Chapter 6

Summary and Conclusion
6.1 SUMMARY

Vital aim of any pharmaceutical research is to contribute in health care of living souls. It can be comprehended by fabricating the formulations capable of sustaining the patient’s prerequisite of efficacious and compliant product. Taking care of lung health through pulmonary delivery has become quite attractive, safe, quick and efficacious technique. Presently, this route of drug delivery is being explored on the fast track for almost all therapeutic applications. Pulmonary arterial hypertension (PAH) is a serious illness characterized by increased pressure in pulmonary arteries, though the systemic blood pressure may be normal. Thus, this is one such medical area that demands the delivery of therapeutically direct into the lungs for safe and quick onset of drug action without affecting the extra-pulmonary vasculature. Moreover, the inhalation treatments available till date for PAH are very short acting and patient has to take Ventavis® (Iloprost solution for inhalation) 6-9 times a day through nebulizer and Tyvaso® (Treprostinil solution for inhalation) four times (3 inhalations per treatment) a day using Tyvaso® inhalation System under medical supervision. There seems great need of self-actuated sustained release inhalation formulation/system to provide quality life to depressed condition of PAH patients. DPI formulations can be taken by patient itself and sustained release of such formulations can obviate the need of multiple administrations per day. Moreover, there would be no troubling unpleasant systemic effects in extra pulmonary vasculature. Iloprost and Treprostinil are stable prostacyclin analogue, but with a very short half-life of 25 min and 55–117 min respectively. Current treatment guidelines from the American College of Chest Physicians (2009) recommend sildenafil as a first-line agent in NYHA class II PAH and as one of the first-line treatments in class III PAH (New York Heart Association, 2010). Its marketed formulation Revatio® is indicated for the treatment of PAH (WHO Group I) in adults to improve exercise ability and delay clinical worsening. Sildenafil citrate, a PDE-5 inhibitor, has a half-life of 3-4h and can be incorporated into delivery systems to sustain its effect to achieve at least once a day formulation.

This study was planned to overcome the problem of frequent administration and systemic side effects associated with the currently available therapy of pulmonary arterial hypertension. This issue was addressed by incorporation of sildenafil citrate into different dry powder formulations to attain practically feasible, scalable and
stable formulations with desired formulation characteristics for better lung deposition and sustained release effect. *In vitro* characterization to determine and compare the aerosolization behaviour and stability and *in vivo* evaluation of sustained release potential of these formulations was also accomplished.

Preformulation studies of sildenafil citrate revealed maximum solubility of 15.74 mg/mL at pH 2 and minimum solubility of 24mg/mL at pH 7.4. Drug distribution studies showed more partitioning towards organic phase and partitioning towards organic phase was increased with phosphate buffer pH 7 as aqueous phase. UV spectroscopic and HPLC method was developed for drug analysis of *in vitro* and *in vivo* samples respectively. The standard curves exhibited good linearity over the range of 2µg/mL to 50µg/mL and 780ng/mL to 200µg/mL respectively.

Conventional dry powder formulations (CDPI) of sildenafil citrate were prepared by mixing different ratios of lactose carriers of different average particle sizes with micronized drug. Formulation (CD3) with Lactohale 200® and Pharmatose® 350M (70:30) showed best aerosolization characteristics with maximum FPF of 36 ± 0.02% with Rotahaler® out of all other combinations and therefore was used in further studies. It showed a geometric diameter of 6.76 µm and mass median aerodynamic diameter (MMAD) of 6.12± 2.23 µm and GSD of 2.26±1.92 (using ACI). In general, all formulations which were aerosolised by the use of Rotahaler® produced significantly higher % FPF (*p* < 0.05) at the same flow rate than those aerosolized with Handihaler®.

In case of co-spray dried drug-sugar composites, mannitol gave non-sticky product with excellent flow at 4% solution concentration and at 1:10 (drug: mannitol) ratio. Optimized spray drying parameters for drug sugar composites were 3mL/min feed rate, atomization at compressed air pressure of 3 bars with -160mm of WC vacuum at 80°C inlet temperature.

Liposomes prepared with drug, HSPC, DPPC and cholesterol (5:18:4.5:2.5 molar ratio) under optimized process conditions showed maximum drug loading with 85% to 88.02% entrapment. Its 3% w/v solution was finally spray dried at optimized parameters of 3 mL/min feed rate, atomized at compressed air pressure of 3 bars with -160 mm of WC vacuum at inlet/outlet temperature of 80°C/52-56°C to get dry powder formulation using mannitol (Lipid: mannitol; 1:3) as a protective carrier.
1.18% w/v of Drug and lipids, in the above optimized molar ratio, were spray dried using trehalose carrier at 1:1 lipid: carrier ratio in methanol solvent to get drug-lipid composites in single step process which showed 96-98% entrapment efficiency at optimized parameters of 3mL/min feed rate, atomized at compressed air pressure of 3 bars with -160mm of WC vacuum at 50°C inlet temperature.

Large porous lipospheres having 95.08 to 97.86 % entrapment efficiency were prepared by spray drying the emulsion of drug and HSPC (1:3 ratio) and 1.5% v/v blowing agent in aqueous solution containing 0.15% w/v DPPC as surfactant and 2.4% w/v mannitol. Final formulation was spray dried at optimized parameters of 3mL/min feed rate, atomized at compressed air pressure of 3 bars with -100mm of WC vacuum at 90°C inlet temperature.

Formation of liposomes and emulsion globules before spray drying was illustrated using Transmission electron microscopy. All the formulations showed almost neutral zeta potential with a maximum of ±4.68mV deviation. Surface topography of dry powder formulations was determined using Scanning electron microscopy. Scanning electron micrographs revealed crystalline nature of CDPI with irregular shape. Surface of spray dried formulations of sildenafil citrate loaded sugar composites and lipid composites were smooth, spherical and non-porous. Spray drying of submicron liposomal dispersion yielded particles with minute pores on the surface. However, spray drying of emulsion feed stock containing a blowing agent led to the formation of highly porous, large sized hollow aerodynamically light particles.

The thermograms of drug-loaded lipid composites did not show any endothermic peak of drug (197°C) revealing the complete entrapment of drug in lipid matrix and protective trehalose covering. Drug-sugar composites, liposomal dry powder for inhalation and large porous lipospheres showed peaks near the melting point and glass transition temperature (166°C) of protective sugar mannitol suggesting the absence of any interactions of drug with the excipients and complete stabilization of lipid surfaces by protective covering of mannitol. Conventional DPI (CD3, prepared with 70:30 LH 200 and P350M) showed diffraction peaks of drug at same intensity (cps) as that of crystalline peaks of sildenafil citrate alone revealing its crystalline nature. However, dry powder formulations prepared using spray drying
technique was amorphous in nature. No crystalline peak representing sildenafil citrate was seen in case of large porous lipospheres supporting the highly amorphous nature of the product.

In case of CDPI, flow parameters were improved on combining fine lactose carriers with coarse carriers and it was particularly best for the formulation CD3 at ratio 70:30 of LH200:P350M. This formulation showed a bulk density of 0.468 ± 0.86 g/cc, tapped density of 0.622 ± 0.99 g/cc and 6.76±1.05 μm geometric diameter which was different from calculated value of 3.16± 2.23 μm due to its asymmetric shape. Drug-sugar composites showed good flow characteristics with <30° (27.03 ± 0.86°) angle of repose, %CI of 14.59%. 1.16 Hausner’s ratio and bulk density and Tapped density of 0.211 ± 2.03g/cc and 0.246 ± 0.18g/cc respectively. Liposomal dry powder for inhalation (DPL7) had a low bulk density of 0.201± 2.05 and a volume mean diameter of 8.99± 1.26 μm thus having a calculated aerodynamic diameter of 4.03 μm. Optimized Drug-lipid composites (DLS7) had a bulk density of 0.207± 0.16g/cc, tapped density 0.232 ± 0.02g/cc with 10.77% CI and 1.22 Hausner’s ratio. Volume mean diameter was 5.12± 0.91 μm and calculated aerodynamic diameter was 2.33 μm. Lowest density particles (LPL16) having 0.101± 1.43g/cc of bulk density and a volume mean diameter >10 μm i.e. 13.28± 0.51 μm were formed with large porous lipospheres. It showed excellent and necessary characteristics for dry powder inhalation formulations.

Moisture content of CDPI formulation was 5.11±1.08% w/w. All spray dried products had lower moisture content when prepared at optimized spray drying process conditions. Drug-sugar composites had a moisture content of 3.07 ± 0.82% w/w and that of drug-lipid composites was 2.69 ± 0.09%. Liposomal dry powder inhalation formulation contained 1.16 ± 2.24% w/w moisture and least moisture content (0.91±1.16% w/w) was found in large porous lipospheres. Residual solvent was not detected using gas chromatography in any of the prepared dry powder formulations.

All spray dried optimized formulations passed the test of Delivered Dose Uniformity performed using Dosage Unit Sampling Apparatus (DUSA by Copley Scientific) for DPIs. Selected formulations were characterized for in vitro deposition using ACI. Optimized conventional DPI, drug sugar composites, drug-lipid composites, liposomes and large porous lipospheres showed FPF of 36±0.02%, 54±
0.33%, 30.05 ± 0.39%, 62.01 ± 0.09% and 82 ± 0.42% respectively. Respective MMAD for the above said formulations was found to be 6.12 ± 2.23μm, 2.96 ± 1.83μm, 2.125± 0.5 μm, 4.095 ± 0.52 μm and, 4.64 ± 0.71μm. Thus the best lung deposition could be achieved with large porous lipospheres followed by liposomal dry powder.

Conventional DPI Formulation F1 and drug-sugar composites F2 showed best fitting with first order release kinetic model with F2 showing faster release than F1. However, all lipid based formulations were able to sustain the release for more than 24 hours. In vitro release profile suggested a biphasic release pattern for liposomal dry powder formulation (F3) and best fitting to Korsmeyer-Peppas kinetic model. Drug lipid composites (F4) formulation could sustain the release for 24h and showed a release pattern according to Hixson-Crowell’s kinetic model. Large porous lipospheres showed in vitro release of more than 34 hours and release pattern was best fit with Higuchi’s model.

Stability of all dry powder formulations F1 to F5 was evaluated by analysing samples at specified times with respect to % assay/ % drug retained, % w/w of moisture content, MMAD, % emitted dose and % FPF to propose the suitable storage condition for each. In case of conventional DPI (Formulation F1), % FPF was dropped from 36 ± 0.02% to 28 ± 0.48% after 12M long term storage stability condition. Spray dried formulation of drug-sugar composites, formulation F2 maintained % FPF (54.04 ± 0.33) even after 6 M (52.04 ± 1.09%) of accelerated stability condition and 12 M (53.11 ± 1.07%) of long term stability. Sildenafil citrate-lipid composites formulation (F4) was found to be stable only at 5±3°C (2-8°C) storage condition and maintained % FPF (28.66 ± 0.44%) even after 12 M when compared to initial value (30.05 ± 0.39%).

Liposomal dry powder formulation (F3) showed 5% drop from the initial assay (100.11 ± 1.83% to 95.11 ± 0.96%) at 6M, 30°C/65% RH accelerated stability condition, the percent drug retained was dropped from 98.79 ± 1.42% to 92.79 ± 1.01. % FPF was also dropped from 62.01 ± 0.09 to 48.18 ± 0.53%. Thus sildenafil citrate loaded liposomal dry powder formulation (F3) is recommended to be stored below 25°C/60% RH or at 5±3°C (2-8°C) to have its excellent storage stability. Similarly, sildenafil citrate loaded large porous liposphere formulation (F5) showed no
significant (p<0.05) change in % assay, % drag retained (99.07± 1.79 % and 99.98± 2.36 %), % moisture content (1.87± 1.98% w/w and 1.06± 2.86% w/w) and any of the aerosolization parameters % FPF 80.17 ± 0.17% and 80.99 ± 1.39%) at 25°C/60% RH and at 5±3°C (2-8°C) respectively and is therefore recommended to be stored below 25°C/60% RH or at 5±3°C (2-8°C) to attain its excellent storage stability.

Fluorescein loaded lipid based formulations were used to compare percent macrophage uptake of these formulations with standard 2 μm polystyrene fluorescent beads. Initial and final average RFU values were recorded and % of the initial observed RFU was determined at each time point to demonstrate the % macrophage uptake of the formulations at that time. Standard beads were completely phagocytised within 4 h. However, only 25.67±1.22% of drug- lipid composites could be taken up by macrophage cells even after 24 h. Further, addition of DPPC in the formulation reduced the macrophage uptake of liposomal formulation which could be observed from the extent of uptake of M2 (with DPPC), that was significantly (p<0.05) lower (3.28±0.99%) as compared to liposomal formulation without DPPC, M1 (11.47±1.19%). In case of large porous lipospheres (M4), no macrophage uptake was detected for 12 h and finally uptake was almost negligible (1.90±1.06%) even after 24h. Results were also supported with the images of alveolar macrophage uptake of different formulations using inverted Olympus microscope with Camera and DP controller software.

Pulmonary delivery of sustained release dry powder formulations of sildenafil citrate was performed using endotracheal intubation technique to evaluate its prolonged local efficacy in monocrotaline-induced pulmonary hypertensive rats. Development of disease was evident with the significant increase in mean right ventricular systolic pressure (RVSP) and increased right ventricular hypertrophy (RVH) after 14 days of MCT injection. Inhalation of sildenafil citrate dry powder formulations could significantly prevent the development of PAH and better protection could be achieved with lipid based formulations. On 28th day of the MCT injection, Only-MCT28 treated animals showed strikingly increased mean RVSP (72.50 mm Hg ± 4.764 vs 40.00 mm Hg ± 4.472) and %RVH (63.607 ± 4.033% vs 46.948 ± 3.845%) when compared to Only-MCT14 treated animals. However, there was no significant difference in mean systemic arterial pressure (within range of 116.9 mm Hg ± 0.516 and 120.0 mm Hg ± 0.813) amongst control, Only-MCT14,
Only-MCT<sub>28</sub> and all formulation treated groups. The above seen trend in hemodynamics and RVH was also supported by the cGMP levels found in the lung homogenates of the rats in different groups. During histopathological studies, liposomes dry powder and large porous lipospheres treated groups showed significantly negligible inflammatory response, medial thickening and muscularization. PDE5 inhibition itself has been shown to reveal anti-inflammatory properties on pulmonary inflammatory processes like influx of macrophages and neutrophils in a rat model of airway hyper-reactivity and similar findings were observed in the results. There was only 56.55\% drug left in the lung homogenates at 2h in case of drug sugar composites as compared to 86.55\% in case of conventional dry powder treated animals. This indicates that the spray drying of sildenafil citrate with suitable sugar carriers, rather than simply mixing it with the dry ingredients, may lead to immediate release of the drug from the formulation. All lipid based formulations were able to sustain the drug levels in rat lungs. In case of large porous lipospheres, 52.3\% of the initial amount reaching the lungs could be detected even after 48h, which was 23.21\% in case of liposomal dry powder and 11.02\% in case of drug lipid composites whereas, no drug could be detected after 12h in case of conventional DPI and drug-sugar composites. The most prolonged pulmonary mean residence time of 21.24 hours was seen with large porous lipospheres.

6.2 Conclusion:

The preceding discussion and consequences attained after the extensive investigation and experimentation support us to state following concluding remarks:

- Inhalation formulations of sildenafil citrate could be suitably formulated by conventional mixing of micronized drug with lactose carrier i.e. conventional DPI method and by spray drying technique with mannitol or/as well as with lipid based carriers.

- Spray drying parameters like feed rate, air pressure, inlet temperature and vacuum have individual as well as combined impact on formulation characteristics. Spray drying parameters showed interaction with each other to influence the formulation characteristics like percent drug retained moisture content, percent yield and aerodynamic diameter.
• Formulations prepared by spray drying technique presented better particle size distribution and aerosolization behaviour as compared to conventional DPI formulations.

• Mannitol imparted better flow and aerosolization behaviour to the dry powder inhalation formulations as compared to Trehalose as a carrier and a stabilizing sugar during spray drying. Drug-sugar composites with mannitol showed better lung deposition than drug-lipid composites with Trehalose, determined by Andersen Cascade Impaction technique.

• Liposomal dry powder for inhalation and large porous lipospheres displayed better lung deposition and dispersibility as compared to conventional DPI, drug-sugar and drug-lipid composites with large porous lipospheres having the best aerosolization characteristics.

• Macrophage uptake was quite less in case of lipid based formulations with bigger geometric size but aerodynamic diameter within favourable 2-5 μm range of inhalation. Aerodynamically light, hollow and bigger porous lipospheres showed almost negligible (1.90±1.06%) macrophage uptake even after 24h due to its bigger 13.28± 0.51 μm geometric size.

• Presence of DPPC in the liposomal formulation reduced the rate and extent of macrophage uptake.

• The pulmonary administration of sildenafil citrate dry powder formulations could significantly prevent and reverse PAH without affecting systemic arterial pressure in monocrotaline treated rats.

• Lipid based formulations showed better prevention and reversal of PAH in monocrotaline injected rats. This connotes the better deposition and longer stay in lungs for lipid based formulations linking the improved hemodynamic and biochemical parameters for better prevention and treatment of PAH in MCT model. This assessment was also supported by the histological demonstration of significant reduction of fully muscularized peripheral pulmonary arteries and reduced inflammatory reaction and medial thickening of the peribronchial arteries after treatment with inhalation formulations.

• Out of all different formulations studied, inhalation of spray dried drug-sugar composites showed the fastest drug action and large porous lipospheres demonstrated the most prolonged localization in the lungs of male wistar rats.
• Thus, lipid based inhalation formulations have revealed the potential to increase the $t_{1/2}$ of the drug in the lungs that can be further explored to reduce the patient inconvenience of frequent administration of currently available therapies while exerting the minimum systemic side effects.

The present results propose the promising innocuous potential of lipid based dry powders as pulmonary delivery system for prolonged localized drug effect to treat pulmonary arterial hypertension.