**Design and Evaluation of Atazanavir Bi Sulfate Nanosuspension**

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**ABSTRACT**

Nano sizing refers to the reducing the size of active Pharmaceutical Ingredient (API) to micron range up to below 1 μm typically a few hundred nanometers. The sub-micron particles are stabilized with surfactants or polymers in NPs, which can be further processed into standard dosage forms, such as capsules or tablets, suitable for oral administration. These nano formulations offer increased dissolution rates and enhance bioavailability of insoluble compounds (BCS Class II and IV drugs). NPs efficiently improve oral absorption of poorly soluble drugs and achieve a higher bioavailability compared to traditional formulation. Present study has been undertaken to develop NS of ATV, by media milling method, with improved oral bioavailability. Formulation of NS requires a careful selection of stabilizers. Steric and electrostatic stabilizers are needed to stabilize the nanoparticles against inter-particle forces and prevent them from aggregating. Steric stabilization is often combined with electrostatic stabilization for additional repulsive contribution. Different pharmaceutical excipients including povidone (PVP K25), poloxamer and steric stabilizer and sodium lauryl sulphate (SLS) as anionic electrosticc stabilizer were used in an effort to develop stable ATV loaded NS.

**Keywords:** Atazanavir Bi Sulfate, Nanosuspension, dissolution, bioavailability.

**INTRODUCTION**

The very low solubility of Atazanavir (ATV) hinders its administration, absorption and bio distribution. Thus, there is need to have some innovative formulation approach to enhance the bioavailability. This can be overcome by increasing the solubility of API and reduction of particle size to nanometer range. Nano suspensions are considered to be the best alternate approach for poor water soluble drugs to enhance oral bioavailability.

Currently, media milling is preferred over HPH technique because it is easy to scale up to industrial pharmaceutical unit operations. Also, crystalline nature of the drug remains largely intact during the media milling processing, thus relieving any stability concerns. Furthermore, no organic solvent or harsh environment is needed. NSs efficiently improve oral absorption of poorly soluble drugs and achieve a higher bioavailability compared to traditional formulation.

**Human Immunodeficiency Virus (HIV)**

Human Immunodeficiency Virus usually attacks the immune system. Lentiviruses are in turn part of a larger group of viruses known as retroviruses. Retroviruses are the exception because their genes are composed of Ribonucleic Acid (RNA). The name 'lentivirus' literally means 'slow virus' because they take such a long time to produce any adverse effects in the body. There are two types of HIV: HIV-1 and HIV-2. HIV-2 is less easily transmitted, and the period between initial infection and illness is longer in the case of HIV-2. Worldwide, the predominant virus is HIV-1, and generally when people refer to HIV without specifying the type of virus they will be referring to HIV-1. The relatively uncommon HIV-2 type is concentrated in West Africa and is rarely found elsewhere.

**Acquired Immune Deficiency Syndrome (AIDS)**

AIDS is a disease of the human immune system caused by the HIV. The illness interferes with the immune system, making people with AIDS much more likely to get infections, including opportunistic infections and tumors that do not affect people with working immune systems. This susceptibility gets worse as the disease continues. HIV is transmitted in many ways, such as: sexual intercourse, contaminated blood transfusions, hypodermic needles, during pregnancy (between mother and baby) and from breastfeeding. It can be transmitted by any contact of a mucous membrane or the bloodstream with a bodily fluid that has the virus in it, such as the blood, semen, vaginal fluid, preseminal fluid, or breast milk from an infected person.

AIDS is a clinical consequence of infection with HIV. HIV primarily infects vital organs of the human immune system such as CD4+ T cells (a subset of T cells), macrophages and dendritic cells. It directly and indirectly destroys CD4+ T cells.

Once the number of CD4+ T cells per microliter of blood drops below 200, cellular immunity is lost. Acute HIV infection usually progresses over time to clinical latent HIV infection and then to early symptomatic HIV infection and later to AIDS, which is identified either on the basis of...
the amount of CD4+ T cells remaining in the blood, and/or the presence of infections. 8

The virus and disease are often referred to together as HIV/AIDS. The disease is a major health problem in many parts of the world, and is considered a pandemic, a disease outbreak that is not only present over a large area but is actively spreading. 9 In 2009, the World Health Organization (WHO) estimated that there are 33.4 million people worldwide living with HIV/AIDS, with 2.7 million new HIV infections per year and 2.0 million annual deaths due to AIDS. 10 In 2007, UNAIDS estimated: 33.2 million people worldwide were HIV positive; AIDS killed 2.1 million people in the course of that year, including 330,000 children, and 76% of those deaths occurred in sub-Saharan Africa. 11 According to UNAIDS 2009 report, worldwide some 60 million people have been infected since the start of the pandemic, with some 25 million deaths, and 14 million orphaned children in southern Africa alone. 11 However, with the adherence to HAART the efficacy rate of the available treatment has increased up to 85% against the AIDS as well as secondary diseases such as Kaposi’s sarcoma. 12,13

**Antiretroviral (ARV) Drug Treatment**

Antiretroviral drugs are medications for the treatment of infection by retroviruses, primarily HIV. The aim of antiretroviral treatment is to keep the amount of HIV in the body at a low level. This stops any weakening of the immune system and allows it to recover from any damage that HIV might have caused already. The treatment consists of drugs that have to be taken every day for the rest of a person’s life. If only one drug was taken, HIV would quickly become resistant to it and the drug would stop working. There are different classes of ARV drugs that act on different stages of the HIV life-cycle. HIV can easily develop resistance to individual ARV therapies, but it is harder for HIV to become drug-resistant when multiple ARV drugs with varied mechanisms of action are combined into a single HIV treatment. Taking two or more ARV at the same time vastly reduces the rate at which resistance would develop, making treatment more effective in the long term.

### Table 1: Classification of ARV drugs

<table>
<thead>
<tr>
<th>Antiretroviral Drug Class</th>
<th>Mechanism of Action</th>
<th>Generic Name of Drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fusion or Entry Inhibitors</td>
<td>Prevent HIV from binding to or entering human immune cells</td>
<td>Enfuvirtide, Maraviroc</td>
</tr>
<tr>
<td>Nucleoside/Nucleotide Reverse Transcriptase Inhibitors (NRTIs)</td>
<td>NRTIs inhibit reverse transcription by being incorporated into the newly synthesized viral DNA strand as faulty nucleotides</td>
<td>Zidovudine, Didanosine, Zalcitabine, Stavudine, Lamivudine, Abcavir, Emtricitabine, Tenofovir</td>
</tr>
<tr>
<td>Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs)</td>
<td>NNRTIs inhibit reverse transcriptase by binding to an allosteric site of the enzyme</td>
<td>Efavarinz, Nevirapine, Loviride, Delavirdine, Etravirine, Rilpivirine, Lersivirine</td>
</tr>
<tr>
<td>Protease Inhibitors (PIs)</td>
<td>PIs target viral assembly by inhibiting the activity of protease, an enzyme used by HIV to cleave nascent proteins for the final assembly of new virions.</td>
<td>Saquinavir, Ritonavir, Indinavir, Nelfinavir, Amprenavir, Tipranavir, Lopinavir, Darunavir, Atazanavir, Nelfinavir</td>
</tr>
<tr>
<td>Integrase Inhibitors</td>
<td>Inhibit the enzyme integrase, which is responsible for integration of viral DNA into the DNA of the infected cell.</td>
<td>Raltegravir</td>
</tr>
</tbody>
</table>

### Table 2: Composition of the Different Formulations

<table>
<thead>
<tr>
<th>Batch No.</th>
<th>Excipient Name</th>
<th>Concentration of Excipient (% w/v)</th>
<th>Concentration of Drug (%w/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Poloxamer 188</td>
<td>0.5 %</td>
<td>0.5 %</td>
</tr>
<tr>
<td>2</td>
<td>Poloxamer 407</td>
<td>1.0 %</td>
<td>0.5 %</td>
</tr>
<tr>
<td>3</td>
<td>Poloxamer 407</td>
<td>2.0%</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Poloxamer 407</td>
<td>0.5 %</td>
<td>0.5 %</td>
</tr>
<tr>
<td>5</td>
<td>Poloxamer 407</td>
<td>1.0 %</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Poloxamer 407</td>
<td>2.0%</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Poloxamer 407</td>
<td>0.5 %</td>
<td></td>
</tr>
<tr>
<td>8</td>
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<td>1.0 %</td>
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</tr>
<tr>
<td>9</td>
<td>Poloxamer 407</td>
<td>2.0%</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Poloxamer 188</td>
<td>0.5%</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Poloxamer 188</td>
<td>1.0%</td>
<td>1.0%</td>
</tr>
<tr>
<td>12</td>
<td>Poloxamer 188</td>
<td>2.0%</td>
<td></td>
</tr>
</tbody>
</table>
Current Limitations of ARV Drug Therapy

ARV drug therapy has contributed significantly to improved patient/disease management, its current use is associated with several disadvantages and inconveniences to the HIV/AIDS patient. Many ARV drugs undergo extensive first pass metabolism and gastrointestinal degradation leading to low and erratic bioavailability. The half-life for several ARV drugs is short, which then requires frequent administration of doses leading to decreased patient compliance. A major limitation is that HIV is localised in certain inaccessible compartments of the body such as the CNS, the lymphatic system and within the macrophages.

These sites cannot be accessed by the majority of drugs in the therapeutic concentrations required and the drugs also cannot be maintained for the necessary duration at the site of HIV localization. These sub-therapeutic drug concentrations and short residence time at the required sites of action contribute significantly to both the failure of eliminating HIV from these reservoirs, and the development of multidrug-resistance against the ARV drugs.

The severe side effects associated with ARV therapy can therefore be attributed to the subsequent large doses essential for achieving a therapeutic effect, due to the inadequate drug concentrations at the site of action, and/or the poor bioavailability of several ARV drugs. These drugs also suffer from physico-chemical problems such as poor solubility that may lead to formulation difficulties. Strategies currently being investigated to overcome these limitations include the identification of new and chemical modification of existing chemical entities, the examination of various dosing regimens, as well as the design and development of novel drug delivery systems (NDDS) that can improve the efficacy of both existing and new ARV drugs.

More specifically, in the past decade there has been an explosion of interest in the development of NDDS for the incorporation of ARV drugs as a way of circumventing the problems described above and optimizing the treatment of HIV/AIDS patients. NDDS present an opportunity for formulation scientists to overcome the many challenges associated with ARV drug therapy.

The nanometer size and high surface area to volume ratio which affect the pharmacokinetics and bio distribution of the associated drug molecule are main features of NDDS.

Atazanavir

Atazanavir is classified as a BCS II drug (high permeability/low solubility). The free base of Atazanavir does not have sufficient bioavailability.

Therefore, quite a number of different acid addition of salts for example: Hydrochloride, Methanesulphonate (mesylate), Sulphates and bisulphate salts have been tested for the purpose of developing an orally administrable drug form. Owing to its good solubility in comparison with the other salts. Atazanavir bisulphate is used for producing the currently available oral drug forms.

Mechanism of Action

Atazanavir is an azapeptide HIV-1 protease inhibitor (PI). The compound selectively inhibits the virus-specific processing of viral Gag-Pol proteins in HIV-1 infected cells, thus preventing formation of mature virions and infection of other cells.

MATERIALS AND METHODS

Atazanavir is a gift sample obtained from Hetero drugs Ltd. Reference capsule was purchased from Zirconium oxide beads were received as gift sample from SPARC, India. Chemicals and reagents used for the preparation of buffers, analytical solutions, and other general experimental purposes are obtained from various resources.
Development of Atazanavir Nanosuspensions Formulation

NS are composed of the pure drug particles plus stabilizing agents in an aqueous medium. NS was prepared by media milling technique. Zirconium oxide beads were used as milling media. In glass vial, weighed quantity of zirconium oxide beads was taken and 5 ml distilled water was added in this vial. Surfactant and drug were incorporated and comminution process was carried out for a specific time period.

Optimization of NS using Factorial Design

Various formulation and process variables relating to effectiveness and usefulness should be optimized simultaneously when developing pharmaceutical formulations. The difficulties in optimizing a pharmaceutical formulation are due to difficulty in understanding the real relationship between dependent and independent responses.

Factorial design has often been applied to optimize the formulation variables with basic need of understanding of interaction of independent variables. Based on the preliminary studies we came to know that concentration of drug, concentration of polymer, concentration of surfactant and milling time are the main factors which affect the mean particle size and Zeta Potential of the nano suspension. Box-Behnken designs is used to determine relationship between different response variables and set of quantitative parameters. We have applied the four-factorial three-level Box–Behnken experimental design for optimization of ATV NS formulation. A four-factor, three-level Box-Behnken design was generated by Design-Expert®8.0 software to conduct the study.

Preparation of Atazanavir Nano-Suspension

ATV NS was prepared by media milling technique A solution of PVP K25 (1% w/v) and SLS (0.5% w/v) in 5 ml double distilled water was prepared in a 20 ml glass vial. ATV (4.0 %w/v) was then dispersed in this stabilizers solution.

Subsequently, 6.0 g of zirconium oxide beads (diameter ranging from 0.4 to 0.7 mm) used as milling media were added. Comminution was carried out on a magnetic stirrer (Remi equipment Pvt Ltd, India) using polygon magnetic stirring bar (Ø 8 mm × l 22 mm) at 800 rpm for 22 hrs at ambient temperature. Subsequently, NS was separated from milling media by decanting the suspension, followed by washing of the beads with double distilled filtered water.

Lyophilization of ATV NS

Mannitol and trehalose were used as cryoprotective agent in NS formulations to form a lyophilized product. It was added to NS after the media milling step but just before the freezing step. The freshly prepared ATV NSs were lyophilized with cryoprotective agent (i.e. trehalose or mannitol) at different concentrations (5%, 10%, 20%, 30% and 40% w/v). Briefly, ATV NSs were cooled down to −70°C for 12 h followed by freeze-dried in a freeze-drier under vacuum for 24 h. The lyophilized product was re-dispersed with the double distilled filtered water.

Evaluation of Nanosuspension

Characterization of ATV NS Formulation

Particle Size Measurement

Figure 1: 3D Response surface plots for mean particle size: (A) Effect of drug concentration (X1) and milling time (X4) on the response Y1, (B) effect of polymer concentration (X2) and surfactant concentration (X3) on the response Y1 (C) effect of Surfactant concentration (X3) and milling time (X4) on the response Y1.

The particle size analysis and PDI of ATV NSs were determined using a Malvern Zeta Sizer Nano ZS 90 (Malvern Instruments, Malvern, UK). The PDI indicates the width of a particle distribution (e.g. 0.0 for a narrow, 0.5 for a very broad distribution). Prior to the measurement, the samples were diluted with double distilled filtered water to a suitable scattering intensity
and re-dispersed by shaking before the measurement. All measurements were performed in triplicate. The results are expressed as mean ± standard deviation (SD). A decrease in the MPS of ATV NS was observed when PVP K25 used as stabilizer. The size of particles in NS stabilized with PVP K25 was ranged from 335.5 nm to 515.5 nm with narrow PDI value ranged from 0.211 to 0.314. PVP K25 was able to reduce the particle size in nano-range even though at higher concentration of drug. PVP K25 is efficient for full coverage of newly generated particles in NS. 3D response surface plots for Mean Particle size as given in Figure 1.

![Figure 1: 3D Response surface plots: (A) Effect of polymer concentration (X2) and surfactant concentration (X3) on the response Y2, (B) effect of polymer concentration (X2) and milling time (X4) on the response Y2.](image)

**Solid State Evaluation**

Changes in the crystalline state can affect the solubility, dissolution velocity, the oral bioavailability as well as the stability of a pharmaceutical formulation. 21,22

Therefore the influence of media milling on the crystalline structure of ATV in nanosuspension was investigated via DSC and XRD analysis.

**DSC Analysis**

Thermal properties of the lyophilized NS (LNS) samples were investigated with a Shimadzu differential scanning calorimeter (Shimadz, Japan). Thermograms of standard ATV powder, PVP K25, SLS, trehalose, their physical mixture (PM) and LNS were recorded in order to characterize the physical state of ATV in the NS.

A heating rate of 10°C/min was employed in the range of 25-300°C with nitrogen atmosphere supplied at 40 ml/min. Each sample was taken (~ 4-8 mg) in an aluminum pan, crimped and sealed. An empty aluminum pan was used as reference.

**XRD Analysis**

XRD diffractograms of standard ATV powder, PVP K25, SLS, trehalose, their PM and LNS were obtained using Bruker AXS D8 Advance X-ray diffractometer. Scans were performed between 5° < 2θ < 80°.
Morphology of NS by SEM and TEM

![SEM images](image)

**Figure 5**: SEM images of (A) standard ATV and (B) LNS showing the particle size reduction with NS formulation. TEM image of (C) LNS showing spherical shape of ATV particles in NS.

Morphological evaluation of LNS was conducted through the SEM (JSM 6380 LV, JEOL, Japan) and TEM (Tecnai 20 Philips). For SEM analysis, LNS and standard ATV were fixed on a brass stub using a carbon double sided tape. Samples were then subjected to conductive coating with Au-Pd (80% - 20%). SEM was operated at an acceleration voltage of 20 kV. For TEM analysis, LNS was diluted with double distilled water and a drop of it was placed on a carbon-coated copper grid (300 mesh, 3mm) and air dried.

Drug Assay

The NS formulation was assayed for ATV by diluting with methanol and further dilution with mobile phase. A 20 μl aliquot was injected into the HPLC for ATV measurement at 225 nm.

Saturation Solubility

Saturation solubility of standard ATV, PM and LNS formulation was carried out in double distilled water. LNS, PM and standard ATV were dispersed in water, 10 ml each, to obtain suspension and placed on a mechanical shaker for 24 h. Samples were centrifuged and the resulting supernatant was analyzed using HPLC (Shimadzu Corporation, Kyoto, Japan) method after suitable dilution with methanol. The experiment was conducted in triplicate. The mean results of three experiments of each sample and SD were reported.

Dissolution Study

In vitro dissolution studies were performed using gelatin capsules containing an amount of the formulation (LNS, PM or standard drug) equivalent to 50 mg of ATV and compared with MF dissolution profile. Tests were performed according to the United States Pharmacopeia (USP) and “Dissolution Methods for Drug Products” guide of FDA using dissolution apparatus II. Experiments were performed using 900 ml media (1% SLS in water) at 37 ± 0.5°C at a rotation speed of 50 rpm. At preselected time intervals, 5 ml samples were withdrawn and replaced with 5 ml of pre-thermostated fresh dissolution medium. Samples were filtered through 0.1 μm syringe filter; filtrate was diluted with mobile phase and 20 μl was injected into HPLC for analysis. Dissolution tests were performed in triplicate. Graph of percent cumulative drug release vs. time was plotted. Dissolution profiles were evaluated on the basis of dissolution efficiency (DE) and percentage of drug dissolved (DP) at 5 min and 60 min, time needed to dissolve 50% of drug (t50%), area under curve (AUC) and mean dissolution time (MDT). An add-in program, DD solver, for comparison of drug dissolution profiles was used to calculate different dissolution parameters.

The dissolution rate was markedly enhanced in the LNS, as 89.25% ± 1.11 of the drug dissolved in 5 min, as compared to only 4.75% ± 2.52, 8.78 ± 2.1 and 19.58% ± 0.65 from standard ATV, PM and MF, respectively. The standard ATV and PM did not achieve complete dissolution during the 60 min test period and only 49.00% ± 5.13 and 64.03% ± 5.05 of the drug dissolved over 60 min, respectively, owing to the large crystal size, while NS showed a significantly enhanced dissolution rate with 99.13% ± 0.21 of the drug dissolving over 60 min.

PAMPA Study

The BD Gentest™ pre-coated PAMPA plates were used to perform permeability assays for standard ATV, MF and LNS. The permeability assay was carried out as per protocol described in references. The 96-well filter
plate, pre-coated with lipids, was used as the permeation acceptor and a matching 96-well receiver plate was used as the permeation donor. Sample solutions were prepared by diluting 10 mM stock solutions in 20% methanolic PBS pH 7.4 (final concentration of 200 μM). Sample solutions were added to the wells (300 μl/well) of receiver plate and 20% methanolic PBS pH 7.4 was added to the wells (200 μl/well) of pre-coated filter plate. Filter plate was then coupled with receiver plate and the plate assembly was incubated at room temperature without agitation for 5 h. The assembled plate was placed into a sealed container with wet paper towels to avoid evaporation. At the end of incubation, samples from the donor and receiver plate were analyzed for ATV concentration by HPLC method. The effective permeability (Pe) values for the standard ATV, MF and LNS are reported in Table 3. The LNS represents significant improvement in permeability than the MF in PAMPA model.

Permeability of the ATV was calculated using the following formula:

\[
Permeability (Pe) = \frac{-\ln\left[\frac{1-C_{\text{eq}}}{C_{\text{eq}}(V_D+V_A)}\right]}{[\Delta x(V_D+V_A)]/V_D+V_A} \tag{1}
\]

A = filter area (0.3 cm²), VA = donor well volume (0.3 ml), VA = acceptor well volume (0.2 ml), t = incubation time (seconds), CA(t) = compound concentration in acceptor well at time t (mM), CD(t) = compound concentration in donor well at time t (mM), and Ceq = [(CD(t)*VD+CA(t)*VA)/(VD+VA)].

**Table 3: Effective permeability value by PAMPA study (n=6)**

<table>
<thead>
<tr>
<th></th>
<th>Effective permeability (Pe) ± S.D. (10-6 cm/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard ATV</td>
<td>15.95 ± 1.02</td>
</tr>
<tr>
<td>MF</td>
<td>16.84 ± 0.82</td>
</tr>
<tr>
<td>LNS</td>
<td>20.81 ± 1.10</td>
</tr>
</tbody>
</table>

Membrane Integrity Test

Lucifer Yellow, a fluorescence dye was selected to study membrane integrity in PAMPA with and without the addition of placebo which contains excipients of same concentrations used to prepare NS. Studies in the literature have shown that Lucifer Yellow CH does not cross the cell membrane as long as the cell lipid membrane remains intact thus, in order to measure the stability of the lipid bilayer, the amount of Lucifer Yellow found in the acceptor well was measured Chromatographic separation was performed using a Phenomenex Hypersil C4 (100 mm x 4.6 mm i.d μm particle size) column. Separation was achieved using a mobile phase consisting of acetonitrile and 100 mM ammonium acetate buffer pH 7.0 in the ratio of 70:30 (v/v), pumped at a flow rate of 1 ml/min. Column was maintained at ambient temperature and an injection volume of 20 μl was used. The wavelength of the excitation filter was 430 nm. The wavelength of the emission filter was 530 nm.

**In-vivo Evaluation of ATV NS Formulation**

**Animals:** Male Albino rats (200 ± 15 g) and New Zealand white male rabbits (2.0 ± 0.2 kg) were used for the in situ intestinal absorption study and in vivo pharmacokinetic studies respectively Animals were maintained at a temperature of 25 ± 2°C and a relative humidity of 70 ± 5% under natural light/dark conditions and were fed with food and water ad libitum. Prior to experiment the animals were kept under overnight fasting. Absorption curve of LNS represented significant improvement in drug absorption than the MF. This enhancement may be attributed to effect of the surfactants, nano-range particle size and adhesion property of NS.

**Table 4: Absorption parameters of LNS and MF**

<table>
<thead>
<tr>
<th>Absorption Parameter</th>
<th>LNS</th>
<th>MF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ka (h⁻¹)</td>
<td>0.6256 ± 0.10</td>
<td>0.2547 ± 0.01</td>
</tr>
<tr>
<td>t1/2 (h)</td>
<td>1.11 ± 0.17</td>
<td>2.72 ± 0.11</td>
</tr>
<tr>
<td>Uptake percentage (%)</td>
<td>98.13 ± 0.78</td>
<td>80.96 ± 0.64</td>
</tr>
<tr>
<td>AUC0-6h in situ (mg·h)</td>
<td>155.68 ± 4.68</td>
<td>96.39 ± 4.50</td>
</tr>
</tbody>
</table>

Data are shown as Mean ± SD, n=3.

**Figure 6:** Amount of ATV absorbed from LNS and MF during in situ single pass intestinal perfusion studies showing higher absorption rate of EFV in NS compared to MF. Data are represented as Mean ± SD; n=3 for each group.

**In-vivo Pharmaco Kinetic Study**

Bioavailability of LNS was compared with MF and standard ATV. Rabbits were allocated at random to three treatment groups and administered standard ATV, MF and LNS. The standard ATV, MF and LNS equivalent to 10 mg/kg dose of ATV were filled in mini hard capsules and administrated orally. Blood samples (1.5 ml) were collected through the marginal ear vein into heparinized tubes at 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 8, 12, 24 and 48 h after administration. Blood samples were centrifuged at 3000 rpm for 10 min using a high speed centrifuge machine and plasma samples were withdrawn and stored at -20°C until analysis. Plasma samples collected from the
rabbits were analyzed using developed reverse phase HPLC. Trapezoidal method was employed to calculate the area under the curve (AUC) of plasma concentration as a function of time (t). Mean residence time (MRT) was calculated as area under the first moment curve (AUMC) divided by AUC. AUMC was determined from the plot of plasma concentration multiplied by time (C X t) versus time.

RESULTS AND DISCUSSION

NSs are useful as oral dosage forms for poorly soluble drugs.29,30 Atazanavir is a very hydrophobic compound with low density, high flow resistance and practically insoluble in water. In this study, ATV was formulated into NS for oral administration using media milling method.

The NS of ATV prepared without any stabilizer showed rapid agglomeration of drug nano particles immediately after preparation. The agglomeration of ATV nano particles is not only due to the attractive forces between the particles in the absence of significant energy barrier, but also it is a result of the so-called hydrophobic effect. The presence of hydrophobic particles or molecules in water causes distortion and re-arrangement of hydrogen bonding in the aqueous medium, therefore, greatly increasing the free energy of the system.31 As a result, these hydrophobic particles tend to agglomerate to reduce the system free energy.

NS formulation of ATV requires a careful selection of stabilizers. Stabilizers are needed to stabilize the nano particles against inter-particle forces and prevent them from aggregating. At the nanometer domain, attractive forces between particles, due to dispersion or Van der Waals forces, come into play. This attractive force increases dramatically as the particles approach each other, ultimately resulting in an irreversible aggregation.32 In the media milling process, comminution continually fractures organic crystals while stabilizer adsorb onto fresh surface and stabilize each broken particle.33

**Abbreviation:** NS – Nano Suspension; ZP – Zeta potential; ATV – ATAZANAVIR; ARV - Anti retroviral; v/v – volume/volume; SEM – Scanning electron microscope TEM - Transmission electron microscopy ; DSC - differential scanning calorimeter.

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