ORIGINAL ARTICLE

Bilayer Tablets: Atorvastatin Calcium Nanoparticles and Clopidogrel Bisulfate Sustained Release Formulation

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Abstract

Background: Bilayer tablets are hopeful to deliver the different drugs in various release patterns to achieve the desired effect on patients with improved patient compliance. Atorvastatin is poor water soluble drug, and nanoformulation was adopted for enhancement of bioavailability whereas clopidogrel was tried with sustained release (SR) to achieve continuous antiplatelet action. **Aim**: The aim of the present study was to formulate and evaluate the bilayered tablets containing clopidogrel bisulfate in the SR portion and atorvastatin calcium nanoparticles in conventional release portion. **Materials and Methods:** To produce a single tablet containing two different classes of drugs and to enhance the bioavailability of atorvastatin calcium nanotechnology was employed using 2% surfactant of sodium lauryl sulfate to the ratio of drug atorvastatin and then subject to high-pressure homogenization and sonication, further, it was evaluated for particle size and zeta potential. The SR layer of clopidogrel bisulfate was prepared using polymers hydroxypropyl methyl cellulose and ethyl cellulose along with other excipients such as magnesium stearate, lactose, and talc by dry granulation technique. Then punched into bilayer tablet by two-run of the machine for each drug separately. Bilayer tablets were evaluated for their physical parameters such as weight variation, friability, hardness, and thickness and *in vitro* drug release study. **Result and Discussion:** Prepared atorvastatin nanoparticle was found to 677 nm in size with zeta potential of −17 mV and weight variation, friability, hardness and thickness that was within the limit. The *in vitr*o drug release study showed that atorvastatin calcium nanoparticle of 99% at 15 min and clopidogrel bisulfate of 93.6% at 7 h and follows anomalous or non-Fickian type release (i.e.) Case-II type of release. *In vivo* study on rabbits proves an increase in relative bioavailability of atorvastatin calcium nanoparticles when compare to marketed product. **Conclusion:** The research outcomes are bilayer tablet of atorvastatin nanoparticles shown an increase in oral bioavailability on rabbits and *in vitro* study of clopidogrel bisulfate SR for better control over antiplatelet activity.

Key words: Atorvastatin calcium, bilayer tablets, clopidogrel bisulfate, nanoparticles, non-Fickian type release, sustained release

INTRODUCTION

Cardiovascular system made up of heart and blood vessels includes numerous problems many of which are related to process called as atherosclerosis. This hardening of arteries or atherosclerosis is speeded up by hypercholesterolemia or hyperlipidemia or high triglyceride levels in the body.[1] Atorvastatin calcium is a BCS Class II drug prevents in the conversion of 3-hydroxy-3-methylglutaryl coenzyme-A to mevalonate, an early rate-limiting step in biosynthesis of cholesterol, but has very low aqueous solubility and very low oral bioavailability of 12%.[2] Poorly water soluble drugs can be converted to nanosuspensions using the top-down technique - high-pressure homogenization technique. To preserve the particle size, stabilization with surfactants or stabilizers is required. Clopidogrel bisulfate is a prodrug and its active metabolite, a thiol derivative is formed by oxidation of clopidogrel to 2-oxo-clopidogrel and subsequent hydrolysis. The active metabolite selectively inhibits the binding of adenosine diphosphate (ADP) to its platelet receptor and the subsequent ADP-mediated activation of glycoprotein II b/III a complex, thereby inhibiting platelet aggregation.[3]

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Clopidogrel bisulfate has quite low oral bioavailability,high risk of bleeding due to the high concentration of active metabolite by the conventional release of drug in the stomach,[4] the drug-drug interaction between clopidogrel bisulfate and atorvastatin calcium. Sustained release (SR) dosage forms of clopidogrel bisulfate provide a better control of plasma levels, reduced drug-drug interactions, less dosing frequency, less side effects, increased efficacy, and constant delivery of drugs. Bilayer tablet is a new era for the successful development of sequential release of two drugs with one layer of drug conventional release and second layer of drug SR.[5]

MATERIALS AND METHODS

Atorvastatin calcium was obtained as gift sample from Pharmafabrikon Pvt. Ltd., Madurai and clopidogrel bisulfate from Saimirra Innopharm Pvt. Ltd., Chennai. Ethyl cellulose (EC) and hydroxypropyl methylcellulose (HPMC) was procured from Himedia Ltd., Mumbai. All other excipients such as sodium lauryl sulfate (SLS), D-mannitol, lactose, magnesium stearate, talc, and ammonium acetate were obtained from a commercial source. Reagents such as acetonitrile, methanol used were of analytical grade.

Preformulation studies

Melting point determination

Digital melting point apparatus was used to determine the melting point of atorvastatin calcium and clopidogrel bisulfate by capillary method.

Solubility studies

The solubility of the drug was predicted by dissolving 1 mg of the drug in proportions of 1 ml, 10 ml, 30 ml, and 100 ml of ethanol, water, and methanol and analyzed by ultraviolet (UV)-spectrophotometer.

Fourier transform infrared (FTIR) drug polymer compatibility study

IR spectra of the drug and its inclusion complexes were recorded by KBr pellet method using FTIR spectrophotometer between 400 and 4000/cm.

Determination of λ_{max}

The prepared drug solution of atorvastatin calcium and clopidogrel bisulfate was scanned using double beam UV spectrophotometer in the spectrum mode between the wavelength ranges of 200-400 nm.

Standard curve of atorvastatin calcium and clopidogrel bisulfate

Atorvastatin calcium was serially diluted to get different concentrations (1, 2, 3, 4, 5 mcg/ml) to determine the linearity range and analyzed at 246 nm using UV spectrophotometer. Clopidogrel bisulfate was serially diluted to get different concentrations (5, 10, 15, 20, 25, and 30 mcg/ml) to determine the linearity range and analyzed at 216 nm using UV spectrophotometer.

Preparation of atorvastatin calcium nanosuspension

Atorvastatin calcium pure drug (500 mg) was taken and dissolved in 50 ml of prepared SLS solution (i.e.) 2% for drug. Then the prepared drug solution was subjected to high-pressure homogenization of single cycle with 20,000 psi. The nanosuspension was stabilized using probe sonication. The prepared nanosuspension was then studied for particle size and zeta potential.

Characterization of atorvastatin calcium nanosuspension

Determination of particle size by photon correlation spectroscopy

The average mean diameters and size distribution of nanosuspension were found out by photon correlation spectroscopy using Zetasizer (nano ZS90, Malvern Instruments) at 25°C. The samples were kept in polystyrene cuvette and the readings were found out at a fixed angle.

Determination of zeta potential

The electrophoretic mobility (zeta potential) measurements of nanosuspension were made using Zetasizer (Nano ZS90, Malvern Instruments). The samples were placed in a polystyrene cuvette (at 25°C), and zeta dip cell was used to measure the potential.

Freeze drying (Lyophilization technique)

Freeze-drying was performed to convert aqueous nanosuspension into powder. The formulation was kept in freezer for 4 h. Then, the formulation was freeze dried using freeze dryer at the temperature of −40°C and −1.12 mbar pressures for 48 h. The lyophilized formulations were used for the further characterization studies (Nakrani *et al*., 2010).

Scanning electron microscope (SEM) analysis

The stub containing sample was placed in SEM chamber and analyzes the surface morphology.

Drug content in the formulation

A volume of 100 mg of the nanoformulation was dissolved in 100 ml of distilled water. From this 1 ml was taken and made up to 10 ml. Further from this 1 ml was taken and made up to 10 ml and absorbance was taken at 246 nm

using UV-spectrophotometer. From this required amount of nanoformulation was taken to be made into bilayer tablet.

Formulation of bilayer tablets

Preparation of bilayer tablets of atorvastatin calcium nanoparticles and clopidogrel bisulfate SR formulation.

First punching layer

A volume of 75 mg of clopidogrel bisulfate pure drug was taken and mixed with polymers (HPMC - 75 mg/150 mg/225 mg) or (EC - 75 mg/150 mg/225 mg) (i.e.) in the ratio of 1:1, 1:2, 1:3 with drug. Then, the drug and polymer are mixed with excipients of talc 3 mg and magnesium stearate 1.5 mg and lactose of required mg to produce tablet of 357 mg.

Second punching layer

33 mg of atorvastatin calcium nanoparticle contains 10 mg of atorvastatin calcium pure drug. It is mixed with 2% of talc (0.6 mg) and 1% of magnesium stearate (0.3 mg). The mixed drug and excipients were bought to the second step of punching.

Formulation of bilayer tablets is mentioned in Table 1.

Evaluation of bilayer tablets

Weight variation test

20 tablets of each formulation were weighed using an electronic balance, and the test was performed according to the USP official limits.

Friability test

10 tablets were taken for each formulation, maximum mean loss from three samples of not more than 1% is considered acceptable for most products.

Hardness of tablets

Hardness of 10 tablets (randomly) was determined by Monsanto hardness tester. Hardness measured in kg/cm^2.

Tablet thickness

10 tablets were selected at random and thickness was measured using vernier calliper scale, which permits accurate measurement in millimeter (mm).

In vivo study

In vitro studies were carried out in dissolution test apparatus (USP, XXIII-type 2 Paddle) using 900 ml of dissolution media (0.1 N HCl pH 1.2, phosphate buffer pH 7.4) maintained at 37°C ± 0.5°C and with sampling intervals of 15 min, 30 min, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 7 h, and 8 h. The samples are filtered, and 1 ml was taken for analysis. Drug release data were plotted and tested with zero order plots, Higuchi plot and Korsemeyer-Peppas plot. The *in vitro* dissolution kinetic parameters, dissolution rate constant, correlation coefficient, and dissolution efficiency were calculated.

In vivo study

The pharmacokinetic study was approved by institutional animal ethical committee (IAEC-325/2016/IAEC) and carried out in male adult rabbits. Each rabbit was anesthetized by an injection of 25% urethane saline dissolution (4 ml/kg). The right marginal ear vein was cannulated by polyethylene tubing for blood sampling. Drug solutions were prepared by adding drug (respect to human dose) to distilled water (10 ml) and stirring for 1h and then administered orally through catheter. Blood samples 0.5 ml are collected from the marginal ear vein at designated time points (15 min, 30 min, and 45 min) into microcentrifuge tubes containing 10 µl of sodium citrate with 30 % concentration. Plasma was harvest by centrifuging blood using Eppendorf at 10,000 rpm for 10 min and stored in a deep freeze condition (−80°C).[6]

Description procedure of standard graph

Standard solutions of atorvastatin calcium were prepared in RP-C18 column and mobile phase of acetonitrile: 0.05 M ammonium acetate (45:55 v/v) (adjusted to pH 6.09) and appropriate dilutions were made with mobile phase 200-400 ng/ml and stored 5°C. Samples were formed by spiking 100 µl of known concentration of solution into 100 µl of rabbit plasma with 1.0 ml/min flow rate and analyzed in photodiode array detector at 247 nm.[7]

Table 1: Formulation table for bilayer tablets

Formulation code	Clopidogrel bisulfate (mg)	HPMC (mg)	EC (mg)	Lactose (mg)
AC1	75	75	-	168.6
AC2	75	-	75	168.6
AC3	75	150	-	93.6
AC4	75	-	150	93.6
AC5	75	225	-	18.6
AC6	75	-	225	18.6

Each formulation contains 33 mg of atorvastatin calcium nanoparticle formulation equivalent to 10 mg of atorvastatin calcium pure drug (with excipients) and SR clopidogrel bisulfate of 75 mg with polymer and excipients. Total weight of each tablet-357 mg. HPMC: Hydroxypropyl methyl cellulose, EC: Ethyl cellulose, SR: Sustained release

Extraction procedure for plasma standard plot

The prepared plasma concentrations were mixed for 10 min using vortex mixer and continuation of centrifugation for 10 min at 10,000 rpm on table top centrifuge. The organic layer was collected into a watch glass to get easy method of evaporation. The solid was remixed with 200 µl of mobile phase and ingested into analytical system.

The area of peak was noted and plotted the graph against the concentration.

Pharmacokinetic data analysis

Peak plasma concentration (C_{max}), time of peak concentration(T_{max}), elimination rate constant, biological half-life, area under curve, area under first-moment curve, mean residence time, and relative bioavailability were calculated.

RESULTS AND DISCUSSION

Preformulation study

Melting point of atorvastatin calcium was found to be 176°C and clopidogrel bisulfate was found to be 158°C. Solubility study confirms that atorvastatin calcium was soluble in methanol (27 ml methanol requires to dissolve 1 g of drug) and clopidogrel bisulfate was freely soluble in methanol (10 ml of methanol require to dissolve 1 g of drug). FTIR spectra of atorvastatin calcium, clopidogrel bisulfate, physical mixture, and other excipients confirm that there is no interaction between the drugs and excipients. Atorvastatin calcium and clopidogrel bisulfate were scanned for the maximum wavelength at 246 and 216 nm, respectively. A standard curve of atorvastatin calcium (1-5 mcg/ml with regression of 0.998) and clopidogrel bisulfate (5-30 mcg/ml with regression of 0.996) were noted.

Preparation and size evaluation of atorvastatin calcium nanoparticles

The atorvastatin calcium nanosuspension was analyzed by Malvern Zetasizer after suitable dilution with water. The particle size was found as 677 nm, and its surface charge of zeta potential was negative (−17 mV) as shown in Table 2. The prepared atorvastatin calcium nanoformulation was subjected to lyophilization using freeze dryer and the temperature was maintained at −80°C for 24 h. The formulation was lyophilized and made into powder. The morphology of the particle was evaluated by scanning electron microscopy.

SEM analysis

Morphology of atorvastatin calcium nanoparticles was confirmed by scanning electron microscopy, and the shape of the particles was found to be needle shaped as shown in Figure 1.

Drug content of atorvastatin calcium nanoparticles

25 mg of the formulation should contain 8.33 mg of drug, but contains 7.6 mg of drug which shows the 91.24%.

Evaluation of bilayer tablets

The prepared bilayer tablets were evaluated for weight variation, friability, hardness, and thickness and were found to be within the limit as shown in Table 3.

Dissolution release study

In vitro dissolution study was carried out for all formulation AC1, AC2, AC3, AC4, AC5, AC6 and was found that all formulations show drug release in 15 min which proves that the drug atorvastatin calcium was formulated as nanoparticles and increase in rate of *in vitro* drug release. AC6 was selected as the best formulation which showed drug release of 93.6%

Table 2: Preparation and size evaluation of atorvastatin calcium nanoparticles

Formulation	Homogenization	Probe sonication (min)	Particle size (nm)	Zeta potential (mV)
Atorvastatin calcium	22,000 psi	15	677	−17

Table 3: Evaluation of bilayer tablets

Formulation code	Weight variation test[a] (mg)	Friability test[b] (%)	Hardness test[b] (kg/cm^2)	Thickness test[b] (mm)
AC1	357.7±	0.95	5.92±0.037	7.12±0.015
AC2	361.6±	0.82	6.14±0.050	7.41±0.051
AC3	356.5±	0.53	6.84±0.036	7.75±0.005
AC4	359.4±	0.70	6.33±0.035	7.12±0.026
AC5	357.5±	0.72	6.51±0.017	7.39±0.115
AC6	360.5±	0.67	6.67±0.023	7.26±0.005

[a]Readings were average of 20 tablets from each formulation, [b]Readings were average of 10 tablets from each formulation

in 7 h for clopidogrel bisulfate with EC in ratio 1:3 and selected for further studies.

In vitro release kinetics data of clopidogrel bisulfate formulation of AC-6 was found by zero order plot, Higuchi plot, Korsemeyer-Peppas plot as shown in Table 4.

The drug release was found to follow anomalous or non-Fickian type release (i.e.) case-II type of release which indicates clopidogrel bisulfate release was combination process such as diffusion or erosion.

Stability study

Stability study was studied for formulation AC6 for 3 months, and the *in vitro* drug release was found to be stable as shown in Table 5.

Determination of pharmacokinetic parameters

Determination of pharmacokinetic parameters for formulation AC6 of atorvastatin calcium nanoparticle and marketed formulation of atorvastatin calcium is shown in Table 6.

$$\text{Relative bioavailability (F)} = \frac{AUC_{test}}{AUC_{std}} = \frac{4800}{1275} = 3.76$$

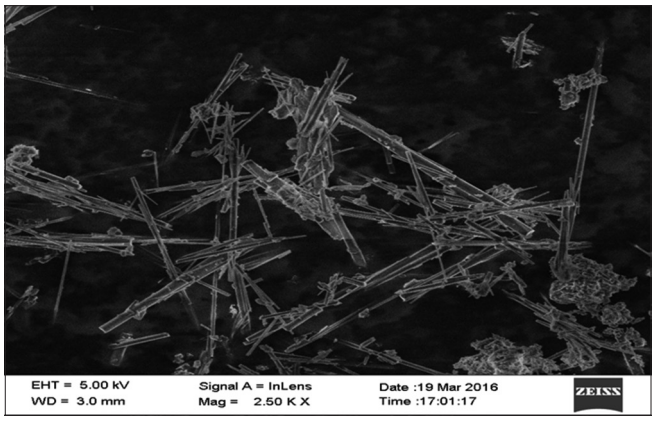

Figure 1: Scanning electron microscope image of atorvastatin calcium nanoparticles

Relative bioavailability of formulation AC-6 of atorvastatin calcium was 3.76-fold increase when compared to marketed formulation of atorvastatin calcium which confirms the nanoformulation of atorvastatin calcium in AC-6 for enhancement of bioavailability.

CONCLUSION

The present study concludes that atorvastatin calcium in nanoparticles drug delivery system by high-pressure homogenization technique yields 677 nm of particles with good zeta potential and SEM analysis also confirms the surface morphology of nanoparticles with needle shape. Bilayer tablets of AC1, AC2, AC3, AC4, AC5, and AC6 was prepared by 75 mg of clopidogrel bisulfate along with polymers HPMC and EC of different ratio with excipients of lactose along with talc and magnesium stearate was taken as first punching layer for SR and 33 mg of atorvastatin calcium nanoparticles formulation contains 10 mg of atorvastatin calcium pure drug was taken along with talc, magnesium stearate, which was the second punching layer for conventional release. weight variation, friability, hardness, and thickness were shown that all the tablets were within test limit. *In vitro* dissolution studies were performed for all six formulations and AC6 which showed the release of 93% at 7 h for sustain release layer of clopidogrel bisulfate and 99% at 15 min for nanoformulation release layer of atorvastatin calcium. This confirms the increase in rate of *in vitro* dissolution of atorvastatin calcium and SR character of clopidogrel bisulfate. Stability study of formulation AC6 also confirms the suitability of the method for bilayered tablets of different formulation approaches. Further *in vivo* pharmacokinetic study on rabbits for atorvastatin calcium proves that 3.76-fold increase in relative bioavailability of nanoformulation when compared to marketed formulation.

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Table 4: *In vitro* release kinetics data of clopidogrel bisulfate formulation of AC-6

Formulation code	Regression for zero order plot	Regression for Higuchi's plot	*n*-value for Korsmeyer-Peppas plot
AC-6	0.998	0.944	0.890

Table 5: Stability study – *in vitro* drug release of formulation AC6

Temperature condition	1st month		2nd month		3rd month	
	A_6 (%)	C_6 (%)	A_6 (%)	C_6 (%)	A_6 (%)	C_6 (%)
25°C/60% RH	99	93.6	98.7	93.2	93	93.1
30°C/65% RH	98.7	93.6	98.7	93.1	93	93.1
40°C/75% RH	98.7	93.2	98	93.1	92.7	93.1

Table 6: Determination of pharmacokinetic parameters for AC6 of atorvastatin calcium nanoparticle formulation

Pharmacokinetic parameters	Nanoparticle formulation	Marketed formulation
C_{max} (ng/ml)	200	50
t_{max} (min)	30	45
K_{el} (min^{-1})	0.092	0.046
$t_{½}$ (min)	7.53	15.6
AUC_{0-t}* (ng.min/ml)	4800	1275
$AUC_{0-}\infty$ (ng.min/ml)	6104	2362
$AUMC_{0-t}$* ($ng.min^2/ml$)	144,000	59,625
$AUMC_{0-\infty}$ ($ng.min^2/ml$)	3,279,602	3,267,473
MRT (min)	560.8 (9.3 h)	1408.6 (24.3 h)

AUC: Area under the curve, MRT: Mean residence time

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