4. Materials and Methods

4.1. Materials

Asenapine maleate (ASM) was a gift sample obtained from Sun Pharmaceutical Industries Ltd., Gurgaon, India. Arteether was provided as gift sample from Edelwiss Life Sciences, Chandigarh, India. Levodopa and Carbidopa were purchased from Intas Pharmaceutical Ltd., Ahmedabad, India. Glyceryl monostearate (GMS) was generously donated by Lupin Research Park, Pune, India. Oleic acid (OA) and Tween 80 (polyoxyethylene sorbitan monooleate, T 80) were purchased from SDFCL, Mumbai, India. Glycol chitosan (GC) and dialysis membranes (molecular weight cut-off between 12000 and 14000) were purchased from HiMedia, Mumbai, India. Potassium dihydrogen orthophosphate and triethyl amine were purchased from Merck, Mumbai, India. Nanosep Centrifugal filter devices (Omega Membrane, MWCO 100 kDa) were purchased from Pall Life Sciences, Mumbai, India. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide), Dulbecco's Modified Eagle medium (DMEM) and Fetal bovine serum (FBS) were purchased from Sigma-Aldrich, India. The water used in all experiments was ultrapure, obtained from a Millipore–DirectQ UV, Millipore, France). The solvents and chemicals used for analysis of drug were HPLC grade. All other chemicals used in the research work were of analytical grade and used as obtained. The solutions were prepared on the day of experiment on daily basis.

4.2. Methods

4.2.1. HPLC analytical method development

The HPLC method is the most efficient method for the estimation of drug in formulation. Here, in present study, a rapid, specific, precise and validated reverse phase high performance liquid chromatography (RP-HPLC) method is developed according to ICH guideline and applied to the assay of ASM in tablets and bulk form (ICHGuideline, 2005).

4.2.1.1. Instrumentation and analytical conditions

HPLC method was performed using Waters 515 HPLC pump having Rheodyne 7725i injector and equipped with photodiode array (PDA) 2998 detector (Waters, USA). The chromatographic separation of ASM was achieved by using a Water C18 spherisorb 5.0 µm ODS2, 4.6 mm x 250 mm column (Waters, USA) connected with guard column (5.0 µm ODS2, 4.6 mm x 10 mm). The mobile phase consists of acetonitrile and phosphate buffer in the ratio of 75:25 and at a flow rate of 1.0 ml/min. The buffer was prepared by dissolving 1.36 g potassium dihydrogen orthophosphate in 1000 ml Milli Q water. Further, above solution was mixed with 10 ml of triethyl amine and pH 3.3±0.05 was adjusted with ortho-phosphoric acid. The mobile phase was filtered through 0.45 µm Nylon filter (Pall Corporation, USA) and degassed in ultrasonic bath (PCI Analytics, Mumbai India) prior to use. An injection volume of 20 µL was used in all experiments and the elution was carried out at controlled room temperature (20–25 °C). Analysis was carried out at λ_{max} 268

nm based on the UV spectroscopy scanned data over 200-400 nm. All the experiments were performed in triplicate.

4.2.1.2. Preparation of standard stock and working solutions

A primary stock solution of asenapine was prepared by dissolving accurately weighed 35.14 mg of the asenapine maleate in 50 ml of HPLC grade methanol which is equivalent to 500 µg/ml of asenapine. From the above stock solution, a secondary stock solution of concentration 100 µg/ml was prepared by diluting with methanol. The stock solution was protected from light by covering with aluminum foil and stored at 4°C. The different concentration of working standard solution 0.1, 0.5, 1, 5, 10, 20, 30, 40, 50 and 100 µg/ml were prepared by suitable dilution of secondary stock solution. The paliperidone (internal standard, [IS]) in concentration of 10 µg/ml was constant in each working solution. The three concentrations 10, 40 and 80 µg/ml were considered as quality control (QC) sample.

4.2.1.3. Method validation

The analytical procedures were validated according to International Conference of Harmonization guidelines (ICHGuideline, 2005). The statistical analysis was used to verify the validity of the method.

System suitability

The system suitability testing is required for analytical method to comply USP NF 32/27. The system suitability was assessed by three replicate analysis of 10 µg/ml

concentration of drug. The acceptance criterion was ±2% for the percent coefficient of variation (%RSD) for the peak area and retention times for both drug and IS.

Linearity

The calibration curve were obtained with six working standard solution (10, 20, 40, 60, 80 and 100 µg/ml). The peak area ratio of the drug to the IS was considered for the plotting the linearity graph. The experiments were performed in triplicate. The linearity was calculated by the linear regression analysis, which was calculated by the least square regression method (Mendez AS *et al.*, 2003).

Accuracy and Precision

Accuracy of the assay method was determined by conducting recovery experiment. The accuracy was evaluated in triplicate analysis of the QC samples. Precision of an analytical procedure expresses the closeness of the agreement between a series of measurement obtained from multiple sampling of the homogeneous sample. Repeatability refers to the use of the analytical procedure within a laboratory over a short period of time that was evaluated by assaying the QC samples during the same day. Intermediate precision or Inter-day precision was assessed by comparing the assays on different days (3 days).

Limit of detection and quantitation (Sensitivity)

ICH Q2R1 defines the limit of detection (LOD) of an individual analytical procedure as the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. Whereas limit of quantitation (LOQ) is the

lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The parameters LOD and LOQ were determined on the basis signal to noise ratio. LOD and LOQ were regarded as the amounts for which signal to noise (S/N) was 3:1 and 10:1, respectively.

Robustness

The robustness of the HPLC method was determined by analysis of samples under a variety of conditions such as small changes in the percentage of mobile phase, flow rate and pH of the buffer solution. The effect of these changes on retention time (RT) and peak parameters were studied for knowing the robustness of the method (Emami J *et al.*, 2006).

4.2.1.4. Application of method

The developed method was applied for assay of asenapine in pharmaceutical dosage form. The sublingual tablet and drug-lipid matrix were prepared in the pharmaceutical laboratories. Ten tablets were weighed, crushed and mixed. A portion of powder equivalent to 10 mg of asenapine was accurately weighed, transferred into a 10 ml volumetric flask, diluted with 8 ml of methanol, sonicated for 15 minutes for complete extraction of drug and finally made the volume upto 10 ml with methanol. Further, it was filtered with 0.45 µm syringe filter. The clear filtrate was taken and diluted with methanol to get 100 µg/ml.

4.2.2. Development of Nanostructured lipid carriers (NLC)

4.2.2.1. Quality target product profile

The quality target product profile (QTPP) is defined as "A prospective summary of the quality characteristics of a drug product that ideally will be achieved to ensure the desired quality, taking into account safety and efficacy of the drug product". The target product profile forms the basis of design for development of the product (Food and Administration D, 2009). Justification for selection of attributes and desired target levels for each product attribute have been tabulated in Table 4.1.

4.2.2.2. Critical material attributes and process parameter

Critical material attribute (CMA) and critical process parameters (CPP) are defined as "A material or process whose variability has an impact on a critical quality attribute and therefore it should be monitored or controlled to ensure desired drug product quality". The Critical Quality Attribute (CQA) of drug product and their QTPP were contributed in selection of excipient and process parameters1. For preparation of ANLC, critical material attributes of drug substance asenapine and excipients including solid lipid, liquid lipid and surfactant were accessed. Further, two critical process parameters: homogenization speed and sonication time, were included based on the selected method for preparation of NLC (high shear homogenization and sonication). The justifications for selection of materials and process parameters have been tabulated in Table 4.2.

Table 4.1: Quality target product profile of NLC

Product Attribute	Desired Target Attribute Level	Justification
Particle Size (nm) (d=50%)	<200 nm	Based on the literature report, the particle size <200 nm have potential to cross blood brain barrier. Since brain is the desired target for action of asenapine, we have decided to make the nanoparticles in size of below 200 nm(Kozlovskaya L *et al.*, 2014; Martins S *et al.*, 2012).
Entrapment Efficiency (% Drug Loaded)	>60%	High drug entrapment permits lower total volume/excipient for administration of formulation. This minimizes the undue exposure of excipients to tissues which may occur due to lower entrapment efficiency or high volume dose.
Polydispersity Index (PDI)	<0.3	Since polydisperse system have greater tendency to aggregation than monodisperse system a lower PDI is desired to target the achievement of a stable nanoparticulate system.
Ionic/steric stabilization	Stable Nanosuspension	Since the lipids and surfactants screened for the formulation are non-ionic, the charge on particles was predicted to be near neutral range with some residual charge from other sources. This was confirmed during the initial trials and evaluation of formulation characteristics (Zeta Potential: -2 to -5.98 mV). It was concluded that the stability of nanosuspension will be achieved by steric hindrance.

Table 4.2: Critical material attributes and critical process characteristics of NLC

Drug substance attribute		
Drug Substance		**Justification**
BCS Class	Class II	Asenapine is classified as a BCS Class II compound (low solubility, high permeability). Low solubility problem may be overcome by nanonization of particles.
Sublingual bioavailability	35%	The low sublingual bioavailability and high gastro instability of asenapine, makes it a suitable candidate for nanoparticulate lipid drug delivery system.
Oral bioavailability	<2%	The lipid coat over the particles prevents metabolism.
Excipients attribute		
Excipient		**Justification**
Selection of Solid Lipid	Glyceryl monostearate	Drug solubility was evaluated in stearic acid, glyceryl monostearate, Compritol 888 ato and Precirol ATO 5. Glyceryl monostearate was selected based on high solubility of asenapine (200 mg/g of GMS).
Selection of Liquid lipid	Oleic acid	The trial batches were prepared with fixed ratio of liquid lipid (oleic acid, caprylic/capric triglyceride and propylene glycol dicaprylate/dicaprate) to solid lipid (GMS). The minimum particle size (<200 nm) with sufficiently stable colloidal dispersion (one month) was obtained with oleic acid.
Selection of Surfactant	Tween-80	Among screened surfactants having brain targeting ability (Tween 80 and Poloxamer 188), it was concluded that Tween 80 stabilized colloidal dispersion (Stable for more than one month without aggregation of particles) resulted in low particle size and had acceptable stability.

Critical process parameters

Process		Justification
Homogenization speed	8000-16000 rpm	The homogenization speed ranges were selected based on instrument limitation and trial batches. The homogenization speed less than 8000 rpm leads to large particle size (> 200 nm) and polydisperse colloidal system (PDI > 0.3). However, the upper range was set at 16000, since no significant difference in particle size was observed above 16000 rpm (Particle size < 200 nm with monodisperse colloidal system).
Sonication time	5-15 min	The time duration for sonication was selected based on the literature and trial batches. Moreover, longer duration of sonication was avoided due to leaching of drug from matrix and possible metal contamination (Betts JN *et al.*, 2013).

4.2.2.3. Formulation development by quality by design

The development of novel dosage form by QbD required in-depth knowledge of product characteristics, source of variability, formulation and manufacturing process variables (including drug substance, excipient and process parameters). This knowledge is then used to implement a flexible and robust manufacturing process that can adapt and produce a consistent product over time. Some of the salient features of QbD include: (a) Defining quality target product profile (b) Identifying potential critical quality attributes (CQAs) of the drug product (c) Determining the critical quality attributes of the drug substance, excipients (d) Selecting an appropriate manufacturing process (e) Defining a control strategy (Yu LX, 2008).

With the exception of few studies (Singare DS *et al.*, 2010) application of experimental design approach for developing novel drug delivery system emphasizes on optimization of composition variables only (Alukda D *et al.*, 2011; Wang F *et al.*, 2014). However, it is already established that process parameters also play a crucial role in novel dosage form. Here, Box-Behnken Design (BBD) was selected to optimize asenapine loaded nanostructure lipid carriers using Design-Expert software (Version 7.0.0, Stat-Ease Inc., Minneapolis, USA). This design was specifically selected for exploration of complete design space with reduced experimental runs, without aliasing interaction factors (Li GY *et al.*, 2011; Singh B *et al.*, 2005a; Singh B *et al.*, 2005b). Five independent variables were selected in which

three were composition variables and two were process variables. The factors and their levels were chosen on the basis of trial batches and data mining. The variables (A) liquid lipid to solid lipid ratio, (B) drug to solid lipid ratio, (C) aqueous surfactant concentration, (D) homogenization speed and (E) sonication time were selected as independent factors. Particle size (Y1) and entrapment efficiency (Y2) were selected as dependent variables (Response). The independent variable and their levels with set constraint for optimization are presented in Table 4.3.

Table 4.3: Investigated dependent, independent variables and their levels in Box-Behnken experimental design

Independent Variables	**Levels**		
	Low (-1)	Medium (0)	High (+1)
A= OA/ GMS (w/w)	0.1	0.15	0.2
B= ASM/ GMS (w/w)	0.1	0.15	0.2
C= Tween-80 (%w/v)	0.5	1.0	1.5
D= Homogenization speed, HS (rpm)	8000	12000	16000
E= Sonication time, ST (min)	5	10	15
Dependent Variables	**Constraint**		
Y1=Particle Size, PS (nm)	Minimum		
Y2= Entrapment efficiency, EE (%)	Maximum		

OA: Oleic acid, GMS: Glyceryl monostearate, ASM: Asenapine maleate, HS: Homogenization speed, ST: Sonication time, PS: Particle size, EE: Entrapment efficiency

4.2.2.4. Optimization and model validation

A suitable model was selected based on the lack of fit test and model statistic data. The response was fitted to linear, two factor interaction, quadratic and cubic model then evaluated by statistical significance of coefficient, PRESS (predicted residual sum of squares) and r^2 values. Based on model, a polynomial equation was generated to describe the effect of factors on response by Design Expert Software. Based on dependent variables constraint, optimized batch was selected by numerical method with maximum desirability factor. This optimized asenapine loaded nanostructure lipid carrier formulation (ANLC) was used for further in-vitro and in-vivo characterization.

4.2.2.5. Preparation method for nanostructure lipid carriers

Nanostructure lipid carriers (NLC) were prepared by high shear homogenization and sonication method (Garg A and Singh S, 2011). Briefly, specific quantity of glyceryl monostearate (solid lipid), oleic acid (liquid lipid) and asenapine maleate (drug) were mixed and kept in molten state at 70 °C. In another beaker, 50 ml of aqueous phase containing Tween-80 as surfactant was kept at 70 °C on magnetic stirrer (RCT basic, IKA). This molten lipid and drug were poured drop wise into aqueous phase under high shear homogenization (Ultra Turrax T25, IKA) using S25-10G probe. The resulting suspension was ultrasonicated at 60% amplitude at 0.5 s frequency using probe Ultrasonicator (UP200H, Hielscher). The final volume of nanosuspension was adjusted to 50 ml and stored at room temperature. The

nanosuspension was evaluated for particle size and entrapment efficiency after 24 h of preparation. A total of 46 experimental batches were prepared from five factors, three levels Box-Behnken statistical experimental design (Table 4.4).

Table 4.4: Composition of experimental batches

Batch No	Composition and process variables				
	OA/ GMS (w/w)	ASM/ GMS (w/w)	T-80 (%w/v)	HS (rpm)	ST (minute)
NLC -1	0.15	0.15	1.00	12000.00	10.00
NLC -2	0.10	0.20	1.00	12000.00	10.00
NLC -3	0.15	0.15	1.00	12000.00	10.00
NLC -4	0.15	0.10	1.50	12000.00	10.00
NLC -5	0.10	0.15	0.50	12000.00	10.00
NLC -6	0.15	0.15	1.00	8000.00	5.00
NLC -7	0.15	0.20	0.50	12000.00	10.00
NLC -8	0.15	0.15	1.00	12000.00	10.00
NLC -9	0.15	0.15	1.00	16000.00	5.00
NLC -10	0.10	0.15	1.00	12000.00	5.00
NLC -11	0.15	0.10	1.00	12000.00	15.00
NLC -12	0.15	0.20	1.00	12000.00	15.00
NLC -13	0.15	0.15	1.00	12000.00	10.00
NLC -14	0.20	0.15	1.00	8000.00	10.00
NLC -15	0.15	0.15	0.50	8000.00	10.00
NLC -16	0.15	0.10	1.00	16000.00	10.00
NLC -17	0.20	0.20	1.00	12000.00	10.00
NLC -18	0.15	0.15	1.50	12000.00	5.00

NLC -19	0.15	0.15	1.50	8000.00	10.00
NLC -20	0.15	0.15	1.50	16000.00	10.00
NLC -21	0.10	0.10	1.00	12000.00	10.00
NLC -22	0.10	0.15	1.00	12000.00	15.00
NLC -23	0.15	0.15	1.00	12000.00	10.00
NLC -24	0.15	0.10	1.00	8000.00	10.00
NLC -25	0.20	0.15	0.50	12000.00	10.00
NLC -26	0.15	0.20	1.50	12000.00	10.00
NLC -27	0.20	0.15	1.50	12000.00	10.00
NLC -28	0.15	0.20	1.00	12000.00	5.00
NLC -29	0.15	0.10	0.50	12000.00	10.00
NLC -30	0.15	0.20	1.00	16000.00	10.00
NLC -31	0.15	0.15	1.00	12000.00	10.00
NLC -32	0.20	0.10	1.00	12000.00	10.00
NLC -33	0.15	0.15	1.00	8000.00	15.00
NLC -34	0.15	0.20	1.00	8000.00	10.00
NLC -35	0.20	0.15	1.00	12000.00	5.00
NLC -36	0.15	0.10	1.00	12000.00	5.00
NLC -37	0.10	0.15	1.50	12000.00	10.00
NLC -38	0.10	0.15	1.00	8000.00	10.00
NLC -39	0.15	0.15	0.50	12000.00	15.00
NLC -40	0.20	0.15	1.00	16000.00	10.00
NLC -41	0.15	0.15	1.50	12000.00	15.00
NLC -42	0.10	0.15	1.00	16000.00	10.00
NLC -43	0.15	0.15	1.00	16000.00	15.00
NLC -44	0.20	0.15	1.00	12000.00	15.00
NLC -45	0.15	0.15	0.50	12000.00	5.00
NLC -46	0.15	0.15	0.50	16000.00	10.00

4.2.3. Preparation of Glycol chitosan coated nanostructured lipid carriers (GC- ANLC)

Asenapine NLC modified with glycol chitosan (GC-ANLC) was prepared by method discussed in section 4.2.2.5 with modification. In preparation and optimization of GC-ANLC, 0.2, and 0.10 w/w ratio of oleic acid, asenapine were mixed with glyceryl monostearate, respectively and kept at molten state at 70 °C. In another beaker, 50 ml of aqueous phase containing Tween-80 (1.5 %w/v) and glycol chitosan was kept at 70 °C on magnetic stirrer (RCT basic, IKA). The molten lipid phase was poured drop wise into aqueous phase under 16000 rpm high shear homogenization (Ultra Turrex T25, IKA) using S25-10G probe. The resulting suspension was ultrasonicated for 5 minute at 60% amplitude and 0.5 s frequency using probe Ultrasonicator (UP200H, Hielscher). Further, different concentration of glycol chitosan (0.01, 0.05, 0.1, 0.2 and 0.4 %w/v) was added to above suspension and then kept at magnetic stirrer for 24 h. Finally, GC-ANLC nanoparticles were collected by centrifugation at 20,000 rpm for 30 min and washed thrice with distilled water to remove unabsorbed glycol chitosan.

4.2.4. Characterization and optimization of ANLC and GC-ANLC

4.2.4.1. Particle size, polydispersity index and zeta potential

Particle size was determined by measuring random changes in intensity of light scattered by suspended particles during their Brownian motion. This technique is commonly known as dynamic light scattering (DLS) or photon correlation

spectroscopy (PCS). The particle size, polydispersity index (PDI) and zeta potential were determined by particle size analyzer (Delsa Nano C, Beckman Coulter) at 25 °C. Polydispersity index indicates the distribution of particle size of nanoparticles which reveal nature of distribution like monodisperse and polydisperse. Zeta potential was determined by electric movement of charged particles under an applied electric field from Doppler shift of scattered light. It is based on the Helmoltz-Smoluchowski equation:

$$\xi = Ue \times 4\pi\eta/\varepsilon$$

where, ξ is zeta potential, Ue is electrophoretic mobility, η is viscosity and ε is dielectric constant of the medium (Jain A et al., 2013). All studies were performed in triplicates and mean value was considered for data analysis and presentation.

4.2.4.2. Entrapment efficiency

The entrapment efficiency (EE) was estimated with method described by Vuddanda et al. 2014 (Vuddanda PR *et al.*, 2015). Accurately measured 500 µl nanosuspension was placed in the upper chamber of Nanosep centrifuge tubes having ultra filter with molecular weight cut-off 100 kDa (Pall Life Sciences). Nanosep was centrifuged at 15000 rpm for 30 min using a cooling centrifuge at 4 °C (C-24, Remi). The free amount of asenapine in the filtrate was collected from lower chamber and estimated by HPLC method. The EE was calculated by the following equation:

$$\mathrm{EE}(\%) = \frac{\text{Total drug} - \text{Free drug}}{\text{Total drug}} \times 100$$

4.2.5. *In-vitro* drug release study

To simulate micro environment of *in-vivo* biological fluid, *in-vitro* drug release study was carried out in phosphate buffer pH 7.4 for 24 h. The release study of ASM, optimized NLC (ANLC) and glycol chitosan coated ANLC (GC-ANLC) were performed using dialysis bag method. The ASM, ANLC and GC-ANLC suspensions equitant to 10 mg were filled in pretreated dialysis bag (Dialysis Membrane-135, Molecular weight cut off between 12-14 kDa, HiMedia, Mumbai, India) and immersed in 100 ml of phosphate buffer pH 7.4 to mimic biological fluid. The phosphate buffer was magnetically stirred at 100 rpm at 37 °C and 1.0 ml aliquots were withdrawn from release medium at predetermined time for 24 h and replaced with fresh phosphate buffer. The solution was filtered by 0.45 µm syringe filter and concentration of asenapine was measured by HPLC method to calculate cumulative % drug release with respect to time. With the help of DDsolver software, in-vitro drug release data was fitted into various release model like zero order, first order, Higuchi, Korsemeyer-Peppas and Hixson-Crowell to understand the mechanism of drug release from lipid matrix (Fazil M *et al.*, 2012; Zhang Y *et al.*, 2010).

4.2.6. Solid state characterization

4.2.6.1. Fourier transform infrared spectroscopy

The IR spectra of ASM, GMS, lyophilized ANLC and GC-ANLC were recorded by Fourier Transform Infrared Spectroscopy (FTIR-8400S, Shimadzu). Sample preparation involved mixing the sample with potassium bromide (KBr) in 1:50

ratio, triturated in glass mortar, pelletized, and finally placed in sample holder. The spectrum was scanned over the wavenumber of 4000–400 cm^{-1}.

4.2.6.2. Differential scanning calorimetry

The DSC was performed to evaluate any change in drug with respect to melting enthalpy, glass transition temperature and any interactions with excipients. The physical state of ASM, GMS, lyophilized nanocarriers (ANLC and GC-ANLC) was characterized by the differential scanning calorimetry (DSC Q1000, TA instrument). About 2-5 mg of sample was placed in standard aluminium pans and scanned in the range from 5 °C to above the melting point with temperature increment speed of 10 °C/min under the dry nitrogen used as effluent gas (flow rate 50 ml/min).

4.2.6.3. X-Ray Diffraction

The physical properties of asenapine in pure form and inside the lipid matrix were measured by X-Ray Powder Diffraction (XRD). X-ray powder scattering measurements were carried out to check the crystallinity of drug in pure and lyophilized nanocarriers (ANLC and GC-ANLC). Study was performed on a Siemens DIFFRACplus 5000 powder diffractometer with CuKα radiation (1.54056 Å). The tube voltage and amperage were set at 40 kV and 40 mA, respectively. Each sample was scanned between 10° and 40° in 2θ with a step size of 0.01° at 1 step/s (Mohammad MA *et al.*, 2011).

4.2.7. Surface characterization

4.2.7.1. Transmission electron microscopy

The size and morphology of ANLC and GC-ANLC were observed using a TEM (TECNAI-12 and TECNAI-G2). One drop of appropriately diluted nanosuspension was spread on 400 mesh gold coated copper grid. The grid was air dried at room temperature under vacuum for 24 h before observation.

4.2.7.2. Atomic force microscopy

The external morphology of ANLC and GC-ANLC was further visualized by Scanning probe microscope (NTEGRA Prima, NT-MDT) in semi contact mode. ANLC suspension was diluted 10 times with distilled water and one drop of nanosuspension was placed on the small microscope slide to form a dry film of suspension for observation.

4.2.8. Stability studies

Stability study of ANLC and GC-ANLC was carried out at 30 ± 2 °C, 65 ± 5% RH for three months. Sealed vials of ANLC and GC-ANLC suspension was placed in stability chamber. The formulations were analyzed for particle size, zeta potential, entrapment efficiency and *in-vitro* drug release profile comparison (f1: difference factor, f2: similarity factor) at each month. The difference factor calculates the percent difference between the two curves at each time point and is a measurement of the relative error between the two curves. It is expressed as:

$$f_1 = \{[\sum_{t=1}^{n}(R_t - T_t)]/[\sum_{t=1}^{n}R_t]\} \times 100$$

The similarity factor is a logarithmic reciprocal square root transformation of the sum of squared error and is a measurement of the similarity in the percent dissolution between the two curves. It is expressed as:

$$f_2 = 50 \times \text{Log}\left\{\left[1 + \frac{1}{n}\sum_{t=1}^{n} w_t(R_t - T_t)^2\right]^{-0.5} \times 100\right\}$$

where n is the number of time points, R is the dissolution value of the reference batch at time t, and T_t is the dissolution value of the test batch t at time t. w_t is a weight factor that can be used to enhance the influence of particular time points. (Costa P and Sousa Lobo JM, 2001; Dash S *et al.*, 2010). The results were expressed as mean ± SD. The student t-test was applied to examine the significance difference.

4.2.9. *In-vitro* cell viability study

Cell viability studies of ASM, ANLC and GC-ANLC in A549 cell line was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay. In this assay, the formation of formazan is directly proportional to number of viable cells. Briefly, A549 cells were seeded in a 96-well plate in 100 µl of Dulbecco's Modified Eagle medium (DMEM) (Sigma-Aldrich, India,) with 5% Fetal bovine serum (FBS) (Sigma-Aldrich, India,) and antibiotic (Penicillin-Streptomycin) and then allowed to be cultured at 37°C for 24 h. After culture, cells were treated with medium containing different concentration of ASM and GC-ANLC (0.001, 0.01, 0.1, 1, 10 µM asenapine) in 200 µl/well for 24 h at 37°C. The untreated cells were used as

control (100% viability) for calculation of cell viability. Further, 20 µl of MTT solution (5 mg/ml) was added to each well and incubated for 4 h at 37°C. The media and unreduced MTT were removed carefully by pipetting and finally 100 µl of DMSO was added in each well to dissolve the formazan crystals. The plate was shaken for 10 min and absorbance was measured at 570 nm in a microplate reader (Model 680, BioRad). All the experiments were performed in triplicate and % cell viability was calculated using following formula:

$$\% \text{ Cell viability } = \frac{(\text{Absorbance})\text{test}}{(\text{Absorbance})\text{control}} \times 100$$

4.2.10. *In-vivo* pharmacokinetic study

4.2.10.1. Animal and study design

The pharmacokinetic parameters of the ASM, ANLC and GC-ANLC via nasal route were determined in Charles foster rats (200-240 g) according to protocol approved by Institutional Animal Ethical Committee (Dean/44094/2013-14). The rats were housed in a polypropylene (421×290×190 mm) cage at normal room temperature in 12-h light/dark cycle with food and water *ad libitum*. All animals were allowed to acclimatize for one week according to their treatment protocol and kept fasting overnight before experimentation. Total four groups, comprising five animals for each time point were assigned for pharmacokinetic study. Three groups were assigned for intranasal delivery of ASM, ANLC and GC-ANLC respectively and one more group for intravenous injection of ASM through intravenous tail vein injection.

All animals from each group administered with 1.0 mg/kg equivalent dose of asenapine. For the intranasal administration, rats were placed in supine position with upright nose and formulations were administered with help of micropipette in equally divided volume for each nostril. Five animals per time point were sacrificed for collection of their blood and brain. The plasma was collected by centrifuging blood sample at 4,000 rpm at 4 °C for 20 min. However, brain samples were taken and homogenized in distilled water using a tissue homogenizer. The plasma and brain homogenate were stored at -40 °C until further processing.

4.2.10.2. Plasma brain homogenate processing

Asenapine was extracted from plasma and brain homogenate by two step liquid–liquid extraction procedure. In this process, 100 µl of plasma and brain homogenate were mixed with 1.5 ml of 2 % isopropanol in n-hexane and arteether (IS) as internal standard. The sample was further kept at vortex mixer (CM 101, Remi) for 3 min before centrifugation at 4000 rpm for 10 min. The supernatant was removed and remaining precipitated protein and tissues of this step were re-extracted in similar way. Both supernatant in two step process was mixed and kept for complete drying at 40 °C under nitrogen environment. The dried residues were reconstituted with 100 µl of mobile phase for analysis of asenapine by LC-MS/MS.

4.2.10.3. Chromatographic condition and LC-MS/MS analysis

The quantification of asenapine in plasma and brain homogenate were performed by partially validated method in Q-trap 5500 LC–MS/MS mass spectrometer

(Applied Biosystems, MDS Sciex, Framingham, MA) with Analyst 1.6 software (Framingham, MA). A 10 µl of reconstituted sample was injected into an ekspert ultra LC 100-XL HPLC system (AB Sciex), consisting of vacuum degasser, ekspert 100 pump with ekspert 100-XL auto sampler. The Q1/Q3 transitions of m/z 286.1/229.0 and 330.3/267.4 were used to quantify asenapine and IS, respectively. The ion source gas 1 (GS1) and ion source gas 2 (GS2) were optimized to 60 and 35 psi. Declustering potential (DP) was optimized to 90V. Flow injection analysis was used to optimize collision energies (CE). The CUR and collision gas were optimized to 35 and 7 psi respectively. Entrance potential (EP) and collision cell exit potential (CXP) were optimized to 10 and 16 V, respectively. The ion source potential was set at 5500 V and source gas temperature was optimized to 450 °C. UFLC elution was carried out in isocratic mode with mobile phase consisting of 85:15 (v/v); ACN: ammonium acetate buffer (10 mM, pH 4) at a flow rate of 0.45 ml/min with injection volume 10 µl. The separation of drug was performed on a Phenomenex, Luna C-18 column (3 µm, 100 mm x 2 mm i.d.) with a C-18 guard column (Phenomenex, Luna C-18, 5 µm, 30 mm x 2 mm, i.d.). The column temperature was maintained at 40 °C using ekspert 100 oven.

4.2.10.4. Pharmacokinetic parameters analysis

The mean plasma concentration – time profile of ASM, ANLC and GC-ANLC was evaluated by non-compartmental pharmacokinetic analysis. The pharmacokinetic parameters, area under the curve (AUC), peak plasma concentration (C_{max}) and its

time (t_{max}) of both ASM and GC-ANLC in plasma and brain homogenate were calculated by Phoenix 64 Software (WinNonlin 6.4, CERTARA). The relative bioavailability of ANLC and GC-ANLC were compared with ASM. The drug targeting efficiency (DTE) to brain of nanocarriers via intranasal route was calculated according to following equation (Serralheiro A et al., 2014):

$$DTE = \frac{(AUC\ brain\ /\ AUC\ plasma)i.n.}{(AUC\ brain\ /\ AUC\ plasma)i.v.}$$

In above equation, AUC_{brain} and AUC_{plasma} are the areas under the drug concentration–time curves for brain and plasma, respectively.

4.2.11. Animal behavioural studies

4.2.11.1. Animal and dose

CF rats (180-220 g) were used for all behaviour studies of ASM, ANLC and GC-ANLC. The rats were divided into separate groups (five animals per group) and housed in a polypropylene (421 × 290 × 190 mm) cage at normal room temperature in 12-h light/dark cycle. They had free access of food and water. ASM, ANLC and GC-ANLC equivalent to 1.0 mg/kg asenapine were given in all studies via nasal route using micropipette. The group administered with intranasal blank nanostructured lipid carrier suspension was considered as vehicle control for induced locomotor, paw test and catalepsy. Further, one more groups administered with intra-peritoneal l-dopa (10 mg/kg) and carbidopa (2.5 mg/kg) was considered as positive control in induced locomotor activity test. In most studies, the animal behaviour models for

screening of developed formulation are evaluated on a one day treatment response, which raises concerns about validity of this therapeutic-like behaviour especially in a disease like schizophrenia where pharmacological effect (therapeutic effect or side effect) are usually manifested after 2-3 weeks of treatment. Thus, our experiments were performed for continuous administration of drug for 21 days and observations were recorded on 1st, 7th, 14th and 21st days of treatment in order to account for any inconsistency (Lieberman JA *et al.*, 2008). The data were reported in mean±SD for each group.

4.2.11.2. Induced locomotor activity test

This behavioural model is based on a hypothesis that increase in locomotor activity is due to an increased dopaminergic activity in the mesolimbic system. Indeed, asenapine have antagonist effects on dopamine agonist induced hyperactivity (Geyer MA and Ellenbroek B, 2003). The locomotor count was determined by Digital Actophotometer (IKON, India). On the day of observation, ASM, ANLC and GC-ANLC groups received the respective formulation followed by intra-peritoneal administration of l-dopa (10 mg/kg) and carbidopa (2.5 mg/kg). The locomotor activity was measured for 5 min by placing the animals in Actophotometer 1 h after drug administration (Kumar M *et al.*, 2008; Marston HM *et al.*, 2009).

4.2.11.3. Paw test

The paw test is model for a prediction of both therapeutic potential as well as extrapyramidal side effects (EPS) associated with any antipsychotic drug. The

increase in hindlimb retraction time (HRT) was associated with the antipsychotic potential, whereas the increase in forelimb retraction time (FRT) was associated with the potential to induce EPS. Also, this model has unique feature for differentiating classical antipsychotics which are equipotent in prolonging both the forelimb retraction time (FRT) and hindlimb retraction time (HRT) and atypical antipsychotics which are much more potent in prolonging HRT than FRT (Ellenbroek BA *et al.*, 1987). The test was performed on a Perspex platform measuring 30 cm × 30 cm, with a height of 20 cm. The top of the platform had two holes of 3.5 cm diameter for the forelimbs and two larger holes of 4.5 cm diameter for hind limbs and a slit for the tail. For both FRT and HRT, the minimum time was set to 1 s and maximum to 60 s. Experiment was performed in triplicate in five minute interval and average FRT and HRT were then calculated for each rat.

4.2.11.4. Catalepsy test

This animal test was performed to evaluate the effect of delivery system on extrapyramidal side effects (acute dyskinesias, dystonic reactions, tardive dyskinesia, parkinsonism, akinesia, akathisia, and neuroleptic malignant syndrome) associated with asenapine in term of catalepsy. Briefly, rat forepaws were placed on horizontal bar fixed at a height of 10 cm above the surface whilst their hind limbs rested on a platform. The amount of time animal remains immobile was calculated. After administration of vehicle control, ASM, ANLC and GC-ANLC; the amount of

time spent maintaining this abnormal position was measured after 1 h dosing and mean values were reported (Franberg O *et al.*, 2008).

4.3. Toxicity study

4.3.1. Nasal toxicity study

Nasal histopathological study was carried out at rat nasal mucosa by intranasal administration of phosphate buffer (pH 6.4) (negative control), isopropyl alcohol (positive control due to its cilio-toxic nature) and GC-ANLC daily during 7 days. At the end of experiment, rats were sacrificed and their nasal tissues with the epithelial cell membrane were taken out according to protocol approved by Institutional Animal Ethical Committee (Dean/44094/2013-14). The tissue samples were fixed in 10% formaldehyde for 24 h and dehydrated with ethanol. Tissue blocks of paraffin beeswax were then prepared for sectioning by slide microtone. The obtained tissue sections were collected, deparaffinized and stained by hematoxylin and eosin stains. The tissue specimens of all three groups were examined using a light microscope (Salama HA et al., 2012).

4.3.2. Embryo fetal toxicity study

4.3.2.1. Animals

The female Charles-Foster rats (180-220 g) were used for the present study. The rats were housed in polypropylene (421 × 290 × 190 mm) cages at standard laboratory conditions (24 ˚C, 60% RH,12h L/D cycle), with free access of food and water. The animals were maintained and used in according to protocol approved by

Institutional Animal Ethical Committee (Dean/2015/CAEC/1425). The nulliparous female rates were allowed to mate with males (1:2 ratio/set) for overnight. On next day, they were checked for presence of sperms in vaginal swab and sperm positive rats were designated as gestation day 0 (GD-0).

4.3.2.2. Study design

All sperms positive female rats were divided into four groups (n=4 /group). In first two groups, ASM and GC-ANLC were administered via nasal route using micropipette. In the remaining two groups, one group was exposed to distilled water as vehicle of asenapine, whereas second group was kept as blank nanoparticles. In this study, asenapine in pure form and in nanoformulation were carried out at dose 1.0 mg/kg/day, which was equivalent to lower dose of human recommended dose (10 mg/day). The required dose volume was administered daily to pregnant rats from gestation day 6-21 in treated (ASM and GC-ANLC) groups. The control rats received an equivalent volume of vehicle only (distilled water and blank nanoparticles). The dose volume was adjusted on every third day based on the current body weight of the individual animal. Both control and treated pregnant rats were sacrificed after deep anesthetization (sodium pentobarbital, 50 mg/kg, ip) on GD 21 (09:00 hr), and their fetuses were collected by uterectomy, examined externally for birth defects, if any; then weighed on electronic balance.