Abstract. The objective of this study was to formulate and evaluate a novel drug solubilization platform (so called nanosuspension prepared by comminution method using High pressure homogenizer; GEAI Niro soavi) and further use the nanosuspension as granulating fluid admixed with excipients for further tablet production. The solid state characterization of Lacidipine along with the excipients reveal compatibility, as confirmed X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR) and Differential scanning calorimetry (DSC). The performance of nanosuspension is highly dependent on the selection of polymers. We have used polymeric suspending agents such as propylene glycol and hydroxy propyl cellulose in the study and evaluated. The results revealed hydroxy propyl cellulose as a suitable stabilizer compared to other polymers. The dissolution studies of Lacidipine shows complete release of drug within 45 minutes using hydroxy propyl cellulose as stabilizer in comparison to available marketed product. Hydroxy propyl cellulose was better adsorbed with the drug compared to other polymers resulting in better mechanical interaction during comminution which causes the drug particle size to reach in nano meter scale. The particle size of the nanosuspension, as confirmed by Zeta revealed a particle size of 293.7 nm. The appearance of the nanosuspension shows a bluish opalescence and the morphology revealed by Transmission Electron Microscope shows a particle size of 200.0 nm. The In-Vivo pharmacokinetic study was performed on rabbits to assess the bioavailability, since the bioavailability is directly proportional to the particle size. The In Vivo pharmacokinetic results revealed that the AUC and Cmax were increased to two and six folds respectively in comparison to conventional market tablet. This may be recognized to increase solubility and permeability of the drug due to this novel formulation approach.

Key words. Lacidipine, nanosuspension, solubility studies, Nanopure® Technology, drug delivery, animal experiments.

Introduction. The objective of the study was to formulate and evaluate BCS class IV cardiovascular drug Lacidipine by nano sized drug suspension. Lacidipine has low solubility in almost all the solvents. Lacidipine belongs to 1,4-dihydropyridine derivatives which are characterized by very poor solubility and low dissolution rate in water. The drug delivery of Lacidipine in new drug delivery form is essential to overcome the low solubility and improve the bioavailability. Several techniques and approaches were commonly used to increase the solubility and bioavailability of Lacidipine such as micro emulsion based transdermal gels as reported by Ganneu R. et al. [1], solid dispersion technique using PVP K 29/32 as reported by Amit Mukharya et al. [2]. Nanosuspension is a novel formulation approach to deliver the drug in nanometer range. The nanosuspension is a unique technique which controls both physic and chemical characteristics of a drug as suggested by Chee Wun How et al [3], Adamson and Gast et al [4], suggests one need to increase the change of Gibbs free energy (ΔG) so as to achieve the objective of reducing the critical size and surface energy perquisite for the development of nano particle. Nanosuspensions are simple, cost effective as reported by Constantinides et al. [5]. The process is feasible in industrial pharmaceutical unit operation. The drug delivery is simple, Lacidipine by nano particles using comminution is an efficient way to overcome the limitation of conventional drug delivery of Lacidipine tablets. Nanosuspension is sensitive to the choice of polymers as well as stabilizer as reported by Lee et al. [6]. The use of surfactants in the nanosuspension aids in decreasing the size of the drug particle. In Nanopure technology (PharmaSol GmbH, Germany), poorly water soluble drugs are transferred to drug nanocrystals via a high pressure homogenization process [7]. The nanosuspensions can be used as granulation fluid for further tablet production by very smart formulation approach. The nanosuspension is admixed to binders and other excipients, and the granules are finally compressed into tablets [8]. In this study we have investigated the formulation technological process and different parameters influencing the performance of nanosuspension. The oral bioavailability of Lacidipine tablets containing nano crystals of Lacidipine was assessed using an animal model.

Material and methods. Lacidipine was obtained as a gift sample from Wockehim Laboratories Ltd, Mumbai. Hydroxy propyl cellulose-L was a kind gift of Aqualon, Signet, Mumbai. Cremophor EL andPEG 20000 was obtained as a gift sample from Sigma Aldrich, Mumbai. Dehydrated ethanol from Merck, Goa. Microcrystalline Cellulose and Lactose Monohydrate from, Signet Mumbai. Butylated hydroxy toluene from Penta manufacturing Co, Mumbai. Magnesium stearate was obtained as a gift sample from Ferro.

Preparation of Lacidipine tablets using Nanopure® Technology. Hydro alcoholic dispersing solvent was taken in a SS vessel, and divided into two equal portions. To one portion surfactant and antioxidant was added under stirring. The drug Lacidipine was added to the other portion under continuous stirring. Both of the portions were mixed under continuous stirring. The suspension was passed through high pressure homogenizer (GEA Niro soavi, Model type NS1001L2K) to get nano sized meter nanosuspension. The nano sized suspension containing nano crystal of Lacidipine was used as a granulating solvent for further tablet production. The nano sized suspension was kept at a temperature range between 2 to 8 °C by ice pack to prevent agglomeration of particles. The hold study was done on nano sized suspension for 72 hours. The initial particle size in the nanosuspension FL4 was found to be 293.7 nm and the size changes to 286.9 nm indicating that there is no significant change in the nano particle during the hold study. The base materials selected for the granulation process were microcrystalline cellulose, Lactose monohydrate, PVP K-25 and Crospovidone CL. The granules were dried followed by lubrication with magnesium stearate. Finally the lubricated granules were compressed into tablets. The pictorial flow chart of the process is displayed in Figure 1, the qualitative and quantitative composition of Lacidipine is displayed in Table 1. Solubility studies. The solubility of Lacidipine was determined by mixing an excess quantity of the drug with approximately 2 ml of the solvent taken in a screw-capped bottle. The bottles were rotated on a rotary shaker (Dolphin, Mumbai) at 100 rpm for 24 hrs at room temperature to attain equilibrium condition. Fourier transforms infrared spectroscopy (FT-IR). Fourier transform infrared spectroscopy was analyzed by Shimadzu, Japan. The drug was weighed and mixed with previously dried potassium bromide KBr. The mixture was carefully ground and the homogenous mixture was placed in an IR pellet die subjected to a pressure of 900 Mpa (9t*cm²) for about 2 minutes to form a disc. The spectral scanning was done in the range of 4000 to 400 cm⁻¹. Differential scanning calorimetry (DSC). The differential scanning calorimeter was done to evaluate the compatibility study of drug Lacidipine with hydroxy propyl cellulose. The binary mixture of Lacidipine and hydroxy propyl cellulose were mixed in the ratio of 1:5 which was subjected to DSC. The sample was heated from 0 to 230 °C at a heating rate of 10 °C/minute under argon atmosphere using a micro calorimeter (DuPont, USA). X-Ray diffraction. To confirm the physical state of the drug in the nanosuspension state, X-ray diffraction was done in an X-ray diffractometer (X’pert, Philips, the Netherlands). The result was observed with a Cu anode used at a voltage of 45 KV at 4Ma. The scan type was continuous and the scan start position was 2.012 to 38.27. The scan step time was 180 seconds. The scattered intensities were measured at ambient temperature and the results were recorded. In-vitro drug release studies. The in vitro drug release from the Lacidipine
tablet 4 mg was carried out in USP II dissolution apparatus with auto sampler containing 500 ml of purified water for a time period of 1 hr. Due to its very low solubility nature of Lacidipine, surfactant i.e. 1% Polysorbate 20 was added in the dissolution media to maintain the sink condition. Dissolution media was maintained at a temperature of 37±0.5 °C. The aliquots of 10 ml dissolution media were auto sampled at specified time points and replaced with fresh media immediately after sampling. Samples were analyzed for drug content by UV Visible spectroscopy at a maximum wavelength of 284 nm.

Particle size and zeta potential.
The Zeta potential and nano particle size distribution of Lacidipine were determined using photon correlation spectroscopy (Zetasizer Nano ZS, ZEN3600; Malvern Instruments, Malvern, UK). The zeta potential was measured using a disposable zeta cuvette. The particle size distribution analysis was performed at angle of 90° degrees at a temperature of 25°C. The zeta potential was measured using a disposable zeta cuvette.

Entrapment efficiency.
Aliquots of quantity 2 ml of the freshly prepared nanosuspensions were centrifuged at 10,000 rpm for 10 min, and the amount of unincorporated drug was measured by UV analysis of the supernatant. The final supernatant was used to measure any unentrapped Lacidipine which might be precipitated in the system.

\[ EE \% = \frac{NL}{TL} \times 100 \]  
[Equ 1]

Where EE is for “Entrapment Efficiency”, NL is for “Nanoparticles Lacidipine” and TL is for “Total Lacidipine”. Moreover Nanoparticles Lacidipine is given by the difference of TL and Lacidipine in supernatant. The final supernatant was used to measure any unentrapped Lacidipine which might be precipitated in the system.

Morphology.
The morphology of the Lacidipine nano particles (FL4) was analyzed using a Transmission Electron Microscope (TEM). The Lacidipine nanosuspension droplet was floated on droplet on to the carbon coated grids on Para film so that the nano crystal of Lacidipine gets fully adsorbed on to the grid. The grid was then dried with filter paper and then dried for 10 minutes. Finally the grid was transferred on to a drop of the negative stain. The negative stain used in these experiments was Uranyl acetate (0.5%). The formulation FL4 was examined with a Jeol Electron microscope to confirm the morphology of Lacidipine.

Table 1. Qualitative and Quantitative composition of Lacidipine Nanosuspension.

<table>
<thead>
<tr>
<th>Sr.no.</th>
<th>Ingredients</th>
<th>Role</th>
<th>FL 1</th>
<th>FL 2</th>
<th>FL 3</th>
<th>FL4</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Lacidipine</td>
<td>API</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>Cremophor ELP</td>
<td>Surfactant</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>HPC-L</td>
<td>Stabilizer</td>
<td>20</td>
<td>-</td>
<td>-</td>
<td>20</td>
</tr>
<tr>
<td>4</td>
<td>PEG 20000</td>
<td>Stabilizer</td>
<td>-</td>
<td>20</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>Polyvinyl pyrrolidone K-25</td>
<td>Stabilizer</td>
<td>-</td>
<td>-</td>
<td>20</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>Butylated Hydroxytoluene</td>
<td>Anti-oxidant</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>7</td>
<td>Purified Water</td>
<td>Dispersion Vehicle</td>
<td>q.s.</td>
<td>q.s.</td>
<td>q.s.</td>
<td>q.s.</td>
</tr>
<tr>
<td>8</td>
<td>Isopropyl Alcohol</td>
<td>Dispersion Vehicle</td>
<td>q.s.</td>
<td>q.s.</td>
<td>q.s.</td>
<td>q.s.</td>
</tr>
</tbody>
</table>

Base Material for granulation

| 9     | Lactose Monohydrate 200M            | Diluent       | 150  | 150  | 150  | 140  |
| 10    | Microcrystalline Cellulose pH 101   | Diluent       | 46   | 46   | 46   | 46   |
| 11    | PVP K-25                            | Binder        | 6    | 6    | 6    | 6    |
| 12    | Crospovidone CL                     | Disintegrant  | 8    | 8    | 8    | 8    |

Extragranular material

| 13    | Aerosil                             | Glidant       | 6    | 6    | 6    | 6    |
| 14    | Magnesium stearate                  | Lubricant     | 6    | 6    | 6    | 6    |

Total tablet weight (mg)

|       |                                      | -             | 250  | 250  | 250  | 250  |

Table 1. Qualitative and Quantitative composition of Lacidipine Nanosuspension.
care and handling. The experiments were carried out on New Zealand White Rabbit of 4 months, of both sexes, weighing between 2.0 to 2.5 kg. They were provided from Sapience Bio-analytical Research Lab and as per CPCSEA guidelines (approval no. 1413/PO/a/11/CPCSEA) under CPCSEA, India. The animals were acclimatized to the standard laboratory conditions in cross ventilated animal house at temperature 25±2°C relative humidity 44–56% and light and dark cycles of 12:12 hours, fed with standard pallet diet, green vegetables and water ad libitum during experiment [10]. Formulations were placed in plastic tube and this tube was attached to a water filled syringe. A wooden biting block with a central opening was placed between the upper and lower teeth of the rabbit and formulations containing tube were inserted in central opening of wooden block. A cannula was inserted into the marginal ear vein for blood sampling and flushed with heparinized normal saline solution. Study design and Sample Processing. Six rabbits were randomly divided into 2 groups, 3 animals each. Groups 1 and 2 received reference tablets (Motens® Tablets 4 mg from Boehringer Ingelheim, US) given po. 3.0mg/kg/day [10]. The Group 2 was subjected to test formulation product (FL4) containing the nanosuspension at the same dose of 3.0 mg/kg/day. Blood samples were taken from ear marginal vein of rabbits at pre-dosing and 0.25, 0.5, 0.75, 1.0, 1.25, 1.50, 1.75, 2.0, 2.25, 2.50, 2.75, 3.0, 4.0, 6.0, 8.0, 12.0, 24.0, 36.0, 48.0 hr for both Groups 1 and 2. Data Analysis. The concentration in plasma of rabbits was measured and the PK parameters were calculated using Win-Nonlin-Pro® software version 4.0.3 (Pharsight Corp-Ro model, US) 2. The area under the plasma concentration curve from time zero to infinity (AUC 0–∞), terminal half-life (t1/2), highest plasma concentration observed (Cmax), and time to Cmax(Tmax) for Lacidipine were determined. Chromatographic Condition. The estimation of Lacidipine from rabbit was performed using Nifedipine as internal standard and finally extracting into absolute ethanol and n-hexane in the ratio of 3:97 solvent system. The resulting solution was centrifuged at 4000 rpm for 7 minutes. The supernatant layer was estimated by HPLC. The chromatographic condition was carried out on a stainless steel column (packed with cyanosil silica gel), using a mixture of 3 volume of absolute ethanol and 97 volume of n-Hexane solvent as mobile phase at a flow rate of 2.0 ml per minute. The pH was maintained at 4.0. The drug was monitored by UV detector at 240 nm at a flow rate of 2.0 ml per minute with a total run time of 15 minutes. This method is previously known method as reported by Ramesh et al. with minor modification [11].

Results and Discussion. Solubility studies. The solubility studies done for Lacidipine in different solvents resulted in a concentration of less than 10 μg/ml. Lacidipine is practically insoluble in 0.1 N HCl, pH 4.5 and pH 6.8 phosphate buffers. From the above study it can be concluded that Lacidipine is poorly soluble in aqueous media. The solvent ideal for the development of nanosuspension using nano pure technique should be water immiscible, hence water and isopropyl alcohol was selected as the choice of solvents. Effect of Stabilizer. The particle size of Lacidipine in the nanosuspension was found to be dependent on stabilizer. The stabilizers used in the formulation FL1, FL2 and FL3 differed primarily in both molecular weight and hydrophilicity. The Specific surface area of the polymer was found to be another crucial factor affecting the particle size of nanosuspension formulation. The greater surface area enhances the tendency of the polymer to better adsorb on the particle surface forming hydrodynamic boundary layer which helps in greater repulsion, steric hindrance and inhibit crystal growth and thus better stability of the nanosuspension. This finding is also in accordance to one of the study reported by Muller et al. [12]. The adsorption of the polymer is influenced by the higher molecular weight. Hydroxy propyl cellulose exhibited highest molecular weight value (MW=7,000–29,000) followed by polyvinyl pyrrolidone K25 (MW=28,000–34,000) and polyethylene glycol (Average Mn is 20). The presence of ether and hydroxyl group of the hydroxy propyl cellulose forms strong adsorption with Lacidipine. Due to high molecular weight, specific surface energy and adsorption of hydroxy propyl cellulose, formulation FL1 was found to perform effective nano comminution. In formulation FL2 due to absence of any hydrophobic unit for adsorption in Polysorbate 80, the formulation was ineffective in nanosuspension. In formulation FL3 polymer PVP proved to be a worst polymer stabilizer due to the presence of amide group. The stabilizer used in the nanosuspension should have hydrophobic moiety so that there is increase reduction in change of Gibbs free energy (Δ G), enhancing the efficiency of formation of nano particles. The effective nanometer size of Lacidipine in the nanosuspension slurry in different formulations can be arranged in the ascending order of PVP<PEG>HPC. The Zeta potential tends to move towards greater negative value with decrease in the particle size. The Polydispersibility index and % transmittance of the formulation trial FL4 is better compared to other formulation trials. The drug content of all the formulations was found to be satisfactory in all the formulation trials. The comparison of the different stabilizer influencing the nanosuspension physiochemical parameters are tabulated in Table 2 and Table 3. Effect of Surfactant interaction. The additional use of surfactant regardless of cationic or anionic affects the particle size of the drug during nano comminution as reported by Park CH et al. [13]. By the results obtained from drug-polymer interaction, HPC used in the formulation FL1 seems to be better stabilizer compared to PEG and povidone. Hence the formulation FL1 with HPC along with surfactant Cremophor EL was evaluated in the subsequent formulation trial. The addition of surfactants appears to have synergistic effect in reducing the nanosuspension slurry to nano meter scale. The formulation FL4 showed a more pronounced reduction in PSD after addition of the Cremophor ELP. The Volume median diameter (d0.9) was further reduced to 20 % after addition of surfactant. The formulation FL4 shows a bluish opalescence after passing through high pressure homogenizer indicating the formation of nanosuspension [14].

Particle size determination. Particle size distribution was carried out by Zeta Sizer. The average cycle maintained was 10 cycles in each of the formulation FL1 to FL4. It was observed that after 10 cycles, the particle size remains unchanged. The average particle sizes of the optimized formulation FL 4 was found to be 293.7 nm and the particle size distribution was determined by Zeta sizer of the optimized nanosuspension in formulation FL4 is represented in Figure 2. Fourier Transformed Infrared (FTIR) Spectroscopic Analysis. The chemical compatibility of Lacidipine with stabilizer
HPC was confirmed by FTIR. The characteristic peak of Lacidipine showed a frequency band of 3347 cm\(^{-1}\), 2979 cm\(^{-1}\), 1675 cm\(^{-1}\) and 1629 cm\(^{-1}\). The group frequency of 3347 cm\(^{-1}\) and 2979 cm\(^{-1}\) corresponds to aliphatic N-H stretch while 1675 cm\(^{-1}\) and 1629 cm\(^{-1}\) corresponds to acetate C=O stretch; and C=C stretch respectively. The characteristic peaks of Lacidipine was found to be 3347 and 1675 cm\(^{-1}\). The FTIR of formulation FL4 showed significant changes in the frequency band of Lacidipine. Both the characteristic peaks shifted from 3347 cm\(^{-1}\) to 3644 and 1675 cm\(^{-1}\) to 1731 cm\(^{-1}\) respectively. The formulation FL4 shows a sharp peak at 744 cm\(^{-1}\) indicating a mono substituted ring. The substitution may have been taken by the ether or hydroxyl group present in the hydroxy propyl cellulose with the amine group present in the Lacidipine molecule. The mechanism is not process degradation rather an hydroxyl bond with the high affinity amine group present in the Lacidipine. (See Supporting Material).

Differential scanning calorimetric (DSC) studies. Compatibility study of Lacidipine and stabilizer hydroxy propyl cellulose was studied with Differential Scanning Calorimeter. The thermogram of Lacidipine exhibited a sharp exothermic peak at 182.95 ºC for Lacidipine \[2\]. HPC Lapicide sample exhibited an exothermic peak at 185.1 °C. It was observed that the onset temperature of Lacidipine in the mixture remains unchanged indicating that the polymer is compatible with Lacidipine. The representative DSC of drug and the excipient are depicted in Figure 3.

XRD. The diffractogram of pure lacidipine shows a characteristic peak at 2θ equal to 7.3 degrees. Lacidipine characteristic peak shifts indicating a change in solid state probably due to the thermal generated by attrition during the process of nano comminution. The other probable reason may be of the interaction of Lacidipine and hydroxy propyl cellulose in molecular level during the comminution process. The representative diffractogram are depicted in Figure 4. HPC and Cremophor do not exhibit any characteristic peaks. In vitro drug release. The reference product (4 mg Tablets), showed incomplete release of drug in 30 minutes. The drug released in 30 minutes was found to be less than 80 %. Whereas, the test formulation with polymer stabilizer HPC in batch No. FL1 showed complete release of drug in 30 minutes. The formulation with polymer HPC along with surfactant Cremophor (FL4) showed complete release of drug compared to reference product as well as FL1. The formulation with polymers such as PVP and PEG showed complete release of drug. It was observed that the release profile of the drug with the polymer PVP and PEG was found to be slower compared to formulation FL1 and FL4. The cumulative dug release Vs time was plotted which is depicted in Figure 5.

In vivo study. The pharmacokinetic parameters for both reference and test formulation are summarized in Table 4. The mean plasma concentrations vs. time graph are depicted in Figure 6. The In vivo bioavailability studies were performed to quantify the nanosuspension in test formulation (T) after oral administration with the marketed reference tablet.
From the Table 2 it can be inferred that the nanosuspension formulation FL4 showed maximum plasma concentration of 6.88 ng/ml while the reference formulation showed only 1.16 ng/ml. The reference formulation took 1.76 hours to reach maximum plasma concentration while the test formulation took 1.0 hours to reach maximum plasma concentration. The AUC 0-∞ for test formulation were nearly 2 fold and 1.5 times respectively than the reference formulation. From the above analysis it is evident that the nanosuspension formulation FL4 showed improved rate of drug release compared to the reference formulations. Since the rate limiting step in the absorption of Lacidipine is dissolution and the results from the study reveals that the nanization of drug in suspension is the rate limiting step which plays a major role for absorption. It can be explained that, following oral administration, nano-crystals disperse spontaneously in the GI due to the increased specific surface area where the active components are present in a solubilized form, and the nano-sized droplets size provides large specific surface area for drug absorption. Additionally high concentration of surfactant used in the nanosuspension may increase permeability of the lipophilic substance across the cell membrane, and lymphatic transport through the trans cellular pathway.

Conclusions. A nanosuspension formulation of Lacidipine was formulated with optimized polymer and surfactant to improve the In Vitro and In Vivo performance of the drug. The important factors concluded in the preparation of Nanosuspension were specific surface area, molecular weight of the polymer, thermal behavior of the drug and the functional groups present in the drug and polymer. HPC proved to be the most successful stabilizer for nanosuspension compared to PEG 20000 and Polymethyl pyrrolidone stabilizer’s studied. From the In vivo bioavailability studies performed on rabbits, it can be concluded that the nanosuspension prepared by high pressure homogenizer exhibits superior pharmacokinetic parameters compared to reference oral conventional tablet. Lacidipine a cardiovascular drug in nanosuspension formulation can overcome the limitation of low solubility, dissolution and bioavailability.

Acknowledgement. The authors wish to thanks and acknowledge Sapiance Bioanalytical Research lab., Bhopal, India for the In-Vivostudy.

Abbreviations. Nanosuspension, comminution, HPC (Hydroxy propyl cellulose), X-ray diffraction(XRD), Fourier transform infrared spectroscopy (FTIR), differential scanning calorimetry (DSC), Transmission Electron microscope (TEM), Stainless steel (SS), Formulation of Lacidipine (FL), PEG (Polyethylene glycol), PVP (Polyvinyl pyrrolidon), Particle size distribution (PSD), MCC (Microcrystalline Cellulose).

References.
7. Möschwitzer J, Müller RH. 2004, from the drug nanocrystal to the final mucosalsheides oral dosage form.  