The Bioscan AN INTERNATIONAL QUARTERLY JOURNAL OF LIFE SCIENCES 20(2): S2: 11-24, 2025 www.thebioscan.com

DESIGN, OPTIMIZATION, FORMULATION AND CHARACTERIZATION OF ATORVASTATIN NANOCRYSTALS

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DOI: https://doi.org/10.63001/tbs.2025.v20.i02.S2.pp11-24

KEYWORDS
Solubility,
Bioavailability,
Nanocrystals,
Atorvastatin,
BCS Class 2,
Lipid lowering agent.
Received on:

12-02-2025

Accepted on:

10-03-2025

Published on:

07-04-2025

ABSTRACT

The most tedious challenge currently faced by formulation scientists is that of formulation of poorly water-soluble drug moieties. According to literature reports, more than 40% of the drugs being introduced into the formulation research pipeline have poor water solubility. Developments of the drug into nanosized formulations have helped overcome the bioavailability problems of many a poorly water soluble drugs belonging to BCS Class II and IV. One category of such nanosized formulations comprise of nanoscaled drug particles stabilized by a suitable stabilizer or surfactant, referred to as "**Nanocrystals."**

Attempts were made to improve the solubility of atorvastatin so as to improve itsoral bioavailability.A very important issue in nanocrystal formulation is the choice of the stabilizers, which are required to prevent nanoparticle aggregation. Pharmaceutical excipients that can be used as stabilizers include the cellulosics, such as hydroxypropyl cellulose (HPC) and hydroxypropylmethyl cellulose (HPMC), povidone (PVP K30), and pluronics (F68 and F127); as well as surfactant stabilizers which can be non-ionic, such as polysorbate (Tween 80), or anionic, such as sodium lauryl sulfate (SLS) and docusate sodium (DOSS).

INTRODUCTION

Atorvastatin, as a synthetic lipid-lowering agent, is an inhibitor of 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase which catalyses the conversion of HMG-Co A to mevalonate, an early rate-limiting step in cholesterol biosynthesis. It is insoluble in an aqueous solution of pH 4 and below, very slightly soluble in water and slightly soluble at pH 7.4 phosphate buffer, slightly soluble in ethanol and freely soluble in methanol. Atorvastatin is readily permeable at the physiological intestinal pH, yet its oral bioavailability following a 40 mg oral dose does not exceed 12% . Thus, attempts were made to improve the solubility of atorvastatin so as to improve itsoral bioavailability.A very important issue in nanocrystal formulation is the choice of the stabilizers, which are required to prevent nanoparticle aggregation. Within this reduced size range, attractive forces between particles appear. These attractive forces arise due to dispersion or van der Waals forces. As particles come close, these attractive forces increase and particle aggregation tends to become irreversible. The added stabilizers act to prevent or minimize such aggregation through steric or electrostatic stabilization. Steric stabilization is achieved by adsorbing polymers onto the particle surface, whereas electrostatic stabilization is obtained by adsorbing charged molecules, which can be ionic surfactants or charged polymers, onto the particle surface. Pharmaceutical excipients that can be used as stabilizers include the cellulosics, such as hydroxypropyl cellulose (HPC) and hydroxypropylmethyl cellulose (HPMC), povidone (PVP K30), and pluronics (F68 and F127); as well as surfactant stabilizers which can be non-ionic, such as polysorbate (Tween 80), or anionic, such as sodium lauryl sulfate (SLS) and docusate sodium (DOSS).

Formulated nanocrystals can be characterized through a number of tests including particle size determination as well as surface charge measurement (zeta potential), particlecrystallization and dissolution. In this study, we aim at improving the poor water solubility of atorvastatin calcium, and hence its bioavailability, through size reduction to the nano-size range. The high-pressure homogenization technique has been employed using a variety of stabilizers at various concentrations.

AIM AND PLAN OF WORK

The most tedious challenge currently faced by formulation scientists is that of formulation of poorly water-soluble drug moieties. Advancement in high throughput screening methods is mainly responsible to an even larger number of newly discovered drugs having poor water solubility. According to literature reports, more than 40% of the drugs being introduced into the formulation research pipeline have poor water solubility. Development of the drug into nanosized formulations have helped overcome the bioavailability problems of many a poorly water soluble drugs belonging to BCS Class II and IV. One category of such nanosized formulations comprise of nano scaled drug particles stabilized by a suitable stabilizer or surfactant, referred to as **"Nanocrystals"**.

Atorvastatin, as a synthetic lipid-lowering agent, is an inhibitor of 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase which catalyzes the conversion of HMG-Co A to mevalonate, an early rate-limiting step in cholesterol biosynthesis. It is insoluble in an aqueous solution of pH 4 and below, very slightly soluble in water and slightly soluble at pH 7.4 phosphate buffer, slightly soluble in ethanol and freely soluble in methanol. Atorvastatin is readily permeable at the physiological intestinal pH, yet its oral bioavailability following a 40 mg oral dose does not exceed 12%. Thus, attempts were made to improve the solubility of atorvastatin so as to improve itsoral bioavailability.A very important issue in nanocrystal formulation is the choice of the stabilizers, which are required to prevent nanoparticle aggregation. Within this reduced size range, attractive forces between particles appear. These attractive forces arise due to dispersion or van der Waals forces. As particles come close, these attractive forces increase and particle aggregation tends to become irreversible. The added stabilizers act to prevent or minimize such aggregation through steric or electrostatic stabilization. Steric stabilization is achieved by adsorbing polymers onto the particle surface, whereas electrostatic stabilization is obtained by adsorbing charged molecules, which can be ionic surfactants or charged polymers, onto the particle surface. Pharmaceutical excipients that can be used as stabilizers include the cellulosic, such as hydroxypropyl cellulose (HPC) and hydroxypropyl methyl cellulose (HPMC), povidone (PVP K30), and pluronics (F68 and F127); as well as surfactant stabilizers which can be non-ionic, such as polysorbate (Tween 80), or anionic, such as sodium lauryl sulfate (SLS) and docusate sodium (DOSS). Formulated nanocrystals can be characterized through a number of tests including particle size determination as well as surface charge measurement (zeta potential), particlecrystallization and dissolution. In this study, we aim at improving the poor water solubility of atorvastatin calcium, and hence its bioavailability, through size reduction to the nano-size range. The high pressure homogenization technique has been employed using a variety of stabilizers at various concentrations.

EXPERIMENTAL METHODOLOGY

Materials

Atorvastatin calcium (ATC) was obtained as a gift sample from Shasun Pharmaceuticals, Pondichery (India). All other chemicals and reagents were of analytical grade purchased from SD fine chemicals, Mumbai. FT-IR spectra were taken using Shimadzu FT-IR-8700 spectrophotometer; DSC thermograms were obtained by differential scanning calorimeter (DSC 60; Shimadzu) where as XRD patterns were recorded using Philips diffractometer (PW3710; Almelo, Netherland).

Melting Point Determination

Melting point was determined by using glass capillary method. The Thiesle tube filled with paraffin oil was used, in to which a capillary containing the drug was placed along with the thermometer. The paraffin oil was then heated continuously till the powder melts and corresponding temperature was noted and recorded as melting point of drug.

Standard Calibration Curve of ATC

Standard Calibration Curve of ATC was carried out in Phosphate buffer (pH 6.8)

Procedure

Accurately weighed 10 mg of ATC was dissolved in required volume of methanol and volume was made up to 100 ml by using Phosphate buffer (pH 6.8) to get 100 µg/ml stock solution. From the above stock, serial dilutions were made by withdrawing 1, 2, 3, 4 and 5 ml solution and diluting up to 10 ml with the same stock solution to get 10, 20, 30, 40 and 50 µg/ml solutions. The solutions were analysed by UV-Spectrophotometer to find the maximum absorption at a particular wavelength. The λ max was found to be 241 nm. Finally, a graph of absorbance vs concentration was plotted.

METHODS

Preparation of nanocrystals [152-154]

Table 1 gives the composition (ratio by weight) of the prepared nanocrystal formulations, using the surfactants sodium lauryl sulphate (SLS) and Tween 80 , as well as the polymers HPMC and HPC .The formulations were prepared by sonoprecipitation technique which involves the mixing of 200 mg of drug with 10ml of methanol and the weighed amounts of stabilizers in 10 ml of water. The drug solution is added with the stabilizers which is suspended in water and these suspensions were mixed and sonicated (Elmasonic S 30 H, Germany) for half an hour to break any agglomerated powder. This pre-milling process was followed by exposure to high pressure homogenization (Standsted SPCH-10-Pressure Cell Homogeniser, UK) for 10 cycles at a pressure of 1000 bar. The resulting suspensions were lyophilized (Savant Novalyphe-NL500, USA) to obtain dry powder .

Determination of Drug content

Sample containing 10 mg equivalent of Atorvastatin Calcium nanocrystals are weighed and dissolved in methanol, and the volume is made upto 100ml with phosphate buffer (pH 6.8). From the above solution 10 ml is pipetted out and made upto 100 ml with phosphate buffer (pH6.8). The absorbance of resulting solution is determined atλmax (241 nm) using Double- beam UV Spectrophotometer (UV-1700 Shimadzu co-operation, Japan) and estimated by its drug content.

EVALUATION OF PREPARED FORMULATIONS

Entrapment efficiency:

The nanoparticles were separated from the aqueous medium by ultracentrifugation at 10,000 rpm for 30 min at 5° C. Then the resulting supernatant solution was decanted and dispersed into phosphate buffer (pH 6.8). Thus the procedure was repeated twice to remove the unentrapped drug molecules completely. The amount of drug entrapped in the nanoparticles was determined as the difference between the total amount of drug used to prepare the nanoparticles and the amount of drug present in the aqueous medium.

$$\text{Entrapment efficiency (\%)} = \frac{\text{Amount of drug released from the lysed nanocrystals}}{\text{Amount of drug initially taken to prepare the nanoparticles}} \times 100$$

Determination of particle size and zeta potential of formulations

Appropriate dilutions of formulations using deionized water were prepared and then these dilutions were examined for their size and zeta potential using a zetasizer (Malvern Zetasizer Nano ZS, UK). The values were compared to those of the drug and a statistical analysis was carried out using a One-Way ANOVA (Post Hoc test used is the LSD) at $p < 0.05$, using SPSS 16 program.

Determination of the saturated solubility of formulation

Saturated solubility of the prepared formulations, the drug as well as the lyophilized drug was determined in water. An excess of the dried powder formulations was suspended in a fixed volume of water and was shaken in a constant temperature water bath (Stuart SBS 40, UK) at 100 rpm at 37 ± 0.5 °C for 48 h (till equilibrium solubility was attained). At the end of the period, the samples were ultra-centrifuged at 15000 rpm (Megafuge 1.0R, Heraeus, Germany) for 15 min to remove the excess solid, appropriately diluted, and the concentration of atorvastatin was determined by UV spectrophotometry at 241 nm. The concentration of the dissolved atorvastatin was calculated using the equation derived from the built calibration curve of the drug in distilled water. The formulation of the highest solubility was compared to that of a physical mixture of the same drug to stabilizer ratio. The experiment was repeated three times for

each formulation. A statistical analysis was carried out using a One-Way ANOVA at $p < 0.05$ to compare the results.

In-vitro dissolution studies

Lyophilized powders, equivalent to 25 mg of atorvastatin were weighted accurately and filled in 00# gelatin capsules. The dissolution rate of pure Atorvastatin nanocrystals were studied in 900 ml of phosphate buffer (pH6.8) using USP type II (Paddle type) dissolution test apparatus with a Paddle stirrer at 50 rpm. A temperature 37±0.5ºC was maintained throughout the study. Drug or nanocrystals equivalent to 25 mg of Atorvastatin was used in each test. Samples of dissolution media (5ml) were withdrawn through a filter (0.45µ) at different intervals of time, suitably diluted and assayed at 241 nm. The samples of dissolution fluid withdrawn at each time were replaced with 5 ml of fresh fluid.Each formulation was repeated three times. The dissolution profile of the plain drug was also determined, as well as that of the physical mixtures of formulations which possessed the highest dissolution rates. The data of the release experiments obtained at 30 min were compared by determination of the dissolution efficiency (%D.E) and a statistical analysis was run using a One-Way ANOVA test (Post Hoc test: LSD).The dissolution efficiency was calculated according to the following equation.

Dissolution efficiency (DE) = t 0∫y.dt / y100 t* 100

Release Kinetics:

Data obtained from *in vitro* release studies were fitted to various kinetic equations. The kinetic models used are zero order equation ($Q=k_0t$),First order equation { ln (100 – Q) = ln Q – k1t},Higuchi equation (Q= kt 1/2), Hixson and Crowell model Qt 1/3 Vs t and Qt 2/3 Vs t -Modified root cube equation. Further, to find out the mechanism of drug release, first 60% drug release was fitted in Korsmeyer and Peppas equation (Q = kpt n). Where, Q is the percent of the drug release at time t and k0 and kt are the coefficients of the equations and n is the release exponent. The n value is used to characterize different release mechanism.

Wettability study [155-157]

The pure drug and formulations were subjected to wettability studies by the Buchner funnel method and water absorption method. In the first method, the pure drug and formulations of about 100 mg were weighed and placed in a Buchner glass funnel. The funnel was plunged into a beaker containing water in a manner that the beaker remains at the same level as the powder in the funnel. Methylene blue powder (100 mg) was layered uniformly on the surface of the powder in the funnel. The time required for wetting methylene blue powder was measured.

Permeation study [155-157]

The permeation study of the pure drug, and optimized nanocrystal drug was carried out using two different membranes, namely egg membrane and cellulose nitrate membrane. The diffusion of the drug through the membranes was analyzed in a diffusion cell. The required length of membrane was cut and attached to the ground bottom layer of the diffusion cell with glue. The cell (donor compartment) was marked for its 10 ml content and was dipped inside a beaker (receptor compartment) containing phosphate buffer pH 6.8. 10 ml of the buffer was added to the donor compartment. It was noted the level of the buffer inside the cell and in beaker was of same level. Weighed amount of the pure drug and formulations equivalent to specified quantity of the drug was added to the cell. The samples at predetermined time intervals were withdrawn and the same volume of fresh buffer was replaced immediately to maintain sink condition. The study was carried out for 1h. The solutions were suitably diluted and the absorbances were measured using UV spectrophotometer at 241 nm.

For the successful fabrication of a nanocrystal formulation, besides selection of the appropriate excipients, equally important is the characterization of the formulation to ensure that the necessary parameters responsible for the performance of nanocrystals are within the specified limits. The following sections discuss in detail the various characterization tests for the evaluation of nanocrystals.

X-ray Diffraction Analysis, Differential scanning calorimeter (DSC) and Fourrier transform infrared spectroscopy (FTIR)

The formulation that proved to possess the best results in the above investigations was tested for the crystalline properties and compared to atorvastatin calcium powder (XRD, X'pert pro, Pan Analytical, Netherland) over a range of 2θ from 5o to 50o with Ni-filtered Cu-Ka radiation. The scan speed was 3 degree. min^{-1}. Differential scanning calorimetric examinations of both the plain drug and the successful formulation were done. The samples were heated at a constant rate of 10 ºC/min, in an atmosphere of nitrogen over a temperature range of 20-250 °C using DSC-50 (Shimadzu, Kyoto, Japan). Similarly, The IR spectra of the pure drug, the successful formulation and its physical mixture were recorded using Infrared spectrophotometer (Shimadzu IR-345-U-04, Japan).

Scanning electron microscopy

The best formulation was examined for its surface properties and compared to atorvastatin calcium powder (Jeol-JSM 5200 Scanning Microscope, Japan).

Short-term stability studies

A short-term stability study was performed on Optimized formulation.. The study was carried out at room temperature and at 4°C for 7 days. The vials containing ATC nanoparticles suspensions were sealed and wrapped in aluminum foil and subdivided into two groups. One group is stored in refrigerator at 4°C and the other group is stored at room temperature 25°C for 7 days. At the predetermined time intervals, aliquots were taken and subjected to particle size and % drug entrapment studies as described above. The change in appearance (presence of aggregates), particle size, span value, and % drug entrapped were recorded and compared to results obtained from freshly prepared nanoparticles.

RESULTS AND DISCUSSION

UV- Spectra (λ max) of ATC in Phosphate buffer(pH6.8)

UV- spectra (λ max) of ATC was carried out inPhosphate buffer(pH6.8).The λ max was found to be 241 nm. The UV-spectra is shown in **Fig.1**.

Standard Calibration Curve of ATC

Standard calibration curve of ATC was carried out in Phosphate buffer(pH6.8) . The readings from calibration curves in Phosphate buffer(pH6.8) was shown in Table 2 and . Calibration curves in Phosphate buffer(pH6.8) was shown in **Fig.2**.

Melting Point Determination

Melting point was determined by using glass capillary method. The melting point of ATC was found to be 179^{0}C. The obtained nanocrystals were examined according to the previously named tests and the results are given in table no:3.

Particle size and zeta potential

Size and size distribution are important characterizations of the nanosuspensions because they direct the other properties, such as physical stability, saturation solubility and dissolution velocity, and even clinical efficacy. The smaller the particle size, the higher the surface energy of the particles,which promotes aggregation. The most frequently used techniques for particle size measurements of nanosized systems are dynamic light scattering techniques, static light scattering techniques and microscopy. The mean particle size of nanosuspensions is typically analyzed by dynamic light scattering also known as photon correlation spectroscopy (PCS). It has advantages of yielding accurate results and fast and easy measurement. However, this technique is not feasible to analyze particles larger than 6µ. Apart from the mean particle diameter, PCS can also yield the width of the particle size distribution, referred to as the "polydispersity index" (PI). The PI value ranges from 0(monodisperse particles) to 0.500 (broad distribution), and is a crucial index that governs the physicalstability. For a long-term stability the PI should be as low as possible (**Table 4**).

Zeta Potential Analysis

The surface charge of the particles is one of the factors influencing the physical stability of nanosuspensions. The higher the particles are equally charged, greater is the electrostatic repulsion between the particles and greater is the physical stability. The particle surface charge is ideally quantified in terms of the "zeta potential", which is measured via the electrophoretic mobility of the particles in an electric field. The particle charge can be measured in surface charge per unit, determined by colloid titration. The zeta potential is determined by measuring the electrophoretic particle velocity in an electrical field. During the particle movement the diffuse layer is shed off, hence the particle acquires a charge due to the loss of the counter ions in the diffuse

layer. This charge at the border of the shedding is termed as the zeta potential. According to the literature, a zeta potential of at least -30 mV for electrostatic and -20 mV for sterically stabilized systems is desired to obtain a physicallystable nanocrystal suspensions. The upper limit for the zeta potential is +30 mV. The investigated nanosuspension showed a value of about ±33mV which indicates good stability of the prepared formulation (**Table 4).**

The obtained formulations showed the following particle size and zeta potential recorded in Table 6.The nanoparticles showed a significant change in particle size compared to the untreated drug with the exception of formulation 11 which did not significantly differ from the drug.As for statistical analysis of the zeta potential, it was found that the drug has a significantly different zeta potential compared to all formulations

The permeation study through egg membrane was done as per the method reported by Mehdi Ansari *et al*. and Giovanna Corti *et al*. The data for egg membrane and cellulose nitrate membrane was given in **(Table 5)**. It was observed that the amount of drug permeated in both membranes was found to be higher for the nanocrystals than the pure drug. Permeation through cellulose nitrate membrane shows better result when compared with egg membrane. The results can be considered as the evident for increase in release rate of the drug.The Buchner funnel method and water absorption method for finding out wettability were investigated as per the method reported by M. C. Gohel *et al*. and Sunil Kumar *et al*. respectively and findings are shown in Table 5. The wetting time and water absorption ratio of the pure drug was found to be 80 min, indicating its poor wettability. The wetting time of prepared nanocrystals found to be very less (30 min) than pure drug indicating its higher wettability.

Saturated Solubility Determination:

The obtained results for the saturated solubility of the twelve formulations, drug and lyophilized drug are listed in Table 3.It was clear that the saturated solubility of formulation 3 was significantly higher than that of the drug, lyophilized drug, the rest of the formulations as well as its physical mixture at $P < 0.05$(**Table 6**).

Thermodynamic solubility implies the solubility of the most stable crystalline form of the drug in a given medium at a specified pressure and temperature. Solubility can temporarily be higher than the thermodynamic solubility. This may be observed with amorphous forms, metastable polymorphic forms, or nanosized drug particles. This enhanced solubility has been designated with diverse terms,such as kinetic or apparent solubility. Since apparent solubility of nanosized particles is higher thanthe thermodynamic solubility of the material, dissolution of nanocrystalline material is likely to lead to a supersaturated solution. This is termed as the "spring effect". Eventually, precipitation may sooner or later occur until the concentration is equivalent to the thermodynamic solubility. Furthermore, changes in the composition and pH of the solution such as in the gastrointestinal tract will affect solubility and hence the tendency for crystallization. Therefore, the supersaturated state should be maintained and precipitation hindered to enhance in vivobioavailability. Some polymers, such as polyvinyl pyrrolidone (PVP), methacrylate co-polymers, hydroxypropyl methylcellulose (HPMC), and hydroxypropyl methylcellulose acetate succinate(HPMC-AS) are effective at maintaining (or at least helping to maintain) supersaturation. This is known as the "Parachute effect".

The parachute effect of the polymer can be due to a combination of mechanisms. First,the polymers can themselves increase the thermodynamic solubility of the drug (also termed as the co-solvency effect), which lowers supersaturation and consequently the thermodynamic driving force for crystallization (this also leads to an additional spring effect with the polymer). Through drug-polymer interaction in solution via electrostatic bonds, van der Waals' forces or hydrogen bonding, even the addition of small amounts of polymers such as PVP and HPMC to solution can significantly increase the aqueous solubility. Second, polymers adsorbed on solid surfaces (e.g., with nanocrystals) can block the interaction of already dissolved drug molecules with crystal surfaces and thereby crystal growth. Electrostatic bonds, van der Waals' forces or hydrogen bonding can all influence the interaction between the polymer and crystal faces, and therefore the degree of crystal growth inhibition. Moreover, the viscosity of the polymer solution may also inhibit the diffusion of the molecules, which limits crystal growth.

Dissolution Studies

The following **Figure 4**show the percentage release from the SLS, Tween 80 and HPMC, HPC formulations, respectively, compared to both the drug, lyophilized drug and the physical mixture of the formulation which showed the highest dissolution rate. The figures showed a significant increase at $p < 0.05$, in the percentage of the drug released after 30 min for the nanocrystal formulations (1-12) compared to the drug, the lyophilized drug and the physical mixtures. The lyophilization did not significanlty affect the dissolution rate of the drug, where after 120 min the percentage of the drug released did not exceed 32%. The nanocrystal formulations of SLS approached 100% release after ten min. They were also significantly higher compared to that of (drug: SLS 10:1) physical mixture. Tween 80 formulations showed an increase in the percentage released as the amount of added Tween 80 increased in the formulation. As for HPMC and HPC formulations, it was clear that both were superior to the drug and its lyophilized form, yet HPC non significantly improved the dissolution rate compared to HPMC after 30 min from the beginning of the experiment. The formulations also showed an increase in the percentage released compared to the physical mixtures.

Table 8 shows the results for the %D.E. for the plain drug, lyophilized drug, physical mixtures and the twelve formulations. The twelve prepared formulations possessed higher values compared to the plain, lyophilized drugs as well as the physical mixtures. The solid state properties (polymorphic crystal form, solvate (especially hydrate) form, degree of crystallinity) influences the apparent solubility and thereby the dissolution rate. Hence, it is crucial to determine these characteristics in nanocrystals. Ideally, thermodynamically most stable crystalline form is desirable to prevent the peril of solid state transformations during production, storage and/or administration. To increase the dissolution and bioavailability of nanocrystals, it is preferable to formulate the nanocrystals in a metastable crystalline form or even prepare the amorphous equivalent of nanocrystals. However, this is not being practiced commonly.

Different nanocrystal manufacturing conditions and procedures can have an impact on the ensuing solid state form. Furthermore, the environmental conditions affect the thermodynamically stable polymorphic form. For e.g., hydrate forms are generally more stable (and therefore less soluble) in aqueous media. Therefore if the drug is susceptible to hydrate formation, then the potential or factors triggering the said conversion should be extensively investigated during stability studies in different conditions . X-ray powder diffraction (XRD), thermal analytical techniques (differential scanning calorimetry, thermogravimetry, etc.) and vibrational spectroscopy (Infrared and Raman) are the most commonly used methods to determine and monitor the solid state form of nanocrystals.

The data obtained from the first 90% release were fitted to various kinetic equations todetermine the mechanism of drug release and release rate as indicated by higher correlation coefficients (r2), the drug release from nanoparticles followed zero order equation and Higuchi model diffusion controlled rather than the first order. Hixson- Crowell and modified cube root equation showed poor correlation.The formulation F3 showed zero order release kinetics, and F11, F14 did not fitted for any other release kinetics. But, release kinetics of F7 indicated that it follow **zero order and Higuchi model** rather than first order.These findings indicated that the drug release from formulated nanoparticles were diffusion controlled(**Table 9)**

X-Ray Diffraction Analysis

X-ray diffraction studies are usually performed for the confirmation of drug crystallinity following its conversion to a nanocrystal formulation. When X-rays interact with a crystalline substance, a diffraction pattern is obtained. Every crystalline substance gives a specific pattern; the same substance always yields the same pattern; and in a mixture of substances each produces its pattern independently of the others. The X-ray

diffraction pattern of a substance therefore represents the unique fingerprint of the substance.

The following **Figure 5** shows the Xray diffraction picture of both the drug and formulation 3 which showed an acceptable release pattern and the highest saturation solubility.The Figure shows that both the drug and Formulation 3 exhibited diffraction peaks characteristic for crystalline Atorvastatin Calcium at the 2θ degree values of 9.37, 11.76, 12.10 and 16.96°. Some additional peaks were observed at 2θ of 6.0169 and 21.9 for formulation 3. Generally, the intensity of peaks was much decreased for formulation 3.The XRD pattern of pure drug ATC produced several diffraction peaks at 2θ=17.078 19.43, 21.58 indicating the crystalline nature of ATC. The XRD of nanocrystals exhibited crystallinity with reduced intensity as compared to pure ATC. X-ray diffraction analysis showed retention of the crystalline state of the drug after high pressure homogenization, since the diffraction pattern was preserved. The additional peaks observed for the formulation 3 are characteristic peaks for SLS which is adsorbed on the surface of crystals (32). Also the observed decrease in the intensity of the peaks for formulation 3 was dueto the decrease of particle size compared.

DSC thermogram

Differential scanning calorimetry (DSC) is one recurrently used method for studying the thermal behaviour of drug and drug nanocrystals. The DSC studies are performed to check the status of crystallinity of drug and interaction of excipients and drug after production of nanocrystals. This is especially important for drugs occurring in different polymorphic forms. Moreover, certain top-down techniques like the high pressure homogenization can lead to particles with an amorphous fraction, thus leading to enhancement of saturation solubility. The DSC of pure drug, physical mixture of drug and excipients (stabilizer) and final formulation which may be in dried form is done.**Figure 6** shows the DSC of both the drug and F3. The drug showed a sharp peak at about 157 °C. For Formulation 3, a peak is observed at 153 °C.

The DSC thermogram of ATC showed an endothermic peak at 172^0C, corresponding to its melting point. This peak indicates the crystallinity of ATC. Only one sharp peak was observed which confirmed that ATC is free from impurities. The DSC thermogram of the nanocrystals involving ATC showed endothermic peak at lower temperature i.e 169 ^{0}C compared to the peak of pure ATC at 172 ^{0}C.This may be attributed to uniform dispersion of ATC into the carrier, maltose. In the DSC, the observed reduction in the endothermic peak of formulation 3 indicates possibly transformation to a more amorphous form of the drug which exhibits polymorphism.

FTIR studies

Chemical properties of drug and interaction with excipients are evaluated by FT-IR studies.**Figure7** shows the FTIR spectroscopicanalysis of the drug, formulation 3 and the physical mixture corresponding to formulation 3. The pure drug showed characteristic peaks at 2941 cm^{-1}(C-H - stretching), 1317 cm^{-1}-(C-N - stretching), 3055 cm^{-1}(C-HO-stretching alcoholic group), 1651 cm^{-1}(C = C-bending), 746 cm^{-1}, 690 cm^{-1}(C-F-stretching), 1109 cm^{-1}(O-H-bending) (26). It is clear that the main drug peaks are retained in both the physical mixture as well as the nanocrystals of the drug indicating no possible interaction between the drug and the stabilizer

Scanning electron microscopy

Ideally, the shape or morphology of the nanocrystals can be determined using a transmission electron microscope (TEM) and/or a scanning electron microscope (SEM). A wet sample of suitable concentration is needed for the TEM analysis. When the formulated nanosuspensions are to be converted into a dried powder (e.g., by spray drying or lyophilization), a SEM analysis is crucial to monitor alterations in the particle shape and size before and following the process of the water removal. Principally, agglomeration may be observed following water removal, leading to an increase in the particles size. Such changes and more can be observed through SEM.

The surface morphology before and after the cationic charge nanonization of ATR was examined using scanning electron microscopy. The scanning electron microscopy images of nanocrystal formulations revealed rough scaffold-like structures with high surface area. The particle size was greatly reduced with smooth surfaces by probe sonication when compared to unmodified ATR or pure drug. The crystals of ATR formulation were different from others and the particles were greatly reduced in size with high surface area indicating multiple scaffold-like structures. This could be the major reason for enhanced aqueous solubility of ATR when compared to other nanocrystal formulations (**Fig:8)**

One of major obstacles that limit the use of nanoparticles is their instability in an aqueous medium and frequently particles aggregation is noticed after long term storage of such systems. Furthermore, drug leakage out of the nanoparticles and non-enzymatic polymer hydrolysis can happen. Formulae F3, F7, F11, and F14 were stored at room temperature and 4°C for 7 days. Results from particle size analysis showed no significant difference in the particle size for formulae F3 and F11 at room temperature and 4°C (p= 0.45 and p= 0.08 respectively) while formulae F11 and F14 showed significantly larger particle size when compared to as before storage (p < 0.05) at both temperatures but the increase was higher at room temperature. This could be due to Ostwald ripening which is known to be highly dependent on temperature . Results also showed that the EE was significantly increased after 7 days storage at room temperature for formulae F11 and F14 (p< 0.05) while formulae F3 and F7 showed no significant difference in the % drug entrapped. The observed increase in EE is in accordance with published data reporting drug adsorption on the surface of the particle increases as the particle size increases. Table no:10 shows the results obtained from stability studies.

CONCLUSION

Drug absorption is directly related to both solubility and permeability and inversely related to lipophilicity. Dissolution from nanocrystals is followed by permeation of the dissolved drug across the gastrointestinal wall (in the same way as drug from a solution formulation). Besides increasing permeation of the drug due to elevated dissolved concentrations, stabilizers present in the formulation themselves interact with cells of the epithelial layers to promote permeation.Hence, Atorvastatin calcium, the lipid lowering agent, is taken as a model drug characterized by poor water solubility and bioavailability. In this study an attempt was made for preparation of nanocrystals using sonoprecipitation method. A number of stabilizers were included as well as polymers at different concentrations, and the formulations were homogenized for ten cycles at a pressure of 1000 bars. The obtained nano crystals were evaluated by determining their size, zeta potential, saturated solubility and dissolution rate. Results revealed that Formulation 3, containing (10: 1) drug to sodium lauryl sulphate ratio, possessed the highest saturated solubility and dissolution rate, and hence was analyzed by X-ray diffraction analysis, differential scanning calorimetry, Fourrier transform infrared spectroscopy and scanning electron microscopy. The increase in drug dissolution rate and solubility can be expected to have a significant impact on the oral bioavailability of the drug. This study demonstrated the usefulness of the sonoprecipitation technique as a method of enhancing the dissolution of poorly soluble drug-like AC(Atorvastatin Calcium)

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TABLES

Table 1: Composition of the Prepared Nanoparticle Formulations

Formulation	Composition
1	20: 1 Drug :SLS
2	15: 1 Drug :SLS
3	10: 1 Drug :SLS
4	5: 1 Drug :SLS
5	20:1 Drug: Tween 80
6	15:1 Drug: Tween 80
7	10:1 Drug: Tween 80
8	5:1 Drug: Tween 80
9	1:1 HPMC : Drug
10	0.5:1 HPMC : Drug
11	1:1 HPC: Drug
12	0.5:1 HPC: Drug

SLS: Sodium lauryl sulphate; HPMC: hydroxypropyl methyl cellulose;
HPC: hydroxypropyl cellulose

Table2: Readings from Calibration Curve in Phosphate Buffer (pH6.8)

Concentration (µg/ml)	Absorbance (at 241 nm)
5	0.212
10	0.422
15	0.612
20	0.805
25	1.015

Table 3: Drug Content and Entrapment Efficiency of Prepared Nanocrystals

Sl No	Formulation Code	Drug Content	Entrapment Efficiency
1.	F1	74.43	39.02
2.	F2	76.21	40.18
3.	F3	78.01	52.04
4.	F4	80.25	54.10
5.	F5	67.65	12.46
6.	F6	65.23	22.45
7.	F7	62.10	14.50
8.	F8	60.72	25.20
9.	F9	89.04	58.52
10.	F10	90.48	63.45
11.	F11	93.72	74.20
12.	F12	91.02	66.56
13.	F13	83.50	44.20
14.	F14	79.43	48.32
15.	F15	86.58	51.22
16.	F16	88.53	62.31

Table 4: Particle Size and Zeta Potential Values of Prepared Formulations Compared to the Drug

Formulation	Particle size (nm)	Zeta potential (mv)
1	583.7(±11.3)	-24.8(±1.5)
2	574.2(±10.04)	-23.2(±0.141)
3	546.1(±11)	-25.7(±0.42)
4	593.1(±9.26)	-26.2(±0.9)
5	611(±6.36)	-7.52(±0.97)
6	539.2(±10.04)	-26.1(±0.97)
7	502.5(±2.47)	-25.4(±1.48)
8	231.7(±12.9)	-27.9(±1.6)
9	931.9(±12.09)	-11.4(±0.63)
10	1404(±10.6)	-3.56(±1.08)
11	709.6(±7.35)	-11.1(±0.6)
12	970.2(±0.46)	-12.4(±0.29)
Drug	708.4(±8.2)	-15.4(±3.1)

Table 5: Permeability and wettability data

Formulation	Permeability (mg/ml/h)		Wettability
	Egg membrane	Cellulose nitrate membrane	Buchner FunnelMethod (min)
PureDrug	0.0030	0.0137	80±0.5
Optimized formulation	0.0103	0.0328	30±0.2

Table 6: Saturated Solubility of Formulations

Formulation	Saturated Solubility (µg/mL)
F1	78.55(±2.97)
F2	93.28(±12.9)
F3	383.95(±4.27)
F4	383.95(±4.27)
F5	79.98(±1.43)
F6	182.78(±3.82)
F7	73.16(±1.29)
F8	76.14(±2.02)
F9	79.25(±6.89)
F10	60.35(±1.66)
F11	71.18(±1.28)
F12	78.75(±0.87)
puredrug	43.67(±4.24)
Lyophilized drug	68.804(±0.85)
Physical Mixture of F3	154.294 (±0.71)

Table 8: Dissolution Efficiency of Nanocrystal Formulations Compared to the Plain Drug

Formulation	% D.E.
1	33.791
2	29.881
3	30.399
4	24.567
5	25.647
6	18.246
7	33.727
8	36.358
9	30.411
10	29.106
11	25.669
12	25.125
Drug	11.095
Lyophilized drug	7.418
Physical mixture of Formulation 03	15.073
Physical mixture of Formulation 08	12.183
Physical mixture of Formulation 09	21.655
Physical mixture of Formulation 11	23.657

Table 9: Parameters of the Model Equations Applied to the Release of Atorvastatin from Selected Nanocrystal Formulations

Formulation	Model	r2	Slope	K
F3	Zero order equation	0.9902	1.1603	0.9902
	First order equation	0.9188	0.0401	0.9585
	Higuchi model	0.9880	4.902	0.9902
	Peppas	0.8863	0.5891	1.1972
	Hixson-Crowell	0.9769	0.0567	2.047
F7	Zero order equation	0.9841	1.1885	0.9920
	First order equation	0.9480	0.0299	0.9737
	Higuchi model	0.9839	5.0019	0.9919
	Peppas	0.8848	0.4356	0.9839
	Hixson-Crowell	0.9658	0.0429	2.3753
F11	Zero order equation	0.9474	0.9733	1.139
	First order equation	0.8878	0.9422	0.032
	Higuchi model	0.9307	0.9647	4.752
	Peppas	0.8867	1.0498	0.4752
	Hixson-Crowell	0.9513	2.2859	0.0452
F14	Zero order equation	0.9685	0.9641	1.499
	First order equation	0.9470	0.9731	0.0241
	Higuchi model	0.9454	0.9723	6.234
	Peppas	0.8721	0.7541	0.3649
	Hixson-Crowell	0.9741	2.7924	0.041

Table 10: Particle Size and EE of ATC Nanoparticles After 7 Days Storage at Room Temperature (RT) and 4°C

Formula	Storage conditions	d(0.5)(nm)	E.E(%)	P-Value*
F3	Fresh	114.6±2.0	74.4±2.87	
	RT	129.0±1.0	78.0±2.67	0.01
	4°C	125.3±2.8	86.4±3.26	0.008
F7	Fresh	108.6±6.3	80.0±1.45	
	RT	152.6±2.5	87.5±2.33	0.01
	4°C	121.6±1.5	84.5±1.56	0.01
F11	Fresh	117.0±1.7	70.5±2.45	
	RT	118.0±1.0	69.8±1.98	0.45
	4°C	119.6±0.5	68.1±2.96	0.13
F14	Fresh	109.0±1.0	67.8±2.03	
	RT	112.0±1.7	66.6±2.34	0.08
	4°C	113.0±2.6	68.2±2.55	0.13

Table 7: Invitro release profile of Formulations F1-F16 (F13-F16-Physical Mixture of Formulations F3, F8, F9 and F11)

Time (min)	%Drug release															
	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12	F13	F14	F15	F16
10	3.1	4.5	10.8	7.9	5.6	8.2	7.8	2.5	4.23	6.4	5.2	1.0	5.6	7.8	5.64	4.32
20	17.5	23.75	31.25	21.25	15	18	10.1	5.50	7.43	8.3	9	3.88	12.5	15.1	9.27	9.8
30	26.42	30.23	37.81	32.7	21.4	23.7	25.5	14.58	13.4	11.6	13.1	6.5	28.1	23.8	17.1	11.99
40	30.30	38.21	45.23	39.1	26.3	28.2	30.12	16.8	15.23	16.3	14.23	8.2	33.2	29.6	21.2	14.52
50	35.43	41.78	59.43	45.5	29.1	32.93	37.3	28.72	19.63	20.9	15.2	10.9	36.4	34.3	24.7	17.13
60	43.28	49.7	68.77	52.0	36.9	40.31	46.5	59.30	32.82	35.3	26.6	15.8	48.7	47.0	31.8	21.02
70	51.21	57.65	73.2	59.1	46.0	47.97	55.45	69.13	44.36	41.0	35.2	30.6	57.7	53.3	42.3	34.83
80	57.95	62	81.4	68.3	52.7	55.43	61.38	72.95	52.16	51.3	42.01	38.5	65.6	62.7	50.2	46.72
90	62.3	68.23	83.4	69.23	58.7	61.27	65.89	73.92	59.61	59.61	44.25	41.8	71.2	71.2	59.8	51.28
100	64.75	70.1	86.3	70.8	60.7	66.53	71.87	75.98	61.92	61.8	46.4	47.8	76.5	76.3	66.2	56.93
110	71.22	74.34	91.0	75.25	68.3	77.69	85.31	86.02	75.41	74.4	52.9	60.2	82.1	82.6	79.1	61.5
120	76.65	78.27	95.65	84.7	70.0	88.91	90.97	91.14	82.41	80.78	61.5	64.9	92.3	89.3	85.8	66.25

FIGURES

Figure 1: λ max of ATC in Phosphate buffer (pH6.8)

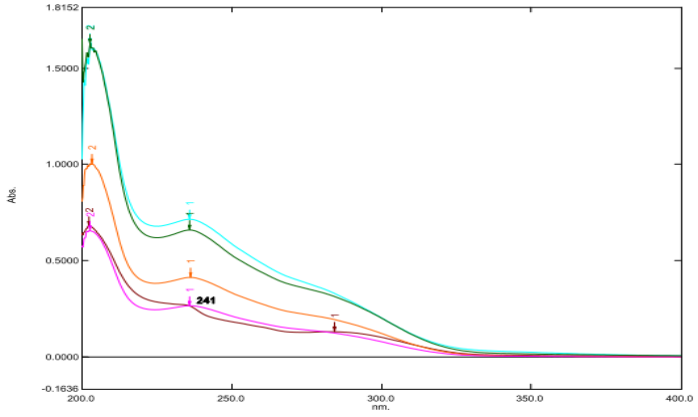

Figure 2: Calibration curve of ATC in Phosphate buffer (pH6.8)

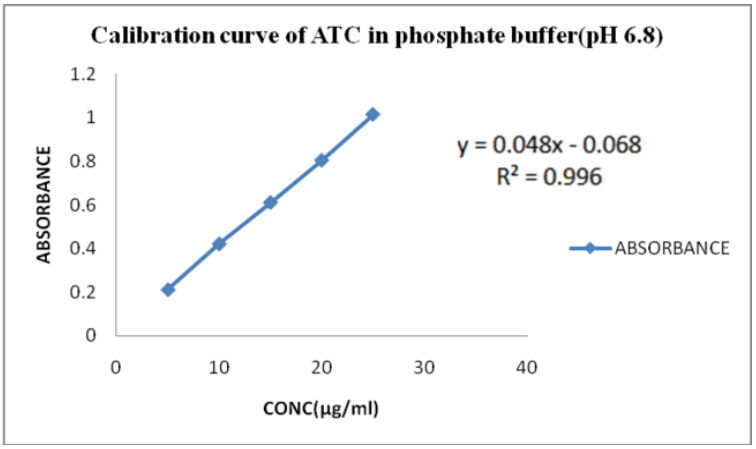

Figure 3: ZETA POTENTIAL

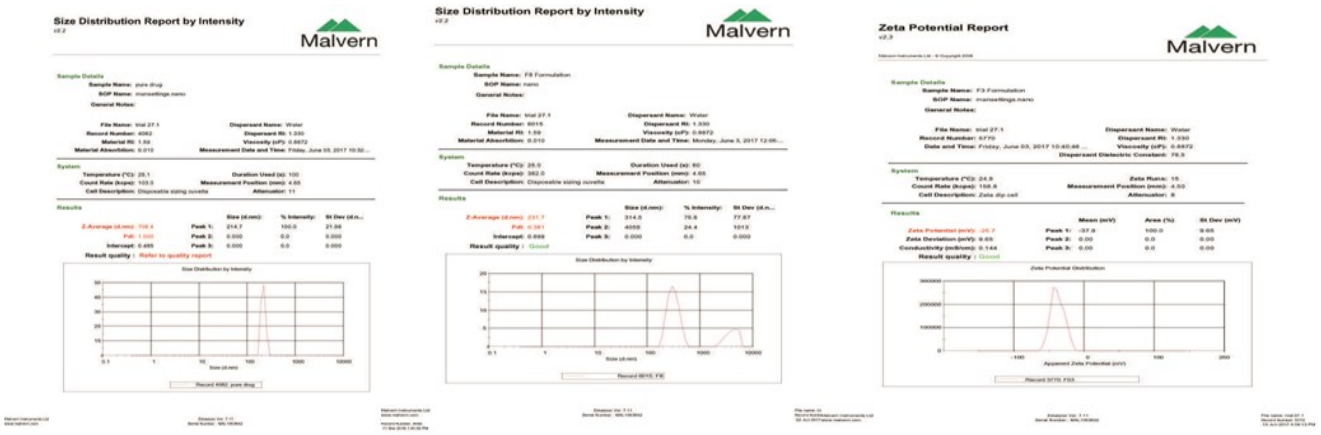

Figure 4: DISSOLUTION STUDIES

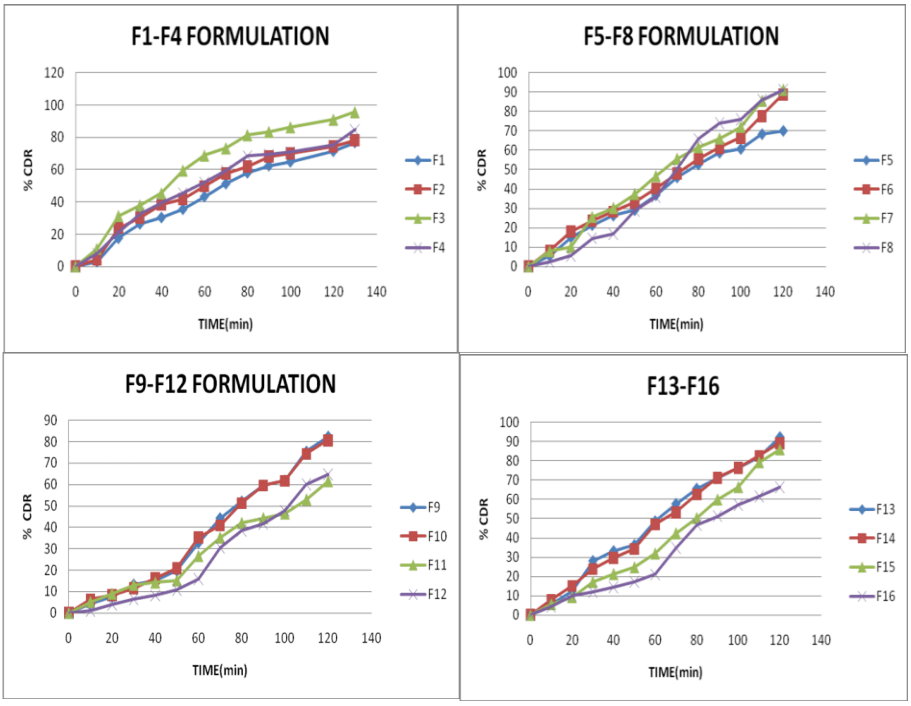

Figure 5: XRD OF PURE DRUG AND OPTIMIZED FORMULATION

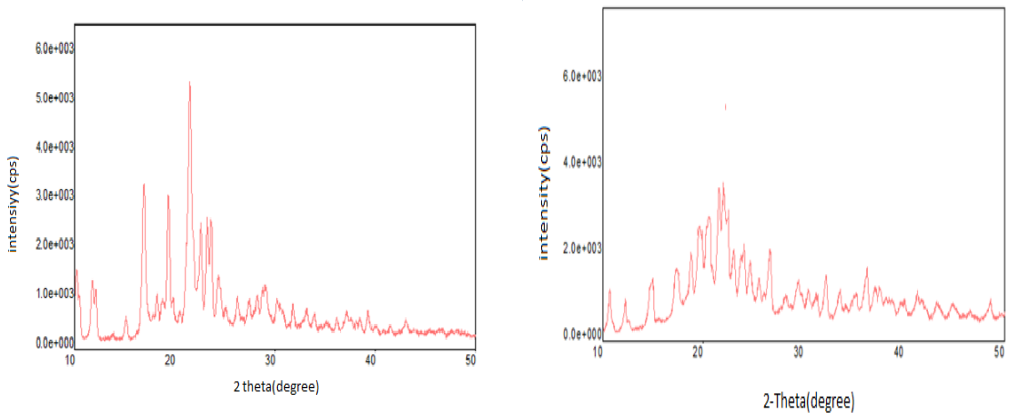

Figure 6: DSC THERMOGRAM OF PURE DRUG AND OPTIMIZED FORMULATION

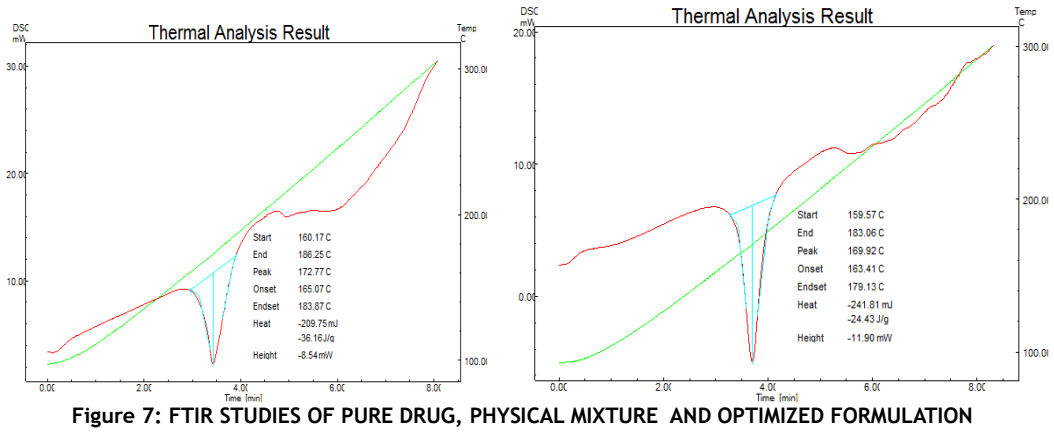

Figure 7: FTIR STUDIES OF PURE DRUG, PHYSICAL MIXTURE AND OPTIMIZED FORMULATION

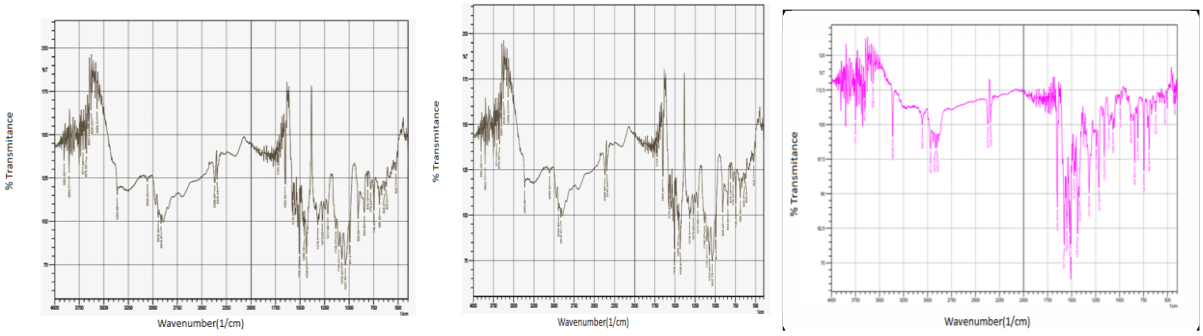

Figure 8: SEM IMAGES OF PURE DRUG AND OPTIMIZED FORMULATION