Chapter-4

Materials and Methods

4.1 Materials

4.1.1 Chemicals

Table 4.1 Chemicals used in study

S. No.	Chemicals	Source
1	Atorvastatin Calcium (ATR)	Ipca Laboratories Limited, Dehradun, India
2	D-α-tocopherol polyethylene glycol 1000 succinate (TPGS)	Antares Health Product Inc., Mumbai, India
3	Polyvinyl alcohol (PVA)	sd fine-chem Ltd., Mumbai, India
4	Eudragit RSPO	Evonic India Pvt. Ltd., Mumbai, India
5	Poly (dl-lactide-co-glycolide) (PLGA)	Evonic India Pvt. Ltd., Mumbai, India
6	Poly (ε-caprolactone) (PCL)	Sigma-Aldrich, India
7	Heparin	Alpha Chemika, Mumbai, India
8	Ortho-phosphoric acid	Hi Media, Mumbai, India
9	Acetonitrile (ACN)	Merck Ltd., Mumbai, India
10	Acetone	Merck Ltd., Mumbai, India
11	Methanol	Merck Ltd., Mumbai, India
12	Rosuvastatin calcium	Jubilant Organosys, Noida, India
13	Sodium acetate	Hi Media, Mumbai, India
14	Formaldehyde	Merck Ltd., Mumbai, India
15	Acetone	Merck Ltd., Mumbai, India
16	Sodium hydroxide	Qualigens Chemicals, Mumbai, India
17	Potassium dihydrogen orthophosphate	Qualigens Chemicals, Mumbai, India

S. No.	Chemicals	Source
18	Disodium hydrogen orthophosphate	Merck Ltd., Mumbai, India
19	Total plasma triglyceride kit	Span Diagnostic Ltd., Surat. India
20	HDL kit	Span Diagnostic Ltd., Surat. India
21	Plasma glucose kit	Span Diagnostic Ltd., Surat. India
22	Plasma creatinine kit	Span Diagnostic Ltd., Surat. India
23	Blood urea nitrogen kit	Span Diagnostic Ltd., Surat. India
24	Plasma creatinine kinase kit	Span Diagnostic Ltd., Surat. India
25	Plasma lactate dehydrogenase kit	Span Diagnostic Ltd., Surat. India
26	Plasma aspartate amino transferase kit	Span Diagnostic Ltd., Surat. India
27	Sodium lauryl sulphate (SLS)	Merck Ltd., Mumbai, India
28	Sodium taurocholate (STC)	Merck Ltd., Mumbai, India
29	Cetyl trimethyl ammonium bromide (CTAB)	Merck Ltd., Mumbai, India
30	Tween 80	Merck Ltd., Mumbai, India

4.1.2 Equipments

Table 4.2 List of major instruments used

S. No.	Instruments	Source
1	Magnetic stirrer	Decibel Instruments, Chandigarh, India
2	Digital electronic balance	Shimadzu, Japan
3	Digital pH meter	IKON Instruments, New Delhi, India
4	Dissection box	Camlin Ltd., Mumbai, India
5	Cooling centrifuge	REMI C20, Mumbai, India
6	Aluminium foil (freshwrapp)	Hindalco Industries Ltd., Mumbai, India

S. No.	Instruments	Source
7	Hot air oven	IKA, Germany
8	Disposable syringes	Hindustan Syringes & Medical Devices Ltd., Faridabad, India
9	Sonicator (bath type)	IKA, Germany
10	Stability chamber	NSW-175, New Delhi, India
11	UV-visible spectrophotometer	Shimadzu 1800, Japan
12	Vortex mixer	REMI Instruments, Mumbai, India
13	HPLC	Shimadzu, UK
14	Foam tape	Camlin Ltd., Mumbai, India
15	Zetasizer	Beckman Coulter, USA
16	Laser particle size analyzer	Akesmind 4800S, Japan
17	Differential scanning calorimeter	Mettler Toledo, Switzerland
18	Powder X-Ray diffractometer	Rigaku, Japan
19	Lyophilizer	Labconco, USA
20	FT-IR spectrophotometer	Shimadzu FT-IR-8400, Japan
21	Transmission electron microscope	PHILIPS TECHNAI-20G^2, Japan
22	Atomic force microscopy	NT-MDT, Russia
23	Camera assisted light microscope	Dewinter Optical, Inc., New Delhi, India
24	High speed homogenizer	IKA Ultra-Turrax (T25), germany
25	-86C ULT upright freezer	Thermoscientific, Germany
26	Membrane filters (0.22 µm and 0.45 µm)	Hi Media, Mumbai, India
27	Dialysis membrane (MWCO 12-14 kDa)	Hi Media, Mumbai, India

4.2. Experimental

4.2.1 Preformulation studies

Preformulation studies include

- Analytical method development by UV-visible spectroscopy
- Analytical method development and validation by HPLC
- Solubility studies

4.2.1.1 UV-visible spectrophotometric analytical method for ATR estimation

Standard calibration plots for quantitative estimation of ATR in phosphate buffer solutions (pH 4.5, pH 5.8, pH 6.8, pH 7.2 and pH 7.4) and in distilled water were established. Stock solution of ATR of strength 50 µg/ml was prepared in respective buffers and distilled water using methanol as co-solvent. Stock solution was further diluted in different concentrations range (2.5–22.5 µg/ml) and absorbance of respective diluted samples were measured at 247 nm wavelength (λ_{max}) on a UV-visible spectrophotometer (Shimadzu 1800, Japan).

4.2.1.2 Analytical method development and validation of ATR by HPLC in plasma samples

4.2.1.2.1 Standard solutions- calibration curve in plasma

Stock solution of ATR and Rosuvastatin calcium (RST) as an internal standard with 100 µg/ml strength were prepared by dissolving accurately weighed amount of respective drug in HPLC mobile phase (containing 25% PBS pH 7.4, 65% methanol and 15% acetonitrile). Various standard dilutions of ATR (ranging from 20 to 1000 ng/ml) were prepared by diluting the stock solutions with mobile phase. The ATR standard solution was stored in an amber colored flask at 4°C and it was found to be stable for at least 4 weeks (Shah et al., 2011). RST stocks solution was added to standard samples to make 200 ng/ml RST concentrations in each standard dilution.

Drug free plasma was spiked with different volume of ATR standard solution and fixed volume of RST standard solution to prepare different diluted working standard of ATR (20, 50, 100, 200, 300, 400, 500 and 1000 ng/ml) in plasma having 200 ng/ml of RST in each.

4.2.1.2.2 Chromatographic systems

ATR in plasma/mobile phase was analyzed by using RP-HPLC (Shimadzu LC 20 AD, Japan). The HPLC system consisted of two pumps (LC-20AD), a UV–visible spectrophotometer detector (SPD-20A) operated at wavelength of 247 nm, a degasser unit (DGU-20A3) and operating software (LC-Solution).

Chromatographic separation was achieved using an Enable (C-18G) C-18 analytical column (250mm × 4.6 mm I.D.) which was packed with 5 µm particles and a guard column (10 mm × 4.0mm I.D., 5µm particle size). Mobile phase comprised of 25 mM sodium phosphate buffer- organic phase (25/75, v/v), adjusted to a pH of 5.0 with ortho-phosphoric acid. Organic phase consisted of 20 part of acetonitrile and 80 part of methanol by volume. The mobile phase was filtered by membrane filter (0.45 µm) unit under low pressure condition, degassed by using bath sonicator and pumped at a flow rate of 1.2 ml/min. Rosuvastatin calcium (200 ng/ml) was used as an internal standard (Kumar et al., 2014).

4.2.1.2.3 Sample preparation procedures

An elementary liquid-liquid extraction method was employed to extract the ATR from plasma samples. To 100 µl aliquot of rat plasma sample (4°C) 100 µl of methanol was added and vortex mixed for 2 minutes for deproteination. Thereafter, 3 ml mixture of ethyl acetate-chloroform (1:1) was added and again vortex mixed for 3 minute. Then the mixture was centrifuged at 4000 rpm for 10 min at 4°C using cooling centrifuge, the organic layer transferred to amber colored glass conical tube and evaporated to complete dryness under gentle steam of nitrogen on a hot plate at 40°C (Bahrami et al., 2005). The residue was reconstituted in 100 µl of mobile phase and injected into sample holder (20 µl) after filtration (Millipore 0.45 µm) for RP-HPLC estimation.

4.2.1.2.4 Extraction ratio

The extraction ratio of ATR from plasma was determined by spiking an equal amount of the ATR into the drug free plasma sample and in mobile phase samples, and comparing their response in HPLC chromatogram peak area as per equation given below.

$$\textbf{Extraction ratio}\ (\%) = \frac{B}{A} \times 100$$

Where B is the peak area ratio of ATR:RST for standard mobile phase and A for the plasma samples.

Extraction at three concentration levels (50, 300 and 1000 ng/ml) were studied in triplicate.

4.2.1.2.5 Linearity, precision and accuracy

Calibration curves of ATR in mobile phase and plasma samples were established over the concentration ranges: 20-1000 ng/ml. Drug concentrations versus the corresponding peak areas ratio of ATR to that of RST were plotted. Precision (intraday and inter day variation) was evaluated by analyzing six replicate plasma samples at the following concentrations: 50, 250 and 750 ng/ml. The acceptability criteria of the data included precision within 15% of relative standard deviation (RSD) (USP, 2009).

Accuracy of the assay method was calculated from the same samples as those used for intraday and interday variation studies. The accuracy was expressed as % bias and to be acceptable, the values should be within ±15% at all concentrations (Bressolle *et al.,* 1996).

$$\% \textbf{ Bias} = \frac{\text{Observed concentration} - \text{Nominal concentration}}{\text{Nominal concentration}} \times 100$$

4.2.1.2.6 Limits of detection (LOD) and quantification (LOQ)

LOD and LOQ were estimated from the signal-to-noise ratios (S/N) (ICH Q2B, 1996). The detection limit was considered as the minimum concentration with a signal to noise ratio of at least 3 ($S/N \geq 3$). The quantification limit was defined as the lowest concentration level that provided a peak area with a signal-to-noise ratio higher than 10 ($S/N \geq 10$), with a precision less than 15% (R.S.D.) and an accuracy within±15% (bias).

4.2.1.3 Solubility studies of ATR in different solvents

As ATR is a calcium salt of an acid, it shows pH dependent aqueous solubility and higher solubility in basic buffer solution due to its ionization. ATR is commercially available in 10 mg, 20 mg, 40 mg and 80 mg in tablet unit dosage. Since, ATR is practically insoluble (<72 µg/ml) in acidic media (pH 1.2 - 4.5) and very slightly soluble (340 µg/ml) in intestinal pH. So the solubility enhancement is required in both the pH (Acidic and intestinal) to maintain sink condition. There is a need to improve solubility of ATR in acidic as well as intestinal dissolution media to overcome the dissolution issue of early stage drug development. In this study, several anionic [sodium lauryl sulphate (SLS) and sodium tauro-cholate (STC)], cationic [cetyl trimethyl ammonium bromide (CTAB)] and non-ionic [Tween 80 and D-α-tocopheryl polyethylene glycol 1000 succinate (TPGS)] surfactants have been employed for solubility enhancement of ATR. Surfactants help in solubility enhancement of poorly soluble drug by micelle formation as shown in **Fig. 4.1**.

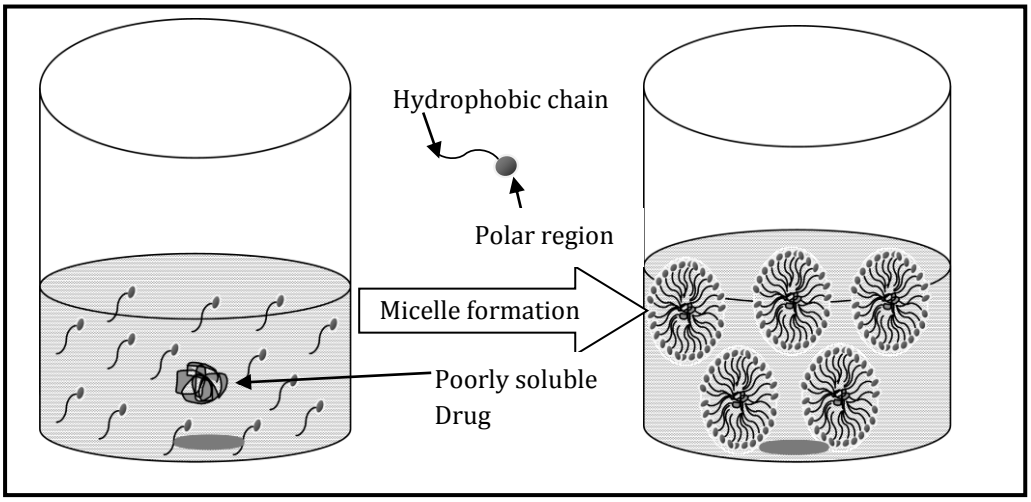

Fig. 4.1 Schematic depiction of micelle formation and solubilization of poorly soluble drug with surfactants under mild agitation (magnetic stirring)

Molar solubilization ratio (MSR), micelle water partition coefficient (P_m), free energy of solubilization (ΔG_s^o) and binding constant (K) are valuable derived solubility characteristics of surfactant-solute system which have been further analyzed in this study.

4.2.1.3.1 Solubility study of ATR in deionized water with and without surfactants

Excess of ATR was added in each amber colored flask containing 20 ml of water with 0/5/10/15/20/25/30/35 mM of an individual surfactant (except TPGS where surfactant concentrations were kept at 0/1/2/3/4/5/6/7/8/14). The flasks were tightly corked and fixed in thermostatically controlled rotary shaker with agitation (60 revolution/min); temperature (37±0.5°C) and kept for 24 h. After 24 h, the flasks were remained kept there for another 6 h without agitation to settle down the insoluble drug. Supernatant solution was withdrawn and ultra-centrifuged at 18000 rpm for 15 min to settle down the suspended insoluble drug. The final supernatant solution was withdrawn; diluted suitably with respective medium and quantified using UV-visible spectrophotometer. Each solubility study was performed in triplicate. All the containers were kept at 37±2°C to maintain the thermostatic condition. The solubility of ATR with different surfactant concentration was plotted by using Microsoft excel 2007. All the solubility characteristics were calculated accordingly as per equations expressed below (Rangel-Yagui et al., 2005; Chakraborty et al., 2009).

MSR is defined as number of moles of solute solubilized by one mole of surfactant and quantify the effectiveness of surfactant in solubilising a solute by following formula.

$$\textbf{MSR}\ (\boldsymbol{\chi}) = \frac{S - S_{cmc}}{C - C_{cmc}}$$

Where, S = Apparent solubility of solute at the surfactant concentration C ($C>C_{cmc}$) and S_{cmc} = Apparent solubility of organic compound at critical micelle concentration (cmc) of surfactant. It is also determined by slope of solute solubility with respect to surfactant concentration above CMC plot.

P_m, which represents the distribution of solute between surfactant micelle and aqueous phase, is expressed as

$$\mathbf{P_m} = \frac{\chi}{S_{cmc} \times V_w(1 + \chi)}$$

Where S_{cmc} is the solute concentration at the surfactant cmc and V_w is the molar volume of water (0.01805 LM-1 at 25°C).

Thermodynamically, the surfactant solubilization behaviour can be measured by the standard free energy of solubilization (ΔG_s^o) and expressed by following equation.

$$\mathbf{\Delta G_s^o} = -RTlnP_m$$

Where, R, T and Pm are the universal gas constant, absolute temperature and the water micelle partition coefficient, respectively.

Binding constant (K) of solute surfactant system assesses the association of surfactant with the solute and can measured by the following formula.

$$\mathbf{K} = \frac{N \times (S - S_{cmc})}{S_{cmc} \times (C - C_{cmc})}$$

Where, S and S_{cmc} are the solubility of solute at concentration C and C_{cmc} of surfactant, respectively. N is the aggregation number which indicates the number of individual surfactant molecules participated in each micelle formation. The CMC and aggregation number of surfactant are enlisted in Table 4.3 (Sadoqi et al., 2009; Sigma Aldrich product information, 2013).

4.2.1.3.2 Solubility study of ATR in buffers with and without surfactants

Different standard buffer solutions (hydrochloric acid buffer pH 1.2, acetate buffer pH 3, phosphate buffer pH 4.5, pH 5.8, pH 6.8 and pH 7.2) were prepared as per USP using deionized water. The solubility study was carried out as mentioned in above section in buffered solutions.

Table 4.3 List of surfactants used and their critical micelle concentration (cmc) with aggregation number (N)

S. No.	Surfactants	CMC (mM)	Aggregation No. (N)
1.	SLS	7-10	62
2.	STC	3-11	4
3.	CTAB	1.0	170
4.	Tween 80	0.012	60
5.	TPGS	0.13	10

SLS: sodium lauryl sulphate, STC: sodium taurocholate, CTAB: cetyl trimethyl ammonium bromide, TPGS: D-α-tocopheryl polyethylene glycol 1000 succinate

4.2.2 Formulation development

4.2.2.1a Preparation of ATR loaded Eudragit RSPO nanoparticles (AERSNs)

ATR loaded eudragit RSPO nanoparticles (AERSNs) were prepared by o/w emulsification solvent evaporation technique as depicted in **Fig. 4.2**. Briefly, 25 mg of ATR and Eudragit RSPO as mentioned in Table 4.4 were accurately weighed and dissolved in chloroform with the use of magnetic stirring. Polyvinyl alcohol (PVA) was dissolved in deionized water by using temperature controlled magnetic stirrer at 60°C temperature. This aqueous solution is allowed to cool to room temperature and emulsified with the aforementioned organic phase using high speed homogenizer (IKA 25, Germany) for 10 min at 8000-16000 rpm (Table 4.4 & 4.7). The prepared emulsion (o/w) was kept under mild stirring for 24 h to evaporate organic phase completely and then AERSNs suspension was obtained. AERSNs were deep-frozen at -50°C for 12 h in deep freezer (-86°C ULT upright Freezer, Thermoscientific, Germany) and then lyophilized using freeze-drier (LABCONCO, USA) at -45°C temperature and 0.08 mbar pressure up to 36 h for solid state characterizations. The samples used for solid state characterization were lyophilized without using any cryoprotectant to avoid possible interference of cryoprotectant with drug (Kumar et al., 2015; Patel et al., 2015).

4.2.2.1b Preparation of ATR loaded PLGA and PCL nanoparticles (APLNs & ALPNs)

ATR loaded PLGA nanoparticles (APLNs) and ATR loaded PCL nanoparticles (ALPNs) were prepared by nanoprecipitation method (**Fig. 4.3**). Concisely, PLGA/PCL and ATR were weighed accurately using electronic balance, and dissolved in organic phase under mild stirring. Precisely, intended amount of D-α-tocopherol polyethylene glycol 1000 succinate (TPGS) was dissolved in deionized water under mild stirring. Furthermore, organic phase was added to aqueous phase at the rate of 2 ml/min with the help of syringe (needle size #23) while keeping the mixture under magnetic stirring. The mixture was kept continuously under magnetic stirring 24 h to evaporate organic phase.

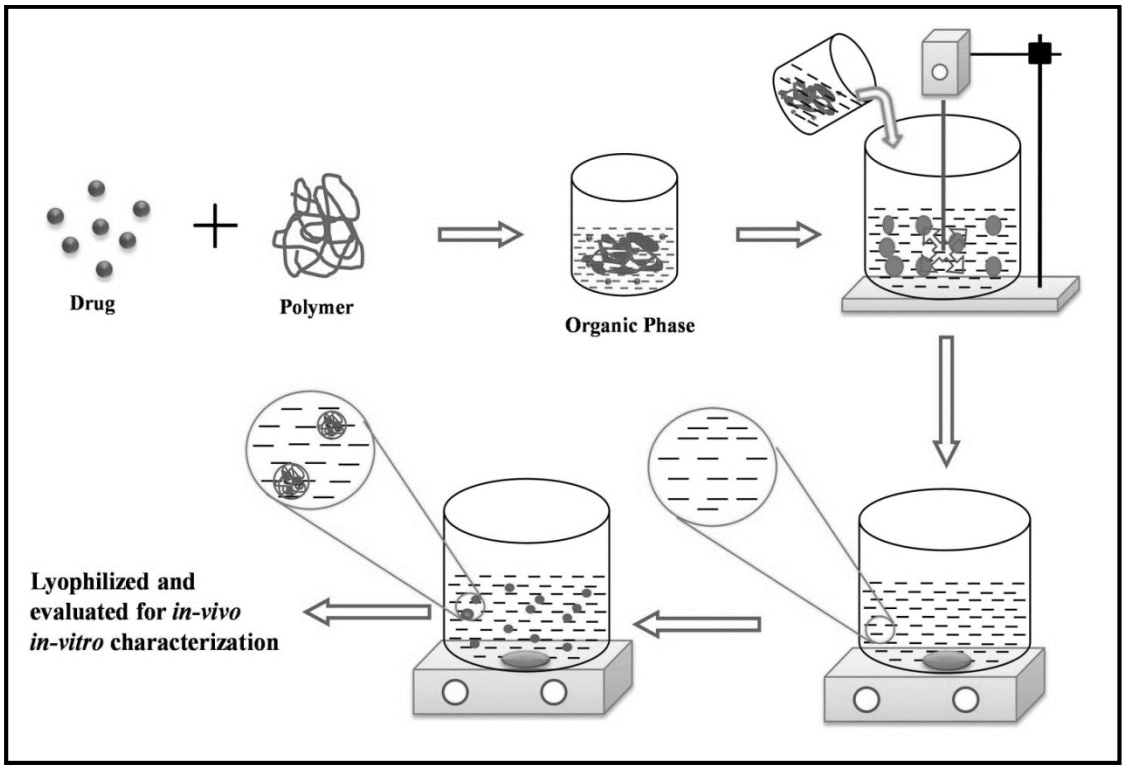

Fig. 4.2 Schematic representation of preparation of AERSNs via emulsification solvent evaporation method

The resulting nanoparticle suspension was centrifuged at 16000 rpm for 30 min and the sediment nanoparticles were re-suspended in freshly prepared 0.2% (w/v) TPGS aqueous solution. The prepared suspension was ultra-frozen at -50°C for 12 h and then lyophilized with 2% (w/v) mannitol by using freeze-

drier (LABCONCO, USA) at -45°C temperature and 0.08 mbar pressure up to 36 hr. Samples of formulations subjected for solid state characterization were lyophilized deliberately without using mannitol to avoid possible interference of mannitol thermogram and diffractogarm with ATR thermogram and diffractogram. Henceforth, prepared formulations were subjected to *in vitro* and *in vivo* evaluation (Kumar et al., 2014; Kumar et al., 2016).

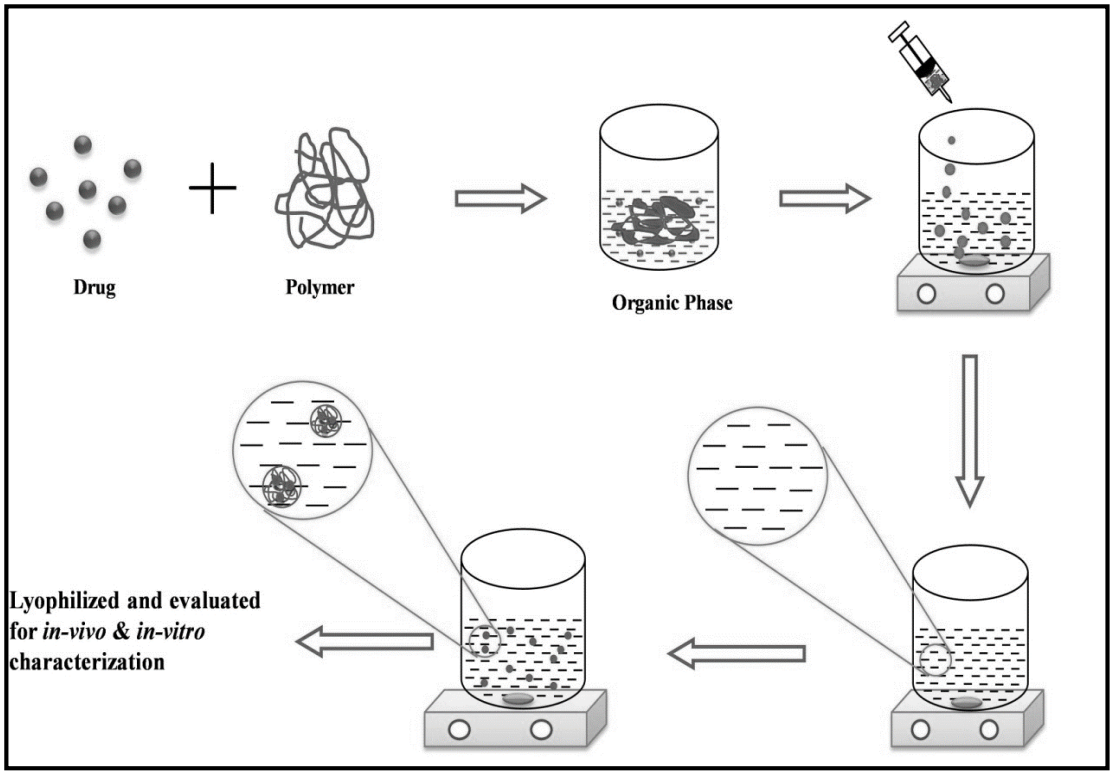

Fig. 4.3 Schematic representation of APLNs and ALPNs preparations by nanoprecipitation method

4.2.2.2 Central composite design and characterization of formulations

Central composite design was used to optimise the formulation and process variables involved in the preparation of formulation. On the basis of extensive literature survey and preliminary formulation study, four factors (X_1) polymer content, (X_2) TPGS/PVA concentration, (X_3) volume of acetone/Chloroform and (X_4) stirring/homogenization speed were selected as formulation/process variables. These four factors with five coded levels (-2, -1, 0, +1 and +2) were selected as independent formulation variables whereas mean

diameter hydrodynamic particle size (R_1) and mean entrapment efficiency (R_2) were selected as depended response variables, respectively. All the independent variables with their actual and coded values are shown in Table 4.4, Table 4.5 and Table 4.6. CCD-RSM was applied for optimization of formulation variables using Design-Expert 7.0 (State-ease Inc., USA) software. 30 batches consisting of 16 factorial points (level '-1' and '+1'), 8 axial points (level '-2' and '+2') and 6 replicated central points (level '0') were developed and prepared accordingly as enlisted in Table 4.7, Table 4.8 and Table 4.9 to establish a mathematical relation which expressed each of response variables as a function of independent formulation variables as shown in following equation.

$$R = A_o + A_1X_1 + A_2X_2 + A_3X_3 + A_4X_4 + A_{12}X_1X_2 + A_{13}X_1X_3 + A_{14}X_1X_4 + A_{23}X_2X_3 + A_{24}X_2X_4 + A_{34}X_3X_4 + A_{11}X_1^2 + A_{22}X_2^2 + A_{33}X_3^2 + A_{44}X_4^2 + A$$

Where R represents the dependent response variables (R_1, R_2 and R_3), A_o is an intercept, Ai are linear terms, A_{ij} (i and j = 1, 2, 3 and 4, i<j) represents the interaction terms, A_{ii} represents the quadratic terms and A is random error.

Table 4.4 List of selected independent and dependent variables of AERSNs with coded and exact value used in CCD

Factors	**Levels**				
Independent formulation variables	**-2**	**-1**	**0**	**1**	**2**
X_1: Eudragit RSPO content (mg)	25	50	75	100	125
X_2: PVA concentration (w/v %)	0.50	0.75	1.00	1.25	1.50
X_3: volume of chloroform (ml)	4	5	6	7	8
X_4: homogenization speed (rpm)	8000	10000	12000	14000	16000
Dependent response variables	Constraints				
R_1 = Mean diameter particle size (nm)	Minimize				
R_2 = Mean entrapment efficiency (%)	Maximize				

PVA: Polyvinyl alcohol

Correlation co-efficient (r^2) indicates quality of mathematical relation whereas analysis of variance ($p<0.05$) indicates the significance of the model as well as its various linear (X_i), interaction (X_{ij}) and quadratic (X_i^2) terms. Optimal

values of independent formulation variables of optimized batch were obtained by setting desired constraint in optimization tool (Kumar et al., 2014; Kumar et al., 2015).

Table 4.5 Experimental variables and their levels with coded and exact value in APLNs formulation preparation

Factors	**Levels**				
Independent variables	**-2**	**-1**	**0**	**1**	**2**
X1: PLGA (mg)	40	80	120	160	200
X2: TPGS (conc. %)	0.1	0.2	0.3	0.4	0.5
X3: Volume of acetone (ml)	2.5	5.0	7.5	10	12.5
X4: Stirring speed (rpm)	400	800	1200	1600	2000
Dependent variables			Constraints		
R_1 = Mean Diameter particle size (nm)			Minimize		
R_2 = Mean entrapment efficiency (EE)			Maximize		

PLGA: dl poly(lactide-co-glycolide), TPGS: D-α-tocopheryl polyethylene glycol 1000 succinate

Table 4.6 List of selected ALPNs formulation/process and response variables with coded and exact value

Factors	**Levels**				
Independent variables	**-2**	**-1**	**0**	**1**	**2**
A: PCL (mg)	30	60	90	120	150
B: TPGS concentration (w/v %)	0.2	0.4	0.6	0.8	1.0
C: Volume of acetone (ml)	3	5	7	9	11
D: Stirring speed (rpm)	500	750	1000	1250	1500
Dependent response variables			Constraints		
R_1 = Mean Diameter particle size (nm)			Minimize		
R_2 = Mean entrapment efficiency (%)			Maximize		

PCL: Poly (ε-caprlocatone), TPGS: D-α-tocopheryl polyethylene glycol 1000 succinate

Table 4.7 Batches of AERSNs designed by central composite experimental design using 4 factors and five levels with coded value (All results are represented as Mean ± S.D. where n = 3)

AERSNs Batches	Independent variables				Dependent variables		PDI
	X_1 (mg)	X_2 (%)	X_3 (ml)	$X_4 \times 10^3$ (rpm)	R_1 (nm)	R_2 (%)	
1	50	0.75	5	10	308 ± 6	75.4 ± 0.8	0.479 ± 0.015
2	100	0.75	5	10	398 ± 9	97.1 ±1.1	0.587 ± 0.021
3	50	1.25	5	10	282 ± 4	68.2 ± 0.8	0.325 ± 0.016
4	100	1.25	5	10	374 ± 7	89.5 ± 1.0	0.342 ± 0.015
5	50	0.75	7	10	239 ± 7	66.9 ± 1.8	0.357 ± 0.014
6	100	0.75	7	10	327 ± 9	88.5 ± 2.6	0.362 ± 0.013
7	50	1.25	7	10	203 ± 7	59.4 ± 1.7	0.301 ± 0.014
8	100	1.25	7	10	305 ± 8	81.1 ± 0.9	0.335 ± 0.16
9	50	0.75	5	14	245 ± 6	65.6 ± 1.0	0.367 ± 0.018
10	100	0.75	5	14	336 ± 7	86.9 ± 1.5	0.412 ± 0.019
11	50	1.25	5	14	219 ± 6	58.1 ± 0.9	0.327 ± 0.012
12	100	1.25	5	14	310 ± 7	79.7 ± 2.1	0.342 ± 0.021
13	50	0.75	7	14	209 ± 5	57.2 ± 1.4	0.323 ± 0.014
14	100	0.75	7	14	294 ± 6	78.5 ± 1.8	0.327 ± 0.020
15	50	1.25	7	14	167 ± 7	49.3 ± 1.6	0.195 ± 0.015
16	100	1.25	7	14	272 ± 6	71.3 ± 0.8	0.247 ± 0.014
17	25	1	6	12	271 ± 9	55.3 ± 1.1	0.364 ± 0.015
18	125	1	6	12	449 ± 13	98.6 ± 1.3	0.486 ± 0.022
19	75	0.5	6	12	279 ± 7	79.5 ± 1.2	0.437 ± 0.016
20	75	1.5	6	12	231 ± 5	64.7 ± 1.1	0.195 ± 0.014
21	75	1	4	12	315 ± 7	80.5 ± 1.3	0.457 ± 0.028
22	75	1	8	12	215 ± 6	60.7 ± 0.8	0.214 ± 0.009
23	75	1	6	8	301 ± 7	82.1 ± 1.4	0.485 ± 0.022
24	75	1	6	16	217 ± 5	62.3 ± 1.9	0.243 ± 0.013
25	75	1	6	12	247 ± 6	72.3 ± 0.7	0.237 ± 0.007
26	75	1	6	12	253 ± 7	71.8 ± 0.8	0.215 ± 0.009
27	75	1	6	12	256 ± 4	71.5 ± 1.0	0.235 ± 0.007
28	75	1	6	12	249 ± 7	72.7 ± 0.6	0.219 ± 0.010
29	75	1	6	12	243 ± 5	70.5 ± 0.5	0.234 ± 0.012
30	75	1	6	12	257 ± 6	71.2 ± 0.8	0.229 ± 0.008

Where X_1, X_2, X_3, X_4, R_1 and R_2 are polymer content (mg), concentration of PVA (%), volume of organic phase (ml) stirring speed (rpm), hydrodynamic particle size (nm) and entrapment efficiency (%) respectively.

Table 4.8 APLNs batches designed by central composite design using four factors and five levels

Batch no.	Independent variables				Dependent variables		Mean PDI
	X_1	X_2	X_3	X_4	R_1	R_2	
1	-1	-1	-1	-1	257	77.3	0.379
2	1	-1	-1	-1	335	89	0.487
3	-1	1	-1	-1	223	69.4	0.245
4	1	1	-1	-1	339	89.8	0.342
5	-1	-1	1	-1	207	69.8	0.256
6	1	-1	1	-1	301	95.3	0.412
7	-1	1	1	-1	176	61.9	0.328
8	1	1	1	-1	284	83.7	0.378
9	-1	-1	-1	1	227	71.6	0.327
10	1	-1	-1	1	301	93	0.476
11	-1	1	-1	1	157	61.3	0.278
12	1	1	-1	1	309	87.3	0.345
13	-1	-1	1	1	191	72.8	0.312
14	1	-1	1	1	274	87.9	0.417
15	-1	1	1	1	140	56.1	0.213
16	1	1	1	1	261	82.6	0.254
17	-2	0	0	0	154	56.4	0.175
18	2	0	0	0	345	93.2	0.567
19	0	-2	0	0	265	88.5	0.517
20	0	2	0	0	164	63.1	0.187
21	0	0	-2	0	269	89.3	0.478
22	0	0	2	0	181	69	0.254
23	0	0	0	-2	234	87	0.435
24	0	0	0	2	169	71	0.213
25	0	0	0	0	194	81	0.231
26	0	0	0	0	197	78	0.217
27	0	0	0	0	196	80.2	0.225
28	0	0	0	0	193	81.7	0.237
29	0	0	0	0	198	79.5	0.224
30	0	0	0	0	195	82.4	.0209

Where X_1, X_2, X_3, X_4, R_1 and R_2 are polymer content (mg), concentration of TPGS (%), volume of organic phase (ml) stirring speed (rpm), hydrodynamic particle size (nm) and entrapment efficiency (%) respectively.

Table 4.9 ALPNs batches designed by central composite design using four factors and five levels

Batch No.	Independent formulation variables with coded value				Dependent response variables		PDI
	X_1	X_2	X_3	X_4	R_1 (nm)	R_2 (%)	
1	1	1	-1	1	235	82.9	0.274
2	0	0	0	-2	241	84.3	0.249
3	1	1	1	1	219	91.2	0.218
4	0	0	0	2	167	72.5	0.207
5	-1	-1	-1	1	211	75.9	0.252
6	0	0	0	0	192	78.6	0.225
7	-2	0	0	0	189	65.7	0.236
8	0	0	0	0	196	76.3	0.226
9	-1	1	-1	1	171	67.3	0.194
10	-1	-1	1	-1	206	69.2	0.24
11	2	0	0	0	324	95.3	0.396
12	1	-1	-1	-1	306	95.7	0.336
13	1	1	-1	-1	278	89.4	0.278
14	0	0	2	0	203	67.2	0.187
15	0	0	-2	0	264	83.1	0.262
16	-1	1	-1	-1	194	77.1	0.198
17	1	-1	1	1	229	87.2	0.276
18	1	-1	-1	1	261	94.7	0.332
19	-1	-1	-1	-1	238	82.6	0.256
20	0	0	0	0	204	74.5	0.221
21	0	0	0	0	197	77.5	0.227
22	0	2	0	0	171	69.7	0.181
23	-1	-1	1	1	183	62.5	0.196
24	0	0	0	0	203	73.4	0.228
25	-1	1	1	1	146	57.3	0.163
26	-1	1	1	-1	163	62.5	0.182
27	0	0	0	0	209	74.8	0.223
28	0	-2	0	0	238	81.7	0.282
29	1	-1	1	-1	273	92.7	0.323
30	1	1	1	-1	249	87.2	0.262

Where X_1, X_2, X_3, X_4, R_1 and R_2 are polymer content (mg), concentration of TPGS (%), volume of organic phase (ml) stirring speed (rpm), hydrodynamic particle size (nm) and entrapment efficiency (%) respectively.

4.2.2.3 Particle size, polydispersity index, zeta potential and entrapment efficiency determination

Hydrodynamic mean diameter particle size (PS) and polydispersity index (PDI) analysis were carried out by photon correlation spectroscopy (PCS) using particle size analyzer (Delsa Nano C, Beckman Coulter Counter, USA) equipped with software N4 plus. The measurements were performed at an angle of 165° at 25°C with proper dilution with Milli-Q water. The zeta potential was analyzed by measuring electrophoretic light scattering using the same particle size analyzer in zeta potential mode.

Entrapment efficiency of formulations were analyzed by indirect method as reported by Patel et al, 2015. Five ml of sample was centrifuged for 30 minutes in cooling centrifuge (Remi, India) at 15000 rpm at 4°C. The supernatant was analyzed for free dissolved drug (FDD) by UV-visible spectrophotometer (Shimadzu 1700, Japan) at 247 nm wavelength.

EE of formulations was determined using following formula

$$\mathbf{EE}\ (\%) = \frac{\mathrm{TAD} - \mathrm{FDD}}{\mathrm{TAD}} \times 100$$

Where TAD: total amount of drug and FDD: free dissolved drug

4.2.2.4 Solid state characterizations of formulations by FT-IR, DSC and PXRD

Compatibility of ATR with polymers in three formulations was examined by Fourier-transform infrared (FT-IR) spectroscopy (SCHIMADZU 8400, Japan) with pressed pellet technique using potassium bromide. Briefly, solid samples were crushed and blended with potassium bromide (IR grade) and compressed using pressed pallet technique and, then mounted in FT-IR in transmittance mode. Samples were scanned in the region of 4,000–400 cm^{-1} while keeping number of reference scan 20 (Patel et al., 2016). All formulation samples, their placebo nanoparticles (without ATR) and their components physical mixture were analyzed by FT-IR and compared to study any interaction between drug and any components of formulation.

Differential scanning calorimetry (DSC) thermograms of ATR, placebo nanoparticles, physical mixture of drug with the polymers and formulations were obtained on a TGA/DSC-1, Star system (Mettler Toledo, Switzerland). Samples (5 mg) were placed in aluminium pan and hermetically sealed. Phase study of samples were investigated at a scanning rate of 10°C/min, covering temperature range of 30–250°C under continuous supply of nitrogen at the rate of 50 ml/min (Patel et al., 2015).

Powder X-ray diffraction (PXRD) patterns were analyzed using a portable X-ray diffraction system (Rigaku, Japan) with Cu rotating anode (K_α radiation; λ = 1.54 nm) generated at 18 kW. The sample was packed into the rotating sample holder and analyzed in the range of 5° to 55° diffraction angle with scanning rate 3°/min (Chaurasia et al., 2015). Formulations, physical mixture of components, placebo nanoparticles (formulations without ATR) and pure ATR diffractograms were analyzed to study any polymorphic change of drug in polymer matrix.

4.2.2.5 Morphological analyses of formulations

Morphological aspects of polymeric nanoparticles have greater influence on its behaviour inside biological system (Kou et al., 2013). Morphological studies were performed using transmission electron microscopy (TEM) and atomic force microscopy (AFM). TEM operates on the same basic principle of light microscope but employs accelerated electrons rather than light. Accelerated electrons possess quite lower wavelength than light and offered even 1000 times higher resolution than light microscope. A drop of freshly prepared formulation suspension on TEM copper grid was placed and air dried at 25°C to prepare a fine film on grid. TEM grid with prepared film was fixed into sample holder to observe the image in TEM instrument (PHILIPS TECHNAI-20G^2, Japan) under low vacuum at high resolution accelerating voltage 200 kV. Observed TEM and electron diffraction (ED) pattern images were recorded (Chaurasia et al., 2015).

AFM is a type of probe scanning microscopy which can have resolution up to the fractions of nanometer. A drop of freshly prepared sample was placed on a glass cover slip and air dried to form thin film. Thereafter, this cover slip was

mounted on sample holder and image was observed as well as recorded through AFM (NT-MDT, Russia) in tapping (intermittent contact) mode using solver next software.

4.2.2.6 In vitro release study

In vitro release study of AERSNs, APLNs and ALPNs were carried out over 24 h, 72 h and 96 h respectively by using dialysis bag (Himedia labs, cutoff weight 12,000–14,000 Da) diffusion method to study the release pattern of ATR from the ATR loaded PNs. In this method, each bag was loaded with formulation equivalent to 2.5 mg of drug, sealed by using cotton threads and dialysed against 100 ml phosphate buffer solution (PBS: 50 mM, pH 7.4) by keeping in 250 ml beaker under magnetic stirring (500 rpm) and thermostatically controlled condition (37±0.5°C). 2 ml of samples were collected at each preset time interval till 96 h and volume of the release media was maintained by adding 2 ml of fresh prewarmed (37±0.5°C) PBS each time. The cumulative percentage release (CPR) was calculated by analyzing the samples on UV spectrophotometer (Shimadzu1800, Japan) at 247 nm. Various release kinetic models as shown below were applied on release profile to examine the release mechanism by using Microsoft excel office 2007 software (Costa & Lobo, 2001).

$$Q_t = Q_o + K_o t \qquad \text{Zero order kinetic}$$

$$\text{Log}\frac{Q_t}{Q_o} = \frac{K_1 \times t}{2.303} \qquad \text{First order kinetic}$$

$$Q = K_H t^{1/2} \qquad \text{Higuchi model}$$

$$\frac{M_t}{M_\infty} = K_{KP} \times t^n \qquad \text{Korsmeyer} - \text{Peppas model}$$

$$W_o^{1/3} - W_t^{1/3} = K_{HC} \times t \qquad \text{Hixson} - \text{Crowell model}$$

Where Q_t: amount of drug released in media, Q_o: initial amount of drug in media at zero time, K_o: zero order release constant, K_1: First order release constant, K_H: Higuchi release constant, M_t/M_∞: fraction of drug release at time t, K_{KP}: a

constant depend upon structural and geometric characteristics of dosage form, n: release exponent indicate release mechanism, W_o: Initial amount of drug in dosage form, W_t: remaining amount of drug in dosage form at time t, K_{HC}: constant incorporating surface volume relation and t: time of release.

4.2.2.7 Storage stability study:

The stability of formulations was evaluated over a period of six months at accelerated condition (40±2°C/75±5% RH), normal condition (25±2°C/60±5% RH) and refrigerated condition (4±1°C) as per ICH guideline. Freshly prepared lyophilized formulation samples were sealed in amber color glass vials and placed in stability chamber at above mentioned conditions. Samples were withdrawn at different time points (0, 1, 2, 3 and 6 month) and analyzed for its properties (PS, EE and PDI).

4.2.2.8 Trace organic solvent estimation in formulation

As organic solvents have been used in preparation of formulations, it is onus to quantify the residual trace amount of organic solvent in formulation so that one can ensure it is within permissible limit. Gas chromatography (GC) methodologies are being employed to estimate the residual organic solvents fraction in the sample. GC (Nucon 5765, New Delhi) equipped with flame ionization detector (FID) and capillary column (30m×0.22mm, ID BP×5 0.25µm) was used to detect the chloroform/acetone in the respective formulation. Mobile phase consists of helium with flow rate of 1.5 ml/minute. The temperature of the detector was set at 220°C and the injector temperature at 220°C. The oven temperature was programmed to 40°C (for 2 min), followed by an increase of 30°C/min until 200°C (Pontes et al., 2009). Briefly, standard stock solutions of chloroform (5000 ppm), acetone (5000 ppm) and 1- propanol (5000 ppm) were prepared in deionized Millipore water (DMW). Dimethyl sulfoxide was used to dissolve chloroform in DMW. Different working dilutions were prepared by stock samples, and standard calibration plots were established for both chloroform as well as acetone using 1-propanol as internal standard. LOD, LOQ, accuracy and precision of this GC methodology were established as mentioned in HPLC

section. Freshly prepared formulations samples were first filtered and aliquot 1 µl of these samples spiked with internal standard (1-propanol 1500 ppm) were injected in GC system preconditioned as mentioned above.

4.2.2.9 In vivo study

4.2.2.9.1 Animals

Male Charles Foster (CF) rats weighing (180-220 g) were procured from central animal house of Institute of Medical Sciences, Banaras Hindu University, Varanasi (India). Each group of rats (n=6) were domiciled in propylene cage at an ambient temperature of 25±1°C, 45–55% relative humidity with a 12 h light/12 h dark cycle fed with normal rat diet and water *ad libitum*. All study protocols were approved by Central Animal Ethical Committee of Banaras Hindu University (Dean/13-14/CAEC/321).

4.2.2.9.2 Pharmacokinetic study

ATR drug suspension (DS) and formulation groups of overnight fasted rats (n=6) were selected for pharmacokinetic study. Experimental male CF rats were divided into four groups each containing six subjects.

Group 1: Drug suspension (DS) group

Group 2: AERSNs group

Group 3: APLNs group

Group 4: ALPNs group

Pure drug and nanoformulation suspensions equivalent to 20 mg/kg of ATR were administered orally to each rat of respective groups. At predetermined time interval (0.5, 1, 2, 4, 8, 16 and 24 h) after dosing, blood samples (0.3 ml) were collected from orbital sinus of each rat into a heparinized micro-centrifuge tube and plasma were separated by cooling centrifugation at 4000 rpm for 5 min at 4°C. ATR concentration in plasma samples were analyzed by validated RP-HPLC method. Pharmacokinetic parameters were analyzed by non-compartmental analysis using Kinetica 5.0 pharmacokinetic software (PK-PD

analysis, Thermofischer) and statistically analyzed by student unpaired *t* test using GraphPad Prism 5.03 (GraphPad Software, USA).

4.2.2.9.3 Efficacy and safety study

Experimental male CF rats were divided into nine groups each containing six subjects.

Group 1: Normal diet control (NDC)

Group 2: High fat-diet control (HFC)

Group 3: High fat-diet placebo-Edragit-nanoparticles treated (HFPET)

Group 4: High fat-diet placebo-PLGA-nanoparticles treated (HFPPLT)

Group 5: High fat-diet placebo-PCL-nanoparticles treated (HFPPCT)

Group 6: High fat-diet ATR suspension treated (HFAST)

Group 7: High fat-diet AERSNs treated (HFAET)

Group 8: High fat-diet APLNs treated (HFAPT)

Group 9: High fat-diet ALPNs treated (HFALT)

The study duration was kept eight weeks and divided into three parts.

Part. 1 Hyperlipidemia induction period (1st to 4th week): Hyperlipidemia was induced in all tested animal group except NDC by feeding high fat diet. No groups were treated with drug/placebo nanoparticles/formulations during this period.

Part. 2 Treatment period (During 4th to 6th week): Different groups were treated with drug, placebo nanoparticles and formulations during this period only. Treatment regimen was shown in Table 4.10.

Part. 3 Washout period (During 6th to 8th week): Treatment with drug/placebo nanoparticles/formulations was withheld during this period. Sustained action of formulations was observed in this period only.

All rats except NDC group were fed with high fat diet consisting of 68% normal rat feed, 10% lard, 10% egg-yolk powder, 10% sugar and 2% cholesterol. Rats encaged in NDC group were fed normal rat pellet diet. After 4 weeks, all

groups were treated accordingly as mentioned in Table 4.10 during fourth to sixth week.

Table 4.10 Treatment regimen during 4th to 6th week to different group

Group	Diet during 8 week	Treatment during 4th to 6th week (mg/kg/day)
NDC	Normal rat diet	....
HFC	High fat diet	....
HFPET, HFPPLT & HFPPCT	High fat diet	Placebo-nanoparticles equivalent of formulation weight treated to respective formulation
HFAST	High fat diet	3mg/kg/day of ATR
HFAET, HFAPT & HFALT	High fat diet	Equivalent weight of formulations containing 1.5 mg of ATR

Blood samples were withdrawn from each tested rat at the end of 2nd, 4th, 6th, 7th and 8th week and analyzed for various biochemical parameters using commercially available span diagnostic kits. Biochemical parameters include PTC (plasma total cholesterol), PTG (plasma triglycerides), HDL_C (high density lipoproteins), CK (creatinine kinase), BUN (blood urea nitrogen), LDH (lactate dehydrogenase), AST (aspartate transaminase) and creatinine. Very low density lipoprotein ($VLDL_C$) and low density lipoprotein (LDL_C) were calculated by using Friedewald formula (Friedewald et al., 1972). The atherogenic Index (AI) was calculated by formula as expressed below (Hou et al., 2009).

$$\mathbf{AI} = \frac{\mathrm{PTC} - \mathrm{HDL}_C}{\mathrm{HDL}_C}$$

Experimental animals were sacrificed and isolated liver tissues were stored in 10% formalin solution. The tissues were further cut in fine slices by microtome, placed over glass slide and stained with hematoxylin and eosin (Young et al., 2014). The snapshot of stained liver tissues were taken with the use of digital microscope assisted with camera (Dewinter, New Delhi, India).

4.2.2.10 Statistical analysis

All the pharmacokinetic parameters comparisons among groups were carried out by using nonparametric unpaired Student's *t* test. The safety and efficacy biochemical parameters were compared among groups with the use of Bonferroni post hoc two ways ANOVA with the use of GraphPad Prism 5.03 (GraphPad Software, USA) software. Difference with a value of $p<0.05$ was considered to be statistically significant.