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# Biodegradable Polymeric Nanoparticles for Delivery of Combination of Antiretroviral Drugs

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**Abstract:** The major shortcoming of antiretroviral therapy was found to be bioavailability and multidrug resistance. In order to overcome these problems the present study has been planned to formulate and evaluate combined nanoparticles of darunavir (DRV) and atazanavir (ATV).Nanoparticles were prepared by nanoprecipitation method using biodegradable polymers like polycaprolactone (PCL) and L-lactide / Ɛ-caprolactone (L-PCL) using  d-α tocopheryl polyethylene glycol succinate 1000 (TPGS) as surfactant. The prepared formulations were optimized based on polymer and surfactant concentration and further evaluated for particle size distribution, entrapment efficiency (EE), scanning electron microscope (SEM), *in vitro* release and *in vivo* pharmacokinetic study. Infrared spectroscopy (IR) and differential scanning calorimetery (DSC) studies were also performed to know any drug excipient interactions. With PCL the particle size was found in range of 138 nm to 196 nm with zeta potential of -22.3 mV(F3) and entrapment efficiency of 91.64% and 83.54% for darunavir and atazanavir respectively. A size range of 160 nm to 186 nm with zeta potential of -32.8 mV(F7) and entrapment efficiency of 60.4% and 85.2% was found for ATV and DRV respectively in case of L-PCL. From IR and DSC studies, formulations were found to be stable with no significant drug-excipient interactions. Surface morphology using SEM analysis showed formation of spherical shaped nanoparticles without aggregation. The prepared formulations demonstrated extended release of DRV and ATV for more than 4 days with 23% and 70 % of drug release from PCL and L-PCL nanoparticles respectively. *In vivo* pharmacokinetic study showed there was twofoldincrease in area under curve (AUC) value of darunavir and atazanavir when administered as PCL and L-PCL nanoparticles as compare to pure drug suspension .Optimized nanoparticle formulations showed stability as per ICH guidelines. Our studies proved for the first time that combined drug delivery can be achieved successfully of antiretroviral drugs through nanoparticle formation.
**Keywords**: Atazanavir (ATV), Darunavir (DRV), Multidrug resistance (MDR), Polycaprolactone and antiretroviral therapy.

## 1. Introduction

Human Immunodeficiency Virus (HIV) is the primary cause of Acquired Immuno Deficiency Syndrome (AIDS) which still remains a significant cause of mortality globally[1]. HIV infection results in compromised immune defence by causing extensive destruction of T-helper cells, macrophages, dendritic cells and other cellular components associated with cell-mediated immunity[2].Globally, an estimated 35.3 (32.2–38.8) million people were living with HIV in 2012[3].The current clinical therapy, known as 'highly active antiretroviral treatment' or HAART, is considered as one of the most significant advances in the field of HIV therapy .The therapyuses combination of drugs that inhibit HIV-1 replication which leads to reduction in viremia and the onset of opportunistic infectionsin most patients and prolonged survival.Anti-retroviral therapy (ARV) significantly results in reduction of morbidity and mortality in patients suffering from AIDS. However there are several limitation linked with the current ARV therapy which includes drug resistance, long-term drug therapy, toxicity, drug interactions, poor bioavailabilityand lack of access to reservoir site [4].

Beauty of nanotechnology-based systems for antiretroviral drug delivery is based on modulation of pharmacokinetics of incorporated molecules[5].It is beneficial to use nanotherapy for antiretroviral drug delivery because of flexibility (all types of drugs can be encapsulated), good toxicity profile, opportunity of drug-release modulation, high drug payloads, relative low cost, easiness to produce and possible scale-up to mass production[6].Protection of incorporated drugs in nanosystems from metabolism is another favourable fact that allows prolonged drug residence in body, thus reducing doses needed for treatment and prolonging the time between dose administrations [7].

Cells of the mononuclear phagocytic system (MPS), such as the monocytes/macrophages (Mo/Mac), act as a reservoir for the HIV virus. Therefore, drug treatment of HIV infection should involve targeting drugs to these cells in addition to the lymphocytes. Several studies involving antiretroviral (ARV) loaded nanoparticles for targeting to the macrophages have consequently emerged [8].

Protease inhibitors like atazanavir (ATV) and darunavir (DRV) used as antiretroviral drugs have low solubility and bioavailability of 37% & 60% respectively. The dose of these drugs ranges from 75 to 600 mg with ritonavir as boosting in conventional formulations. These drugs are extensively metabolised by CYP P450 3A4 when given alone. Researchers have been able to fabricate single antiretroviral drug into a nanoparticle delivery system. But use of a single antiretroviral drug in the treatment of HIV-1 results in development of resistant strains andtreatmentfailures[9,10].So fabricating two drugs in combination in nanoparticles can solve the limitations with single drug delivery and offer synergistic therapeutic effect. Present study was aimed to achievereduce toxicity, longer intracellular drug residence,smaller drug doses and decreased dosing variability.

## 2. Experimental

### 2.1. Materials

Darunavir and atazanavir were kindly gifted by Hetero Pharma Pvt. Ltd. (Hyderabad, India). Verapamil hydrochloride was a kind gift from Fleming Laboratories Ltd. (Hyderabad, India). Poly caprolactone (molecular weight 14000), TPGS (d-Alpha Tocopheryl Poly (ethylene glycol) 1000 succinate)were purchased from (Sigma Aldrich, India).Potassium dihydrogen phosphate, methanol, d-mannitol, disodium hydrogen phosphate, dextrose anhydrous AR and sodium CMC were purchased from Sd Fine-Chem Ltd. (Mumbai, India) All other chemicals and reagents used were of analytical grade.

### 2.2. Preparation of polymeric nanoparticles

DRV and ATV loaded polymeric nanoparticles were prepared by nanoprecipitation techniqueas described in literature [11].Briefly 50 mg of PCL was dissolved in 9 ml of organic solvent containing methanol:acetone (1:2) mixture and sonicated (Sonics & materials, Inc, Vibra cell VCX 750) for 5 min to completely dissolve PCL.10 mg of each drug (darunavir and atazanavir) were dissolved in polymeric solvent system. The organic phase containing PCL and drugswas added slowly to 20 ml TPGS solution (0.5%w/v) under mechanical stirring at 2,000 rpm and stirring was continued for 2 h to evaporate the solvent system.

### 2.3. Optimisation of process parameters

To study the effect of different process parameters on nanoparticle formulations in terms of particle size, polydispersity entrapment efficacy and drug loading capacity each set of formulations were prepared by varying one variable while other was kept constant (Table 1).Formulation parameters studied for optimization were:

a) Polymer concentration (50 mg and 100 mg).
b) TPGS surfactant concentration (0.5 % and0.75%).
c) Grade of PCL (Ɛ- polycaprolactum and l-lactide caprolactum).

**Table 1 Prepared formulation of ATV-DRV nanoparticles**

| S.No | Batch code | Organic phase volume (ml) | Polymer weight (mg) | | TPGS concentration (%w/v) |
|---|---|---|---|---|---|
| | | | PCL | L-PCL | |
| 1 | F1 | 9 | 50 | - | 0.5 |
| 2 | F2 | 9 | 100 | - | 0.5 |
| 3 | F3 | 9 | 50 | - | 0.75 |
| 4 | F4 | 9 | 100 | - | 0.75 |
| 5 | F5 | 9 | - | 50 | 0.5 |
| 6 | F6 | 9 | - | 50 | 0.75 |
| 7 | F7 | 9 | - | 100 | 0.5 |
| 8 | F8 | 9 | - | 100 | 0.75 |

### 2.4. Particle size and Polydispersity index

Prepared nanoparticles suspension was suitably diluted in millipore water. Particle size and polydispersity index (PDI) was performed at 25°C by using zeta sizer (Nano ZS Malvern Instruments, UK).The mean particle size was determined and each experiment was repeated in triplicates.

### 2.5. Entrapment efficiency (E.E.) and loading capacity (L.C.):

The amount of entrapped drugs in nanoparticles was determined by separating the nanoparticles from the aqueous suspension and subjecting it to centrifugation at 12,000 rpm for 1 h (REMI electrotechnik Ltd.R-24).The amount of free DRV and ATV in the supernatant was determined by HPLC analysis (SHIMADZU CORP). Entrapment efficiency and drug loading capacity were calculated according to the given equations:

$$\text{Entrapment efficiency} = \frac{amount\ of\ drug\ in\ nanoparticles}{\text{total amount of drug}} \times 100 \quad Eq\ .....1$$

$$\text{Drug loading capacity} = \frac{\text{The amount of drug in the nanoparticle}}{\text{amount of polymer weight}} \times 100 \quad Eq\ .....2$$

### 2.6. Scanning electron microscopy (SEM) analysis

Shape and surface topography of formulated combined retroviral drugs loaded nanoparticles of PCL and L-PCL was observed under scanning electron microscopy (Shimadzu, s-3700N).For analysis sample was mounted on an aluminium stub and were coated with gold-palladium alloy to thickness of 400Å, viewed at a working distance of 10 mm at an accelerating voltage of 15 kV and observed at 13500 X magnification.

### 2.7. *In vitro* release study

*In vitro* release study ofDRV and ATV loaded NP's was carried out in phosphate buffer pH 7.4 by using dialysis membrane.Equivalent amount of nanoparticles containing 20 mg of drugs were added into the dialysis bag (molecular wt. cut off 12,000 - 14,000 Daltons and was placed in a container with 150 ml of phosphate buffer pH 7.4 at constant agitation of 150 rpm maintained at 37° ± 1°C. An aliquot of 5 ml sample was withdrawn at various time intervals over a period of 96 hr and replenished with equal amount of buffer

immediately .The amount of drug released at each time interval was determined by taking absorbance of filtered samples spectrophotometrically at $\lambda_{max}$ of 263 and 278 nm simultaneously.

## 2.8. Fourier Transform Infraredspectroscopy (FT-IR)

The drug-excipient interaction study was investigated by FT-IR spectroscopy (Perkin-Elmer 1600). The FT-IR spectra of pure drugs, polymers and drug loaded nanoparticles were performed in IR range from 2000cm$^{-1}$ to 200cm$^{-1}$.

## 2.9. Differential scanning calorimetry (DSC)

Differential scanning calorimetry was carried out to study effect of nanoencapsulation of drugs on crystallinity.The possibility of any interaction between darunavir, atazanavir and polycaprolactone was assessed by carrying out the thermal analysis of drugs, physical mixture and nanoparticles by using differential scanning calorimeter (DSCShimadzu,DSC-60,Kyoto, Japan) at heating rate of 5°C/min at a heating range of 40 to 300°C under nitrogen atmosphere.

## 2.10. Stability studies of drug loaded nanoparticles

Stability Studieswerecarried out for optimised freeze dried NP's formulations containing 5% mannitol (F3and F7) as per ICH guidelines[12] (stability testing of new drug substances and products) over a period of 30 days. Optimized formulations were transferred in 5 ml glass vials sealed with plastic caps and kept in stability chambers at temperature and relative humidity of 2-8°C, 45±5% RH,25 ± 2° C, 60 ± 5 % RH and 40 ± 2° Cat 75 ± 5 % RH. The formulations were monitored for changes in particle size, PDI and encapsulation efficiency.

## 2.11. *InVivo*Studies

*In Vivo* pharmacokinetic studieswas carried in malewistar rats of 160-170 g (4-5 weeks old) supplied by the Teena Lab's, India duly approved by IAEC with CPCSEA number (1548/PO/1/11CPCSEA15.02.2012).The animals were housed in separate cages at temperature of 25±2°C and relative humidity of 50±5% for one week. Twelve animals were randomly distributed into two groups each containing 6 animals. First group of animals received oral pure darunavir and atazanavir (suspension) while the second group of animals received combined drug loaded NP's. All the formulations were administered orally at a dose of 70 mg/ kg (darunavir) and 35 mg/kg (atazanavir) body weight. After dosing blood samples (0.25 ml) were collected from the retro orbital eye. Plasma was separated by centrifuging the blood samples at 4000 rpm for 10 min and stored at -20°C for further analysis.

To 100 µl of plasma, 300 µl of methanol and 20µl of internal standard (containing 25µg/ml of verapamil hydrochloride) were added to precipitate the proteins. The samples were vortexed and centrifuged at 5,000 rpm for 15 min. The supernatants were separated and analysed for drug content by validated RP-HPLC (Chromolith High Resolution RP-18 end capped 100 - 4.6 mm using mixture of acetonitrile and 50 mM potassium dihydrogen phosphate with pH adjusted to 5.66 (40:60).

The intra and inter day accuracy and precision for the bioanalytical method was carried out.

The calibration curve for drugs concentration in plasma was linear ($R^2$ =0.997 for ATV and 0.993 for DRV) over the range of 100–1000 ng/mL. Standard non-compartmental analysis were performed to determine all the pharmacokinetic parameters including maximum plasma concentration ($C_{max}$), time taken for its occurrence ($t_{max}$), half-life ($t_{1/2}$)and area under the curve (AUC) for each rat by using thestandard trial version of kinetica software 5.0.The AUC was calculated from zero to infinity via the linear trapezoidal rule. The AUC was calculated from zero to infinity via the linear trapezoidal rule. The relative bioavailability was calculated using the following equation 3

$$F = \frac{AUC\ Test}{AUC\ control} \times 100 \quad eq\ldots 3$$

Student's *t*-test was performed to evaluate significant differences between the optimized formulation and the control. All values were considered statistically significant at *p* <0.05.

## 3. Results and Discussions

**Preparation of nanoparticles:**

Duringpreliminary studies nanoparticles were not formed without using proper surfactant. Initially trials were done using different surfactants like spans 60, tween 80, poloxamer and TPGS. The nanoparticles with narrow size range were found to be with TPGS. Hence for further studies nanoparticles were prepared with TPGS as a surfactant.

### 3.1. Characterisation of nanoparticles

The results of various formulations for particle size, polydispersity, entrapment efficiency and loading capacity were given in Table 2. Mean particle size of combined drug nanoparticles was ranging from 138 nm to 196 nm. Particle size of prepared nanoparticles was affected by concentration of surfactant and polymer used. Increased surfactant concentration with a decrease in polymer concentration resulted in smaller size. Increasing the TPGS concentration from 0.5% to 0.75%,a significant decrease in mean particles size of nanoparticles from 169 to 138 nm was observed. At high stabiliser concentration reduction in particle size was noted due to new area for formation of globules as reported[13].Minimum particle size obtained for formulation F3 was found to be 128 nm. Increased polymer concentration leads to increased in size which can be explained as an effect of viscosity of polymer. The mechanism attributed to the fact that higher viscosity results in viscous organic phase with more diffusion resistance to aqueous phase and thus producing larger NP's[14].The polydispersity index values of the all the formulations were less than 0.2 indicating a narrow and homogenous size distribution.

Zeta potential is an important parameter for determination of stability of nanoparticles. The zeta potential of optimised PCL nanoparticle formulation (F3) and L-PCL nanoparticle was found to be -22.3 mV and -32.8 mV respectively. The entrapment efficiency was determined as 35.2 % to 83.54 % for ATV and 56.4 % to 91.64 % for DRV in prepared formulations. The formulation F3 showed entrapment efficiency of 83.54 % and 91.64 % corresponding to atazanavir and darunavir respectively with lowest particle size. The entrapment efficiency was slightly less in F7 formulation with L-PCL nanoparticles due to its slightly hydrophilic nature[15].The above results indicatethat nanoprecipitation method is very suitable for encapsulations of both antiretroviral drugs. Drug loading efficiency was ranging from 7.04 % to 16.7 % for ATV and 13.6 % to 18.36 % for DRV. Based on the good entrapment efficiency and loading capacity formulation F3 and F7 fabricated with PCL and l-lactide PCL respectively were selected for further characterization.

**Table 2:The mean particle size,PDI,entrapment efficiency (EE) and loading capacity of ATV and DRV loaded PCL and L-PCL nanoparticles**.

| S.No | Batch code | Particle size (nm) | PDI | Entrapment efficiency (%) | | Loading capacity (%) | |
|------|------------|--------------------|-----|-------|-------|-------|-------|
| | | | | ATV | ATV | DRV | DRV |
| 1 | F1 | 169 (±2.25) | 0.233(±0.023) | 35.2 | 7.04 | 17.6 | 80.8 |
| 2 | F2 | 195.5(±15.62) | 0.247 (±0.054) | 48.77 | 9.74 | 11.28 | 56.4 |
| 3 | F3 | 137.5 (±26.45) | 0.112(±0.009) | 83.54 | 9.24 | 13.6 | 91.64 |
| 4 | F4 | 170.55(±35.14) | 0.063 (±0.164) | 59.21 | 11.84 | 16.6 | 83.01 |
| 5 | F5 | 161.6 (±40.51) | 0.239 (±0.094) | 46.2 | 16.7 | 18.36 | 68.8 |
| 6 | F6 | 186.2 (±10.85) | 0.149 (±0.071) | 69.82 | 13.96 | 17.22 | 86.11 |
| 7 | F7 | 160.3 (±39.45) | 0.206 (±0.084) | 60.4 | 12.08 | 17.04 | 85.2 |
| 8 | F8 | 181.4 (±25.87) | 0.247(±0.168) | 76.35 | 15.26 | 17.78 | 88.98 |

### 3.2 Scanning Electron Microscopy (SEM) analysis

SEM analysis was performed for optimised formulations (F3 and F7).All images of drug loaded nanoparticles showed uniform appearance with smooth surface morphology (Figure 1).Pictographs revealed particle size distribution was monodisperse with spherical shape without aggregation.
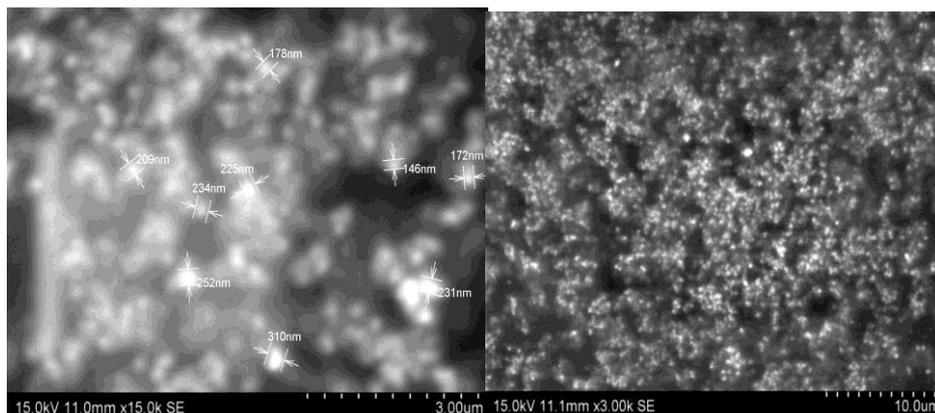
**Figure 1: SEM images of optimised PCL (F3) and L-PCL (F7) formulations**

### 3.3. Fourier Transform Infraredspectroscopy (FT-IR)

To investigate any chemical interaction between drug and polymer FT-IR spectroscopy was done. The overlay spectrum of DRV, ATV loaded PCL and L-PCL nanoparticles was shown in (Figure 2) and characteristic peaks for DRV noted were aromatic  C=C  stretching (1598 cm$^{-1}$),C=O stretching (1709 cm$^{-1}$), aliphatic C-H stretching (2960 cm$^{-1}$), hydroxy (1090 cm$^{-1}$),aromatic C-H(3028,3062cm$^{-1}$), –NH2 group (3464 cm$^{-1}$) and  for ATV aromatic  C=C  stretching (1598 cm$^{-1}$),C=O stretching (1715 cm$^{-1}$), aliphatic C-H stretching (3300 cm$^{-1}$), hydroxy (3600 cm$^{-1}$),aromatic C-H(3300$^{-1}$), –NH2 group (3300 cm$^{-1}$)were found to be similar with literature[15].Spectral analysis for physical mixture retained peaks of pure drug which suggests absence of any chemical interactions and confirmed compatibility between drugs and polymers.
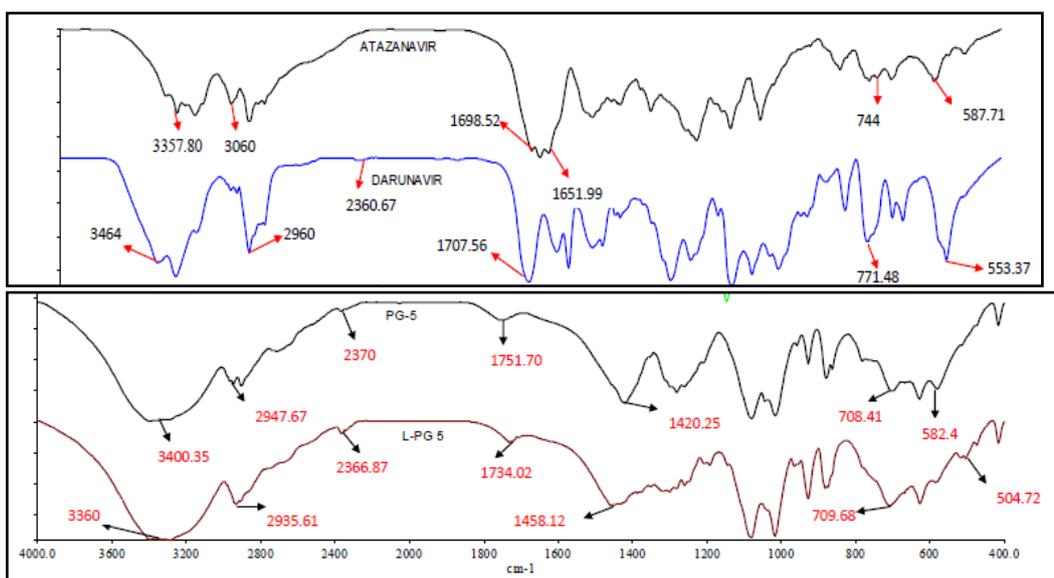


**Figure 2 Overlay of IR spectra of ATV, DRV, drug and PCL or L-PCL mixture, F3 and F7 nanoparticles formulation**

### 3.4. Differential scanning calorimetry

The fate of incorporated drug in nanoparticles is very important for a drug delivery system. Physical status of the drug encapsulated in polymeric matrix was investigated by DSC. As shown in Figure 3, DSC thermograms showed an endothermic peak at 76.38°C, 201.4°C and 60°C for DRV, ATV, PCL and L-PCL respectively. The melting peaks of pure drug i.e. darunavir and atazanavir were slightly reduced in the thermogram of loaded nanoparticles which shows formulation and drugs were entrapped in an amorphous or a solid solution state within the polymer matrix.
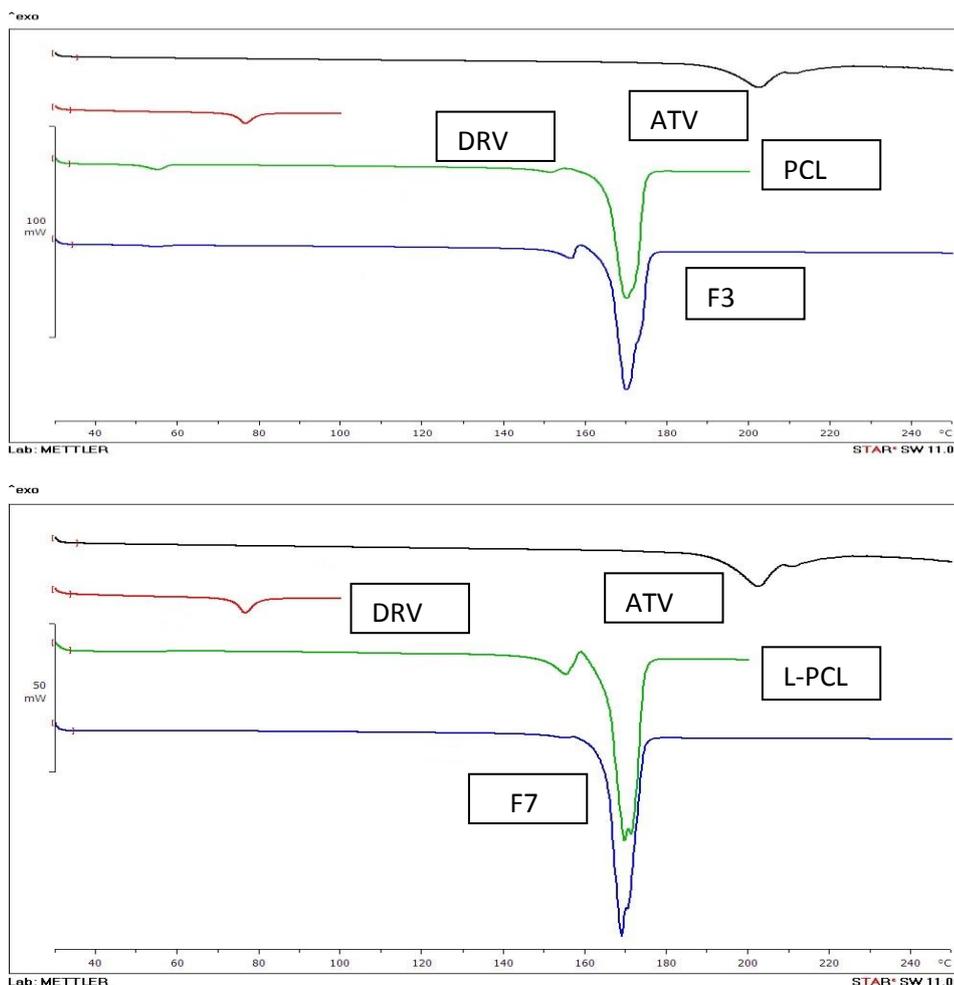
**Figure 3 Overlay of DSC thermogram of ATV, DRV, PCL, L-PCL and optimised F3 and F7 nanoparticle formulations.**

### 3.5. *In vitro* release study:

The release rate of darunavir and atazanavir from the nanoparticles was measured in PBS medium (pH 7.4) by RP-HPLC (Chromolith High Resolution RP-18 end capped 100 - 4.6 mm using mixture of acetonitrile and 50 mM potassium dihydrogen phosphate with pH adjusted to 5.66 (40:60). in triplicate. The *in vitro* drug release for F3 and F7 formulations were carried out for 96 hours and results are depicted in Figure 4.The ATV-DRV loaded PCL nanoparticles showed sustained release upto 4 days. About 70 % and 23.48 % of ATV and DRV release was observed within 96 h with l-lactide polycaprolactone grade of PCL nanoparticles respectively. While the release was comparatively slow for PCL fabricated nanoparticles showing 8.3 % and 17.36 % release for DRV and ATV respectively.

### 3.6. *In vivo* release from DRV-ATV nanoparticles

The encapsulation of two antiretroviral drugs in NP system was explored to provide sustained release of drugs in plasma. The plasma-concentration time profile of drugs from NP's of optimised formulations F3 and F7 and pure DRV-ATV suspension in rats was shown in Figure 5.The $C_{max}$ values of DRV-ATV PCL NP (DRV-7702 ng/ml, ATV-8877.6 ng/ml) and L-PCL NP (DRV-8956 ng/ml , ATV- 9856 ng/ml ) was higher than pure DRV ATV suspension (DRV- 4356 ng/ml, ATV- 4852.9 ng/ml, Table 4).Results showed sustained release of drugs over 24 hr period and twofold increase in area under curve (AUC) value of darunavir and atazanavir when administered as PCL and L-PCL nanoparticles as compare to pure drug suspension.
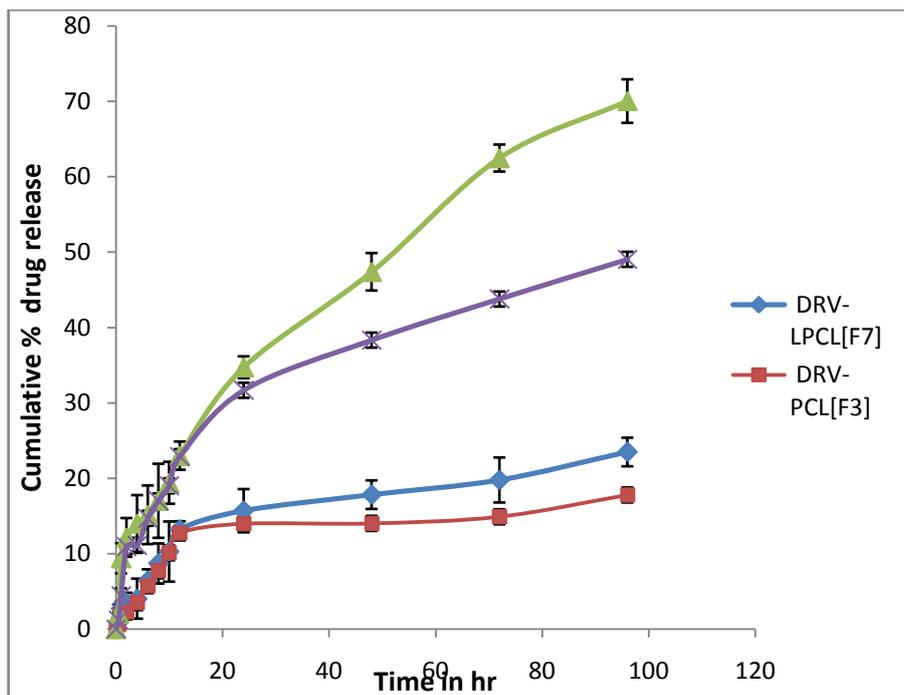
**Figure 4:** *In vitro* **release profile from PCL nanoparticles (F3) and lactide-PCL (F7) nanoparticles of darunavir (DRV) and atazanavir (ATV)**
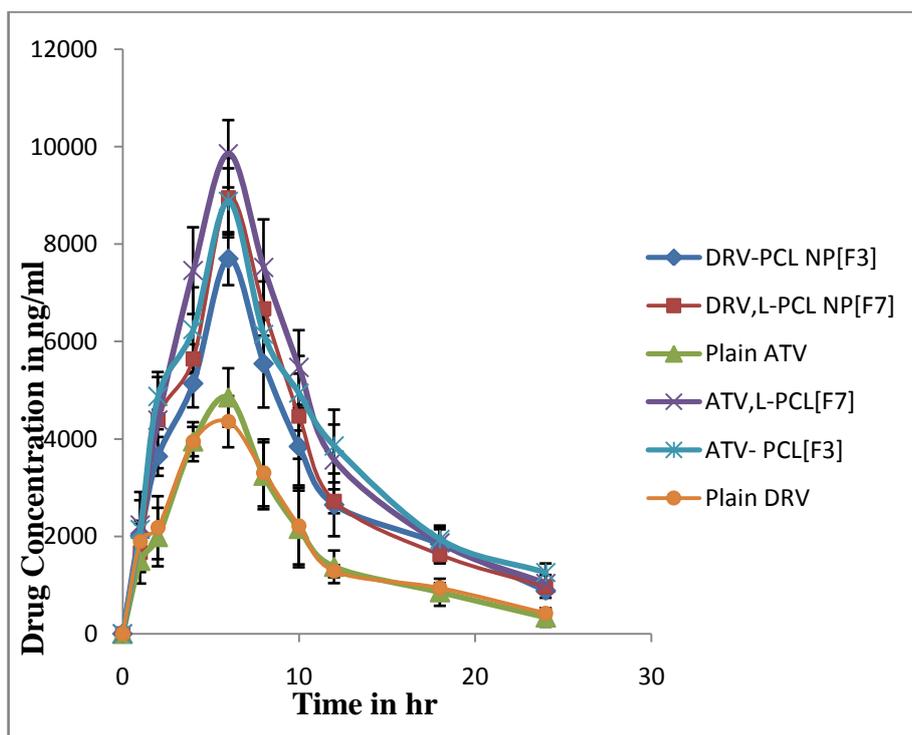


**Figure 5**: *In vivo* **release of atazanavir and darunavir from F3, F7nanoparticles and drug suspension**

## 4. Conclusion

In conclusion atazanavir and darunavir loaded drug nanoparticles were successfully prepared for sustained release with high entrapment efficacy using biodegradable polymers (PCL and L-PCL) by nanoprecipitation method.Concentration of surfactant and polymer affected the particle size and entrapment efficacy.In vitro release studies in phosphate buffer pH 7.4 showed sustained release during 96 hr study compared to plain drug. Stability studies indicates that nanoparticles were stable at 2-8ºC after 30 days and increase in particle size was observed with nanoparticles stored at 25 ± 2 ºC,60 ± 5 RH,40 ± 2 ºC,75 ± 5 RH. *In vivo* studies suggested that darunavir-atazanavir NP's improved the bioavailability of drugs as compared to pure

drug suspension. The results of this experimental work clearly demonstrate that PCL and L-PCL polymer can be used to fabricate nanoparticles by nanoprecipitation method for combination antiretroviral drugs to develop an effective drug delivery system which may be a new promising drug delivery system for the management of HIV-1 infected patients.

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