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NANOPARTICLES OF ATORVASTATIN – A NOVEL WAY OF COLON TARGETING

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ABSTRACT

The present study involves the formulation, optimisation and evaluation of Atorvastatin loaded chitosan nanoparticles by ionotropic gelation method using sodium tripolyphosphate as ionic cross-linker and enteric coating of the optimised nanoparticles for colon targeted drug delivery. The five nanoparticulate formulations prepared were evaluated for various parameters like particle size, zeta potential, % yield,% drug content,% entrapment efficiency, surface morphology, drug-polymer compatibility studies include (FT-IR,DSC), *in vitro* dissolution studies. The particle size and zeta potential of finalised ACT4 formulation was 384.4 nm , -32.2 mV respectively.SEM analysis revealed that the particles were of spherical in shape having smooth surface. The drug-polymer compatibility studies depicted that there was no interaction of between drug and polymer. The % entrapment efficiency was from 47.4% to 69.7 %. *In vitro* dissolution studies showed highest % release for ACT4 (88.4%) in 24 hrs. Kinetic modelling revealed that the *in vitro* drug release follows first order kinetics and non-fickian drug release. The optimised nanoparticulate formulation from *in-vitro* dissolution studies were subjected to enteric coating. Pharmacokinetic studies revealed that the AUC.AUMC and MRT has been significantly increased for E-ACT4 nanoparticles showing evidence in enhancement of bioavailability and T_{max} of 16.8 hrs indicates successful colon targeted drug delivery of E-ACT4 formulation. The data obtained from this study proved that enteric coated chitosan nanoparticles are potential candidates for colon specific drug delivery of Atorvastatin and thereby enhancement of bioavailability, safety and efficacy.

INTRODUCTION:

Colon targeted drug delivery system (CSDDS) refers to targeting of drugs into the lower part of GIT, which occurs primarily in the large intestine or also referred as colon. The delivery of drugs to the colon has number of therapeutic implications in the field of drug delivery. The oral route is preferred but absorption and degradation in upper GIT is major obstacle and must be circumvented for successful colonic delivery (Krishnaiah et al., 2002). Treatment might be more effective if the

novel drug delivery technology such as nanoparticles were targeted directly on the site of action in the colon (Agnihotri et al., 2004). Lower doses might be adequate and if so systemic side effects may be reduced. The region of colon is recognized as having a somewhat less hostile environment with less diversity and intensity of activity than in the stomach and small intestine. Additionally, the colon has a longer retention time and appears highly responsive to agents that enhance the absorption of poorly soluble drugs. The special placement of drugs into selected locations in the GIT is quite difficult due to physiological constraints, namely, motility and mucus turnover. In some cases drugs may be unstable in upper GIT and are generally not well absorbed from the lumen of the GIT due to their relatively large molecular size and high peptidase activity. Protecting drugs from hydrolysis in GIT and subsequently releasing these drugs in the ileum or colon may result in better systemic bioavailability.

Atorvastatin is inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase. This enzyme catalyzes the conversion of HMG-CoA to mevalonate, an early and rate-limiting step in cholesterol biosynthesis, used for the treatment of hyperlipidemia. Statins are the first line treatment for the reduction of cholesterol in the fight against cardiovascular disease. Atorvastatin is a commonly prescribed statin, however it also has numerous side effects resulting in poor patient compliance such as myopathy (muscle dysfunction). Atorvastatin undergoes rapid oral absorption, with an approximate time to maximum plasma concentration (Tmax) of 1–2 hours. The absolute bioavailability of the drug is approximately 14%; however, the systemic availability for HMG-CoA reductase activity is approximately 30%. Atorvastatin undergoes high intestinal clearance and first-pass metabolism, which is the main cause for the low systemic availability. Targeted delivery to the colon assists to develop a safer, less variable drug delivery solution for Atorvastatin. The colonic delivery of Atorvastatin will reduce Cytochrome P450 ("CYPP450") metabolism and hence increase bioavailability, efficacy and improve safety of Atorvastatin through reduced inter and intra-patient variability and lower risk of drug-drug interactions with medications that are CYP450 inhibitors (Marcelo Bravo Oxford Pharmascience Group Plc)

MATERIALS AND METHOD

Materials: Atorvastatin was provided as a gift sample from Smilax Laboratories, Hyderabad. Eudragit S100 and L 100 were provided as gift samples from Evonik Ltd Mumbai, Enteric capsules were provided as gift sample from Nature Capsules Limited Bengaluru. Sodium tripolyphophate was purchased from Sigma Aldrich, chitosan was purchased from Himedia. All the other chemicals were of analytical grade procured from Himedia and Merck. Double distilled water was used throughout the study.

Preparation of drug loaded nanoparticles:

The chitosan nanoparticles (CH-NPs) were prepared according to the technique first reported by (Calvo et al., 1997) with suitable modification, based on the ionotropic gelation of chitosan (CH) with sodium tripolyphosphate (TPP) . Briefly, TPP aqueous solution was added to CH solution (in 1.5% v/v acetic acid) kept in a beaker. The solution was stirred for 30 min at 500 rpm with the help of magnetic stirrer (Remi Instruments, India) at room temperature. The CH to TPP ratio was varied in order to optimize the particle size and drug entrapment efficiency. The drug solution was added in to the CH solution in order to get drug loaded CH-NPs. Free unentrapped drug was removed by ultracentrifugation (Hitachi, Japan) at 30,000 rpm for 30 min. Supernatant of dispersion was discarded and NPs were

washed with distilled water at least 3 times for complete removal of free drug. The Atorvastatin loaded CH-NPs were then dispersed in to distilled water, lyophilized and preserved till further use.

CHARACTERIZATION OF NANOPARTICLES:

Particle size distribution:

The particle size of the nanoparticles was measured by Horiba scientific instruments(particle size analyser).Samples were prepared by diluting nanoparticles with sufficient amount of water in concentration of 0.0001% to 0.1%.The average particle size was determined by Laser Diffractometer .

Zeta potential:

The zeta potential was measured using Laser Doppler Microelectrophoresis (Horiba Scientific)

Scanning electron microscopy: (SEM)

The morphology and surface of nanoparticles were observed using SEM(S-3700)

The samples of freeze dried nanoparticles were dispersed on a glass slide, and kept under a vaccum.The samples were coated with a thin gold/palladium layer using a sputter coat unit.

Percentage yield:

The percentage yield can be determined by calculating initial weight of raw materials and final weight of nanoparticles.

Percentage yield = Practical yield/Theoretical yield X 100. In case of ACT1 to ACT5 ,Theoretical yield =Total solids weight = Weight of Atorvastatin (drug)+ Weight of chitosan (polymer) +Weight of STPP (cross-linker)

Drug content:

The drug content in each formulation was determined by weighing nanoparticles equivalent to 10 mg of Atorvastatin and dissolving in 100 ml of phosphate buffer followed by stirring .The solution was filtered through 0.45µ membrane filter diluted suitably and absorbance of resultant solution was measured spectrophotometrically at 266nm using methanol as blank. The drug content of prepared NPs was determined by the formula.

Drug content% = Weight of drug in nanoparticles/ Weight of nanoparticles X 100

Drug entrapment efficiency:

Also known as association efficiency. The drug loaded NPs were centrifuged at a high speed of 15000 rpm at -4ºC for about 30 min and supernatant was assayed for non bound drug concentration by UV spectrophotometer

Entrapment efficiency% = Bound drug- unbound drug/ Bound drug X 100

Drug-polymer compatibility studies:

i) Differential scanning calorimetry (DSC):

The differential scanning calorimetry thermograms were recorded using differencial scanning calorimeter (DSC; Mettler Toledo,Japan).Indium standard were used to calibrate the DSC temperature and enthalpy scale.Approximately 2-5 mg of each sample was heated in a pierced aluminium pan from 30ºC to 300ºC at a heating rate of 10ºC/min under a stream of nitrogen at a flow rate of 50 ml/min. Thermal data analyses of the DSC thermogram were conducted using STARe software(version 5.21).

ii) Fourier transforms infrared spectroscopy (FT-IR):

The FT-IR spectra of Atorvastatin, Chitosan, Sodium tripolyphosphate and optimised formulation were recorded using Fourier transform infrared spectrophotometer (Bruker Alpha-T, Switzerland) to investigate any interaction between Atorvastatin and polymers in formulated nanoparticles.The samples were grounded with KBr and pressed into a disk shape for measurement. The prepared pellets were scanned over a frequency range of 4000-400 cm^{-1}

***In-vitro* drug release studies:**

The release of drug from the optimized nanoparticles was studied by using dialysis bag. Nanoparticles (equivalent to 10 mg of Atorvastatin) taken in a dialysis bag (cut –off 12,000) were placed in a beaker containing 100 mL of dissolution medium. In order to simulate the pH changes along the GI tract, pH 1.2, 7.4 and 6.8 were sequentially used as dissolution medium (Cheng G et al., 2004). When performing the experiments the pH 1.2 medium was first used for 2 hrs(since the average gastric emptying time is 2hrs), then removed and the fresh pH 7.4 phosphate buffer was added .After 4 hrs the medium was removed and the colonic fluid pH 6.8 buffer was added for subsequent hours(18hrs). Rotation speed was 100 rpm, temperature was maintained at 37±0.5ºC. At specified time intervals (1, 2, 3,4,6,8,10,12,18 and 24 hrs) 2 mL aliquot of each sample was withdrawn and replaced by an equal volume of the release medium. Samples were filtered and amounts of drug released were determined spectrophotometrically at wavelength of 266nm.The data were presented as mean ± SD of at least triplicates.

Enteric coating of Atorvastatin nanoparticles:

The coating of optimised NP's was performed by a simple solvent evaporation method.The enteric coating solution was composed of Eudragit S 100 (6%w/v) and Eudragit L100 (6%w/v).Coating was obtained by dispersing 100 mg of ACT4 in coating solution with a core:coat ratio of 1:10 followed by solvent evaporation in a rotary evaporator. Samples of coated nanoparticles (E-ACT4) were then dried and weighed (Sanjay K Jain et al., 2015).

Pharmacokinetic studies: Studies were carried out as per guidelines of the Institutional Animal Ethical committee (Regd. No. 1677/PO/Re/S/2012/CPCSEA).Male albino rats (150-200 gm) were used for the study .The rats were weighed, labelled and divided into four groups, namely the control group - administered only with the plain vehicle, second group –administered with pure drug (Atorvastatin) suspension prepared using 1% sodium carboxy methyl cellulose. Third group – administered with optimised enteric coated nanosuspension and fourth group- administered with marketed formulation.

Instrumentation and chromatographic conditions:

The HPLC system (Shimadzu, Kyoto,Japan) with a UV detector set at a wavelength of 266 nm. The samples were chromatographed on a reverse phase C18 column . The mobile phase consists of Acetonitrile:Water (80:20v/v) pH 2 adjusted with orthophosporic acid acetonitrile was filtered through a 0.45 µm membrane filter and degassed under vacuum before use. It was pumped at a flow rate of 1 mL/min for the run time of 15 min under these experimental conditions with an injection volume of 25 µL of the sample solution.

Experimental procedure: The animals were fasted overnight. The preparations were (5 mg / kg bodyweight) administered to rats using oral feeding tubes. Blood was collected from the retro-orbital plexus in EDTA-treated tubes. The time intervals for blood withdrawal were of 1,2,4,6,8 ,12,and 24 h. Plasma was separated by centrifuging the blood samples at the speed of 3,000 rpm for 10 min at -4ºC .Methanol was added in a ratio of 1:4 to separate proteins and was centrifuged at 10,000 rpm at -4ºC.The supernatant was collected and the concentration of the drug was estimated using RP-HPLC as explained earlier.

Determination of pharmacokinetics parameters

The pharmacokinetic parameters were determined from the data of plasma

drug concentration at different time points by using MS-Excel 2003 software according to the procedure described elsewhere (Jambhekar, Breen, 2009; Bourne, 2001).

Stability studies and storage condition

The selected formulation of tablets were stored in amber colored glass bottles at 45˚ C + 75% RH for a period of 3 months as per ICH tripartite guideline for stability testing of new drug substances and product framed by European agency for the evaluation of medicinal products and was observed for any changes in color, odour and percentage drug content and cumulative drug release in various simulated gastric fluids (SGF, SIF and SCF) (Kotwal et al., 2007).

Kinetic modelling of drug release profiles

The drug release data were subjected to zero order, first order, Higuchi model (Higuchi et al., 1963), Korsmeyer model and Peppas model for analyzing the mechanism of drug release and release kinetics from the dosage form using MS Excel 2007. The model with the highest correlation coefficient was considered to be the best fitting one (Dorozynski et al., 2004).

Zero-order release kinetics

Zero-order release kinetics, cumulative amount of drug released vs time and the release rate data are fitted to the following equation:

$C = K_0 .t$

First–order release kinetics

First-order release kinetics, log cumulative percentage of drug remaining Vs time and the release rate data are fitted to the following equation:

$C = 100\times(1 – e^{-Kt})$

Higuchi release model

The Higuchi release, cumulative percentage of drug released vs square root of time and the release rate data are fitted to the following equation: $Q = Kt_{1/2}$ Where, K is the constant reflecting the design variables of the system and t is the time in hours. Hence, drug release rate is proportional to the reciprocal of the square root of time (Hixson et al., 1931).

Mechanism of drug release

The drug release were plotted in Korsmeyer et al's equation, as log cumulative percentage of drug released vs. log time, and the exponent n was calculated through the slope of the straight line.

$M_t / M_\infty = Kt^n$

Where M_t/M_∞ is the fractional solute release, t is the release time, K is a kinetic constant characteristic of the drug/polymer system, and n is an exponent that characterizes the mechanism of release of tracers (Korsmeyer et al., 1983). If the exponent n = 0.45, then the drug release mechanism is Fickian diffusion, and if 0.45 < n < 0.89, then it is non-Fickian or anomalous diffusion. An exponent value of 0.89 is indicative of Case-II Transport or typical zero-order release (Siepmann et al., 2001)

RESULTS AND DISCUSSION

Preparation of nanoparticles and optimization:

Different formulations of Atorvastatin-loaded chitosan nanoparticles (ACT1–ACT5) were prepared by modified ionic gelation technique. The formation of CHNPs is governed by the controlled gelation of the CH/TPP crosslinking and evaluated to select the nanoparticles with the optimal composition. The evaluation criteria were the particle size, zeta potential, %yield, % EE, drug loading, morphological characters, and the drug release profiles.

Optimization of CH to TPP ratio:

Optimization was done by preparing five different weight ratios of CH: TPP (1:1, 2:1, 3:1, 4:1 and 5:1) on the basis of size, zeta potential, and entrapment efficiency,% yield and drug loading (Table). Results showed that formulation ACT4 (CH: TPP ratio 4:1) is the optimum ratio for colon delivery. This formulation showed particle size of about

384.2±7.1 nm (Fig),zeta potential of -32.2mv (Fig), and acceptable entrapment efficiency (69.7±4.3 %). During optimization of CH:TPP ratio, it was noted that a high concentration of TPP was unfavourable as formulations ACT1, ACT2 and ACT3 showed larger particle size,which may be due to low zeta potential value and resulted in aggregation of NPs leading to increase in particle size as compared to formulation ACT4 .

Particle shape and morphology:

The shape and morphology was examined using Scanning electron microscopy (SEM) (Zeiss EVOMa 15). The SEM images of the optimized formulation ACT4 are shown in fig 3. The view of optimised nanoparticles of ACT4 formulation showed a spherical shape and smooth surface. From the results formulation ACT4 (4:1 ratio chitosan: sodiumtripolyphosphate) was selected as optimised one for colon delivery.

FTIR studies:

The characteristic bands of Atorvastatin, chitosan and sodium-tripolyphosphate (Table 2) were observed on the spectrum of the Atorvastatin-loaded chitosan nanoparticles (Fig 6) almost at the same frequencies, and thus indicating the absence of chemical interactions between the drug and polymer.

DSC studies:

DSC thermogram of pure Atorvastatin showed sharp peak at 181.5°C corresponding to its melting point. The thermogram of Atorvastatin nanoparticles showed a similar endothermic peak at 180.8°C which confirms that there was no polymer drug interaction.

***In-vitro* drug release studies:**

The *in-vitro* drug release profiles of Atorvastatin loaded nanoparticles showed extended release up to 12 hrs for ACT1, ACT2, ACT3 formulations. This may be due to chitosan:sodium tripolyphosphate concentration was insufficient to coat drug. Hence the release was found to be retarded. Further stability of these formulations was also poor. The drug release from ACT4 and ACT5 formulations were found to be, controlled and extended for a period of 24hrs which may be due to high concentration of chitosan. ACT4 formulation was subjected to enteric coating based on particle size, zeta potential, % yield, entrapment efficiency, drug loading, drug release profile which were found to be superior when compared to ACT5. *In-vitro* release data obtained was fit to different kinetic models like zero-order; first-order, Higuchi, Hixson-crowell, Korsmeyer peppas plot and results were shown in the table. The coefficient of correlation (R^2) was considered to be main parameter for interpreting the release kinetics of drug from the matrix systems. In case of optimised formulation, the higher values R^2 of the linear regression curves in the first order plot than zero order plot showed that the drug release followed first order kinetics. As the matrix forming polymers were used, Higuchi model was applied which showed good linearity with regression R^2 value of 0.9718 suggested that the release mechanism was diffusion controlled. In order to know the release mechanism, data so obtained was fitted to Korsmeyer peppas law. The release exponent value n= 0.6998, indicates the dominant mechanism of drug release of drug release from the drug matrix of optimised formulation was swelling and erosion which is always associated with diffusion mechanism. It can be anomalous transportation i.e., non- fickian kinetics (combination of pure diffusion controlled coupled with dissolution controlled drug release). At the end of 4 hrs formulation ACT4 was found to release 30% of drug which is not acceptable due to probable release in the upper part of GIT. In the case of E-ACT4, there was no significant drug release up to 5^{th} h. Drug release

began only after 5th h in simulated intestinal fluid (pH 6.8). This can be explained by the fact that the Eudragit S and L 100 polymers contain carboxyl groups that ionize when the pH switches from acidic to alkaline. As the ionization takes place, the integrity of the coat is disturbed and the drug starts leaching from the nanoparticles thus depicting enteric coated formulation has protected the drug release in upper part of GIT. In the case of ACT4, drug release was found to be 51% ± 3.2%and 78.92% ± 4.20% at the end of 8th and 24th h, respectively while E-CPNs showed drug release of 33.64% ± 2.1% and 94.5.24% ± 3.15% at the end of 8th and 24th h, respectively. This clearly justifies the successful formulation development of Atorvastatin (E-ACT4) for colon targeting.

Stability studies:

Stability study revealed that there is no significant change in physiochemical properties, drug entrapment efficiency and *in-vitro* drug release.

Pharmacokinetic studies:

The results of pharmacokinetic studies are shown below. In the bioanalytical method for estimation of Atorvastatin Fig 11 shows the HPLC chromatogram with internal standard. From Fig 11. The retention time for Atorvastatin was found to be 3.033 min. The method followed was reproducible, robust and simple.

Plasma concentration vs time profiles are shown in the following Fig 12. In order to evaluate drug absorption, plasma drug profile was established in terms of the plasma concentration *vs.* time profiles of Atorvastatin after oral administration of various formulations to the rats (5 mg/kg) and is depicted in Figure. After oral administration of the Atorvastatin suspension, the drug was detected rapidly in plasma in the initial hours. Thereafter, the drug-plasma concentration was found to be decreased quickly to undetectable levels after 10 h. These results were in accordance with the reports published by Zinutti *et al.* (1998) and Li *et al.* (2008). In the case of marketed formulation drug-plasma concentrations levels were similar to pure drug with slight improvement in AUC, MRT which is not significant.

A significant improvement in bioavailability is evident from the graph. A significant increase in AUC_{0-24} is a clear indicative of enhancement in bioavailability in the form of nanoparticles. The AUMC was found to be 5736.56, 53094.7 and 9799.2(μg/ml.hr$^{2)}$ for pure drug, EACT4 and marketed formulation respectively. The mean residence time was calculated as 4.65 h for pure drug, 14.62 h for E-ACT4 formulation and 6.62h for marketed formulation. Thus, an increase of 2.33 times was evident in MRT for the prepared nanosuspension. Decrease in particle size increases the surface to volume ratio and specific surface area. The use of particle size reduction to increase the surface area for dissolution and thereby increase in bioavailability of poorly-water soluble drugs has been widely investigated and accepted world-wide. The increase in AUC of EACT4 formulation could be attributed to the greater dissolution rates owing to its reduced particle size with increase in surface area. In the case of EACT4, the maximum Atorvastatin level was reached after 12 h of oral administration and then gradually decreased over the next 12 h, which indicated the prolonged residence time of the released drug in the colon with slow leaching of the drug to systemic circulation due to low permeability and compromised surface area. The colon, acts like a homogeneous reservoir to elicit slow and constant drug input which is beneficial for sustained and controlled drug delivery.

Table 1: Composition of chitosan nanoparticles

Ingredients	A CT1	ACT2	ACT3	ACT4	ACT5
Atorvastatin (mg)	10	10	10	10	10
Chitosan :TPP	1:1	2:1	3:1	4:1	5:1
Acetic acid %w/v	1.5	1.5	1.5	1.5	1.5

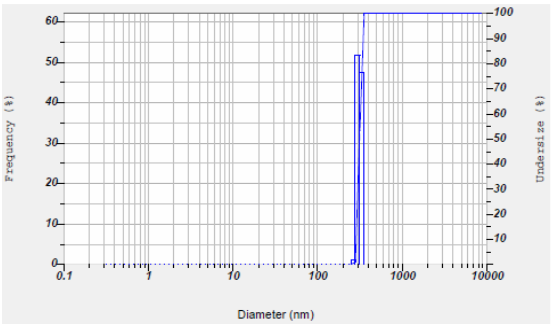


Fig1 :Particle size of ACT4- 384.2nm

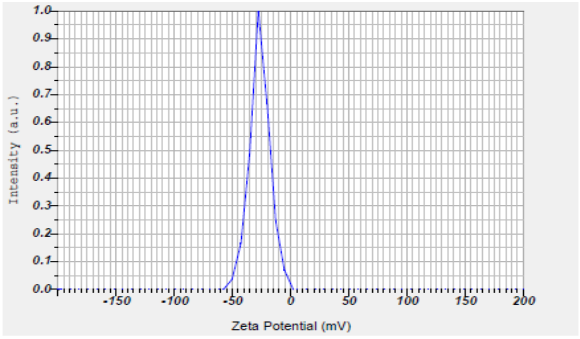


Fig 2: Zeta potential of ACT4- -32.2mv

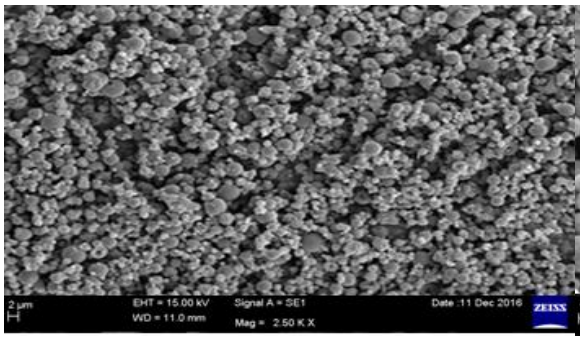


Fig 3 : SEM image with Mag = 2.50 KX

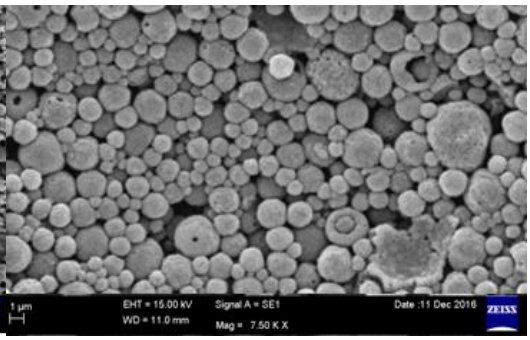


Fig 4 : SEM image with Mag = 7.50 KX

Fig 5 FTIR spectra of Atorvastatin

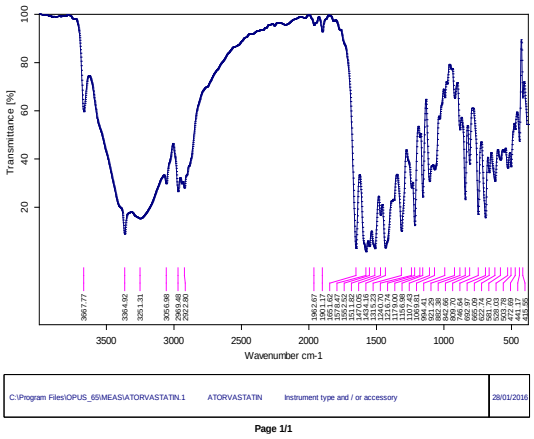


Fig 6: FTIR spectra of formulation

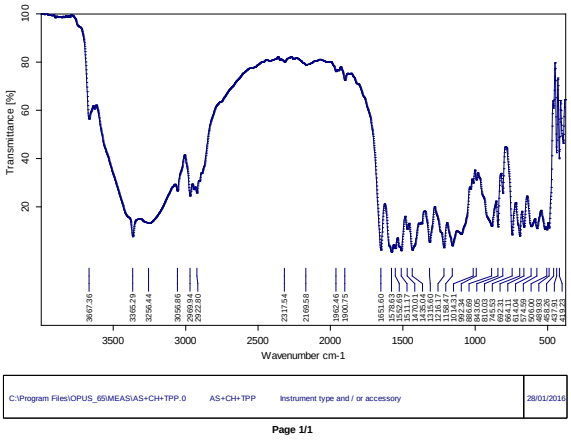


Table 2: FT-IR data of Atorvastatin, chitosan, sodium-tripolyphosphate and Atorvastatin loaded nanoparticles

Group frequency (in cm^{-1})	Frequency of Atorvastatin (in cm^{-1})	Frequency Atorvastatin nanoparticles (in cm^{-1})	Frequency of chitosan (in cm^{-1})	Frequency of sodium tripoly phosphate (in cm^{-1})
Aromatic N-H	3508	3607	3391	3631
Aromatic free O-H stretch	3667	3586	---	3367
Aromatic O-H bonded stretch	3364	3323	---	---
C=O stretching	1650	1641	1664	1653
C-F 4475stretching	1435	1463	---	---
C-O 4475stretching	1316	1294	---	---
C-N stretching	1217	1208	---	---
C-H stretching	2927	2904	2830	2926

Fig 7: DSC thermogram of Atorvastatin

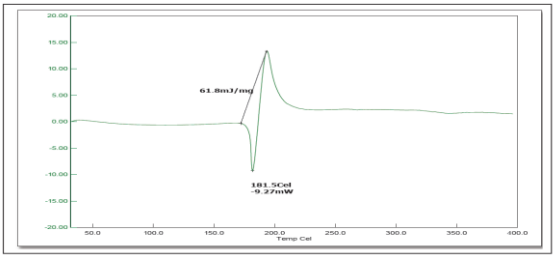
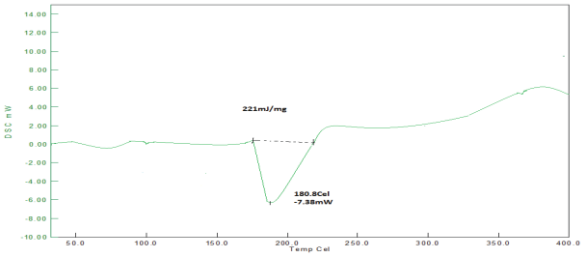


Fig 8: DSC Thermogram of formulation



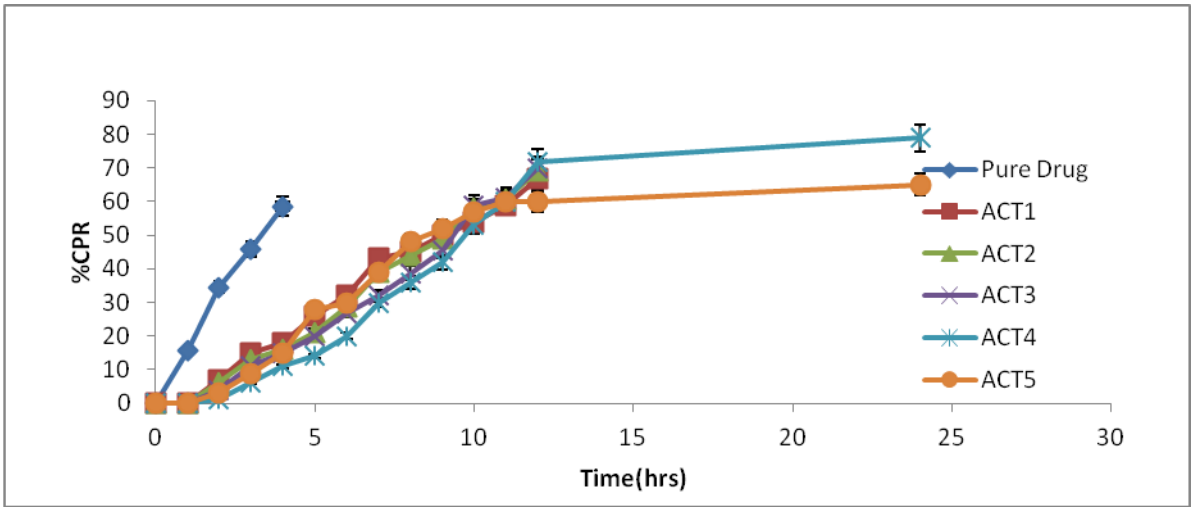


Fig 9: *In-vitro* drug release profile of pure Atorvastatin, ACT1, ACT2, ACT3, ACT4 and ACT5 formulations

Table 3: Kinetic models and corresponding regression values of ACT4 formulation

Plot	Regression values	
Zero order plot	0.9009	
First order plot	0.9862	
Higuchi plot	0.9718	
Hixson crowell plot	0.9101	
Korsmeyer plot	0.9815	0.6998(n)

CONCLUSION:

In the light of these findings, it can be concluded that Atorvastatin loaded chitosan nanoparticles are of appropriate particle size obtained by ionotropic gelation method. All the results of characterization tests indicated that the drug is encapsulated within the nanocore. This nanoparticle based formulation possesess good % yield, drug content,%entrapment efficiency as well as acceptable drug release profile. The release of drug from the enteric coated nanoparticles was found to be controlled over a period of 24 hrs with absolutely no release in acidic medium. Pharmacokinetic study in rats proved that the formulation EACT4 holds a better potential for release of drug in colonic pH and thereby enhancement in absorption and bioavailability.

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