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Research Article

IN-VITRO STUDIES AND CHARACTERIZATION OF SOLID LIPID NANOPARTICLES OF PROTEASE INHIBITOR, ATAZANAVIR

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Abstract

Protease Inhibitors are the new class of anti-HIV agent. Protease Inhibitors, e.g. Atazanavir, important constituents of the Highly Active Antiretroviral Therapy regimen, have dramatically impacted outcome of HIV treatment. In this research study, the Solid Lipid Nanoparticles of Protease Inhibitor, Atazanavir was characterized by entrapment efficacy, drug loading, particle size, zeta potential and drug release.

Keywords: Protease Inhibitors, Atazanavir, Solid Lipid Nanoparticles, Entrapment Efficacy, Drug Loading, Particle size, Drug Release, TEM imaging, diffusion studies using dialysis membrane

Introduction

With the advances of medicines and research we are trying to tame the menace of HIV-AIDs. When the addition/invention of newer ANTI-RETROVIRAL DRUGS is becoming difficult, and more resistant cases of existing molecules are appearing, there is a big onus on all the researchers to come out with the newer techniques to be applied on existing ANTI-RETROVIRAL DRUGS. For example one of the methods may be to increase the efficacy e.g. they can alter the Pharmacokinetic properties of drug molecule to enhance the bioavailability to those parts of human body where existing molecules are not reaching optimum levels to treat the disease completely. One of such site is brain of human body. For the protection of our brain, we are empowered with Blood Brain Barrier. Sometimes this defense facility hinders the treatment part as it prevents the crossing of most of the Drugs from body blood plasma to brain.

The anti-HIV drugs are active against human immunodeficiency virus (HIV). The main objective of Anti-retroviral therapy (ART) is to prolong human life and postpone treatment complications.

Protease Inhibitors are the new class of anti-HIV agent and are important constituents of the HAART regimen, have dramatically impacted outcome of HIV infection. However

they have limited ability to reach the CNS, with the majority of this class of drugs are not detected in human CSF after administration.[1]

Nanoparticle drug delivery system, due to their extreme small size range, has been proposed as potential brain targeting systems. Several studies have been conducted to access the ability of Nanoparticles to our Blood Brain Barrier.

Recently it was researched & concluded that poly (alkyl cynoacrylate) nanoparticles coated with surfactants such as PlurenicR F68 and polysorbate 20, 40, 60 & so on. I.V. administration can be transported across Blood Brain Barrier.

Atazanavir is an attractive candidate for brain targeting. In this research study, the Solid Lipid Nanoparticles of Protease Inhibitor, Atazanavir was characterized by entrapment efficacy, drug loading, particle size, zeta potential and drug release.

Materials and Methods:

Atazanavir (ATZ) was gifted as sample by Ranbaxy Research Laboratory, Gurgaon, India. Compritol 888 ATO was gifted as sample by Gattefosse, Mumbai, India**.** Palmitic Acid was gifted as sample by Gattefosse, Mumbai, India. Polysorbate 80 (P80) (Tween 80) was obtained from Merck, India. Poloxamer 188 was obtained from HiMedia, Mumbai, India.

Solid lipid nanoparticles loaded with atazanavir sulfate, were formulated by hot homogenization followed by ultrasonication method. Compritol 888 ATO and Palmitic Acid were used as lipids and Tween 80 and Poloxamer 188 were used as surfactants.

Determination of In-vitro drug release

Dialysis bag diffusion technique[(2,3)] was used to determine in-vitro drug release. Dialysis bag was made from dialysis membrane 70 (HiMedia, Mumbai, India) has molecular weight cut off between 12000 to 14000 and pore size of 2.4 nm.

Characterization Methods of SLNs

Determination of Entrapment Efficiency (%)

The EE% of the Atazanavir sulfate in the freshly prepared SLNs was determined by centrifugation followed by filtration method. The entrapment efficiency could be calculated using the following equation, [(4)]

$$(EE\ \%) = \frac{W_{initial\ drug} - W_{free\ drug}}{W_{initial\ drug}} \times 100$$

Where, $W_{initial\ drug}$, mass of initial drug used for the assay

$W_{free\ drug}$, mass of free drug detected in the supernatant

Table 1: EE% of all the ATZ-sulfate-SLN formulations

Batch number coding	EE%	Batch number coding	EE (%)
ATZ-sulfate-SLNs A	88.52	ATZ-sulfate-SLNs G	87.80
ATZ-sulfate-SLNs B	62.06	ATZ-sulfate-SLNs H	59.63
ATZ-sulfate-SLNs C	38.58	ATZ-sulfate-SLNs I	36.37
ATZ-sulfate-SLNs D	91.91	ATZ-sulfate-SLNs J	90.46
ATZ-sulfate-SLNs E	61.71	ATZ-sulfate-SLNs K	58.05
ATZ-sulfate-SLNs F	48.07	ATZ-sulfate-SLNs L	44.80

ATZ-sulfate-SLN: Atazanavir sulfate Solid Lipid Nanoparticle

It was observed that formulation D exhibited the maximum entrapment efficiency. As we observed in partitioning behaviour, Atazanavir sulfate was less partitioned in Palmitic Acid as compared to compritol 888 ATO, like that we observed same results for EE% in Palmitic Acid SLNs as compared to Compritol 888 ATO.

Determination of Drug Loading (%)

Drug loading capacity was calculated as drug analysed in the nanoparticles versus the total amount of the drug added and the lipid during preparation, according to the following equation (5)

$$DL\ (\%) = \frac{\text{Amount of drug analysed in SLN}}{\text{Total drug added + Amount of Lipid}} \times 100$$

Table 2: % drug loading in ATZ-sulphate-SLNs formulations

Batch number coding	DL (%)	Batch number coding	DL (%)
ATZ-sulfate-SLNs A	4.78	ATZ-sulfate-SLNs G	6.13
ATZ-sulfate-SLNs B	5.70	ATZ-sulfate-SLNs H	5.87
ATZ-sulfate-SLNs C	6.16	ATZ-sulfate-SLNs I	5.93
ATZ-sulfate-SLNs D	6.28	ATZ-sulfate-SLNs J	6.15
ATZ-sulfate-SLNs E	6.11	ATZ-sulfate-SLNs K	5.97
ATZ-sulfate-SLNs F	6.13	ATZ-sulfate-SLNs L	5.51

It was observed that formulation ATZ-sulfate-SLN D exhibited maximum drug loading. Formulation D was found with maximum entrapment efficiency and drug loading so we left all other formulation here only and only evaluated formulation D for further characterization, permeation study, lyophillization and accelerated stability study. Formulation ATZ-sulfate-SLNs D is composed of Compritol 888 ATO lipid.

Determination of Particle size and zeta potential

Photon Correlation Spectroscopy (PCS) and Laser Differaction (LD) are the techniques to determine the Particle size of the SLNs. It was done by Malvern Mastersizer 2000S.

Particle Size Analysis: Laser Diffraction

Average diameter of ATZ-sulfate-SLNs formulations D were measured using LD (Malvern, Mastersizer 2000S) at a fixed angle of 90. Table 3 shows the detail Laser diffraction particle size of three ATZ-sulfate-SLNs formulation D (D1, D2, and D3). Particle size was reported in three different'd' value i.e. d (0.1), d (0.5) and d (0.9). The median volume particle size, d (0.5), i.e. the size in nanometer at which 50% of the sample is smaller and 50% is larger, d (0.1) (means 10% of the particles are below this nanometer and rest are above this size) and d (0.9) (means 90% of the particles are below this size and rest are above this size) were used as characterization parameters.

Table 3: Laser Diffraction particle size of three different ATZ-sulfate-SLNs formulation D and average of three along with Standard deviation (D1, D2, D3) *(Particle size data are based on average value obtained by 3 time analysis of each preparation of ATZ-sulfate-SLNs D).*

ATZ-sulfate SLN	Particle size (nm)		
	d(0.1)	d(0.5)	d(0.9)
D1	133	176	246
D2	132	173	237
D3	132	173	237
D	132.33±0.58	174.00±1.73	240.00±5.20

We observed that 10% of SLN were below 132 nm, 50 % of the particles were below 174 nm and 90 % of particles were below 240 nm.

Table 4: Particle size distribution of ATZ-sulfate-SLN formulation D1, D2 and D3 *(Data are based on average value obtained by three time analysis of each preparation of formulations)*

ATZ-SLN formulation	Particle size in nanometer divided in various size ranges									
	105.0	112.5	129.0	148.0	170.0	195.5	224.5	257.5	295.5	339.5
	105 only	105-120	120-138	138-158	158-182	182-209	209-240	240-275	275-316	316-363
D1 volume (%)	0	3.49	10.53	19.44	22.07	19.47	13.23	7.35	3.52	0.93
D2 volume (%)	0	3.94	11.64	20.44	22.36	19.49	13.03	6.81	2.2	0.09
D3 volume (%)	0	3.90	11.57	20.36	22.32	19.5	13.1	6.82	2.4	0.03
D volume (%) ± SD	0	3.78 ±0.25	11.25±0.63	20.08±0.55	22.25±0.16	19.48±0.03	13.12±0.11	6.99 ±0.31	2.71 ±0.71	0.35 ±0.50

Maximum % of ATZ-sulfate-SLNs formulation D was found in range of 158 nm -182 nm. This formulation exhibited narrow particle size distribution.

Zeta Potential

Zeta potential is a key factor to evaluate the stability of a colloidal dispersion[6]. Zeta potential of the one of the drug compound, reported in the literature, a SLNs suspension was measured by the electrophoretic mobility of the nanoparticles in a U-type tube at 20°C[7]. Zeta potential value observed was -10 mV.

Transmission Electron microscopy

The particle size, shape of ATZ-sulfate-SLNs formulation D was examined by TEM [8] (Hitachi, H7500, Japan). Various SLNs loaded with ATZ-sulfate was prepared as described previously but only the optimized one was examined by TEM for confirming shape and size of the particles. 1 ml of SLN suspension was taken and diluted to 10 ml with double distilled water. No staining of the sample was done. The sample was passively adsorbed onto the copper grid and then dried prior to observation. The particle size observed was correlated with that obtained from LD.

In-vitro drug release

Atazanavir sulfate from the Compritol 888 ATO nanoparticles was determined by dialysis bag diffusion method with PBS pH 7.4 as a releasing medium at 37±0.5°C. [5] Dialysis bag was made from dialysis membrane 70 has molecular weight cut off between 12000 to 14000 and pore size of 2.4 nm. USP dissolution type II was used for in-vitro drug release study.

Table 5: Observed data for release study of Atazanavir sulfate from three different ATZ-sulfate-SLNs formulation D1, D2 and D3 at various time points *(D avg. and SD are calculated on the bases of results obtained from drug release of D1, D2 and D3)*

Time Point (Hrs.)	% Atazanavir sulfate from ATZ-sulfate-SLNs formulation D				Standard deviation (SD) for ATZ-sulfate-SLNs formulation D
	D1	D2	D3	D avg. (D1+D2+D3 / 3)	
0	0.00	0.00	0.00	0.00	0
0.25	6.80	8.98	8.78	8.19	1.21
0.50	13.59	15.27	11.88	13.58	1.70
0.75	17.90	17.33	16.74	17.32	0.58

01	22.49	17.75	17.70	19.31	2.75
02	35.26	30.27	42.89	36.14	6.36
04	47.11	47.22	45.99	46.77	0.68
06	49.14	48.45	47.78	48.46	0.68
09	53.24	49.66	49.80	50.90	2.03
12	56.42	51.62	50.99	53.01	2.97
24	58.64	52.70	52.33	54.56	3.54

Very controlled release was observed, only 54% up to 24 hr.

Sterilization of Atazanavir sulfate-SLN

ATZ-sulfate-SLNs formulation D were prepared as described previously and sterilized by steam sterilization (Autoclaving) at 121°C for 15 minutes in aseptic environment. 5 ml sterilized ATZ-sulfate-SLNs suspensions was kept at various temperature (20°C, 25°C, and 30°C) for 7, 14, and 30 days to evaluate, whether it shows any microbial growth or not. Sample was evaluated visually as well as microscopically, for bacterial and fungal growth. No microbial growth was observed.

Lyophillization of ATZ-sulfate-SLNs

The ATZ-sulfate-SLNs formulation D were prepared as described previously and lyophilized using Alpha 1-2 LD plus Freeze Dryer. 2% Sucrose was added as cryoprotectant before freezing. Slow freezing was carried out at -30°C and subsequently lyophilized for 24 hr. Lyophilized SLNs were kept for stability study.

Accelerated stability study

Freeze dried formulation of SLNs were kept at accelerated stability conditions i.e. 40°C temperature and 75% relative humidity. Three Lyophilized Atazanavir sulfate SLNs sample (equivalent 100mg) was kept in well closed container for the accelerated stability study. According to ICH guidelines sampling time point in accelerated stability study are 0, 3, and 6 months, but here in this study the sample was only sampled at 3 months. Particle size and in-vitro drug release are two parameters on which the results of study depends. Stable formulation should not show any change in particle size and drug release study.

Accelerated Stability Study

Stability is a very big issue with the SLNs. Table 6 show accelerated stability study conditions and sampling time point.

Table 6: Accelerated stability study conditions and sampling time point.

Formulation (lyophillized)	Temperature (°C)	Relative Humidity (%)	Sampling point
ATZ-sulfate-SLNs D1	40	75	3 month
ATZ-sulfate-SLNs D2	40	75	3 month
ATZ-sulfate-SLNs D3	40	75	3 month

Three Lyophilized ATZ-sulfate-SLNs of formulation D i.e. D1, D2, and D3 was kept for stability at 40°C temperature and 75% RH. The sampling was done only at 3 month. Only two characteristics i.e. Particle size and in-vitro drug release were evaluated to check the stability of the SLNs. Particle size of the SLNs were performed by LD and in-vitro drug release was performed by dialysis bag diffusion method. Increase in particle size and more controlled release was observed.

Results and Discussion:

Entrapment Efficiency

The EE% of the drug in solid lipid nanoparticles was measured by centrifugation of solid lipid nanoparticles suspension

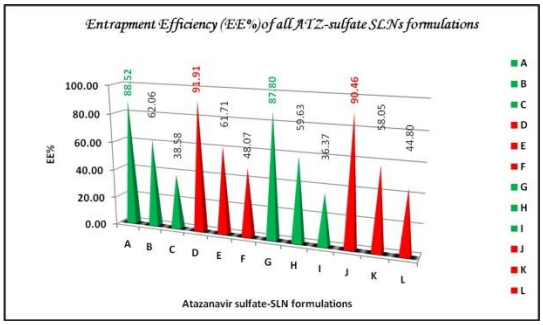

Figure 1: Entrapment Efficiency of all the formulations of ATZ-sulfate-SLNs formulations.

HPLC method for Atazanavir sulfate

There are a number of analytical methods developed for Atazanavir. Analytical method used here was successfully reproduced from the already existing method[9], produced good sensitive results for the drug. This method was tested for linearity which results in good linearity from range of 0.01ppm to 500ppm i.e. 1. There was a slight difference observed in chromatogram as compared to chromatogram produced by Seshachalam et al. The retention time was increased from nearly 10 minute to 12 minute.

followed by filtration and analysis of drug in the supernatant was measured by HPLC method of drug analysis.

Entrapment efficiency[10] in formulations with ratio 1:4 was observed less as compared to the

formulations with ratio 1: 3.5; it is because of higher % of surfactant.

Drug loading

Drug loading was achieved from 4.78% to 6.28%. Formulation D achieved maximum drug loading. Figure 2 shows drug loading of all formulations. For efficacy and efficiency reasons, the amount of drug loading is very important.

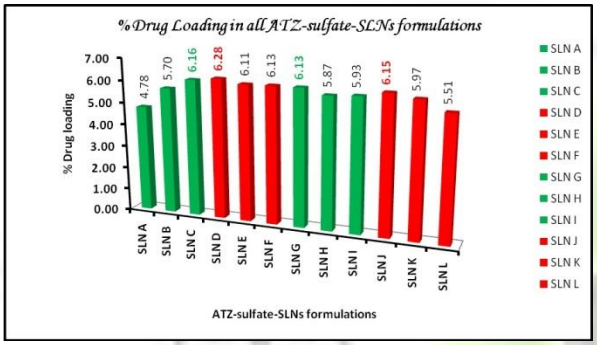

Figure 2 : % Atazanavir sulfate loading in various ATZ-sulfate-SLNs formulations

Particle Size analysis: Laser diffraction

The average particle size distribution pattern, shown in Figure 3 below;

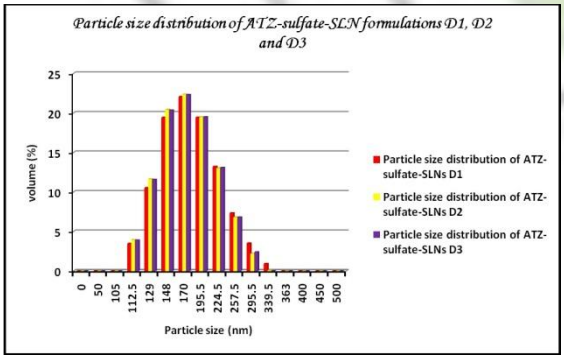

Figure 3: Laser diffraction particle size distribution of various formulations of ATZ-sulfate-SLNs D

The method hot homogenization followed by ultrasonication could produces uniform particle size pattern. Figure 5 show the average d values for ATZ-sulfate-SLNs D. it shows that 90 % of SLNs were below 240 nm while 50 % were above 174 nm and 10 % were below 132 nm.

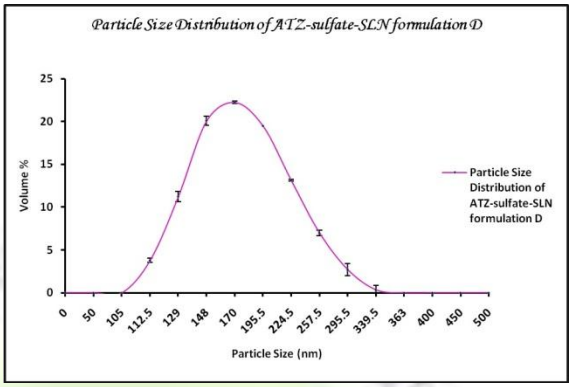

Figure 4: Laser Diffraction particles size distribution of ATZ-sulfate-SLNs formulation D

The size distribution of particles is represented by polydispersity index and a smaller value for the polydispersity index indicates that the particles have a smaller distribution range, which is critical for regulatory purposes. SLNs preferably have size range of 50 to 400 nm and polydispersity index of below 1. Figure 4 show that ATZ-sulfate-SLN D has desirable results.

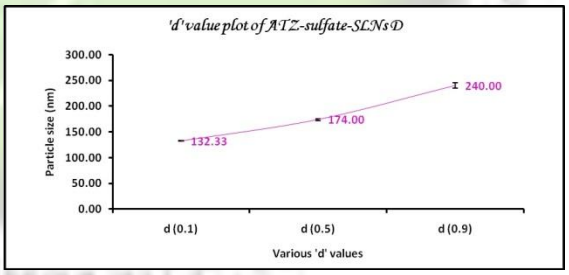

Figure 5: 'd' values of ATZ-sulfate-SLNs formulation D

Transmission Electron Microscopy

Only freshly prepared ATZ-sulfate-SLNs formulation D was examined for TEM images, because this one shows most expected results among the other formulations. Results showed that shape of the ATZ-sulfate-SLNs was

spherical and had a narrow size distribution approximately 92 nm to 250 nm.

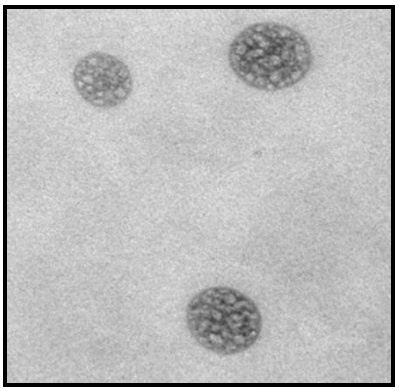

Figure 6: TEM images of ATZ-sulfate SLNs formulation D. Particles were imaged using an accelerating voltage of 80kv and at a magnification of 150000X.

Zeta Potential

Weak negative charge of ~-10.77 ±0.45 mV was observed for ATZ-sulfate-SLNs formulation D (This result is average of three different ATZ-sulfate-SLNs formulation D1, D2, D3 Figure 7 shows the zeta potential of three formulations). We know that, as the zeta potential increases, electrostatic repulsion between two particles increases. If, the electrostatic repulsion exceeds the attractive forces than the colloidal system will remain be stable[11-16].

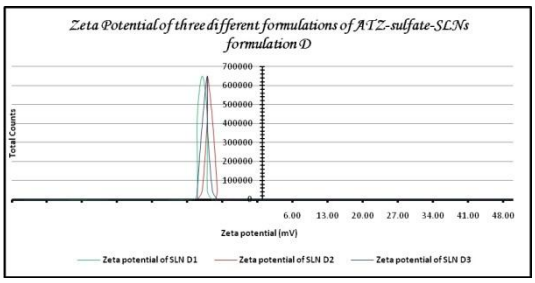

Figure 7: Zeta Potential distribution of three different ATZ-sulfate-SLNs formulations D1, D2, D3.

(Middle vertical line showing vertical axis with total counts value just in left of it. Negative values of zeta potential (mV) from -50 to 0 are now shown in the graph. The values from 0 to -50 starts from 0 and proceeds in descending order up to -50 on horizontal axis).

In-vitro drug Release

The cumulative percent release of drug from SLN was evaluated over 24 hrs time period, using PBS as release medium. ATZ-sulfate release from SLNs demonstrated an initial burst release of approximately 19% by one hour with gradual release up to approximately 54% after 24 hrs. Figure 8 shows the release pattern of Atazanavir sulfate from freshly prepared ATZ-sulfate-SLNs formulation D.

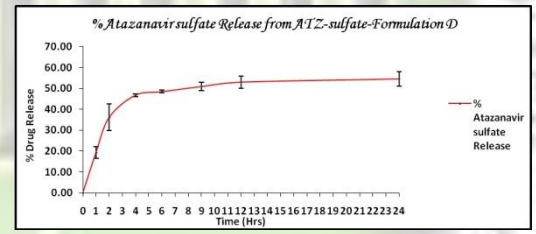

Figure 8: Percentage Atazanavir sulfate release from freshly prepared compritol 888 ATO SLN formulation D using PBS (pH 7.4 at 37°C) as releasing medium. *Atazanavir sulfate release from SLNs demonstrated an initial burst release of approximately 19% by one hr. with gradual release up to 54% after 24 hrs.*

Conclusion:

In this study SLNs of Atazanavir Sulphate were studied for entrapment efficiency and drug loading by preparing different formulations by varying the concentration of atazanavir sulfate. ATZ-sulfate-SLNs D was

the optimized formulation. Three formulations of ATZ-sulfate-SLNs D, namely ATZ-sulfate-SLN D1, D2, D3 were prepared and particle size of all three was determined with laser diffraction (LD), which was observed in range of 105nm to 363nm. TEM images showed that, SLNs were spherical in shape and its size was identical to that obtained from LD. Entrapment efficiency of 91.91% and drug loading of 6.28% was achieved with optimized formulation. TEM images showed that the SLNs were spherical in shape and particle size obtained was identical with that obtained from Laser diffraction. In-vitro drug release study was done by dialysis bag diffusion technique, shows controlled release up to 54% after 24 hrs using phosphate buffer saline pH 7.4 at 37°C. Particle size of SLNs increased two times after sterilization by autoclave. This study shows the preparation and characterization of controlled release formulation of solid lipid for the enhanced delivery of Atazanavir sulfate.

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