mJ/g). The thermogram of nanosuspension formulation showed endothermic melting peak at 120.5 °C which is close to the expected value for the drug addition, melting enthalpies of endotherm were at a lower-energy state as compared to a crystalline form of the drug. The shift in the drug peak to a lower temperature and the decrease in the area of the peak in the nanosuspension compared to pure drug might be due to smaller drug crystals. Additionally, this decrease in enthalpy value indicates low lattice energy, and it was very well reported that the particles with lower lattice energy are easier to dissolve (Wei L et.al.,2011).

***In vivo* pharmacokinetics study**

A pharmacokinetic study conducted in mice proved that the bioavailability was enhanced when nanosuspension formulation of nifedipine was compared to the market formula. There was a statistically significant difference in the T max, C max, AUC (0-24) and MRT data between the market formula and the nanosuspension optimized formulation. The C max value of nifedipine nanosuspension formulation was significant ($p<0.05$) higher than market formulation. The AUC (0-24) value of nifedipine nanosuspensions after oral administration was almost 2 folds higher than those obtained of the marked formulation.

**CONCLUSION**

Nanosuspensions of nifedipine were prepared successfully by nanoprecipitaion method. The prepared nanosuspenions were found stable with appropriate concentrations of stabilizer. It has been concluded that this methodology is a novel and more reproducible for developing novel drug delivery system for nifedipine to overcome the problem of solubility and to enhance its oral bioavailability. The prepared formulations were found significantly enhanced dissolution characteristics in comparison to the available marketed formulation. Hence nifedipine nanosuspensions proved to be most promising new drug formulation for oral drug delivery with enhanced oral bioavailability.

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