"TREATMENT OF PULMONARY ARTERIAL HYPERTENSION BY PULMONARY DRUG DELIVERY"

A Thesis Submitted to The Maharaja Sayajirao University of Baroda for the award of degree of

DOCTOR OF PHILOSOPHY
IN
PHARMACY

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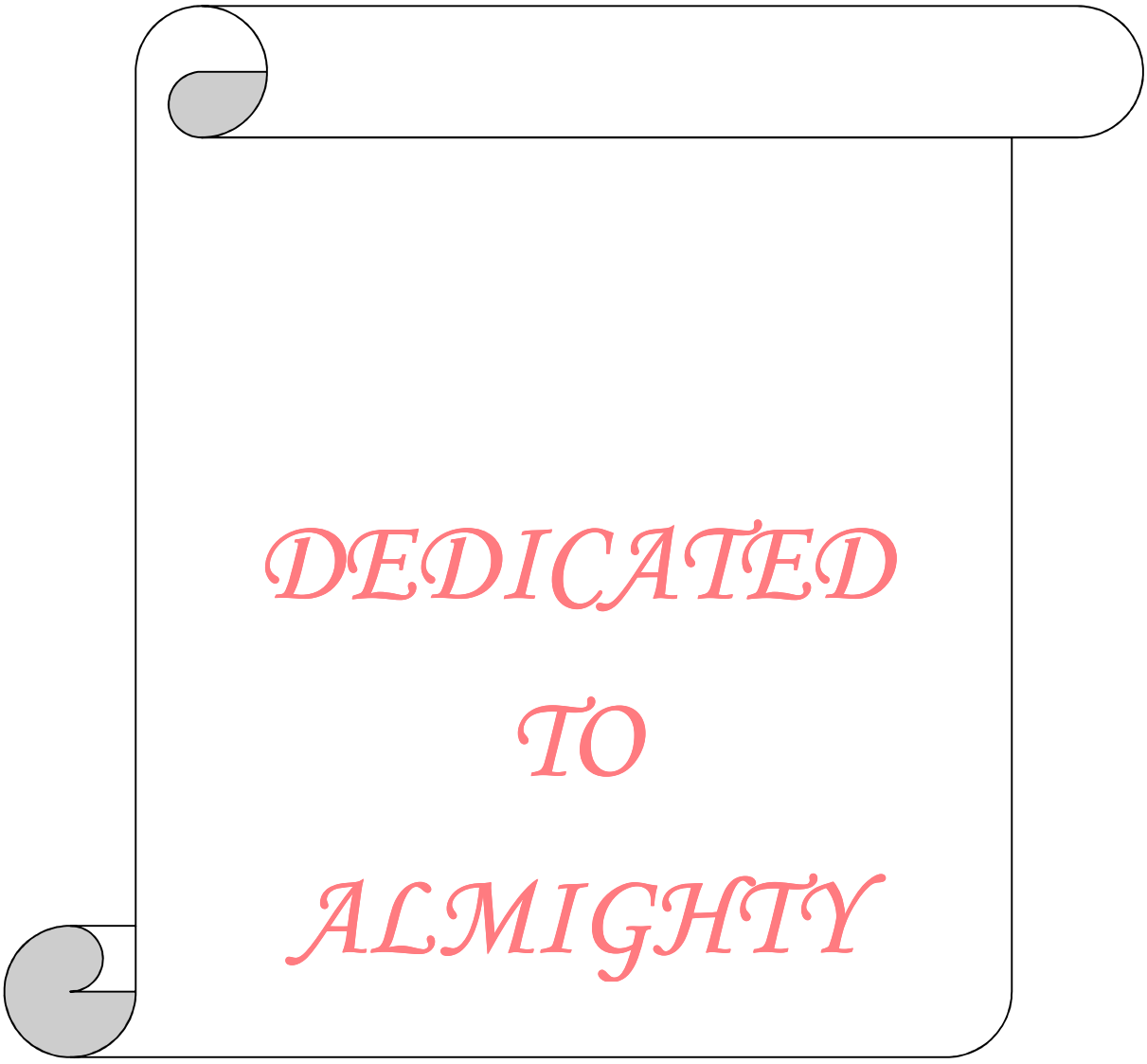
December, 2012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CERTIFICATE

In accordance with the University Ordinance number ACED/FTE/1590-4, I the undersigned state that the work presented in this thesis titled **"Treatment of Pulmonary Arterial Hypertension by Pulmonary Drug Delivery"**, comprises of independent investigations carried out by me. Whenever references have been made to the work of others, it has been clearly indicated with the source of information under the chapters of references.

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DECLARATION

I hereby declare that the topic entitled **"Treatment of Pulmonary Arterial Hypertension by Pulmonary Drug Delivery"** which is submitted herewith to The Maharaja Sayajirao University of Baroda, Vadodara for the award of Ph.D. in Pharmacy is the result of work done by me in Pharmacy Department, Faculty of Technology & Engineering, The M.S. University of Baroda, under the guidance of Prof. Ambikanandan Misra, Pharmacy Department, Faculty of Technology & Engineering, The M. S. University of Baroda, Vadodara.

I further declare that the results of this work have not been previously submitted for any degree.

Sonia Trehan

Place : Vadodara
Date : 26th Dec, 2012

Acknowledgement

With vital devotion, *I express my foremost heartfelt gratitude in the name of* ***Almighty*** *for directing and navigating me to and through this project to the successful accomplishment of my long cherished dream of this thesis with strength, assets, confidence and faith.*

Words are too trivial and scarce to convey my profound sense of indebtedness, gratefulness and honour for being driven by revered guidance of ***Professor Ambikanandan Misra****, Pharmacy department, Dean, Faculty of Technology and Engineering, The Maharaja Sayajirao University of Baroda. His encouraging, patient and insightful guidance towards innovative and quality work has pervaded in me the essence of dedication, hard work, morality and sincerity towards research and investigation. His constructive ideas, intelligent, determined and qualitative approach has suaved my way not only to complete this project but also to face further challenges of life. I would always ruminate to his genuine concern and support during hard times and right direction during difficult decisions. I take this golden opportunity to express the feel of pride and good fortune to have such a kind and preeminent individuality as my supervisor.*

Special thanks to Prof. M. R. Yadav, Head of Pharmacy department for kind support and for providing me necessary facilities for research work. I shall thank Dr. (Mrs) K. K.Sawant, co-ordinator, G.H. Patel, Pharmacy Department, Prof. S. H. Misra, Mrs. S. Rajput and Prof. R. Balaraman for their helpful suggestions and encouragement.

I feel a tremendous paucity of words to explicate my fortuity for unconditional love, sacrifices, invigorating inspiration and earnest care provided by my parents throughout the period of toil, turmoil and tribulations. They always stood by my side in all the phases of acid tests of life. I shall always remain indebted to them for their genuine concern and timely support without which this write up would have been a wild goose chase for me. I take this rare opportunity to express exult in extending appreciation to my in-laws for their encouragement and blessings.

No one walks alone on the journey of life. Right from where I started, one who joined me, walked beside me and helped me along the way continuously, kept me going and urged me to accomplish this project is none other than my husband, Dr. Aman Trehan. I take this opportunity to express profound respect, admiration and a deep sense of gratitude for the affection, strength, motivation and guidance I received from him. I am sincerely thankful to my husband for his love, cooperation, understanding, patience and moral support throughout my research work which has helped in the successful completion of this thesis. Above all, it is impossible to account for the wealth of consideration, kindness, sympathy, care and generosity hidden in the soul of my loving son Ansh Trehan. This work

and write-up is the honest compensation of sacrifices realized by Ansh through the age of six to nine years and innocent assent of my sweet, little and affectionate one year old daughter, Saanvi. In fact, anything I inscribe here will be inadequate to express my feelings for them.

There is no suitable word to convey my regards to my sister-in-law, Mrs. Rekha Abrol for her ever selfless affection and devotion. Special thanks to my dear elder brothers for their forever implicit and heartfelt well wishes and blessings. I owe my special thanks to my sister-in-laws; lovely niece and nephews for their warm care, concern and admiration.

Special thanks are extended to Dr. Veena Gandotra, Mr. Sonu Gandotra and their family for their sincere support, genuine concern and timely help whenever needed. Exceptional regards and words in memories of blessings showered by my uncle late Sh. Ashok Tuli who always wanted me to be doctorate.

I owe a debt of gratitude to Dr. Mukul Jain, Vice president, Zydus Research Centre (ZRC), Ahmedabad for allowing me to avail the facilities of ZRC for research purpose. I wish to convey my sincere thanks to Dr. Shafiq Shaikh for providing a favourable and encouraging environment during formulation development and characterization at NDDS lab of ZRC. I also express my special thanks to Dr. Dutta, of cell culture lab at ZRC for providing all the required materials, equipments and facilities to carry out macrophage uptake studies. I take a special note of thanks to Jinesh Pancholi and Abhishek for their untoward help to carry out different research activities at ZRC. I also thank Jitender Patel, Ameesh and Mukesh, the research scientists, NDDS lab, ZRC for their timely help.

My special thanks to Dr. Mahesh Patel, Director, NDD, Wockhardt Research Centre, Aurangabad, for allowing me to assess the esteemed premises and facilities to carry out in-vivo studies. I acknowledge my gratitude to Dr. Manohar Nandanwar, Head, NDD-Toxicology, for his kind support and sincere thanks to Dr. Atul Kansagara, NDD, Toxicology for his genuine interest, concern and attention for designing the protocols for precise and suitable efforts towards utility consequences. I owe a great deal of appreciation and gratitude to Ms. Anusuya Patel, Analytical Pharmacokinetic Lab, NDD for taking out her precious time even on weekends to guide and assist me with dedication throughout execution and compilation of the animal studies revealed in this project. Without her devoted involvement, this exhaustive work would have not been possible in a systematic manner. It would be remiss if I don't thank Mr. Rajesh Chavan for helping in biochemical studies and another word of gratitude to Amit Patel, Vineet Zope and Harshal Segade for their constant help. I am thankful to Dr. Shrikant Satale for his sincere help and supervision for histopathological studies. Another word of thanks for all laboratory and technical staff for their help and assistance. Special thanks are due to Mr. Ganga Saheb Kale for helping me in animal experiments.

I offer sincere thanks to Mr. Brahmanand Dube for constant help and co-operation during the tenure of research work. I am also grateful to Mr. Vijay Pawar for helping me and joining hands in the final stages of editing. I would like to thank my all colleagues, Manisha Lalan, Bhavik Shah, Gaurang Patel, Jigar Lalani, Deepa Patel, Rakhi, Garima, Himanshu, Gitanjali Kher, Kaustubh, Bisvaroop Nanda, Kailash, Sandeep, Dipesh Baradia, Nirav Khatri, Mohan Rathi and all research Scholars who had help me directly or indirectly, for their fruitful advice and constant co-operation during tenure of research work.

Thanks are also due to Office staff of Faculty of Technology and Engineering and The Maharaja Sayajirao University of Baroda for their support and cooperation.

Sonia (Abrol) Trehan

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ABBREVIATIONS

SDC	Sildenafil citrate
GAGR	Compound annual growth rate
COPD	Chronic obstructive pulmonary disease
MDI's	Meter dose inhalers
DPI's	Dry powder inhaler
PH	Pulmonary hypertension
PAH	Pulmonary arterial hypertension
PAP	Pulmonary arterial pressure
PCP	Pulmonary capillary pressure
PVR	Pulmonary vascular resistance
CDPI	Conventional dry powder for inhalation
DS	Drug sugar composites
DL	Drug-lipid composites
DPL	Dry powder liposomes
LPL	Large porous lipospheres
DPPC	Dipalmitoyl phosphotidylcholine
HSPC	Hydrogenated soya phosphatidylcholine
HDDS	Hydrophobic drug delivery system
PLGA	poly(lactic acid co-glycolic acid)
PLGA-b-PEG	poly(lactic-coglycolic acid) - poly(ethylene glycol)-block-co polymer
HA	Hyaluronic acid
ILD	Interstitial lung disease
WHO	World Health Organization
GARD	Global Alliance Against Chronic Respiratory Diseases
MCT	Monocrotaline
VIP	Vasoactive Intestinal Peptide
CCB's	Calcium channel blockers
ERA	Endothelin receptor antagonist
IPAH	Idiopathic pulmonary arterial hypertension
PDE-5	Phosphodiesterase type 5
PDGF	Platelet-derived growth factor
CTDs	Connective tissue diseases
SCF	Supercritical fluid
SFV	Spray freezing into vapour
SFL	Spray freezing into liquid
SFD	Spray-freeze-drying

Chapter: 1

Introduction

1. Introduction:

Existence of almost all the pharmaceutical industries till date is founded on the conventional delivery forms through oral or parenteral delivery routes. Conventional delivery systems and routes are simple to design and formulate with fast acting responses. But this approach is associated with enormous complications counting reduced potencies due to partial degradation during first pass metabolism, bioavailability problems, toxic side effects and compliance issue due to frequency of administration and painful parenteral route. Thus increased cost and complexity of individual drug molecules' development and formulation has diverted the attention of research organizations towards the strategy of delivering the existing molecules in well-designed delivery systems. Moreover, since most of the patents are reaching expiry in near future, the forte drug delivery techniques are the next target of grosses for pharmaceutical research establishments.

Goal of these sophisticated drug delivery techniques is to sail the drug to the right and exact location for efficacious drug delivery while by-passing the first pass effect and limiting the other toxicities. Other key motive is to maintain the therapeutic drug level at the desired site for longer period of time. These niche products have the potential to contribute towards maximum benefit to the industries and most important the ultimate health assistance to indigent client, the patient. These products impart better patient acceptance and compliance with reduced intake frequency, less plasma fluctuations, reduced side effect and uniform effect at a comparatively lower dose than conventional dosage forms.

Controlled drug delivery systems release the drug by different mechanisms like dissolution controlled, diffusion controlled, both dissolution and diffusion controlled, ion-exchange, degradation or erosion, pH independent, osmotically controlled or altered density for prolonged release etc. Broadly, these may be complexes, association supramolecular structures; reservoir or matrix type formulations prepared using release controlling materials like biodegradable polymers, lipids or dendrimers.

BCC Research report, Jan 2012 **(Fig.1.1)** highlighted the revenues of advanced drug delivery systems: technologies and global markets. It was estimated to reach nearly $137.9 billion in 2011 and $175.6 billion by 2016 at a compound annual growth rate (CAGR) of 5%. The U.S. region accounted for $81.6 billion in 2011 and

in 2016 should be worth $102.6 billion, a CAGR of 4.7%. The European region, worth nearly $37.5 billion in 2011, should be worth $49.1 billion in 2016, a CAGR of 5.6%. The rest of the world accounted for nearly $18.8 billion in 2011 and in 2016 should be worth nearly $23.8 billion, a CAGR of 4.8%. Amongst all these delivery techniques, the global pulmonary drug delivery technologies market (BBC Research 2012), was $19.6 billion in 2010, was expected to be $22.5 billion by 2011 and had been further projected to reach nearly $44 billion by 2016 increasing at a compound annual growth rate (CAGR) of 14.3%.

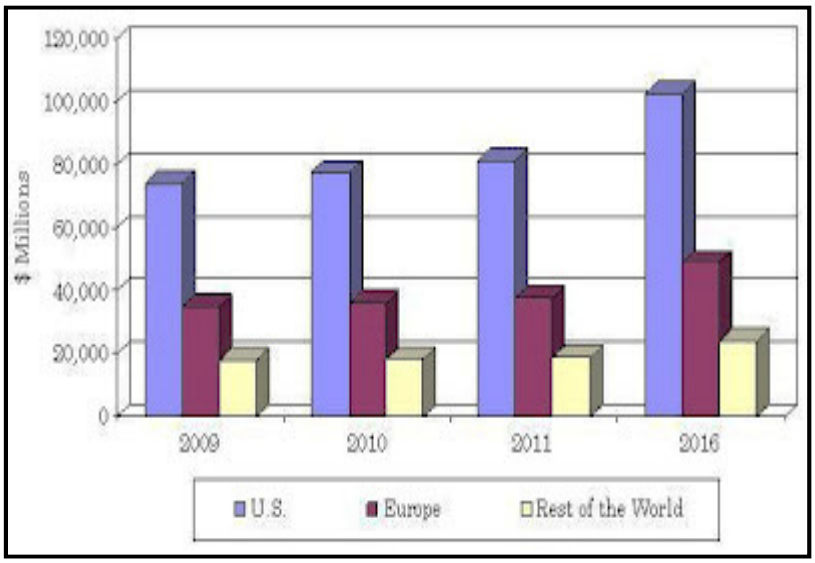


Fig. 1.1: Global revenue of advanced drug delivery systems by region, 2009-2016
(BBC Research, 2012)

Pulmonary drug delivery has attracted interest in past few years **(Fig.1.2)** and has advanced substantially for local treatment for lung diseases due to superior local targeting and reduced systemic side effects with the administration of low drug dosages by pain/needle free technology. Pulmonary tract seems very promising and attractive route for the administration of active substances intended to treat local pulmonary diseases like asthma, chronic obstructive pulmonary disease (COPD), microbial infections and pulmonary arterial hypertension as well as systemic diseases like diabetes. The most recurrent use of inhalation therapy is for the asthma with drugs such as salbutamol sulphate, anticholinergic agents and corticosteroids. Technologies used for pulmonary delivery involve the inhalation using nebulizers,

Meter dose inhalers (MDIs) and dry powder inhaler (DPI) devices (Karhale *et al.* 2012).

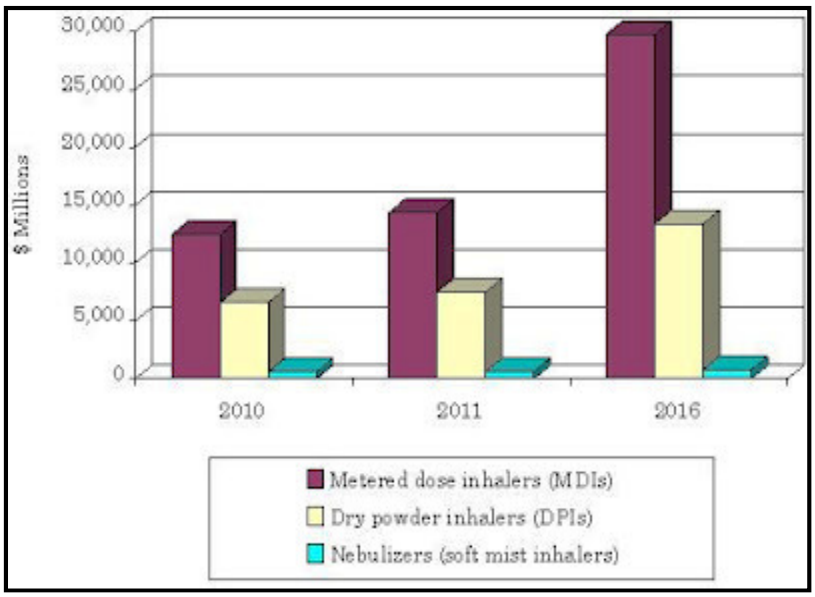


Fig. 1.2: Global revenue of pulmonary drug delivery technologies by region, 2010-2016 (BBC Research, 2012)

Nebulizers are best for emergency care units but require assistance and larger doses to achieve therapeutic effect. MDIs are most commonly used out-patient technique but involve the use of chlorofluorocarbons (CFCs) as propellants, now largely replaced by hydrofluoroalkane 134a. However, a newer technology of DPI owes many advantages over the use of nebuizers and MDIs. DPIs comprise lower doses, no spacer with minimum patient coordination of breathing following actuation, devoid of CFCs and have better chemical stability than liquid dosage in MDIs and nebulizers (Shah N.D. *et.al.* 2012).

Owing to many advantages of this delivery route, remarkable research has progressed in this field which require not only the knowledge of drug delivery technology but also the lung physiology. A lot of challenges are posed to the effective, safe and reproducible pulmonary delivery of DPIs. Mucosal barrier, macrophage uptake of the formulations as a host defense mechanism, oropharyngeal deposition, training in using delivery devices, powder retention in devices, desired formulation characteristics like aerodynamic particle size, moisture content,

maximum and reproducible respirable fraction are the major challenges in pulmonary drug delivery. These challenges can be confronted by the use of biocompatible and non-immunogenic technologies like lipid based delivery systems. Liposomes, lipid-composites and large porous lipospheres using spray drying technology have been shown to have sustained release pulmonary delivery potential and efficacy. Applications of pulmonary delivery (Karhale *et.al.* 2012) have sheltered immense area of health sector for the past few years **(Table 1.1)**. Lot of research is in progress pertaining to sustained delivery of DPIs **(Table 1.2)** and the increasing demand of this field has procreated special pulmonary divisions in pharma zone.

Table 1.1: Therapeutic applications of pulmonary drug delivery

No.	Application	Active pharmaceutical agent/ Device
1	Patients on Ventilators	Baby masks
2	Vaccination in emphysema	Flu vaccines
3	Transplantation	Aerosolized cyclosporine
4	Diagnosis	Methacholine and Histamine responsiveness in asthma
5	Lung injury	Prostaglandin E/ Nebulization
6	Asthma and COPD	Levosalbutamol and Tiotropium bromide, Cortecosteroids
7	Cystic fibrosis	N-Acetylcysteine Recombinant human deoxyribonuclease
8	Diabetes	Insulin/pMDI
9	Angina pectoris	Isosorbide aerosol
10	Bone Disorders	Calcitonin and parathyroid hormone
11	Cancer chemotherapy	5-fluorouracil
12	Antibiotics	Ribavirin, Pentamidine, Amphotericin
13	Smoking cessation	Nicotine aerosol
14	Tuberculosis therapy	Isoniazid and Rifabutin
15	pulmonary embolism	Lower molecular weight heparin
16	Pulmonary arterial hypertension	Iloprost/ Nebulization

Pulmonary arterial hypertension (PAH), a specific subgroup of pulmonary hypertension (PH), is a rare and progressive disease making it extremely important to seek care at specialized centres in pulmonary hypertension. Although pulmonary arterial hypertension was revealed in 1891 there were no recognised treatments for this disorder till 1994 with introduction of Flolan® (epoprostenol sodium). It increased the life expectancy from 2 years to 5 years for pulmonary hypertension patients. Currently there is no predicted life expectancy for pulmonary hypertension patients as treatments continue to expand.

PAH is a progressive disease affecting the arteries of the lungs. The pulmonary arteries are the vessels that carry the blood from the heart to the lungs. It affects all age groups from new-borns to geriatric patients with female predominance. Unlike general hypertension otherwise known as high blood pressure, PAH cannot be easily measured with a blood pressure cuff. There are several tests such as echocardiograms that may point towards a diagnosis of PAH but right heart catheterization is the only way to definitely measure the required hemodynamics necessary to make the diagnosis.

The hemodynamic definition of PAH is a mean pulmonary artery pressure at rest greater than 25mm Hg in the presence of a pulmonary capillary wedge pressure less than or equal to 15mm Hg. A normal mean pulmonary artery pressures for a healthy patient is 12-16mm Hg and a normal wedge pressures is 6-12mm Hg. Basically the pressures in the right side of the heart and the pulmonary arteries are elevated while the pressures in the left side of the heart are normal. Vasoconstriction and cell proliferation lead to thick walls of pulmonary arteries in PAH patients and there are about 146,000 sufferers across the US, EU and Japan.

Commercially available treatments include parenteral dosage forms like like Flolan®, Revatio® and Remodulin®; oral dosage forms like Tracleer®, Revatio®, Thelin®, Letairis® and Adcirca®. The only available pulmonary dosage forms include Ventavis® (iloprost sterile solution) and Tyvaso® that require very frequent dosing of 6-9 times and four times per day respectively. The major challenges associated with oral and parenteral dosage forms include non-specific vasodilation and other toxic effects in whole vasculature.

Thus, there exists an utmost need to have suitable pulmonary dosage form which can circumvent all the above mentioned challenges associated with currently available therapy. Sildenafil citrate, a selective phosphodiesterase-5 inhibitor, has been extensively explored to demonstrate its preventive and chronic potential for the treatment of PAH. This study was planned to overcome the problem of frequent administration and systemic side effects associated with the currently available therapy of PAH by design and development of sustained release sildenafil citrate (SDC) dry powder formulations to treat PAH directly through pulmonary delivery, hence potentially minimizing systemic adverse effects and patient non-compliance.

Different dry powder formulations like conventional dry powder for inhalation (CDPI), drug sugar composites (DS), drug-lipid composites (DL), dry powder liposomes (DPL) and large porous lipospheres (LPL) were technically evaluated and compared with respect to their aerosolization behaviour, morphology, release characteristics and stability. This issue was further addressed by pulmonary delivery of sustained release dry powder formulations of sildenafil citrate to evaluate its prolonged local efficacy in monocrotaline-induced pulmonary hypertensive rats.

Table 1.2: Research on sustained release dry powder formulations for pulmonary drug delivery

S.No.	Drug	Delivery carrier	Components of the carrier	Method of preparation	Type of delivery	Reference
1	Budesonide	Nanoparticles	Poly lactic acid	Pulse laser ablation technique	Local/ Sustained release	Arya *et al*. 2006
		Porous particles	Chitosan	Spray drying	Local/ Sustained release	Naikwade *et al.* 2009
		Liposomes	Lipids	Lyophilization	Local/ Sustained release	Joshi *et al.* 2001
2	Terbutaline sulphate	Microspheres	Phospholipids	Spray drying	Local/ Sustained release	Cook *et al*. 2005
3	Albuterol	Large porous lipospheres	DPPC	Spray drying	Local/ Sustained release	Vanbever *et al*. 1999
4	Tacrolimus	Liposomes	Hydrogenated soya phosphatidylcholine (HSPC)	Spray drying	Local/ Sustained release	Chogule *et al*. 2006
5	AI-128™ (Anti asthmatic DPI) by Acusphere	Porous microparticle technology/ hydrophobic drug delivery system HDDS (TM)	-	-	Local/ Sustained release	http://www.acusphere.com/technology/tech_pdds.html

Table 1.2: Research on sustained release dry powder formulations for pulmonary drug delivery (Continued….)

S.No.	Drug	Delivery carrier	Components of the carrier	Method of preparation	Type of delivery	Reference
6	Salbutamol sulphate	Microparticles	PLGA-b-PEG and poly lactide - poly(ethylene glycol) - poly lactide-	Spray drying	Local/ Sustained release	Cartier *et al.*
		Liposomes	Soya phosphatidylcholine	Lyophilization	Local/ Sustained release	Huang *et al*. 2010
7	Amphotericin B	Liposomes	HSPC	Lyophilization	Local/ Sustained release	Shah *et al*. 2004
8	Amiloride Hydrochloride	Liposomes	Hydrogenated soya phosphatidylcholine	Spray drying	Local/ Sustained release	Chogule *et al*. 2006
9	Fluticasone propionate and Salmeterol xinafoate	Microparticles	HPMC	Spray drying	Local/ Sustained release	Nutan *et al.* 2011
10	Insulin	Microparticles	Hyaluronic acid (HA)	Spray drying	Systemic/ Sustained release	Surendrakumar *et al.*
		Liposomes	Phospholipids	Spray-freeze drying		Bi *et al*. 2008
		Large porous particles	PLGA/cyclodextrin	Double emulsion Technique		Ungaro *et al*. 2009

Table 1.2: Research on sustained release dry powder formulations for pulmonary drug delivery (Continued….)

S.No.	Drug	Delivery carrier	Components of the carrier	Method of preparation	Type of delivery	Reference
11	Ketotifen fumarate	Liposomes	Egg phosphatidylcholine	Lyophilization	Systemic/ Sustained release	Joshi *et al*. 2001
12	Isoniazid and rifabutin	Microparticles	poly(l-lactic acid)	Spray drying	Local/ Sustained release	Muttil *et al*. 2007
13	Dapsone	Liposomes	Dipalmitoyl phosphotidylcholine	Spray drying	Local/ Sustained release	Chogule *et al*. 2008
14	Fluticasone propionate	Liposomes	Phospholipids	Spray drying	Local/ Sustained release	Nirale *et al*. 2009
15	Amikacin	Liposomes	HSPC	Lyophilization	Local/ Sustained release	Shah *et al*. 2004 (1)

Chapter: 2

Literature Review

2. LITERATURE REVIEW

2.1 PULMONARY HYPERTENSION

2.1.1 Definition

Pulmonary hypertension (PH) is characterized by an increase of the arterial pressure and vascular resistance within the pulmonary circulation. Precapillary PH is differentiated from postcapillary PH and is depicted in **Fig. 2.1.** Patients suffering from postcapillary PH, diseases of the left heart (atrial, ventricular, valvular) cause pulmonary venous congestion, resulting in an increase of pulmonary capillary pressure (PCP) and pulmonary arterial pressure (PAP). On the other hand, precapillary PH is characterized by an isolated increase in PAP with normal PCP. Precapillary PH may lead to corpulmonale, right heart failure, and death (Badesch et al. 2004).

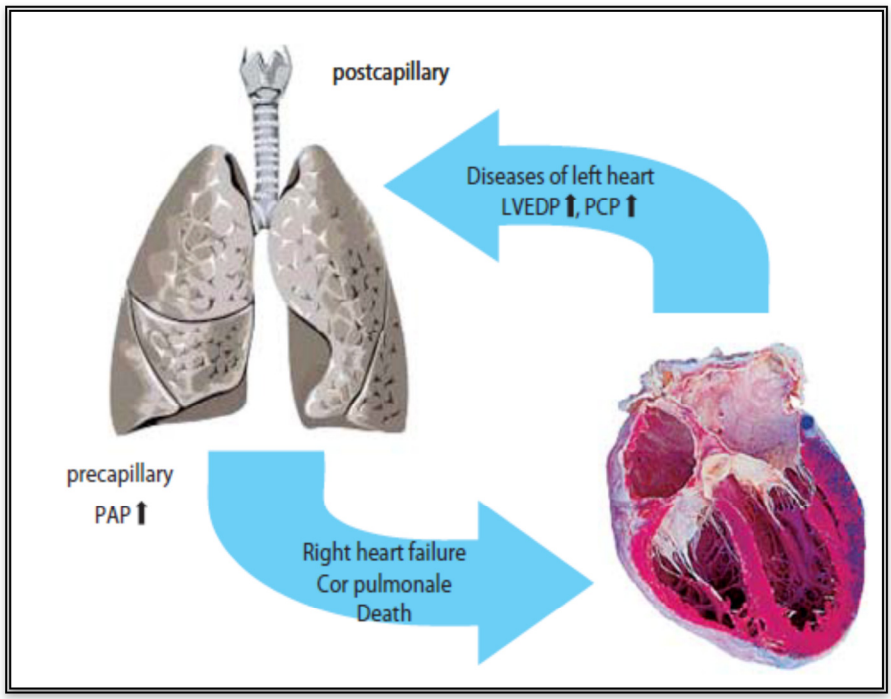


Fig. 2.1: Pathogenesis of pulmonary hypertension (Rosenkranz 2007)

PH is a haemodynamic and pathophysiological state as shown in **Table 2.1** that can be seen in multiple clinical conditions. PH has been defined as an increase in mean pulmonary arterial pressure (PAP) 25 mmHg at rest as measured by right heart catheterization (RHC) shown in **Table 2.1** (Humbert *et al.* 2006; Peacock *et al.* 2007; Hatano *et al.* 1975; D'Alonzo *et al.* 1991). Recent revisit of available data has revealed that the normal mean PAP at rest is 14+3 mmHg, with an upper limit of

normal of 20 mmHg. The importance of a mean PAP between 21 and 24 mmHg is still unclear. Patients having PAP in this range need further evaluation in epidemiological studies. The definition of PH on exercise as a mean PAP is not supported by published data and healthy individuals can reach much higher values (Kovacs *et al.* 2009; Badesch *et al.* 2009; Naeije *et al.* 1993)

Table 2.1: Haemodynamic definitions of pulmonary hypertension[a]

(Nazzareno *et al.* 2009)

Definition	Characteristics	Clinical group(s)[b]
Pulmonary hypertension (PH)	Mean PAP ≥25 mmHg	All
Pre-capillary PH	Mean PAP ≥25 mmHg PWP ≤15 mmHg CO normal or reduced[c]	1. Pulmonary arterial hypertension 3. PH due to lung diseases 4. Chronic thromboembolic PH 5. PH with unclear and/or multifactorial mechanisms
Post-capillary PH	Mean PAP ≥25 mmHg PWP >15 mmHg CO normal or reduced[c]	2. PH due to left heart disease
Passive	TPG ≤12 mmHg	
Reactive (out of proportion)	TPG >12 mmHg	

a: All values are measured at rest.

b: According to Table 2.2

c: High CO can be present in cases of hyperkinetic conditions such as systemic-to-pulmonary shunts (only in the pulmonary circulation), anemia, hyperthyroidism, etc.

CO=cardiac output; PAP= pulmonary arterial pressure; PH=pulmonary hypertension; PWP=Pulmonary wedge pressure; TPG=Transpulmonary pressure gradient (mean PAP-mean PWP)

2.1.2 Classification of Pulmonary Hypertension

The classification of PH has undergone a series of multiple changes since the first classification which was drafted in 1973 (Hatano *et al.* 1975). At Dana Point, Silicon Valley, US, during the Fourth World Symposium on Pulmonary Hypertension, the consensus of an international group of experts maintained the general philosophy and structural organization of the previous Venice classification which was held in 2003 (Simonneau *et al.* 2004). Though the five major categories of PH were retained in the Dana Point classification, a number of important amendments were made to accurately reflect the information published over the past five years as well as to clarify certain areas that were ambiguous in the previous classification. The Venice classification is listed in **Table 2.2** and the new Dana Point classification is listed in **Table 2.3.**

Table 2.2: Venice Classification 2003 (Rosenkranz 2007)

1. **Pulmonary arterial hypertension (PAH)**
   - 1.1. Idiopathic (IPAH)-cause unknown
   - 1.2. Familial (FPAH)-genetic background
   - 1.3. Associated with other diseases (APAH): collagen vascular disease (e.g.scleroderma), congenital shunts between the systemic and pulmonary circulation, portal hypertension, HIV infection, drugs, toxins, or other diseases or disorders
   - 1.4. Associated with significant venous or capillary disease
   - 1.5. Persistent pulmonary hypertension of the newborn (PPHN)
2. **Pulmonary hypertension associated with left heart disease**
   Left sided Atrial or ventricular disease, valvular disease (e.g. mitral stenosis)
3. **Pulmonary hypertension associated with lung diseases and/or hypoxemia**
   Chronic obstructive pulmonary disease (COPD), interstitial lung disease (ILD), Sleep-disordered breathing, alveolar hypoventilation, chronic exposure to high altitude, Developmental lung abnormalities
4. **Pulmonary hypertension due to chronic thrombotic and/or embolic disease**
   Pulmonary embolism in the proximal or distal pulmonary arteries;
   Embolization of other matter, such as tumor cells or parasites

5. **Miscellaneous**

   Sarcoidosis, pulmonary Langerhans cell histiocytosis, lymphangioleiomyomatosis and compression of pulmonary vessels (adenopathy, tumour, and fibrosing mediastinitis etc.)

Table 2.3: Dana Point Clinical Classification 2008 (Simonneau *et al.* 2009)

Group I - Pulmonary arterial hypertension (PAH)

- 5.1. Idiopathic (IPAH)
- 5.2. Heritable Familial (FPAH)
  - 5.2.1. BMPR2
  - 5.2.2. ALK1, endoglin
  - 5.2.3. unknown
- 5.3. Drug and toxin induced
- 5.4. Associated with other diseases (APAH):
  - 5.4.1. collagen vascular disease (e.g. scleroderma),
  - 5.4.2. HIV infection,
  - 5.4.3. portal hypertension
  - 5.4.4. congenital shunts between the systemic and pulmonary circulation,
  - 5.4.5. Schistosomiasis
  - 5.4.6. Chronic hemolytic anemia
- 5.5. Persistent pulmonary hypertension of new born

Group I' Pulmonary veno-occlusive disease and/or pulmonary capillary haemangiomatosis

Group II - Pulmonary hypertension associated with left heart disease

- 5.6.Systolic dysfunction
- 5.7.Diastolic dysfunction
- 5.8.Valvular disease (e.g. mitral stenosis)

Group III - Pulmonary hypertension associated with lung diseases and/or hypoxemia

- 5.9. Chronic obstructive pulmonary disease (COPD),
- 5.10. Interstitial lung disease (ILD)
- 5.11. Other pulmonary disease with mixed restrictive and obstructive pattern

5.12.Sleep-disordered breathing,

5.13. alveolar hypoventilation

5.14.Chronic exposure to high altitude

5.15.Developmental lung abnormalities

Group IV - Pulmonary hypertension due to chronic thrombotic and/or embolic disease

5.16.Pulmonary embolism in the proximal or distal pulmonary arteries

5.17.Embolization of other matter, such as tumor cells or parasites

Group V – PH with unclear and/or multifactorial mechanisms

5.18.Hematological disorders

5.19.Systemic disorders

5.20.Metabolic disorders

5.21.Others: tumoral obstruction, fibrosing mediastinitis, chronic renal failure or dialysis

(*ALK-1=activin receptor-like kinase 1 gene; APAH= associated pulmonary arterial hypertension; BMPR2=bone morphogenetic protein receptor, type 2; HIV=human immunodeficiency virus; PAH=pulmonary arterial hypertension.*)

Various pathological features define the diverse clinical PH groups (Pietra *et al.* 2004; Tuder *et al.* 2009).

Group 1. PAH: Pulmonary veins are normally not affected. Specifically, pathological lesions affect the distal pulmonary arteries (less than 500 mm of diameter). Characteristic features include medial hypertrophy, intimal proliferative and fibrotic changes (concentric, eccentric), adventitial thickening with moderate perivascular inflammatory infiltrates, complex lesions (plexiform, dilated lesions), and thrombotic lesions.

Group 1': Distal pulmonary arteries are usually affected by medial hypertrophy, intimal fibrosis, and uncommon complex lesions. It includes mainly PVOD which involves septal veins and pre-septal venules (constant involvement) with occlusive fibrotic lesions, venous muscularization, frequent capillary proliferation (patchy), pulmonary oedema, occult alveolar haemorrhage, lymphatic dilatation and lymph node enlargement (vascular transformation of the sinus), and inflammatory infiltrates.

Group 2: Left Heart Disease Group: Distal pulmonary arteries can be affected by medial hypertrophy and intimal fibrosis. Pathological changes in PH due to left heart disease group are characterized by enlarged and thickened pulmonary veins, pulmonary capillary dilatation, interstitial oedema, alveolar haemorrhage, and lymphatic vessel and lymph node enlargement.

Group 3: Lung Diseases and/or hypoxia: A variable degree of destruction of the vascular bed in emphysematous or fibrotic areas can also be present. Pathological changes in PH due to lung diseases and/or hypoxia include medial hypertrophy and intimal obstructive proliferation of the distal pulmonary arteries.

Group 4: CTEPH: Collateral vessels from the systemic circulation (from bronchial, costal, diaphragmatic and coronary arteries) can grow to reperfuse at least partially the areas distal to complete obstructions (Galie *et al.* 2006). In CTEPH, pathological lesions are characterized by organized thrombi tightly attached to the pulmonary arterial medial layer in the elastic pulmonary arteries, replacing the normal intima. These may completely occlude the lumen or form different grades of stenosis, webs, and bands (Humbert *et al.* 2004). Interestingly, in the non-occluded areas, a pulmonary arteriopathy indistinguishable from that of PAH (including plexiform lesions) can be there.

Group 5: PH with unclear and/or multifactorial mechanisms: It includes heterogeneous conditions with different pathological features for which the aetiology is ambiguous or multifactorial.

Exertional intolerance (severity grading) is quantitated by the classification of the World Health Organization (WHO) as shown in **Table 2.4.**

Table 2.4: WHO Classification of the functional status with pulmonary hypertension (Barst *et al.* 2004)

Class	Signs and symptoms
I	Patients with pulmonary hypertension **without limitation of physical activity**. Ordinary physical activity does not cause undue dyspnea or fatigue, chest pain or near syncope.
II	Patients with pulmonary hypertension **resulting in slight limitation of physical activity**. They are comfortable at rest. Ordinary physical activity causes undue dyspnea or fatigue, chest pain or near syncope.
III	Patients with pulmonary hypertension **resulting in marked limitation of physical activity**. They are comfortable at rest. Less than ordinary physical activity causes undue dyspnea or fatigue, chest pain or near syncope.
IV	Patients with pulmonary hypertension **with inability to carry out any physical activity without any signs or symptoms**. These patients have indications of right ventricular failure. Dyspnea and fatigue may even be present at rest. Discomfort is increased at any physical activity.

2.1.3 Epidemiology of PAH

PAH is a rare disease that affects 15 to 50 subjects/ million inhabitants in the Western world (Humbert 2007). A pioneer national registry conducted in the United States in the early 1980s describes the natural history of PAH, where 187 patients with "primary" pulmonary hypertension (corresponding to idiopathic and familial PAH in the recent classification) were described and followed for up to five years (Rich *et al.* 1987; D'Alonzo *et al.* 1991). Recently, the French PAH Registry has gathered data on PAH patients in the modern management era (Humbert *et al.* 2006; Humbert *et al.* 2004; Barst *et al.* 2004), and studied survival during a three-year follow-up. In this recent registry established in 2002 to 2003, 674 patients were included with a strict catheter diagnosis of PAH in a network of about 20 university pulmonary vascular centers spread throughout the country (Humbert *et al.* 2006).

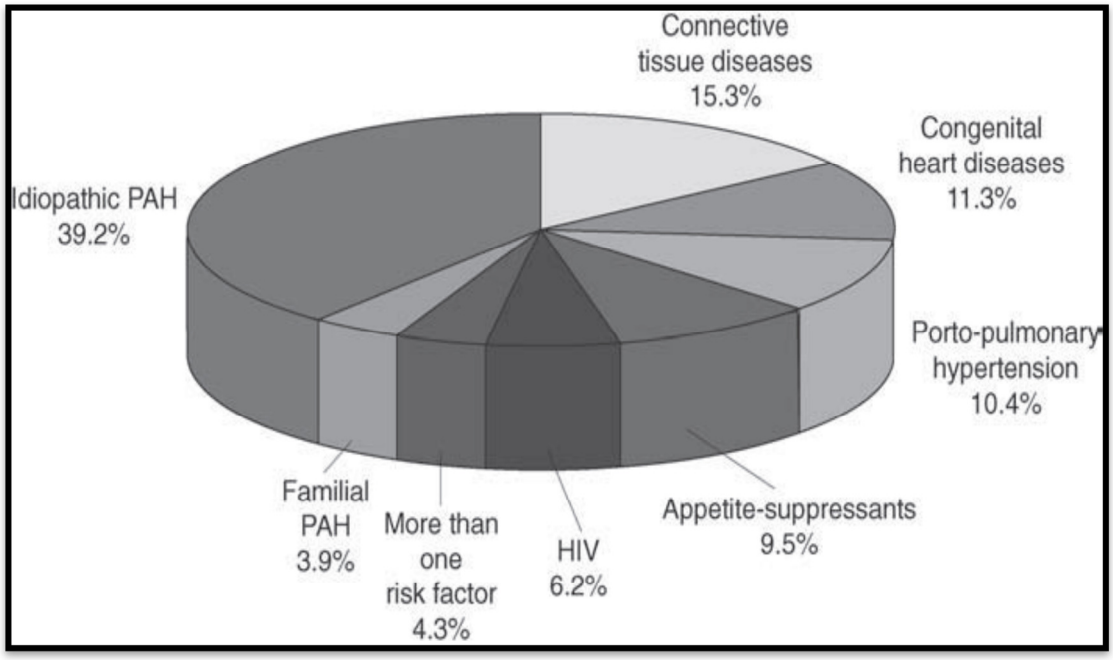


Fig. 2.2: Distribution of patients with PAH in the 2002 to 2003 French Registry (Humbert *et al.* 2006).

About 53% of enrolled patients presented with idiopathic (39.2%), familial (3.9%), or anorexigen-associated PAH (9.5%). The remaining patients had PAH associated with another disease such as CTD, congenital heart diseases, portal hypertension, or HIV infection **(Fig. 2.2)**. Particularly of interest, 29 patients (4.3%) displayed two coexisting conditions known to be associated with PAH **(Fig. 2.2)**, HIV infection and portal hypertension being the most common coexisting conditions.

Idiopathic PAH corresponds to sporadic disease, without any familial history of PAH or any other known trigger (Runo *et al.* 2003; Simmoneau *et al.* 2004; Chin *et al.* 2008). In the French Registry, idiopathic PAH was seen very commonly in women, with a female to male ratio of 1.6:1.

PH is obviously much more prevalent than reported in developing countries where relatively common diseases such as schistosomiasis, sickle cell disease, HIV infection, liver cirrhosis, and others including autoimmune and congenital heart diseases may trigger pulmonary vascular disease (Humbert 2007; Simmoneau *et al.* 2004; Humbert *et al.* 2007; Lapa *et al.* 2009). In addition, hypoxia is a major risk factor for pulmonary hypertension with more than 140 million individuals living above 2500 m worldwide, including 80 millions in asia and 35 millions in south

america (Penaloza *et al.* 2007). Of note, chronic mountain sickness is a public health problem in the andean mountains and other mountainous regions around the world (Humbert *et al.* 2007; Lapa *et al.* 2009; Penaloza *et al.* 2007). In China and Brazil, PH is now being formally studied (Lapa *et al.* 2009, Bousquet *et al.* 2007; Jing *et al.* 2007; Souza *et al.* 2006).

WHO program of the Global Alliance Against Chronic Respiratory Diseases (GARD) currently supports improving awareness, diagnosis, prevention, and treatment of pulmonary hypertension in developing countries (Bousquet *et al.* 2007). In the developing world, highly prevalent diseases such as schistosomiasis in parts of South America, Asia, and Africa, or sickle cell disease in populations of African origin are associated with a marked risk of pulmonary hypertension (Lapa *et al.* 2009). It remains widely believed that PH is a rare condition (Humbert 2007). Although true for PAH (Humbert 2007; Humbert *et al.* 2006; Rich *et al.* 1987; Peacock *et al.* 2007; Thenappan *et al.* 2007), the global burden of pulmonary hypertension as a whole is currently unknown and largely underestimated. In addition, hypoxia is a major worldwide risk factor for pulmonary hypertension (Penaloza *et al.* 2007; Chaouat *et al.* 2008).

Last, up to 4% of all patients with acute pulmonary embolism may develop chronic thromboembolic disease and pulmonary hypertension (Fedullo *et al.* 2001; Pengo *et al.* 2004; Hoeper *et al.* 2006; Tapson *et al.* 2006). Altogether pulmonary hypertension is certainly underestimated both in developing and in developed countries, and further well-designed studies are mandatory to better approach the burden of the disease in populations exposed to different risk factors (Humbert 2007).

2.1.4 Pathogenesis

PH is a heterogeneous disease, in which cardiac and/or pulmonary diseases as well as primary pathological changes of the pulmonary vasculature lead to an increase of pulmonary vascular resistance (PVR) and pulmonary artery pressure (PAP). PAH has a multifactorial pathobiology. Vasoconstriction, vascular remodeling and thrombosis all contribute to increased PVR. Recent genetic and pathophysiological studies have revealed that the combination of genetic factors and associated diseases and/or trigger mechanisms lead to the manifestation of the disease.

The early stages of PAH are characterized by primarily functional alterations of the pulmonary vasculature. At later stages, progression of the disease is associated

with morphological changes specifically of the small pulmonary arteries ("vascular remodeling") that share a relatively homogeneous histomorphological pattern. Entire process of pulmonary vascular remodeling involves all layers of the vessel wall and is complicated by cellular heterogeneity within each compartment (Kaul *et al.* 1984; Humbert *et al.* 2004b). Indeed, each cell type (endothelial cells, smooth muscle cells, fibroblasts), as well as inflammatory cells and platelets, may play an important role in this condition. The pathogenic process being mediated by several mediators including endothelin-1, prostacyclin, and nitric oxide-mediated signals, that all play a central role (McLaughlin *et al.* 2006). These factors represent the therapeutic targets of the currently available medical treatment options as represented in **Fig. 2.3.**

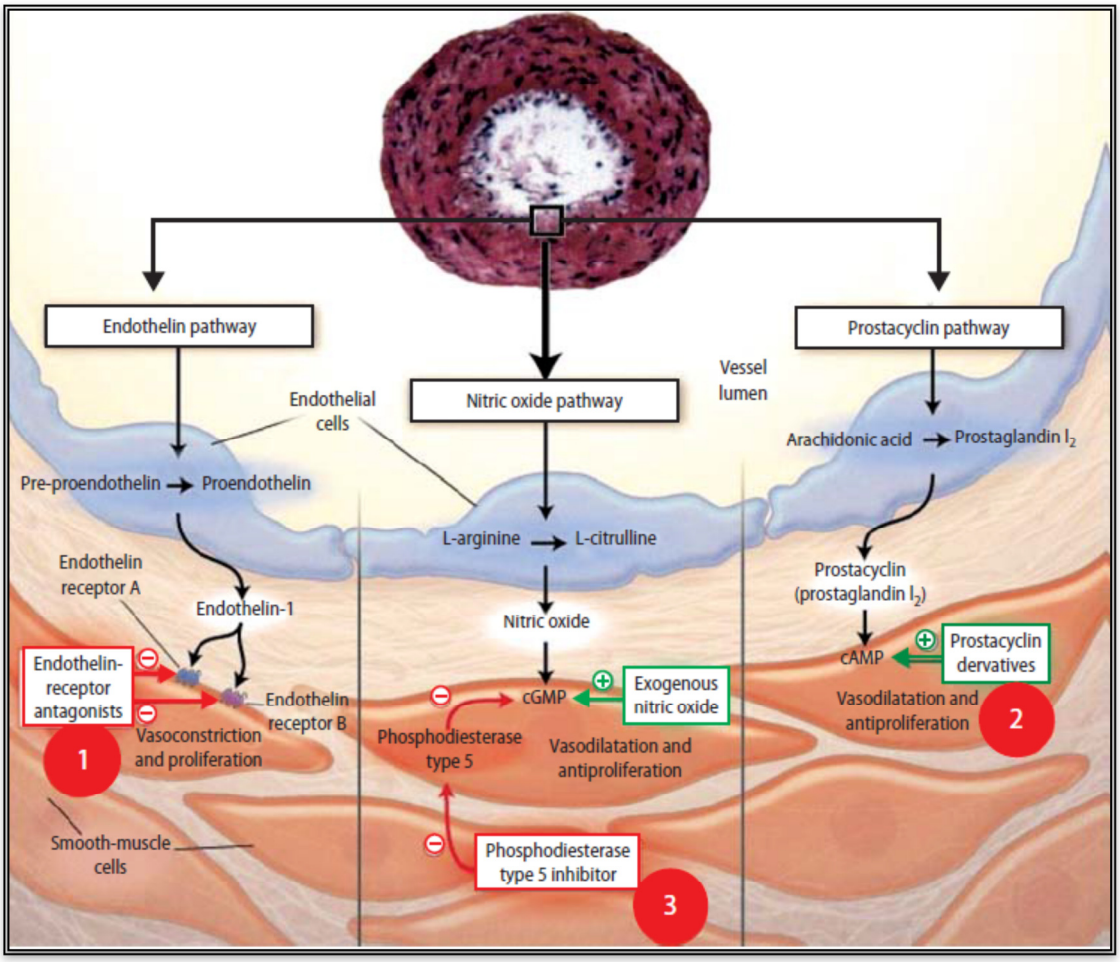


Fig. 2.3: Pathogenesis of pulmonary hypertension (Humbert *et al.* 2004a; 2004b)

2.1.5 Diagnosis

In patients with dyspnea, establishment of the diagnosis of "pulmonary hypertension" is frequently delayed by several months or years (Zahn *et al.* 2006). Hence, the alertness for this disease is required along with improvements in its symptoms. In dyspnea patients the differential diagnosis must include pulmonary hypertension, and a proper further diagnostic work-up should be initiated (McGoon *et al.* 2004). Patients with PH show no specific signs and symptoms. Symptoms indicating PH include exertional dyspnea, decline of physical performance, fatigue, weakness, angina, syncope, peripheral edema, and abdominal distension.

During evaluation of patients, physical examination should focus on further signs and symptoms, because this additional information may be the clue to possible linked diseases that represent the underlying cause of pulmonary hypertension. If the physical examination (e.g., accentuated pulmonary component of S2, pansystolic murmur of tricuspid regurgitation, jugular vein distension, peripheral edema, hepatomegaly, ascites), clinical presentation (dyspnea on exertion/at rest, fatigue, weakness), and unspecific tests such as ECG (e.g., right ventricular hypertrophy and strain) or chest X-ray (e.g., central pulmonary arterial and/or right ventricular enlargement) indicates pulmonary hypertension, both non-invasive and invasive tests may be applied to confirm the diagnosis as shown in **Table 2.5.**

Table 2.5: Diagnostic work-up of patients with pulmonary hypertension

(Rosenkranz 2007)

Clinical presentation
- Dyspnea on exertion, functional status (WHO/NYHA), severity

Non-invasive diagnostic testing
- Echocardiography (transthoracal): right ventricular size and function, tricuspid valve regurgitation, PAPs, Tei Index, TAPSE
- 6-min walk test (6MWT): reliable parameter regarding severity, evaluation of therapeutic efficiency, and prognosis
- Pulmonary function testing: FC, FEV1, FEV1/FC, DLCO, ABGs
- Laboratory tests: BNP/NT-Pro BNP, troponin T
- Cardiopulmonary exercise testing (CPET): peak VO_2, VE/VCO_2
- Ventilation-perfusion scintigraphy: pulmonary embolism?
- High resolution computed tomography: interstitial lung disease?
- Detection or ruling out of relevant underlying diseases: Collagen vascular disease, systemic lupus erythematosus, HIV, congenital heart disease, etc.

Invasive diagnostic testing
- Right heart catheter: PAPs, PAPm, PCP, PVR, heart index, etc.
- Vasoreactivity testing: nitric oxide, iloprost, prostanoids

2.1.6 Animal Models for PAH

The pathogenesis of PAH arteriopathy remains ambiguous despite exhaustive and intensive investigation in a variety of animal model systems. Numerous animal models of PH are currently available and the most commonly used animal models are the chronic hypoxic model and the monocrotaline injury model. These animal models have been used for quite some time and have undoubtedly contributed to a better understanding of the said process. Newer models have been developed which involve modification of classic approaches that exhibit more severe PH and vascular lesions including neointimal proliferation and occlusion of small vessels. In addition, genetically manipulated mice have been generated that have provided insight into the role of specific molecules in the pulmonary hypertensive process. Unfortunately, at present, there is no perfect preclinical model that completely recapitulates human PAH. All models, however, have provided and will continue to provide invaluable insight into the numerous pathways that contribute to the development and maintenance of PAH.

2.1.6.1 Chronic Hypoxia Model

In this model, normo and hypobaric hypoxia are frequently utilized to study PAH. Most commonly utilized hypoxic animal systems are rat (mainly), mice (minimal vascular remodeling) and fawn-hooded (FH) rats. Fawn hooded rats develop more severe PH due to immature lungs with decreased number of alveoli, increased endothelin production in pulmonary artery SMCs, and develops systemic hypertension too, unlike human PAH. Histological changes that occur include rapid muscularization of small, normally non-muscular arteries in the alveolar wall, appearance of cells expressing -smooth muscle actin (-SM actin), increased thickening of the previously muscularized precapillary pulmonary arteries and pulmonary artery-specific vascular inflammatory response (Burke *et al.* 2009).

Pathophysiological changes include doubling of mean pulmonary artery pressure (after 2wk of hypoxia), right ventricular hypertrophy. Chronic hypoxic exposure in the rat leads to increased expression of genes of endothelial cell proliferation and decreased expression of those associated with apoptosis.

It's a useful model as it is predictable and reproducible. Limitations of this model (Bauer *et al.* 2007; Heath 1992; Voelkel *et al.* 2000; White 2004; Zaiman *et al.*

2005) include variability in responses to chronic hypoxia between species; responses are significantly affected by age, no evidence of nonreversible intimal fibrosis or plexogenic lesions, similar to those in human PAH and cause systemic hypertension too. This model is suitable for less severe PH (not PAH), relevant to human PH associated with hypoxia as it occurs in pulmonary parenchymal disease, sleep disordered breathing, severe chronic obstructive pulmonary disease (COPD), and residence at high altitude.

2.1.6.2 Monocrotaline Model

Monocrotaline (MCT) is a toxic pyrrolizidine alkaloid in the plant Crotalaria spectabilis. It is activated *in vivo* to form reactive monocrotaline pyrrole (MCTP) which causes direct endothelial damage leading to vascular injury and resulting in progressive development of PAH in species. It leads to inexorable development and progression of severe and eventually lethal PAH.

MCT model is characterized by acute damage to the peripheral vasculature of the lung and other organs. The preferred species is rats as varied results are obtained in mice even with active MCTP. Histological changes include direct endothelial damage (Stenmark *et al.* 2006; Wilson *et al.* 1989a), dramatic accumulation of mononuclear inflammatory cells in the adventitial sheath of the small intraacinar arteries (Wilson *et al.* 1989b), smooth muscle hypertrophy in media and vascular remodeling. The pathophysiological changes include increased mean pulmonary artery pressure and right ventricular hypertrophy (Miyauchi *et al.* 1993). ECG changes are detected with 3D instruments.

Limitations include response is variable among species strains and even animals, onset is delayed until 1-2 wk after the initiating dose, mortality increased at higher doses of 60mg/kg, “acute toxic model” unlike in humans with severe PAH and costly toxin. It’s a useful model as PAH is achieved in rats by single s/c or i/p injection of MCTP, technically simple for investigators, most widely studied compared to other models and mortality can be decreased at lower doses like 40mg/kg. This model is suitable for acute/ subacute PAH and is relevant to human PH associated with hepatic venoocclusive disease.

2.1.6.3 Monocrotaline and Pneumonectomy

It's a modified MCT model. MCT injection causes induce endothelial injury, pneumonectomy causes high blood flow and both combined together results in intimal remodelling in the distal PAHs. In 1996, Tanaka *et al.* tested the hypothesis that by changing the hemodynamic conditions in the pulmonary arteries, the effects of monocrotaline on the pulmonary vasculature would be increased.

Most commonly used model is rat. Histological changes include neointimal pulmonary vascular occlusive lesions, proliferating endothelial and SM-like cells, medial thickening, inflammation, BMPR signaling is apparently inactivated and development of more severe PAH. It's a useful model as intimal remodeling is closer to human PAH and severe PAH including perivascular proliferative lesion can be induced in younger rats.

Limitations include absence of plexiform lesions, absence of genetic mutation, involvement of veins and other organs, rate of disease progression in weeks compared to years in humans. Still thorough study is required in this case to understand it more deeply.

2.1.6.4 Sugen 5416+Hypoxia Model

This is a model of severe and irreversible PAH. It inhibits VEGF signaling in rats exposed to chronic hypoxia. In 2001, Taraseviciene-Stewart *et al.* developed a model of severe PAH to better address the etiologic mechanisms involved in the endothelial cell hyperproliferation that they believe characterizes the plexogenic arteriopathy of human PAH. Based on the concept that VEGF is an important maintenance and differentiating factor for vascular endothelial cells, these investigators designed experiments to inhibit VEGF signaling in rats exposed to normoxia or chronic hypoxiaVEGF is an important maintenance and differentiating factor for vascular endothelial cells. VEGF inhibitor i.e. SU5416 causes severe and irreversible PAH associated with pre-capillary arterial endothelial proliferation in hypoxic rats. Histological and pathophysiological changes include endothelial cell hyperproliferation, plexogenic arteriopathy similar to human PAH, increased vascular SMC proliferation, medial wall thickening and no perivascular infiltration of monocytes/macrophages. It causes severe and irreversible PAH, persisted and progressed even after removal from the hypoxic stimulus, affects only lung and not other organs. This model is useful for severe and irreversible PAH.

2.1.6.5 BMPR2 Model

Genetic studies have shown that BMPR2 signaling plays a critical role in the pathogenesis of IPAH and familial PAH (FPAH). FPAH accounts for approx 6% of all cases of PAH and shows an autosomal dominant manner of inheritance. Almost 80% of FPAH patients carry germline BMPR2 mutations. Finding of various studies in mice reveals that SM specific downregulation of dominant-negative form of BMPR2 leads to elevated RV systolic pressure, minimal remodeling, and inflammation, no intimal fibrotic occlusion of arteries or plexiform lesions. BMPR2 mutation in the tail domain in SMC leads to development of PH, significant vascular remodeling, pruning, and adventitial perivascular inflammation. BMPR2 gene deleted in pulmonary endothelial cells leads to accumulation of SM actin-positive cells, marked inflammatory infiltrate, concentric fibrosis and marked thrombosis in many of the vessels. West *et al.* 2008 recently reported that mice expressing a BMPR2 mutation in the tail domain in SMC [SM22-rtTAxTet0 (7)-BMPR2 (R899x)] develop PH, significant vascular remodeling, pruning, and adventitial perivascular inflammation.

Thus these genetic models of BMPR2 mutations, although in some ways disappointing in that they do not recapitulate human disease, serve as useful genetic resources to further the knowledge regarding gene mutations in PAH. The incomplete penetrance could be considered a limitation for studying PAH pathogenesis, but it also presents opportunities to further identify environmental and genetic factors that influence PAH pathogenesis in terms of frequency, time of onset, and severity.

2.1.6.6 Overexpression of S100A4/MTS1

The S100A4/Mts1 gene was identified because of its differential expression in highly metastatic mouse mammary adenocarcinoma cells (Ebralidze *et al.* 1989). It is a stimulator of angiogenesis and inflammatory responses (Ambartsumian *et al.* 2001). When studying the tumor biology of this gene, it was noted that a subset (5%) of transgenic mice overexpressing S100A4 in all tissues developed pulmonary arterial changes resembling plexogenic lesions.

In this model, there was seemingly no progressive development of vascular lesions, as the mice either lacked pulmonary lesions altogether or developed plexiform-like arteriopathy. The investigators also reported minimal S100A4 expression in human lungs with no PH or with evidence of early-stage disease but

marked expression of S100A4 in late-stage plexogenic arteriopathy, suggesting that S100A4 is not involved in the initial responses but may be functionally significant in the development of the more severe arterial lesions seen in end-stage disease. The specific roles of S100A4 in lesion development remains unclear, as S100A4 overexpression has also been noted in hypoxic models of PH lesions found in these S100A4-overexpressing mice are composed of SM-like and endothelial-like cells. However, in mice it was noted that S100A4 is expressed in the endothelial cell, whereas in human vessels S100A4 is expressed in the neointimal SMC. Furthermore, in those animals that develop plexogenic arteriopathy, there is a marked periarterial inflammatory response, suggesting again that an inflammatory insult triggers the development of plexogenic arteriopathy in these mice.

When exposed to hypoxia, S100A4-overexpressing mice had greater pulmonary arterial pressure increases and more RV hypertrophy, which, unlike in control mice exposed to hypoxia, was sustained, even 3 month after return to room air. The S100A4 mice did not develop more severe peripheral vascular disease than control mice in response to hypoxia, but those changes that did develop did not regress on return to room air.

2.1.6.7 IL-6 Overexpression in mice

Several reports document that IL-6 is elevated in the serum and lungs of patients with PAH. In 2009, Steiner *et al.* investigated the role of IL-6 in the pathogenesis of pulmonary vascular disease using lung-specific, IL-6-overexpressing transgenic mice under both normoxic and chronic hypoxic conditions. Transgenic mice exhibited elevated RV systolic pressures, RV hypertrophy with corresponding vasculopathic changes, endothelial cells and excessive accumulation of T lymphocytes, activation of the proangiogenic factor VEGF, proproliferative transcription factors c-Myc and Max, upregulation of the antiapoptotic proteins survivin and Bcl-2 and downregulation of the growth inhibitor TGF-B. Interestingly, in the IL-6 overexpressing mice exposed to chronic hypoxia, a proliferative arteriopathy with inflammation was observed in the distal arterial vessels occlusive vascular lesions and dramatic increases in the number of elastic lamellae.

2.1.6.8 Neprilysin and VIP knockout Mice

Neprilysin is a transmembrane metalloendopeptidase that degrades neural peptides important for both growth and contraction of SMC. In 2009, Dempsey *et al.* demonstrated that neprilysin null mice exhibit exaggerated PAH and striking increases in muscularization of distal vessels in response to chronic hypoxia. Vasoactive Intestinal Peptide (VIP) is emerging as a critical regulator of tone and structure in the pulmonary circulation. Recently, it was shown that deletion of the VIP gene leads to significant increases in RV systolic pressures, vascular remodelling and inflammation in mice breathing room air. Genetic manipulation of mice is providing important insights regarding the role of specific genes in the PAH process. Continues work in this area will undoubtedly lead to further insights into mechanisms involved in PAH.

It is clear that there is no currently available perfect preclinical model of human PAH. Arguably, there is probably no animal model that accurately reproduces all the clinical pathological features of any of the groups of human PH. However, it is also clear that animal models have provided, and will continue to provide, valuable insight into the numerous pathways that contribute to the development and maintenance of PH. These models will allow us to investigate important interactions between the various triggers which have been implicated in PH, their impact on signaling pathways, and their temporal evolution into the structural and functional abnormalities, which characterize the pulmonary hypertensive disease process. Use of both classic and newly developed animal models will allow us to continue to rigorously test new hypotheses regarding pathogenesis and also evaluate the ability of newly developed agents to not only prevent, but also, more clinically important, reverse established disease.

Limitations of the model used should always be acknowledged. For instance, studies have clearly demonstrated interspecies differences in the responses to stimuli capable of promoting PH. Furthermore, for each given biological pathway studied to date, the relative importance in a specific animal strain appears influenced by not only the inciting stimulus and/or the disease, but also by age, sex, environment, and species-specific counterregulatory modifications in cells and tissues. Precise comparisons between animal species and the human condition are therefore difficult.

Several additional points are worth considering as work continues in animal models:

- When considering new treatment approaches, prevention studies provide useful information but the more clinically relevant experiment is to determine if treatment reverses neointimal arteriopathy and hypertension once they are established.
- Complete hemodynamic assessment of the animals utilized is essential including assessment of both static and dynamic parameters of the vasculature and the relative roles of vasoconstriction and structure remodeling in determining the magnitude of these parameters.
- Standards for defining PAH in the models should be considered and care must be taken when defining the conditions under which hemodynamics are assessed.
- Assessment of the functional capacity of both diseased and treated animals to determine whether improvement in hemodynamics and cardiac function afforded by the pharmacologic intervention is "clinically" significant.
- Longer term studies on the effects of treatment must be performed with effects on survival and toxicity being included.
- It must always be kept in mind that cellular and molecular pathogenesis of even the obstructive vascular lesions described in new animal models may not duplicate that which occurs over months, and more likely, years in human PAH.
- Careful and rigorous clinical trials will be required to establish safety and efficacy of any new PAH therapy in human patients (Stenmark *et al.* 2009).

2.1.7 Treatment of PAH

Over the last few years, there has been a considerable improvement in treatment options for patients with pulmonary hypertension. In patients with PH secondary to other diseases, the underlying cause of PH should be treated. Specific treatment strategies depend on the type and classification of PH. Symptomatic patients with PAH who are in functional class III or IV should receive specific medical therapy. The definition of "conventional therapy" for pulmonary arterial hypertension (PAH) is sometimes ambiguous for clinicians. Depending on a disease's awareness, availability of therapies, and a clinician's habits, what is "conventional" to one may be considered differently by another. Indeed, it would be better to consider

conventional therapy as the combination of general measures (first of all, not to harm) and supportive therapies (anticoagulants, diuretics, supplemental oxygen, etc.). Historically, treatment with calcium channel blockers (CCBs) was also considered as conventional therapy as it was the only vasodilator that has demonstrated clinical efficacy (in a small subset of patients with idiopathic PAH) before availability of treatments targeting endothelial dysfunction (Channick 2008)

2.1.7.1 General Measures:

An important general measure for patients with PAH is the avoidance of circumstances and substances that may aggravate the disease (**Table 2.6**). Although there is no evidence-based guidance regarding physical activity, patients with PAH requires sensible advice about general activities of daily living and need to adapt to the uncertainty associated with a serious chronic life-threatening disease. PAH patients have a restricted pulmonary circulation, and peripheral vasodilatation or increased cardiac demand can precipitate worsening of pulmonary hypertension (PH) and put patients with PAH at risk of syncope and acute right-heart failure (Rubin 1997; Giane *et al.* 1998; Humbert *et al.* 2004).

2.1.7.1.1 Physical Activity and Lifestyle

Nevertheless, the appropriate level of physical activity is difficult to define as too much rest also has its attendant risks, physical deconditioning, and muscular involution, leading to functional limitation and worsened symptoms. Exercise can aggravate or trigger symptoms of PH including dyspnea, fatigue, chest pain, presyncope, or syncope. Also, physicians traditionally advise patients against physical activity. They are taught to stay active while adapting effort according to their symptoms. In addition to limitation of heavy physical activity, hot baths should be avoided and patients are advised to be cautious on hot days because induced cutaneous vasodilation may significantly decrease right ventricular preload and cardiac output (Naeije *et al.* 2001). A compromise should be found to encourage patients to have a moderate exercise activity while avoiding heavy physical activity that would be potentially dangerous (Humbert *et al.* 2004; Naeije *et al.* 2001).

2.1.7.1.2 Altitude and Travel

An aggravating factor in PAH is hypoxic vasoconstriction. In these circumstances, supplemental oxygen therapy may be indicated for symptomatic purposes as well as to avoid PAH deterioration (Rubin 1997; Gaine *et al.* 1998). In flight oxygen administration should be considered for patients in functional classes III and IV and those with an oxygen saturation <92%. A flow rate of 2 L/min will raise inspired PO2 to values found at sea level. Nevertheless, the usefulness of this supplemental oxygen therapy is debatable. Finally, patients should be guided how to contact local PH clinics in close proximity to where they are traveling and should be guided to travel with written information about their disease.

2.1.7.1.3 Pregnancy, birth control, and post-menopausal hormonal therapy

The hemodynamic and hormonal changes occurring during pregnancy and peripartum period can lead to severe, and sometimes fatal, right-heart failure. Consequently, a safe and effective method of contraception is always recommended in PAH women of childbearing age (Bonnin *et al.* 2005; Sitbon *et al.* 2002). Pregnancy is associated with 30% to 50% mortality in patients with PAH. As a consequence, PAH is a formal contraindication of pregnancy. There is less consensus relating to the most appropriate methods of birth control. Barrier contraceptive methods are safe for the patient but with an unpredictable effect. Progesterone preparations such as medroxyprogesterone acetate and etonogestrel are effective approaches to contraception and avoid potential issues of oestrogens as those included in the old generation mini-pill (Thorne *et al.* 2006). It should be remembered that the endothelin receptor antagonist (ERA) bosentan may reduce the efficacy of oral contraceptive agents.

The Mirena coil is also effective but rarely leads to a vasovagal reaction when inserted, which may be poorly tolerated in severe PAH. A combination of two methods may also be utilized. The patient who becomes pregnant should be informed of the high risk of pregnancy, and termination of pregnancy discussed. Those patients who choose to continue pregnancy should be treated with disease-targeted therapies, planned elective delivery, and effective close collaboration between obstetricians and the PAH team.105,106 It is not clear if the use of hormonal therapy in post-menopausal women with PAH is advisable or not. It may be considered in cases of intolerable menopausal symptoms in conjunction with oral anticoagulation.

2.1.7.1.4 Infection Prevention

Patients with PAH are susceptible to developing pulmonary infections, which may aggravate PH and be the cause of death in about 7% of cases. While there are no controlled trials, it is recommended to consider routine immunizations against influenza and pneumococcal infection in patients with PAH.

2.1.7.1.5 Elective surgery

In patients with PAH, elective surgery is expected to have an increased risk. It is not clear as to which form of anaesthesia is preferable but epidural is probably better tolerated than general anaesthesia. Patients usually maintained on oral therapy may require temporary conversion to i.v. or nebulized treatment until they are able both to swallow and to absorb drugs taken orally.

2.1.7.1.6 Psychosocial Support

Feeling of isolation is common with Patients with PAH leading to development of anxiety and depression resulting in impairment of quality of life. Timely referral to a psychiatrist or psychologist should be made when appropriate.

Table 2.6: Recommendations for general measures (Rosenkranz 2007)

Statement	Class[a]
It is recommended to avoid pregnancy in patients with PAH	I
Immunization of PAH patients against influenza and pneumococcal infection is recommended	I
Physically deconditioned PAH patients should be considered for supervised exercise rehabilitation	IIa
Psychosocial support should be considered in patients with PAH	IIa
In-flight O_2 administration should be considered for patients in WHO-FC III and IV and those with arterial blood O_2 pressure consistently less than 8 kPa (60 mmHg)	IIa
Epidural anaesthesia instead of general anaesthesia should be utilised, if possible, for elective surgery	IIa
Excessive physical activity that leads to distressing symptoms is not recommended in patients with PAH	III

Information on the severity of the disease is available from many nonprofessional sources, and an important role of the PAH multidisciplinary team is

to support patients with accurate and up-to-date information. Patient support groups may also play an important role in this area. Encouraging patients and their family members to join patient support groups can have positive effects on coping, confidence, and outlook.

2.1.7.2 Supportive Therapy

2.1.7.2.1 Anticoagulation

Anticoagulant therapy is used in patients with PAH. Rationale of this is based on the observation, reported in large pathological series, of pulmonary arteriopathy with thrombotic lesions defined by the presence of eccentric intimal fibrosis and recanalized thrombi (Bjornsson *et al.* 1985; Palevsky *et al.* 1989). Endothelial dysfunction that predisposes patients to pulmonary arteriopathy also promotes intravascular thrombosis (Christman *et al.* 1992). Abnormalities in coagulation and fibrinolytic pathways have also been reported (Huber *et al.* 1994; Hoeper *et al.* 1998; Herve *et al.* 2001). In addition, the presence of an indwelling central venous catheter in patients treated with continuous intravenous prostacyclin infusion may also be considered as a risk factor for venous thromboembolism **(Table 2.7)**. Moreover, the presence of right-heart failure and immobility are risk factors for acute venous thromboembolism, and in patients with PAH who die suddenly, fresh intrapulmonary clots may be found at autopsy (Fuster *et al.* 1984).

Oral warfarin is the most widely used anticoagulant therapy in patients with PAH. Advice regarding the target international normalized ratio (INR) in patients with idiopathic PAH varies from 1.5 to 2.5 in most centers of North America and 2.0 to 3.0 in European centers. Generally, patients with PAH receiving therapy with long-term intravenous prostaglandins are anticoagulated in the absence of contraindications due, in part, to the additional risk of catheter-associated thrombosis. In some cases, curative doses of low molecular weight heparin or unfractionated heparin can be used. However, the use of these anticoagulant drugs has not been studied.

2.1.7.2.2 Oxygen

There is no randomized data to suggest that long-term O2 therapy is beneficial to reduce the PVR in patients with PAH. Most patients with PAH except those with CHD and pulmonary-to-systemic shunts have minor degrees of arterial hypoxaemia at rest unless they have a patent foramen ovale. There are data showing that nocturnal

O2 therapy does not modify the natural history of advanced Eisenmenger's syndrome (Sandoval *et al.* 2001). Guidance may be based on evidence in patients with COPD; when arterial blood O2 pressure is consistently less than 8 kPa (60 mmHg) patients are advised to take O2 to achieve a arterial blood O2 pressure of 8 kPa for at least 15 h/day (Weitzenblum *et al.* 1985). Ambulatory O2 may be considered when there is evidence of symptomatic benefit and correctable desaturation on exercise.

2.1.7.2.3 Diuretics

Right ventricular overload is an integral part of PAH clinical presentation which has been determined as a negative prognostic factor (D' Alonzo *et al.* 1991; Sitbon *et al.* 2002). Although there are no randomized controlled trials of diuretics in PAH, clinical experience shows clear symptomatic benefit in fluidoverloaded patients treated with this therapy. Diuretics and sodium-restricted diet (<2400 mg/day) relieve hypervolemia and associated symptoms such as hepatic congestion and peripheral edema. Whether this strategy improves prognosis is unknown. Furthermore, careful dosage adjustment is needed, on the basis of clinical, echocardiographic, and hemodynamic findings, because hypovolemia can lead to a reduction in the right ventricular preload and decrease in cardiac output.

It is also important to monitor renal function and blood biochemistry in patients to avoid hypokalemia and the effects of decreased intravascular volume leading to prerenal failure. Furosemide and/or spironolactone may be prescribed and increased as needed. Large doses of furosemide, up to 500 mg/day, may be sometimes necessary (Sitbon *et al.* 2002).

2.1.7.2.4 Cardiac Glycosides

Its long-term effectiveness is unknown, though digoxin has been shown to improve cardiac output acutely in patients with idiopathic PAH (Rich *et al.* 1998). Furthermore, digitalis toxicity may be enhanced if hypoxemia and diuretic-induced hypokalemia are also present. Therefore, digoxin is now reserved for patients with PAH who have atrial fibrillation secondary to atrial dilatation.

Table 2.7: Recommendations for supportive therapy (Nazzareno Gaile *et al.* 2009)

Statement	Class[a]
Diuretic treatment is indicated in PAH patients with signs of RV failure and fluid retention	I
Continuous long-term O_2 therapy is indicated in PAH patients when arterial blood O_2 pressure is consistently less than 8 kPa (60 mmHg)[c]	I
Oral anticoagulant treatment should be considered in patients with IPAH, heritable PAH, and PAH due to use of anorexigens	IIa
Oral anticoagulant treatment may be considered in patients with APAH	IIb
Digoxin may be considered in patients with PAH who develop atrial tachyarrhythmias to slow ventricular rate	IIb

2.1.7.3 Drug Therapy

2.1.7.3.1 Calcium channel blockers

Calcium channel blockers (CCBs) have been the only option for medical treatment of pulmonary hypertension for long. The CCBs that have been predominantly used in reported studies are nifedipine, diltiazem, and amlodipine, with particular emphasis on the first two (Rich *et al.* 1992; Sitbon *et al.* 2005). The choice of CCB is based upon the patient's heart rate at baseline, with a relative bradycardia favouring nifedipine and amlodipine and a relative tachycardia favouring diltiazem. Favorable clinical and prognostic effects have been shown in patients with IPAH in non-controlled, non-randomized trials. High dose CCBs are recommended in patients with IPAH and FPAH who demonstrate a positive vasoreactivity test (reduction of PAPm by >10 mmHg to reach a PAPm <40 mmHg) (C, I).

However, only 10–15% of IPAH patients meet these criteria, and only approximately one-half of them benefit from chronic treatment with high dose CCBs as defined by long-term hemodynamic and functional improvement (Forfia *et al.* 2006). High dose CCBs are not recommended in patients with associated forms of PAH or other forms of PH and in patients who did not meet the vasoreactivity response criteria or without prior vasoreactivity testing (C, III). It is advisable to start with a low dose, e.g. 30 mg of slow release nifedipine twice a day, 60 mg of diltiazem

three times a day (t.i.d.), or 2.5 mg of amlodipine once a day and increase cautiously and progressively to the maximum tolerated dose. Hence, CCBs should only be given to the few patients with IPAH and FPAH with a positive vasoreactivity test, and the total long-term response rate in IPAH patients is only 7–10%. The daily doses of these drugs that have shown efficacy in IPAH are relatively high, 120–240 mg for nifedipine, 240–720 mg for diltiazem, and up to 20 mg for amlodipine.

2.1.7.3.2 Prostacyclins

cAMP (adenylate cyclase) is the mediator through which prostanoids cause vasodilation via liberation of cAMP. Furthermore, they exert antiproliferative effects on fibroblasts and vascular smooth muscle cells. Some analogues of prostacyclin (PGI2) which is very unstable have been developed and are used for medical treatment of PAH. Attention and interest in the use of prostacyclins as a treatment for PAH started in the early 1980s, even before the recognition of the relative imbalance between vasodilating and vasoconstricting prostacyclin metabolites in this population (Christman *et al.* 1992; Tuder *et al.* 1999). Several smaller observations during the 1980s paved the way for the landmark trials that established the role of prostanoids for PAH. These include epoprostenol, iloprost, treprostinil, and beraprost.

a. ***Epoprostenol***

Hemodynamic effects of escalating doses of epoprostenol were studies in 1982 by Rubin *et al.* Rubin *et al.* studied effects of increasing doses of intravenous (IV) epoprostenol (2–12 ng/kg/min) in seven patients with idiopathic pulmonary arterial hypertension (IPAH) and severe hemodynamic compromise. Epoprostenol (synthetic prostacyclin) is available as a stable freeze-dried preparation that needs to be dissolved in alkaline buffer for i.v. infusion. Epoprostenol has a short half-life (3–5 min) and is stable at room temperature for only 8 h. This explains why it needs to be administered continuously by means of an infusion pump and a permanent tunnelled catheter.

The efficacy of continuous i.v. administration of epoprostenol has been tested in three unblinded RCTs in patients with IPAH (Rubin *et al.* 1990; Barst *et al.* 1996) and in those with PAH associated with the scleroderma spectrum of diseases (Badesch *et al.* 2000). Epoprostenol improves symptoms, exercise capacity, and haemodynamics in both clinical conditions, and is the only treatment shown to improve survival in IPAH in a randomized study. Long-term persistence of efficacy

has also been shown (Sitbon *et al.* 2002; Paciocco *et al.* 2001) in IPAH, as well as in other APAH conditions (Rosenweig *et al.* 1999; Krowa *et al.* 1999; Nunes *et al.* 2003) and in non-operable CTEPH.

b. Iloprost

Currently delivered via inhalation route, Iloprost is a stable synthetic prostacyclin analogue with a half-life of 20 to 30 minutes. This inhaled formulation was developed as an alternative to epoprostenol with hopes of greater pulmonary vascular selectivity, less systemic vasodilatory effects, and a less invasive and potentially safer delivery mechanism. The pivotal randomized, placebo-controlled trial with iloprost was a 12-week study that assessed the combined clinical endpoint of (*i*) improvement by at least one functional class, (*ii*) a >10% improvement in 6MWD, and (*iii*) no death or deterioration (Olschewski *et al.* 2002). The study enrolled 203 patients with PAH or CTEPH, all of whom were functional class III or IV. The combined clinical endpoint was met by 16.8% of the patients randomized to iloprost and 4.9% in those randomized to placebo (p ¼ 0.007). Cough, headache, and syncope were more common in the iloprost group.

c. Treprostinil

In the first study, the acute hemodynamic effects of IV treprostinil were demonstrated to be similar to IV epoprostenol. Importantly, the acute hemodynamic effects of subcutaneously delivered treprostinil were also similar to IV epoprostenol. Developed with the hopes of obviating the need for IV infusion, the stable prostacyclin analogue treprostinil was initially tested in a series of three pilot studies in patients with IPAH (McLaughlin *et al.* 2003). Lastly, a randomized trial in 26 patients with IPAH demonstrated that continuous subcutaneous (SC) treprostinil infusion led to significant increases in MWD (þ37 m) versus placebo group (6 m) over an eight-week follow-up period. These findings led to a large scale, placebo-controlled randomized international trial of SC treprostinil infusion in 470 patients with PAH with class II to IV symptoms over a 12-week period (Simonneau *et al.* 2002).

Taken together, these findings suggested that relative underdosing of treprostinil and inclusion of a diverse, and overall less impaired PAH cohort may have

led to the relatively modest improvements in MWD. In clinical practice, target treprostinil doses are routinely two to four times higher than epoprostenol doses.

d. Beraprost

Beraprost is the first chemically stable and orally active prostacyclin analogue. The RCT ALPHABET (Galie *et al.* 2002) in Europe and a second in the USA (Barst *et al.* 2003) with this compound have shown an improvement in exercise capacity that unfortunately persists only up to 3-6 months. There were no haemodynamic benefits. The most frequent adverse events were headache, flushing, jaw pain, and diarrhoea.

In a prospective, double-blind placebo-controlled randomized study of beraprost, Galie and colleagues studied 130 patients with PAH for 12 weeks (Galie *et al.* 2002). The primary endpoint of 6MWD improved by 25 m (p ¼ 0.036). However, a year long, double-blind placebo-controlled trial of 116 PAH patients led by Barst *et al.* did not demonstrate sustained improvements with beraprost (Barst *et al.* 2003). Benefits noted in 6MWD and disease progression at 3 and 6 months were not sustained at 9 and 12 months.

In the most critically ill patients, IV epoprostenol is the drug of choice, as the most rapid acting, reliable, and potent prostanoid for which there is a wealth of evidence. There is excellent rationale for the use of prostacyclin analogues in PAH based on the basic mechanisms of the disease, and indeed, clinical trials have demonstrated the benefit of this class of therapy in PAH patients. Parenteral (IV and SC) prostanoids are complicated therapies and are most appropriately managed by practices with considerable experience in their use. Investigational trials of additional prostacyclin analogues and prostacyclin receptor agonists are under way.

2.1.7.3.3 PDE-5 Inhibitors

Impaired release of nitric oxide is associated with pulmonary arterial hypertension (PAH) (1) due, at least in part, to reduced expression of nitric oxide synthase in the vascular endothelium of pulmonary arteries (Giaid *et al.* 1995). Downstream activation of soluble guanylate cyclase is thus reduced in patients with PAH with less cellular synthesis of cyclic guanosine monophosphate (cGMP), the second messenger of nitric oxide. Phosphodiesterase type 5 (PDE-5) inactivates cGMP in the pulmonary vasculature, and it appears to be upregulated in pulmonary hypertension (Wharton *et al.* 2005; Corbin *et al.* 2005). Inhibition of PDE-5 increases

cGMP levels, which may mediate the antiproliferative (Tantini *et al.* 2005) and vasodilating (Michelakis *et al.* 2002) effects of endogenous nitric oxide. These data represent the rationale for the development of PDE-5 inhibitors to treat PAH. The upregulation of PDE-5 in the lung vasculature of patients with PAH may exert (theoretically) a preferential effect of the PDE-5 inhibitors on these vessels. All three PDE-5 inhibitors approved for the treatment of erectile dysfunction, sildenafil, tadalafil, and vardenafil, cause significant pulmonary vasodilation with maximum effects observed after 60, 75 to 90, and 40 to 45 minutes, respectively (Ghofrani *et al.* 2004).

a. Sildenafil

Sildenafil is an orally active, potent, and selective inhibitor of PDE-5. In a study on human pulmonary artery smooth muscle cells treated with the platelet-derived growth factor (PDGF), sildenafil exerted an antiproliferative effect (Tantini *et al.* 2005). Animal studies in the classical model of monocrotalin-induced pulmonary hypertension in rats have shown favorable effects of sildenafil on pulmonary arterial pressure, right ventricular hypertrophy, and survival (Schermuly *et al.* 2004; Schafer *et al.* 2009). In particular sildenafil prevented myocardial remodeling in pulmonary hypertension through an indirect action via right ventricular unloading.

Five randomized controlled studies of sildenafil in the treatment of PAH patients cited improvement in exercise capacity, hemodynamic parameters, and clinical status (Sastry *et al.* 2004; Galie *et al.* 2005; Singh *et al.* 2006; Wilkins *et al.* 2005). A number of uncontrolled studies have reported favorable effects of sildenafil in patients with idiopathic PAH, PAH associated with connective tissue diseases (CTDs), congenital heart diseases, and chronic thromboembolic pulmonary hypertension (CTEPH).

The approved dose of sildenafil is 20 mg TID, but the durability of effect up to one year has been demonstrated only with the dose of 80 mg TID (Galie *et al.* 2005). In clinical practice, uptitration beyond 20 mg TID (mainly 40–80 mg TID) may be needed. The Sildenafil Use in Pulmonary arterial hypertension (SUPER) trial of 278 PAH patients treated with sildenafil 20, 40, or 80 mg three times daily, compared with placebo, confirmed favorable results on exercise capacity, symptoms, and hemodynamics (Galie *et al.* 2005). The durability of the effect over time was shown only for the dose of 80 mg TID .

b. Tadalafil

Tadalafil, an orally administered, once-daily dosing, selective inhibitor of PDE-5, is currently approved for the treatment of erectile dysfunction. Preliminary data on tadalafil for the treatment of PAH are limited to a single-dose hemodynamic evaluation (Ghofrani *et al.* 2004), anecdotal clinical use (Palmieri *et al.* 2004), and a trial on Eisenmenger syndrome patients (Mukhopadhyay *et al.* 2006). An RCT (PHIRST) on 406 PAH patients treated with tadalafil 5, 10, 20, or 40 mg once daily has shown favourable results on exercise capacity, symptoms, haemodynamics, and time to clinical worsening at the largest dose (Galie *et al.* 2009)

c. Vardenafil

Vardenafil is an orally administered, once-daily dosed, selective inhibitor of PDE-5, which is currently approved for the treatment of erectile dysfunction. Preliminary data on the efficacy of vardenafil to treat PAH are limited to a single-dose hemodynamic evaluation and a small study on five patients with different types of pulmonary hypertension (Aizawa *et al.* 2006). In this study, the maintenance dose of vardenafil was 10 to 15 mg daily.

2.1.7.3.4 Endothelin Receptor Antagonists

A vasoconstrictor produced by vascular endothelial cells is ET-1 (Humbert *et al.* 2004; Yanagisawa *et al.* 1998). It is a 21-amino acid peptide that was first characterized in 1988. Big ET, the 38–amino acid precursor, is activated when hydrolyzed by ET-converting enzymes present within the lungs (Takahashi *et al.* 1993; Inoue *et al.* 1989). Once activated, ET acts in an autocrine/paracrine role (Eguchi *et al.* 1995; Galie *et al.* 2004). ET-1 plasma concentrations correlate with indices of disease severity as assessed by exercise capacity and hemodynamic parameters (Montani *et al.* 2007). Therefore, there has been interest in modulating the actions of ET-1 for treatment of PAH via receptor antagonism. Not only is ET-1 one of the most potent vasoconstrictors, it also has proliferative effects and is involved in inflammation and fibrosis (Davie *et al.* 2002; Shi-Wen *et al.* 2004; Prefontaine *et al.* 2008).

A variety of factors can stimulate the biosynthesis of ET, including hypoxia, ischemia, angiotensin II, vasopressin, catecholamines, cytokines, growth factors, and thrombin. ET-1 has been implicated in the pathophysiology of PAH. Patients with

PAH have been shown to have increased synthesis and expression of this important molecule particularly within the pulmonary microvasculature (Giaid *et al.* 1993).
Presently, there are several ET receptor antagonists available for treatment of PAH. They are differentiated by their affinities for ETA and ETB blockade. Bosentan was the first available ET receptor antagonist and is a dual or nonselective blocker, with an ETA/ETB affinity ratio of approximately 40:1 (Clozel *et al.* 2003). Sitaxsentan is considered an ETA selective antagonist with ETA/ETB affinity ratio of approximately 6000:1 (Wu *et al.* 1997). Ambrisentan is another ETA selective antagonist with ETA/ETB affinity ratio of 77:1 (Vatter *et al.* 2002).

a. Bosentan

The first molecule of its class that was synthesized is Bosentan which is an oral active dual endothelin-A and endothelin-B receptor antagonist. Bosentan has been evaluated in PAH (idiopathic, associated with CTD, and Eisenmenger's syndrome) in five RCTs (Pilot, BREATHE-1, BREATHE-2, BREATHE-5, and EARLY) that have shown improvement in exercise capacity, functional class, haemodynamics, echocardiographic and Doppler variables, and time to clinical worsening (Channick *et al.* 2001; Rubin *et al.* 2002; Humbert *et al.* 2004; Galie *et al.* 2008; Galie *et al.* 2006). This has resulted in regulatory authority approval for the use of bosentan in the treatment of PAH patients in WHO-FC II and also in patients with PAH associated with congenital systemic-to-pulmonary shunts and Eisenmenger's syndrome. Bosentan treatment is started at the dose of 62.5 mg twice daily and uptitrated to 125 mg twice daily after 4 weeks. In paediatric patients doses are reduced according to the body weight.

b. Sitaxsentan

An open-label pilot study showed beneficial effects of sitaxsentan on 6MWD exercise capacity and hemodynamics (Barst *et al.* 2002). Sitaxsentan is a selective ETA receptor antagonist. However, an important safety signal of acute hepatitis was identified, with one fatality, which was thought to represent nonlinear pharmacokinetics of sitaxsentan at higher doses.

The most frequently reported laboratory adverse event was increased international normalized ratio (INR) or prothrombin time (PT), related to sitaxsentan's inhibitory effect on the CYP2C9 P450 enzyme, the principal hepatic enzyme involved in the metabolism of warfarin. The most frequently reported clinical

adverse events with sitaxsentan treatment, experienced more frequently by the treatment group than by the placebo group, were headache, peripheral edema, nausea, nasal congestion, and dizziness.

c. Ambrisentan

Ambrisentan has been evaluated in a pilot study (Galie *et al.* 2005) and in two large RCTs (ARIES 1 and 2), which have demonstrated efficacy on symptoms, exercise capacity, haemodynamics, and time to clinical worsening of patients with IPAH and PAH associated with CTD and HIV infection (Galie *et al.* 2008). Ambrisentan is a non-sulfonamide, propanoic acid class, ERA that is selective for the endothelin-A receptor. The open-label continuation study has demonstrated the durability of the effects of ambrisentan for at least 1 year. Ambrisentan has been approved for the treatment of WHO-FC II and III patients. The current approved dose is 5 mg once daily which can be increased to 10 mg once daily when the drug is tolerated at the initial dose.

2.1.7.3.5 Combination Therapy

The rationale for combination therapy is the fact that all drug classes target distinct pathogenic mechanisms (Badesch *et al.* 2007). Presently, an increasing number of patients with PAH is treated with combinations of the above drug classes. When potential interactions between the various compounds are considered, combination therapy appears to be safe, and additive effects have been demonstrated for all possible combinations in small studies.

However, further research is needed to convincingly demonstrate the superiority of certain combinations compared to mono therapy. Recently, the results of a randomized trial of adding inhaled iloprost to existing bosentan (STEP) have been published. This study demonstrated that additional iloprost significantly improved exercise capacity, functional class, time to clinical worsening, and hemodynamic parameters, as compared to bosentan alone.

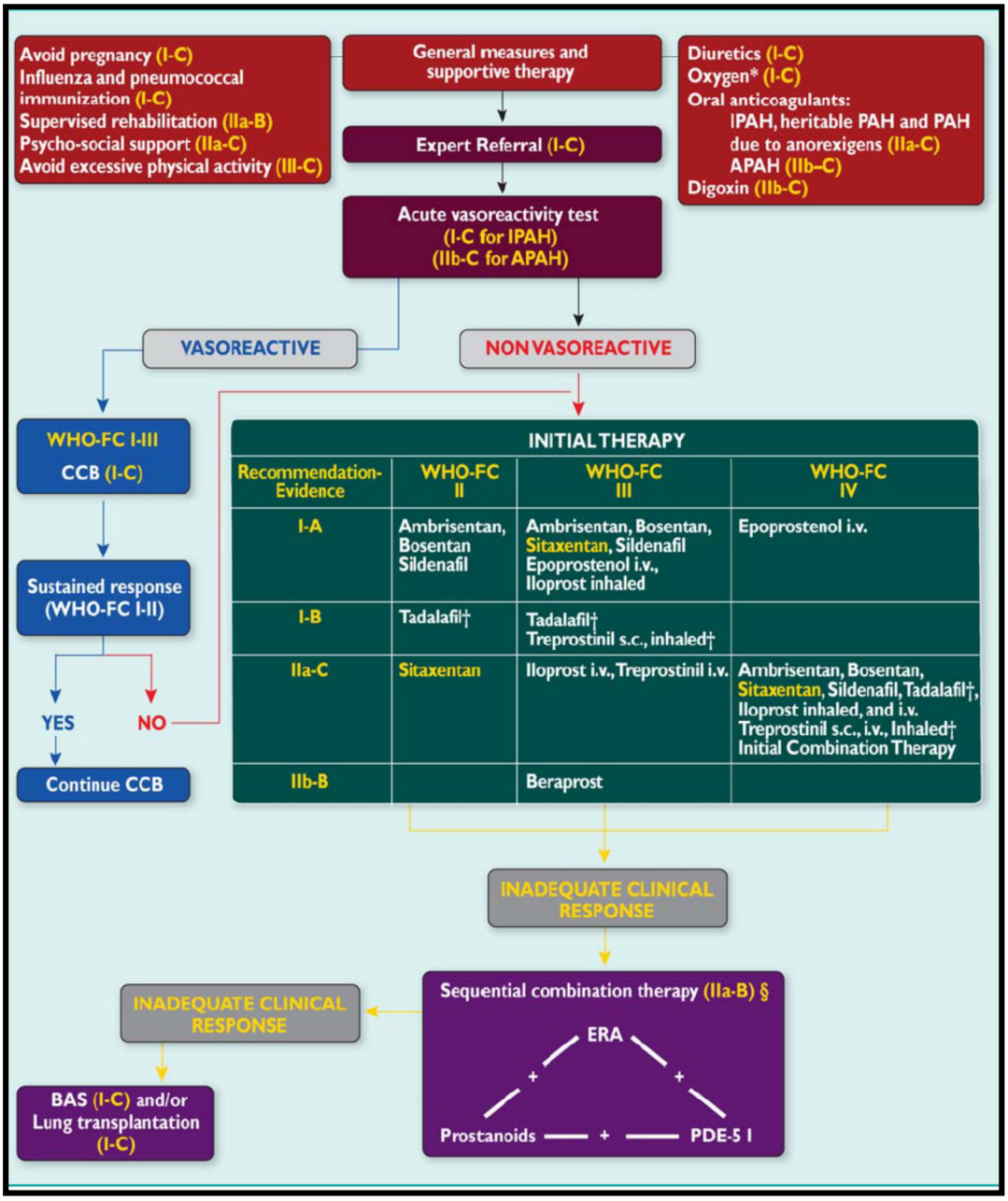


Fig.2.8: Treatment algorithm for PAH patients. *To maintain arterial blood oxygen pressure greater than 8kPa (60mmHg). †Under regulatory review in the European Union. §IIa-C for WHO-FC IL APAH=associated pulmonary arterial hypertension; BAS=balloon atrial septostomy; CCB= calcium channel blocker; ERA=endothelin receptor antagonist; IPAH=Idiopathic pulmonary arterial hypertension; PDE5=Phosphodiesterase type-5 inhibitor; WHO-FC=World Health Organization functional class. (Nazzareno Gaile *et al.* 2009)**.**

2.1.7.4 Surgical Procedures

a. *Balloon atrioseptostomy*

Balloon atrial septostomy (BAS) can be carried out in severely ill patients and is mostly used as bridging to lung transplantation (Sandoval et al 1998). At present, it is restricted to individuals in functional class IV who are refractory to all available medical treatments, and for whom other options are not available (C, II a). BSA aims to create an interatrial right-to-left shunt in order to increase cardiac output, which may increase systemic oxygen transport despite a reduction of systemic SaO2. Mortality of this procedure is reported to be high (5– 15%).

b. *Lung transplantation*

In PAH patients, the three- and five-year survival rate after lung transplantation is approximately 55 and 45%, respectively (Hertz *et al.* 2002). In patients with severe PAH who are in functional class III or IV and are refractory to best medical treatment (i.e., intravenous prostanoids), single (SLTx) or double lung transplantation (DLTx) may be considered (C, I). As the results of SLTx and DLTx are worse than modern drug therapy in stable patients, the necessity of lung transplantation in patients with PAH may decline in the future.

2.1.7.5 Future Therapies

Future studies targeting newly identified alterations in endothelial and smooth muscle cell function may provide novel treatments. Several of the most promising targets are discussed below:

2.1.7.5.1. Serotonin Receptor and Transporter Function

A potent vasoconstrictor and mitogen that has long been suspected to play a pathogenic role in PAH is Serotonin (5-hydroxytryptamine, 5-HT) (Fanburg *et al.* 1997). In 2000, MacLean *et al.* indicated that 5-HT receptors may be upregulated in PAH, providing a novel therapeutic target since antagonists to these receptors. Others have shown that the serotonin transporter (SERT), a molecule that facilitates transmembrane transport of serotonin into the cell, is upregulated in PAH (Dempsie *et al.* 2008); additionally, the fenfluramine anorexigens, which are known to increase the risk of developing PAH, produce an upregulation of the SERT in vitro, supporting a pathogenic mechanism for this system in PAH (Eddhaibi *et al.* 2001). Drugs that downregulate the SERT, such as the selective serotonin reuptake inhibitors (SSRIs), used to treat depression, may be worth exploring as treatment options in the future.

2.1.7.5.2 Vasoactive Intestinal Polypeptide

Vasoactive intestinal polypeptide (VIP) receptors appear to be upregulated in PAH, suggesting that this may be a compensatory mechanism in this disease. In a preliminary case series, eight patients with IPAH who were treated with inhaled VIP at doses of 200 mg four times daily showed marked clinical and hemodynamic improvement (Petkov *et al.* 2003). VIP is a neuropeptide that is produced by a variety of cells that has both potent vasodilating properties and cellular antiproliferative effects (Said *et al.* 2006). Further studies confirming these encouraging preliminary findings and clarifying optimal dosing and long-term safety are being undertaken presently.

2.1.7.5.3. Rho Kinase Inhibitors

Rho kinase is part of a family of enzymes that is involved in the processes of cellular growth and regulation of smooth muscle tone (Oka *et al.* 2007). Studies in animal models of pulmonary hypertension suggest that fasudil, and inhibitor of Rho kinase, may ameliorate the hemodynamic and pathologic severity of pulmonary vascular injury (Abe *et al.* 2004), and provide a rationale for clinical development of this agent in PAH.

2.1.7.5.4 Guanylate Cyclase Activators

Nitric oxide induces the synthesis of cyclic guanosine monophosphate (cGMP) by activation of the soluble guanylate cyclase. Soluble guanylate cyclase stimulators have been shown to produce pulmonary vasodilation and augment the response to nitric oxide in experimental pulmonary hypertension (Evgenov *et al.* 2004), and a preliminary acute clinical study with BAY 63-2521 (riociguat) demonstrated improved pulmonary hemodynamic parameters (Grimminger *et al.* 2009) that warrant further investigation in larger studies.

2.2 PULMONARY DRUG DELIVERY

Pulmonary drug delivery has developed into an attractive target in the pharma and healthcare industry as the lung is capable of absorbing pharmaceuticals either for local deposition or for systemic delivery. The origin of inhaled therapies seen in back 4000 years ago to India, where people smoked the leaves of the Atropa belladonna plant for suppression of cough (Siraj 2011). Pulmonary delivery of drugs has attracted tremendous scientific and biomedical interest in recent years and has progressed considerably within the context of local treatment for lung diseases, by virtue of enhanced local targeting and reduced systemic side effects with the administration of minute drug dosages.

The 21st century has seen a paradigm shift to inhaled therapy due to high surface area and permeability of the lung. The inhalation delivery of therapeutic agents has been known, though poorly understood, for many years. But the pulmonary tract tends to be considered as very promising and attractive route for the administration of active substances intended to treat local pulmonary (e.g., asthma, chronic obstructive pulmonary disease (COPD), microbial infections) as well as systemic diseases (e.g., diabetes). A wide variety of agents has been administered to the lung via oral inhalation, for the treatment of diverse disease states.

By facilitating the systemic delivery of large and small molecule drugs through inhalation into the lung, this advanced pulmonary technology provides an exclusive and inventive delivery alternative in favour of therapies which have to be currently administered by intravenous, intramuscular, and subcutaneous injection, or by oral delivery that causes adverse effects or the drugs are poorly absorbed and also they are associated with pain.

2.2.1 Human Pulmonary System

Human respiratory/pulmonary system is a complex organ system having a close structure-function relationships. This system mainly consists of two vital regions: the conducting airways and the respiratory region. The airway is further divided into nasal cavity, and associated sinuses, and the nasopharynx, oropharynx, larynx, trachea, bronchi and bronchioles. The human lung consists of 5 lobules and 10 bronchopulmonary segments. Arranged adjacent to each segment are lung lobules composed of 3-5 terminal bronchioles. Each bronchiole supplies the smallest structural unit of the lung, the acinus, which consists of alveolar ducts, alveolar sacs,

and alveoli. Alveolar epithelial type I cells typically represents the principle cell type lining the surface of the alveoli. The main functions of these cells, which cover 93% of the alveolar space, are to provide a surface for as exchange and to serve as a permeability barrier. Alveolar epithelial type II cells have a much smaller surface area per cell and they represent 16% of the total cells in the lung. They play a basic role in synthesis, secretion and recycling of surface-active material (lung surfactant).

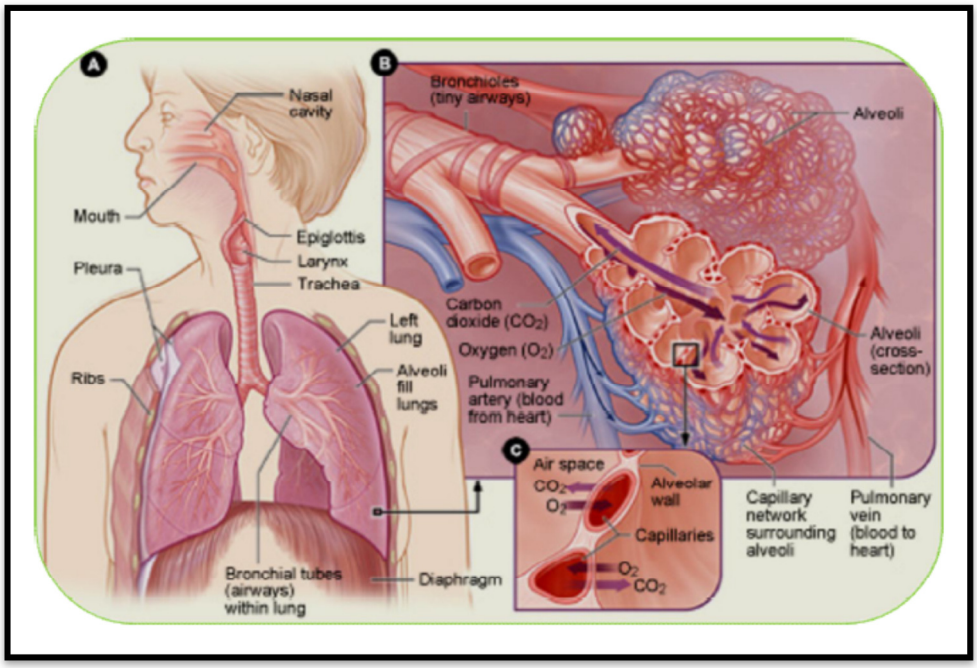


Fig. 2.9: Anatomy and Physiology of pulmonary system (Hickey J A, 2003)

Simply, the alveolar blood barrier consists of a single epithelial cell, a basement membrane, and a single endothelial cell. While this morphologic arrangement readily facilitates the exchange, it can still represent a major obstacle to large molecules. Before entering the systemic circulation, solutes must traverse a thin layer of fluid, the epithelial lining fluid. This layer tends to collect at the corners of the alveoli and is surrounded by an attenuated layer of surfactant. Unlike the larger airways, the alveolar region is lined with a surface active layer consisting of phospholipids (mainly phosphatidylcholine and phosphatidylglycerol) and several key apoproteins. Recent studies indicate that the surfactant may slow down diffusion out of the alveoli.

The respiratory airways, from the upper airways to the terminal bronchioles, are lined with a viscoelastic, gel-like mucus layer whose thickness is 0.5–5.0 mm. The secretion lining consists of two layers: a fluid layer of low viscosity, which surrounds

the cilia (periciliary fluid layer), and a more viscous layer on top, the mucus. The mucus is a protective layer comprising of a complex mixture of glycoprotein's released primarily by the goblet cells and local glands. The mucus blanket removes inhaled particles from the airways by entrapment and mucociliary transport at a rate dependent on viscosity and elasticity. High vascularization of lung tissue makes pulmonary targeting difficult because of fast absorption of most drugs.

2.2.2 Advantages of Pulmonary Delivery (Hindle *et al.* 1999)

1. Rapid onset of action comparable to the parenteral route and quicker than either oral delivery or parenteral delivery.
2. Non-invasive method of delivering drugs into the bloodstream for those molecules that currently can only be delivered parenterally.
3. Low fraction of oral dose i.e. drug content of one 4 mg tablet of salbutamol equals to 40 doses of meter doses.
4. Efficient drug targeting to the lungs for common pulmonary diseases like asthma, emphysema, and chronic bronchitis.
5. Negligible side effects since rest of body are not exposed to drug.
6. Pulmonary delivery circumvents gastrointestinal tract problems such as poor solubility, low bioavailability, irritability, side effects and food effects.

2.2.3 Disadvantages of Pulmonary Delivery

1. More frequent dosing (Ozer *et al.* 2007) specifically in case of IR formulations.
2. Lung barrier i.e aerodynamic filter, which must be circumvented for effective drug deposition to occur.
3. Short-lived duration of action due to the rapid removal of drug from the lungs or due to drug metabolism and clearance by mucous lining the pulmonary airways.
4. Generally, 0-40% of the drug leaving an inhalation device is usually deposited in the lungs by using conventional devices.

2.2.4 Challenges in Pulmonary Delivery

1. Dose reproducibility
2. Optimum particle size: Very large or very small particles lead to reduced efficiency of pulmonary delivery. Optimum particle size for efficient lung deposition is 1-5microns.
3. Device parameters like device shape, geometry, mechanism etc.
4. Less amount of drug per puff: Oral delivery of drugs generally requires doses in milligrams whereas pulmonary delivery involves doses in micrograms for achieving same or better effect.

2.2.5 Parameters determining particle deposition in deep lung

Different biophysical parameters determine regional drug deposition in the human lungs:

1. Anatomy of the respiratory tract.
2. Aerodynamic particle behaviour (e.g. size, density, hygroscopicity, shape, electrical charge).
3. Time of aerosol pulse injection into the breathing cycle.
4. Breathing pattern of the patients (e.g. flow rate, ventilation volume, end-inspiratory breath holding).

Of these factors, most influential one is aerosol particle size and size distribution on aerosol deposition. The aerodynamic particle diameter (AD) is the diameter of a sphere with a density of 1 g/cm3 that has the same aerodynamic behaviour as the particle which shall be characterized. In that way, aerosol particles with different density and shape can be characterized depending on their aerodynamic properties.

2.2.6 Mechanism of drug deposition

There are many different mechanisms by which particles deposit in the respiratory tract viz, gravitational deposition (sedimentation), inertial deposition (impaction), electrostatic precipitation and brownian diffusion. The relative contribution of each depends on the respiratory tract anatomy, characteristics of the inhaled particles as well as on breathing patterns. All mechanisms act generally act simultaneously.

Impaction occurs when a particle's momentum prevents it from changing course in an area where there is a change in the direction of bulk air flow.

Sedimentation results when the gravitational force acting on a particle circumvents the total force of the air resistance. The probability of *sedimentation* is proportional to residence time in the airway and to particle size, and decreases with increasing breathing rate.

Diffusion occurs when the collision of gas molecules with small aerosol particles exerts discrete non-uniform pressures at the particles' surfaces, resulting in random *Brownian motion*. *Diffusion*, however, is the main determinant of deposition of smaller particles in peripheral regions of the lung. The effectiveness of *Brownian motion* in depositing particles is inversely proportional to particle diameters of those particles, 0.5 μm, and is important in bronchioles, alveoli, and at bronchial airway bifurcations. Molecule-size particles may deposit by *diffusion* in the upper respiratory tract, trachea, and larger bronchi.

2.2.7 Pulmonary barriers to drug delivery

The various pulmonary barriers to drug delivery include mucociliary transport system, mucus, complicated anatomy, degradative enzymes and tight junctions between epithelial cells. Some barriers to the absorption of substances in the alveoli are:

1. The most significant barrier being the single layer of epithelial cells
2. Surfactant, a thin layer at the air/water interface, which leads to entrapment of the large molecules
3. The extracellular space inside the tissues and the basement membrane
4. Surface lining fluid which is a reservoir for the surfactant and contains many components of plasma as well as mucous.
5. Vascular endothelium, which is the final barrier to systemic absorption

2.2.8 Factors Affecting Pulmonary Drug Delivery

Various physiological factors which affect drug delivery through lungs include inspiratory flow rate, tidal volume, lung morphology, breathing, synchronization of aerosol generation with inspiration, breath holding, pathological states etc and

pharmaceutical factors viz, design of device, droplet size or powder size, density, shape, stability, partition coefficient etc which affect delivery of drugs via pulmonary route and these are summarized in figure mentioned below. These can be broadly categorized into:

1. Physicochemical properties of drug
2. Delivery systems
3. Modes of pulmonary delivery

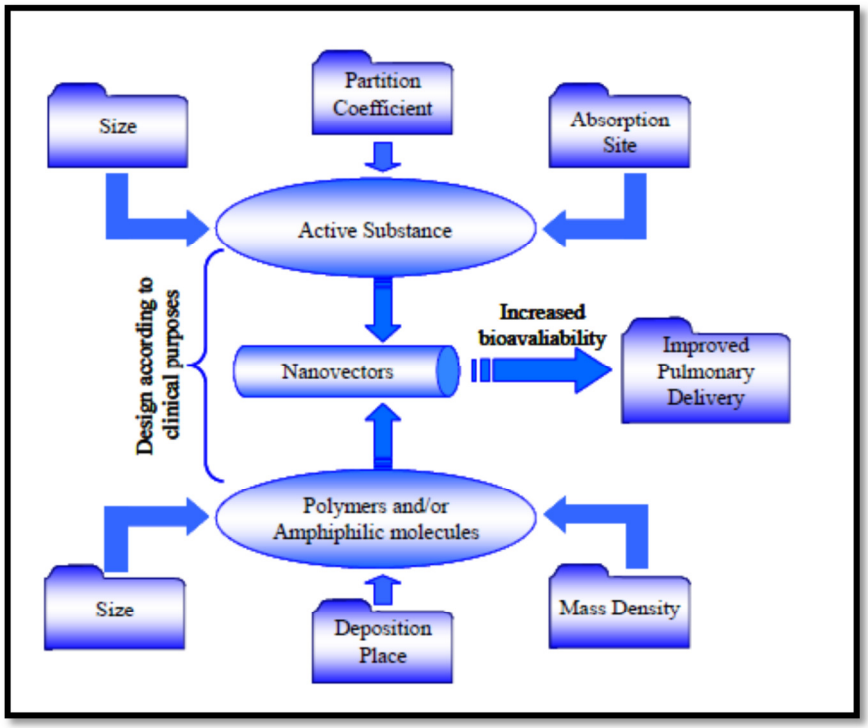


Fig. 2.10: Factors affecting pulmonary delivery (Labiris NR *et al.* 2003)

2.2.8.1 Physicochemical properties of drug:

It includes:

1. Aerodynamic particle diameter,
2. Density of drug particles
3. Shape of drug particles,
4. Physical stability of drug particles,
5. Hygroscopicity and aggregation characteristics
6. Electrical charge
7. Particle engineering etc.

Effect of particle size on deposition efficiency is shown in figure 2.11

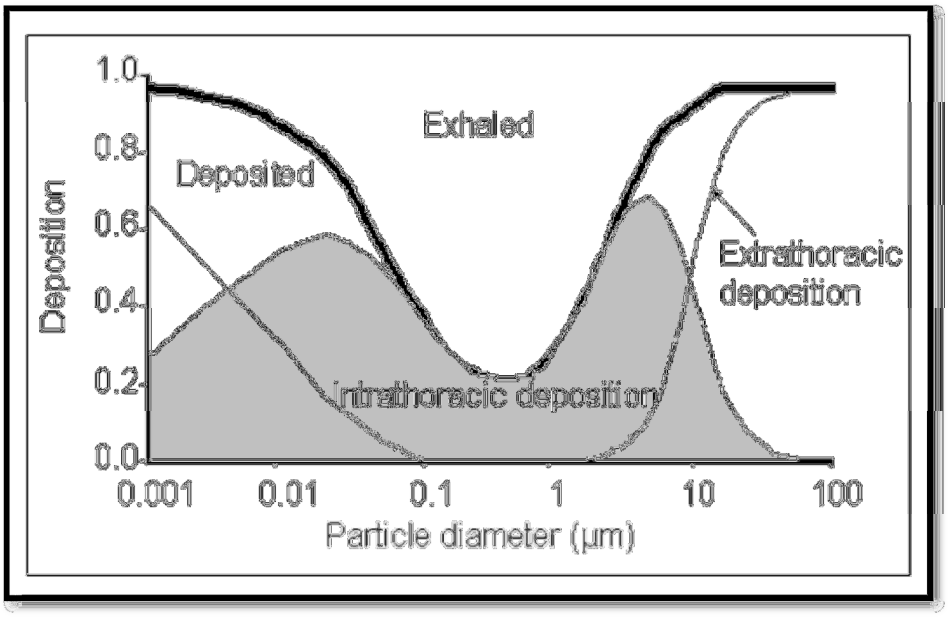


Fig.2.11: Deposition Efficiency as a function of particle size

(Scheuch *et al.* 2007)

2.2.8.2 Delivery Systems

Due to fast growing popularity and sophistication of inhalation therapy, there is an increasing demand for tailor-made inhalable drug particles capable of affording the most efficient delivery to the lungs. To deal with this formulation demand, a wide variety of novel particle technologies have emerged over the past decade. Recent advances in inhalation therapy have sparked considerable biomedical interest in the development of novel particle technologies for pulmonary delivery.

2.2.8.2.1 Nebulizers

Nebulizers convert solutions or suspensions into aerosols of a size that can be inhaled into the respiratory tract. Pneumatic jet nebulizers are the oldest form of aerosol generator, and their basic design and performance have changed little in the past 30 years. Ultrasonic nebulizers, which have been available for many years but are not commonly used for inhaled drug delivery, use electricity to convert a liquid into respirable droplets. The newest generation of nebulizers uses mesh technology (Atkins *et al* 2006).

Mesh Nebulizers: There are certain aerosol devices that use a mesh or plate with multiple apertures to produce a liquid aerosol. This operating principle uses an

aperture plate attached to a piezoelectric material vibrating at high frequency. The rapid vibration of the aperture plate creates a pumping action to produce the aerosol from a liquid solution. Alternatively, the solution can be forced through the mesh to create the aerosol.

Pneumatic Jet Nebulizers: A pneumatic nebulizer delivers compressed gas through a jet, causing a region of negative pressure. The solution to be aerosolized is entrained into the gas stream and is sheared into a liquid film. This film is unstable and breaks into droplets due to surface tension forces. A baffle in the aerosol stream produces smaller particles.

Ultrasonic Nebulizers: The ultrasonic nebulizer converts electrical energy to high frequency ultrasonic waves. The transducer vibrates at the frequency of the ultrasonic waves applied to it (piezoelectric effect). Ultrasonic waves are transmitted to the surface of the solution to create an aerosol

General advantages and disadvantages with use of small volume nebulizers are listed below:

Advantages

1. Capability of aerosolizing many drug solutions
2. Normal breathing patterns can be used
3. Capability of aerosolizing drug mixtures (>1 drug)
4. Useful in very young, very old, debilitated, or distressed patients
5. Drug concentrations can be modified
6. Breath-hold is not required for efficacy

Disadvantages

1. Large and cumbersome equipment required.
2. Lengthy treatment times for pneumatically-powered nebulizers
3. Variability in performance characteristics among different brands
4. Power source is required i.e. electricity, battery etc.
5. Possible contamination with inadequate cleaning
6. Facemask delivery is required
7. Accidental drug delivery into the eyes with facemask delivery

Fig. 2.12: Nebulizer (Sunitha *et al.* 2011)

2.2.8.2.2 Metered Dose Inhalers (MDI)

A metered-dose inhaler (MDI) is a system designed to provide a fine mist of drug, generally with an aerodynamic particle size of less than 5 microns, for inhalation directly to the airways for the treatment of respiratory diseases like asthma and COPD. The MDI is designed to provide a precise metered dose of drug in a fine mist to be inhaled directly into the airways for the treatment of respiratory diseases. The original MDI in 1955 was conceived by Dr George Maison, the president of Riker Labs (now 3M Pharmaceuticals, St Paul, Minnesota) in response to his teenage asthmatic daughter's request for a better delivery system. One year later, Medihaler-Iso (isoproterenol) and Medihaler-Epi (epinephrine) were approved as new drug applications by the FDA. Today, a number of inhalation formulations are available as MDIs (Atkins *et al* 2006).

The propellant-driven MDI is currently the most frequently used device for asthma and COPD. Since the MDI is pressurized, the components of the device are protected from contamination by pathogens and moisture. As with other respiratory drug delivery systems, even when used correctly the MDI only delivers approximately 10-20% of the nominal dose per actuation or puff (Ogden *et al.* 1996; Kohler 1994). Deposition may be lower in children due to differences in breathing pattern or when technique is less than optimal. Most MDIs are designed to deliver a drug dose in the range of 100-200 μg per actuation. The basic necessity of any MDI drug formulation is that the device accurately and reproducibly delivers an aerosol dose containing a significant fraction of drug particles in the fine particle fraction range (aerodynamic

diameter <5 μm). MDIs, regardless of manufacturer or active ingredient (drug), typically consist of some standard components that have very specific functions in the drug delivery system. The components of the MDI include the container (usually aluminum), the propellant (CFC or HFA), the formulation (suspension or solution), the metering valve, and the actuator (press-and-breathe or breath-actuated). The actuator nozzle is MDI specific and is a key determinant of aerosol dose and particle size. The amount of medication delivered is related to nozzle size, cleanliness, and lack of moisture.

Container: Able to withstand high internal pressures, inert and utilize a coating to prevent drug adherence

Propellants: Liquefied compressed gases in which the drug is dissolved or suspended

Drug Formulary: Particulate suspensions or solutions, in the presence of surfactants or alcohol that allocate the drug dose and the specific particle size

Metering Valve: It is most significant component that is crimped onto the container and is responsible for metering a reproducible dose. Elastomeric valves are responsible for sealing and prevention of drug loss or leakage.

Actuator: Normally, referred to as the "boot," partially responsible for particle size, based on the length and diameter of the nozzle for the various MDIs

Dose Counter: Nowadays, newest component of the MDI delivery system. This component provides a visual tracking of the number of doses remaining in the MDI.

Advantages and Disadvantages of Metered-Dose Inhalers:

Advantages

1. Short treatment time
2. Compact and portable and compact
3. Emission of reproducible dose

Disadvantages

1. Coordination due to hand–breathing coordination is difficult for many patients
2. Breath-hold and proper inhalation pattern is difficult

3. High oropharyngeal impaction unless a holding chamber or spacer is used
4. Fixed drug concentrations
5. Alteration of dose due to improper shaking
6. Reaction to propellants
7. Limited range of drugs, only those drugs which are stable in solution form
8. Foreign body aspiration from debris-filled mouthpiece

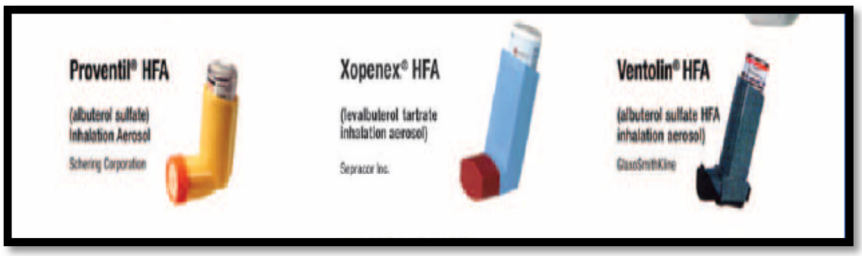


Fig. 2.13: MDIs (Sunitha *et al.* 2011)

2.2.8.2.3 Dry Powder Inhalers

In 1949, Krasno and Rhoads described the administration of penicillin dust using the Aerohalor for treatment of respiratory infections, particularly sinusitis. The first report of a dry powder inhaler (DPI) dates back to the introduction of the MDI. The introduction of the Spinhaler by Fisons for oral inhalation of cromolyn sodium (Intal), as described in 1971 by Bell, Hartley, and Cox, is usually cited as the first DPI in common clinical use.

The introduction of the Spinhaler was partly based on the problem of hand-breath coordination frequently observed with incorrect use of MDIs. With regard to DPIs, two important stimuli have specifically increased the interest in this dosage form and driven the technology forward. DPIs are breath-actuated, thereby ensuring coordination between release of drug and inhalation. The first stimulus came from the Montreal Protocol in 1987, calling for signatory countries to phase out the production of CFC propellants by January 1, 1996, in order to stop depletion of the ozone layer. A more recent stimulus came from the advice not to use nebulizers for severe acute respiratory syndrome patients as their use could be one of the transmission causes of the disease. Replacement of CFC driven MDIs by DPIs was one of the strategies to reach this goal.

Advantages

1. Propellant-free
2. Breath-actuated
3. Compact, small and portable
4. Built-in dose counter
5. Short preparation and administration time

Disadvantages

1. Relatively high oropharyngeal impaction
2. Patient's inspiratory flow dependent
3. Vulnerable to ambient humidity/exhaled humidity

Principle of Operation

All DPIs are breath actuated and do not contain propellants. The patient's inspiratory effort, both inspiratory flow and volume, provide the energy to disperse and deliver the drug powder. All DPIs have an intrinsic resistance to airflow that differs among devices. For example, the HandiHaler has a higher resistance than the Turbuhaler, and both have higher resistances than the Diskus. The resistance determines how much inspiratory flow occurs through the device for a given inspiratory effort. As airflow occurs, a pressure drop between the intake and exiting mouthpiece occurs, thus lifting the powder from the drug reservoir, blister or capsule. Excessive inspiratory flow, however, can increase impaction on the oral cavity and theoretically decrease lung deposition, although for current DPIs this is higher than the patient's capability. The patient's inspiratory effort also deaggregates the powder into finer particles. Higher inspiratory flows generally improve drug deaggregation, fine particle production, and lung delivery.

Currently there are two types (Atkins *et al.* 2006; Ogden *et al.* 1996)

Unit-Dose Devices: Single-dose powder inhalers are devices in which a drug powder contained capsule is placed in a holder. The capsule is opened within the device and the drug powder is inhaled.

Multi-dose Devices: Multi-dose device uses a circular disk that contains either four or eight drug powder doses on a single disk. The doses are maintained in separate aluminium blister reservoirs until just before inspiration.

A major disadvantage of unit dose DPIs is the time needed to load a dose for each use. All DPIs are potentially vulnerable to humidity and moisture, which can cause powder clumping and reduce deaggregation and fine particle development during inhalation. However, capsules and drug blisters generally offer more protection from ambient humidity than does a reservoir chamber containing multiple doses for dispensing when the device is primed for use.

Table 2.8: Currently available DPI in US (categorized by design features)

Design	Device	Drug	Dosing System
Unit Dose	Aerolizer	formoterol	capsule
	HandiHaler	tiotropium	capsule
Multidose	Diskus	salmeterol & fluticasone	blister strip
		salmeterol	
		fluticasone	
	Turbuhaler	budesonide	drug reservoir
	Twisthaler	mometasone	drug reservoir

Performance of the DPI can be changed through changes in the design of the device and also changes in the powder formulation. Supercritical fluid technology is applied to improve the surface properties of the drug substance. Large porous particles have reduced inter-particulate forces due to their low density, the irregular surface structure and/or reduced surface free energy.

Additionally, these particles are reported to have improved aerodynamic behavior in the airways, whereas phagocytises of the deposited particles in the alveoli is reduced. In another approach, smaller porous particles (3-5 mm) have been used to improve de-agglomeration and lung deposition. Air classifier Technology has been recently used in the devices to prevent agglomeration in devices (Kohler *et al* 1994; Kinnula *et al.* 1990; Martin *et al.* 1993)

Figure 2.14: DPI (Advair Diskus) (Sunitha *et al.* 2011)

Table 2.9: Different principles for powder de-agglomeration used in DPIs

Dispersion Principle	Example(s)
Aerosol passage through narrow passages (e.g venture tubes)	Easyhaler® (Orion)
Aerosol conducted against impact bodies (baffles, plates, internal inhaler surfaces)	Clickhaler® (Innovata Biomed) (Parry Billings *et al.* 2000) Skyehaler™ (Skyepharma)
Aerosol conducted through specifically shaped channels or channels with inserts	Turbuhaler® (Astra) (Wetterlin *et al.* 1988) Twisthaler® (Schering) (Fan *et al.* 2000) Directhaler™ (Direct-Haler AS)
Circulation, whirl or cyclone chambers	Pulvinal® (Chiesei) (Meakin *et al.* 1998) Airmax™ (Ivax) (Keatings *et al.* 2002) Novolizer® (Viatris) Taifun® (Focus)
Pressurized air or vacuum chambers	Inhance™ (Nektar) Aspiair™ (Vectura)
Battery powered systems	Spiros® (Dura Pharm)
Miscellaneous	Ratiopharm Jethaler® (Ratiopharm) Ultrahaler™ (Aventis) Eclipse™ (Aventis)

Variables that affect the de-agglomeration forces and adhesion forces and thereby the aerosol generation in a DPI:

1. Drug

a. Conditioning

b. Particle Size Distribution

c. Drug Characteristics

d. Carrier payload

2. Carrier

a. Bulk properties of carrier

b. Surface characteristics

c. Conditioning

d. Ageing

3. Blending Technique of Drug and carrier

a. Type of blender

b. Blending time

c. Batch size

4. Blend

a. Type of blend

b. Homogeneity and conditioning

5. Test for Inhalation

a. Inhaler type

b. Test system

Major Variables and interactions in DPI performance include

1. DPI design i.e. characteristics of powder formulation, dosing system and de-agglomeration principle

2. Resistance to air-flow

3. Patient factors like age, gender, adequate training, smoker/non-smoker and clinical parameters

4. Inhalation Effort

5. Flow characteristics like peak flow rate, flow increase rate and inhalation Time

6. Performance characteristics like dose entrainment, lung deposition and fine particle fraction

Table 2.10: Air flow resistances of some marketed DPIs ($kPa^{0.5}min/l_N$)

Inhaler	Resistance
Rotahaler (GSK)	0.015
Spinhaler (Aventis)	0.016
ISF Inhaler (Cyclohaler; Pharmachemie)	0.019
Novolizer (Viatris)	0.028
Diskhaler (GSK)	0.032
Diskus (GSK)	0.034
Ratiopharm Jethaler (Ratiopharm)	0.036
Handihaler (Boehringer)	0.042
Turbuhaler (Astra)	0.043
Inhalator (Boehringer)	0.051-0.062*

*Depending on capsule position

In nutshell, DPI can be considered as an attractive drug delivery system, both for drugs that are to be administered for local therapy in lungs as well as for drugs that act systemically and for which the lung is only a port of entry to the body.

2.2.9 Developments in Formulation aspects of Pulmonary Drug Delivery

Successful integration of novel technologies with inhalation devices has resulted in a proven track record for inhalation as a route of administration that limits systemic exposure and provides targeted delivery.

The most promising technologies being employed in area of DPIs are lipid based carrier systems, liposomes, polymer based systems, microspheres and large porous spheres. Drug targeting can also be achieved by further tagging the liposomes for specific ligands like monoclonal antibodies and lectins or my making pH sensitive lipoosmes.

2.2.9.1 Co-spray dried Sugars- and lipid-composites

Besides convetional mixing of micronized drug with carriers, the use of sugar and polols has been extensively explored for preparing dry powders by co-spray drying these carriers with the drug. Polyols like mannitol has the capbility to impart

excellent flow and dipersibility to the powder while acting as stabilizers too during processing like lyophilization or spray drying. Sugars carrier like lactose has also been found to improve intracellular targeting of poly-lysine in alveolar cells. But lactose being reducing sugar can influence the stability of proteins and peptides lactose. Nonreducing sugars like trehalose have been considered for their stabilizing action for proteins during dry powder formulation. But hygroscopic nature of trehalose makes it unsuitable due to poor dispersability of the powder. Other substitute carriers like mannitol, glucose, sorbitol, malitol, and xylitol have also been assessed for possible use in DPIs (Naini V *et al*. 1998). Mannitol performed to be the best contestant for excellent aerosolization behavior of DPI formulations.

Many spray-dried sugars and polyols such as lactose, sucrose, mannitol, and trehalose have been assessed as promising excipients for DPIs. Spray-drying is the most prevalent method and is a particularly interesting option for the formulation of proteins and peptides as it is a single-step, single-unit process and escapes certain technical difficulties associated with freeze-drying and spray freeze drying like coalescence of the excipients and crystallization. Cyclodextrins like dimethyl-β-CD can enhance the systemic absorption of certain drugs like salmon calcitonin after intratracheal administration in rats, and recent studies have shown that it is also capable of enhancing the pulmonary absorption of insulin in rats. CDs are suitable absorption enhancing excipients in DPIs rather than carriers, in the systemic delivery of proteins through lungs.

Lipids have also been explored as suitable sustained release carriers for formulating into dry powders for inhalation by single step co-spray drying with drug rather than first preparing liposomes or lipospheres and then spray drying its dispersion to achieve dry powder. Lipid composites of Amphotericin B with egg phosphatidylcholine were prepared by single step co-spray drying its methanol solution to obtain dry powder for inhalation (Kim *et al.* 2001). These may also be considered as proliposomes as these have been shown to form vesicles upon hydration with phosphate buffer saline or physiological fluid.

2.2.9.2 Liposomes

Liposomes are phospholipids vesicles which are single multi layered lipids encapsulating one or more aqueous compartments in which drugs and other substances might be included. Recently, they have been investigated as a vehicle for sustained-release therapy in the treatment of pulmonary disorders, gene therapy and as a method of delivering therapeutic agents to the alveolar surface for the treatment of systemic diseases. Liposomal systems offer many advantages such as the effective encapsulation of small and large molecules with a wide range of hydrophilicity and pKa values. In fact, they have been found to increase nasal absorption of peptides such as insulin and calcitonin by increasing their membrane permeability.

This has been attributed to the protection of the entrapped peptides from enzymatic degradation, increasing nasal retention of peptides, and disruption of mucosal membrane disruption. Jain *et al.* encapsulated insulin in liposomes coated with chitosan and carbapol and administered them intranasally to rats. The results demonstrated that this formulation was effective and that it's a viable option for a sustained release of insulin (Jain *et al.* 2007; Law *et al.* 2001).

Liposomes may be formulated as liquid dosage form for inhalation using nebulizer which may lead to drug leakage during nebulization. The drug loading, release kinetics, and deposition of liposomes in the lungs can be tailored by altering lipid composition, size, charge, drug/lipid ratio, and method of delivery. Liposome-mediated pulmonary delivery may increase in drug retention-time in the lungs, and more significantly, a reduction in extrapulmonary side-effects which invariably results in enhanced therapeutic efficacies.

Incorporation of liposomes into pulmonary delivery systems provides an attractive means for sustained, non-invasive delivery to facilitate the treatments of respiratory diseases like asthma, COPD etc. Formulation of proteins and peptides is often more challenging than formulation of small conventional molecules because of the important role of protein conformation as well as the potential for chemical degradation pathways. The more advanced technique is the fabrication of liposomes into dry powder for inhlation prepared using lyophilization and more preferably using spray drying for better size control.

2.2.9.3 Polymer Based Microspheres and Nanoparticles

Preparation of microparticles for aerosol delivery can be performed using various biodegradable synthetic polymers like PLGA and PLA or natural polymers like chitosan, albumin, dextran and gelatin. Various preparation techniques like emulsion-solvent evaporation, supercritical fluid technology, phase separation, spray-drying alone or combination of spray with freeze drying and emulsion-solvent diffusion may be used.

Nanoparticles are solid colloidal particles with diameters ranging from 1-1000 nm. Nanoparticle systems are being analyzed to improve drug delivery and intranasal drug delivery. They consist of macromolecular materials and which are therapeutically used as excipient in vaccines or as drug carriers, in which the drug is dissolved, encapsulated, adsorbed or chemically attached. Nanoparticles generally offer several advantages due to their small size, but only the smallest nanoparticles penetrate the mucosal membrane by paracellular route and in a limited amount, since the tight junctions are in the order of 3.9-8.4 Å. Many studies have been suggested that nanoparticle systems can be preferably suited as a vehicle for sustained release therapy.

Controlled release from a therapeutic aerosol can prolong the residence of an administered active substance in the alveolar region, minimize the risk of adverse effects by decreasing its systemic absorption rate, and increase patient compliance by reducing dosing frequency.

Microsphere technology has also been widely useful in designing of formulations for pulmonary drug delivery (Cartier R *et al.* 2001). Microspheres are usually based on muco-adhesive polymers like chitosan, alginate, which provide various benefits for pulmonary drug delivery. Moreover, microspheres may protect the drug from enzymatic metabolism and gives controlled drug release, thereby prolonging its effect. Scientists have investigated the aminated gelatin microspheres as a nasal drug delivery system for insulin. They have analyzed nasal mucosa after its exposure to microspheres of alginate/chitosan containing metoclopramide. Many other identical studies have been carried out and positive results are found for nasal delivery of carbamazepine using chitosan microspheres, cyclodextrins, alginate as mucoadhesive polymers and carvedilol using alginate mucoadhesive microspheres.

Scientists have improved the bioavailability of calcitonin by encapsulating the drug in PLGA [poly(lactic acid co-glycolic acid)] and subsequently coating the

nanospheres with chitosan. *In vivo* experiments in guinea pigs revealed that the elimination rate constants of chitosan-coated PLGA nanospheres from various regions of the lung were reduced compared to non-coated nanospheres.

The improved absorption was due to the combined effect of mucoadhesion and opening of epithelial tight junction by chitosan. This concept of augmenting the desirable properties of microparticulate DDS by coating them with mucoadhesive polymers have been extended to liposomal systems also. Many other mucoadhesive polymers have been evaluated in drug delivery by the pulmonary, nasal or oral routes including cellulose derivatives, polyacrylic acids, hyaluronic acids, gelatin, albumin, chitosan, dextran, polyvinyl alcohol and their derivatives.

2.2.9.3 Large porous Particles

The human lungs have efficient means of removing deposited particles over periods ranging from minutes to hours. In the upper airways, ciliated epithelia act like "mucociliary escalator", by which particles are swept from the airways toward the mouth. In the deep lungs, an army of alveolar macrophages is capable of phagocytosing drug particles soon after their deposition in lungs. An effective sustained-release inhalation therapy therefore requires a means of avoiding or suspending the lungs' natural clearance mechanisms until encapsulated drugs have been effectively delivered. Until now, therapeutic dry powder inhalers have been made with particle mass densities (ρ) of 1 to 0.5 g/cm3 and particles of 1 to 3 μm mean geometric diameter (d_g) to avoid excessive deposition in the dry powder inhaler (DPI) and oropharyngeal impaction of bigger particles and exhalation of small particles. Particles of this size range have the limitations of agglomeration as well as rapid clearance by alveolar macrophages.

A recent technlogy of inhalation aerosol has recognized and addresses the shortcomings of the current inhalation therapies by formulating particles possessing low mass density and, large mean geometric size, such that the mean aerodynamic diameter (d_a) remain 1-3 μm for favourable alveolar deposition. The dual benefit of large size and low mass density is the increased particle size results in decreased tendency to agglomerate; hence, in combination with low mass density, improves aerosolization; second, reduced phagocytosis of particles by macrophages due to increased particle size beyond ~2–3 μm. Very large particles deposited in the

pulmonary region may escape clearance by alveolar macrophages and, hence permit efficient drug release for prolonged periods of time (Edwards D.A. *et al.* 1998)

To justify the potential for large and porous aerosols to increase systemic bioavailability as well as to provide controlled release capability in the lungs, scientists encapsulated insulin into biodegradable copolymers. Poly (lactic-co-glycolic) acid particles were prepared with encapsulated insulin in two forms a small, nonporous aerosol particle and a large, porous aerosol particle of similar d_a (2.15 μm). Relative bioavailability of the conventional aerosol particle after aerosolization as a dry powder into the lungs of rats was 12%, and the release time was~4 h.

The relative bioavailability of the large and porous insulin particle was 87.5%, approximately seven times greater than that of the small and nonporous insulin particle. Probable reason for the relatively high bioavailability of the inhaled large-particle insulin is more effective delivery of drug to the lung as a consequence of less powder aggregation. An increase in the size of aerosol particles results in a reduced fractional surface area of particle-particle contact in DPI and thus in fewer tendencies to agglomerate.

This diminished agglomeration means that less energy is required to aerosolize particles or that particles are more effectively aerosolized with a given energy of aerosolization. However, perhaps the predominant cause of the controlled insulin delivery is the role of large particle size in discouraging phagocytosis. The potential of large, porous particles for inhalation of a variety of drugs is presently being investigated. In addition to insulin and testosterone, promising results in animals have recently been attained with long-lasting formulations of estradiol, for hormone therapy, and albuterol, for asthma. In the estradiol study, particles were produced by spray drying of estradiol and other combinations of dipalmitoylphosphatidylcholine, lactose and human albumin.

Large, porous particles with a mean geometric diameter of 10 μm and tapped density 0.09 g/cm^3 were prepared, as were nonporous particles possessing a mean geometric diameter of 3 μm and bulk (tapped) density 0.48 g/cm^3. After being inhaled as an aerosol into the lungs of rats, increased systemic levels of estradiol for 5 days were observed in the large porous particles, whereas the small nonporous particles produced increased systemic estradiol levels for only 1 day. Relative bioavailability in the case of the large, porous estradiol particles was ~87%. In the albuterol study,

large, porous particles prepared by spray drying with albuterol and a combination of dipalmitoylphosphatidylcholine, albumin and lactose resulted in controlled bronchodilation in guinea pigs for ~1 day, at relatively low inhaled albuterol doses, compared with several hours in the case of small, non-sustained-release aerosol particles.

Still considerable work remains to clarify the potential bioavailability and efficiency gains that can be attained after inhalation of large porous aerosol formulations in humans. The study carried out till date suggests that such particles may play a significant role in the development and optimization of new inhalation therapies in the near future.

2.2.10 Methods for preparation of DPIs

Many conventional techniques have been reported to produce DPI formulations. However, these methods have number of limitations, such as particle size, size distribution, shape and poor control over powder crystallinity. To rectify such problems, some of the important techniques are discussed below:

2.2.10.1. Conventional physical mixing with inhalable carriers

The particle size distribution affects the deposition of drug in the respiratory tract. To create particles in the respirable size range (less than 5 microns in diameter), the drug particle size must be reduced in a separate unit operation. The first size-reduction technique the formulation scientist will typically turn to is milling. There are many different mills, but only a few are able to mill powder to the required particle size range of 2-5 μm. The 3 main types of mills used in Active Pharmaceutical Ingredient manufacture are fluid-energy mills, such as the jet mill; high-peripheral-speed mills, such as the pin-mill; and the ball mill.

Jet milling (Cheng *et al.* 1985) (or air-attrition milling) is the most useful technique; it reduces particle size via high-velocity particle-particle collisions. High-pressure nitrogen is fed through nozzles and accelerates the solid particles to sonic velocities. The particles collide and fracture.

A pin mill uses mechanical impact to grind material, both by particle-particle and particle-solid collisions. A pin mill is equipped with a series of concentrically mounted pins located on a spinning rotor and stationary stator plate. The pin mill can produce 1micron particles, but not as small as the jet mill (Drogemeier *et al.* 1996).

The ball mill (Hu *et al.* 2001) is essentially a rotating cylinder loaded with drug and "milling media" (ie, balls that grind the drug between each other as they tumble inside the mill). The size and material of the milling media can be varied. Ball milling is very slow and the process is poorly scalable.

One way to improve the non-pharmacologic properties of a drug is through the addition of excipients. In general, excipients are used to enhance the physical or chemical stability of the active pharmaceutical ingredient, its mechanical properties, and/or its pharmaceutical properties, such as dissolution and permeation. Currently, lactose is the only excipient used in DPIs marketed in the United States. The reasons for this are as much historical as they are physicochemical/pharmaceutical in nature. Lactose had long been used as an excipient in oral dosage forms before being deployed in DPIs. It had an established safety and stability profile, manufacturing process with tight controls over purity and physical properties, and was available and inexpensive. Lactose is highly crystalline and has the smooth surfaces and satisfactory flow properties desirable for a DPI carrier particle.

Excipients can makes up over 99% of the product by weight, making them crucial determinants of overall DPI Performance. It should also be noted that excipients are not always required; the Pulmicort (budesonide) Turbuhaler (AstraZeneca, Wilmington, Delaware) is an example of an excipient-free formulation. After drug and excipient(s) have individually been brought to their desired forms, they are combined in the blending process. When mixing powders with different properties, particle sizes, and ratios, as is the case with DPI formulations, inadequate mixing can cause poor dose uniformity. In many cases, inadequate mixing cannot be overcome simply by increasing the mixing time. Mixer selection, rotation speed, capacity, and fill level are all subject to optimization, as they can all affect the blend homoge- neity (Sudah *et al.* 2002; Alexander *et al.* 2004) After the formulation has been blended, it is filled into capsules, multi-dose blisters, or reservoirs for use with the inhaler device. The filling process is automated and depends on the nature of the metering system.

However, micronized particles, particularly those resulting from high-energy operations such as jet milling, have high surface areas and surface energies, which result in poor flow and a high tendency to aggregate. There are many alternative formulation strategies which aim at alleviating these problems are mentioned below.

2.2.10.2 Supercritical fluid technology

Drugs are rarely crystallised directly to meet the criterion of particle size (1-5 μm) to reach the respiratory airways, therefore additional processing steps usually include re-crystallization, filtering, drying, micronisation or high-energy milling and may also involve granulation before the micronisation and solid-state or surface conditioning after the micronisation stage. This processing sequence only provides limited opportunity for control over particle characteristics such as size, shape and morphology and introduces uncontrolled structural variations (decreased crystallinity, polymorphism) and surface modifications (increased surface free energy, adhesion, cohesiveness and charge) which have an adverse effect on dry powder formulation and may even render the formulation ineffective.

An alternative is to use supercritical fluid (SCF) processing which enables formation of micron sized particulate products in a single step operation, with a significant benefit of selective crystallization, separation of impurities and control of crystalline forms, leading to a potentially clean and recyclable technology. The benefits of SCF-processed particles in both dry-powder (DPI) and multi-dose (MDI) inhaler formulations have been demonstrated for particles of several steroid drugs. An increase of fine particle fraction (*FPF*) for steroid formulations prepared with lecithin was observed. The SEDSÔ method (Solution Enhanced Dispersion By Supercritical Fluids) has an added advantage of very fast, homogeneous nozzle mixing between supercritical antisolvent and drug solution leading to consistent production of micron-sized particulate products.

In recent years, supercritical fluid crystallization (SFC) technologies have gained increasing attention in the pharmaceutical industry due to their capability and versatility of producing micro-fine particles to predetermined specifications. Supercritical fluids (SFs) are those gases and liquid at temperatures and pressures above their critical points (TC - critical temperature; PC - critical pressure). SFs have density values that afford appreciable solvation power. In addition, the viscosity of solutes is lower in SFs than in liquids while the reverse is true for the diffusitivity of solutes, which facilitates mass transfer. More importantly, SFs are highly compressible, particularly near the critical point, and thus their density and solvation power can be carefully controlled by slight adjustment of temperature or pressure.

Broadly speaking, supercritical fluid crystallization technologies can be divided into two categories: 1. precipitation from supercritical solutions, e.g. Rapid Expansion of

Supercritical Solution (RESS); 2. precipitation using SFs as non-solvents or antisolvents, e.g., Gas AntiSolvent (GAS), Supercritical AntiSolvent (SAS), Precipitation with Compressed Antisolvents (PCA), Aerosol Spray Extraction System (ASES), Solution Enhanced Dispersion by Supercritical fluids (SEDSTM).

Of all the gases available for use as SFs in industry, carbon dioxide is the most widely used one because of its low critical temperature (31.1degrees), which makes it particularly suitable for heat sensitive materials, such as biologicals. In addition, it is non-flammable, non-toxic, inexpensive, recyclable and environment friendly. The use of SFs to process pharmaceutical materials has proved to be a cost-efficient approach in generating high purity, micron-sized particles with defined morphology in a single-step operation (Palakodaty *et al.* 1999; Shekunov *et al.* 2000). SFs, by virtue of their attractive physical properties such as variable density and transport properties such as viscosity and diffusivity, and the relative ease by which these properties can be manipulated with temperature and pressure have created tremendous formulation opportunities for engineering drug particles with specific biological applications.

2.2.10.3. Spray Drying

Its an advanced pharmaceutical manufacturing technique used to efficiently produce respirable colloidal particles in the solid state. It was explored in the 1980s as an alternative means of producing fine particles for pulmonary delivery.

Spray drying is a one-step process that converts a liquid feed into a dried particulate form via the following stages: (a) atomization of the feed solution to form a spray; (b) spray-air contact; (c) drying of the spray; and (d) separation of the dried product from the gas stream. The heating and drying of the droplets are usually performed in a chamber to which a stream of hot dry air is admitted in a co-current (i.e., in the same direction as spray) or counter-current (i.e., in the opposite direction to spray) manner. Spray drying has been used to produce solid drug particles for inhalation. The resulting materials are usually amorphous in nature (Brittain *et al.* 1999).

Since the drying is normally accomplished at elevated temperatures inside the drying chamber, chemical degradation and accompanying loss of biological activity could be a problem with thermolabile compounds, for which low-temperature drying or the alternative freeze spray drying may be considered. Examples of spraydried

pharmaceutical materials are insulin (Stahl *et al.* 2002), salbutamol sulphate (Chawla *et al.* 1994), sodium cromoglycate, formoterol fumarate (Dellamary *et al.* 2000) and budesonide (Duddu *et al.* 2002)

Recently, drug particles with improved pulmonary delivery have been developed using specially formulated feed solutions for the spray-drying process. Corrugated bovine serum albumin powders thus produced display better aerosol performance than spherical particles produced under similar conditions but at higher atomizing pressure (Chew *et al.* 2001). It was concluded that the surface asperities of the corrugated particles could lower the area of contact between particles, and thus reduce the particle cohesiveness. Production of large hollow porous particles with improved aerosol performance by spray drying has also been reported for albuterol, estradiol and insulin (Vanbever *et al.* 1999). These spray dried samples have high respirable fractions, ranging from 49% to 92%, depending on the measurement techniques.

Spray-dried hollow porous particles based on similar concept have been commercialized as PulmoSphere™ (Dellamary *et al.* 2000). This system has been tested with cromolyn sodium, albuterol sulfate and formoterol fumarate, and they all show improved physical stability, content uniformity and aerosolization efficiency. Clinical data also demonstrated that delivery of spraydried budesonide PulmoSphere™ powder is more efficient and reproducible than that of the micronized drug from passive DPIs (Duddu *et al.* 2002). Interestingly, it is suitable not only for indirect systemic delivery of drug molecules via the lungs but also for more efficient and reproducible direct delivery of the drug to the lungs from passive DPIs (Hirst *et al.* 2002).

Spray drying, an extremely rapid particle formation process, can be used to control the polymorphic forms of pharmaceuticals, provided that the operating parameters are carefully optimized. For instance, polymorphic forms (A, B and C) of abecarnil, a *β*-carboline derivative and a partial agonist of CNS benzodiazepine receptor, can be produced by spray dr ying using dif ferent solvents in the feed solutions (Beckmann *et al.* 1996). PXRD and DSC data showed that the resulting polymorphs are free of amorphous regions.

Co-spraying with additives has also been explored to improve the delivery of antiasthmatic drugs to the lung, e.g., salbutamol sulphate with L-leucine in DPI formulations (Lucas *et al.* 1999).

2.2.10.4. Freeze Drying

Freeze drying is a process used to dry extremely heat sensitive materials. It allows the drying without excessive damage, of proteins, blood products and even microorganisms, which retain a small but significant viability. In this process the initial liquid solution or suspension is frozen, the pressure above the frozen state is reduced and the water removed by sublimation. Thus a liquid to vapour transition takes place, but here three states of matter involved: liquid to solid, then solid to vapour. Freeze drying or lyophilization is a drying process where water is removed from a product below the freezing point.

Freeze drying requires a high energy supply at low gas pressure. Rate and temperature of the freezing determines the structure of the frozen product. The primary drying occurs at conditions below the triple point. By freezing at low rates crystals are formed. However, at higher rates or when the crystallization is hampered by e.g. anti-freezing peptides, a glass may form instead, which is a solid having the molecular structure and the energy level of a liquid. Different freeze drying techniques differ mainly in terms of rate of freezing. The freezing rate on a shelf occurs in the range 1-2 K/min. In vials that are submersed into a cooling liquid the freezing rate is about 6 K/min. If liquid nitrogen is used the rate can be as high as 160 K/min. So called shock freezing is obtained by spraying the solution as drops into liquid nitrogen or in contact with solid carbon dioxide by which the cooling rate may reach about 200 K/min. In dynamic freezing the shelf or the container is rotated by which thin layers are obtained.

The sublimation energy of water is about 380 kJ/kg, and the vapour pressure of ice at 0 °C is 6, 11 mbar and at –18 °C it is 1,25 mbar and at –40 °C it is 0,13 mbar. These values explain why freeze drying is a slow process. The requirements for an effective process include low partial pressure (not necessarily low total pressure), a short distance between the product and the condenser surface and sufficient energy transfer to the product. Temperature must be maintained low enough to avoid melting. At very low temperature however the drying vanishes. Strongly bound water therefore has to be removed at higher temperature called the secondary drying.

Stages involved in freeze drying: Freezing stage; vacuum application stage and sublimation stage; secondary drying and packaging.

Limitations of freeze drying include:

- The depression of the **freezing point** caused by the presence of **dissolved solutes** means that the solution must be **cooled below** the normal freezing temperature for pure water (-10-30).
- Sublimation can only occur at the **frozen surface** and is **slow** process (1mm thickness of ice per hour). So, the **surface area** must therefore be **increased** and
- The **liquid thickness** prior to freezing be **reduced** in order to reduce the thickness of ice to be **sublimated.**
- At low pressure **large** volumes of **water vapour** are produced which must be removed to **prevent** the **pressure rising** above the **triple point** pressure.
- The dry material often needs to be **sterile,** and it must also be **prevented** from **regaining** moisture prior to the final packaging.

Advantages of freeze drying include:

- Drying takes place at very low temperatures, so the chemical decomposition, particularly hydrolysis is minimized.
- The solution is frozen occupying the same volume as the original solution, thus , the product is light and porous.
- The porous form of the product gives ready solubility.
- There is no concentration of solution prior to drying. Hence, salts do not concentrate and denature proteins, as occurs with other drying methods.
- As the process takes place under high vacuum there is little contact with air, and oxidation is minimized

Disadvantages include:

- The porosity, ready solubility and complete dryness yield a very hygroscopic product. Unless products are dried in their final container and sealed in situ, packing require special conditions.
- The process is very slow and uses complicated plant, which is very expensive .It is not a general method of drying but limited to certain types of valuable products.

2.2.10.5. Spray Freeze Drying

Spray freeze drying was explored for pharmaceutical applications in early 90s. It is an advanced particle engineering method, which combines spray drying and freeze drying processing steps. SFD process consists of:

1. Atomization of liquid solutions or suspension using ultrasound, one-or two fluid nozzles or vibrating orifice droplet generators
2. Freezing of the droplets in a cryogenic liquid or cryogenic vapour
3. Ice sublimation at low temperature and pressure or alternatively atmospheric freeze-drying using a cold desiccant gas stream

The term spray freeze drying is a general description for different techniques and set ups:

1. Spray freezing into vapour (SFV)
2. Spray freezing into vapour over liquid (SFV/L)
3. Spray freezing into liquid (SFL)

SFV is characterized by atomization of liquid feed into cryogenic gas within flow chamber. SFV/L is characterized by direct atomization of the liquid solution beneath the surface of liquid nitrogen. SFL is characterized by atomization of the liquid solution or suspension into cold gas phase of the evaporation cryogen.
This method produces light and porous particles and high fine particle fraction with improved aerosol performance and almost 100% yield at subambient temperatures. Thermolabile protein and peptide substances, DNA etc can be formulated into DPI products. However, this is an expensive process restricted for only expensive drugs.

Advantages of Spray Freeze Drying include:

•Production of a micronized pharmaceutical powder formulation for dry powder inhalation, controlled release drug delivery systems, parenteral applications and intradermal delivery
•Cooling rates of 103K/s with liquid nitrogen achievable at a droplet size of 10μm
•Rapid cooling rates support the formation of glassy water before the protein undergoes aggregation in the freeze-concentrated solution

•Relaxation processes that lead to denaturation of proteins may be prevented by the formation of a glass
•High process yield (>95%) compared to spray-drying (for SFV/L, SFL)
•Better control of final particle size compared to spray-drying

Limitations:
•Time-consuming, often discontinuous process
•Inconvenient handling of some cryogenic liquids
•Complicated scalability
•Sterility of liquid nitrogen?

In nutshell, Spray-freeze-drying (SFD) in general is a feasible method to produce peptide and protein loaded powders for pulmonary and parenteral applications. Different SFD methods have been developed for powder production. The right choices of the atomization and liquid delivery system as well as the right atomization conditions have a strong impact on the final product quality. Spray-frozen solution behave different to regular frozen solutions and need under the same primary drying condition a longer drying time. Peptides and proteins suffer stress during atomization that can be reduced by addition of suitable excipients to the formulation, like sugars or interfacial active substances. SFV/L has shown to be a suitable method to produce powders for ballistic protein and vaccine delivery.

2.3 Regulatory requirements (US) for dry powder inhalers

DPIs are complex drug products and are different in many aspects from more conventional drug products as well as from MDIs. The main characteristics of DPIs should be considered during development, particularly with respect to formulation, manufacturing, container and closure system and both in-process and final controls.

A. Active Ingredient

Information regarding the comprehensive characterization of the physical and chemical properties of the drug substance to be used in inhalation drug products should be included in the application to FDA for DPI product. Important properties of the drug substance may include, density, solvates and hydrates, clathrates, morphic forms particle size distribution, particle morphology, amorphous forms, solubility

profile, microbial quality, moisture and/or residual solvent content, pH profile and pKa(s), and specific rotation.

Appropriate acceptance criteria and tests should be instituted to control those drug substance parameters considered key to ensuring reproducibility of the physicochemical properties of the drug substance. Specifications for control of particle size distribution and crystalline forms (e.g., shape, texture, surface) of the drug substance, parameters often critical for reproducible drug product performance, should be included in the application.

The purity of the drug substance and its impurity profile should be controlled and characterized with appropriate specifications. Important impurity-related parameters may include organic volatile impurities, residual solvents, heavy metals, residual organics, related substances, and inorganics (e.g., reagents, catalysts), (synthetic and degradants). Relative to parent drug any recurring impurity found in the drug substance at a concentration of 0.1 percent or greater should be identified and qualified. In addition to toxicological considerations, justification of acceptance criteria for the drug substance impurities should be based on levels of impurities found in the submitted batches.

B. Excipients

Excipients (when used) comprise a major portion of the formulation content by weight and their quality has a substantial effect on the safety, stability, performance, quality, and effectiveness of such drug products. The source of each excipient should be identified in the application. Each source should be assessed, and the material supplied should meet appropriate acceptance criteria based on test results for several batches of excipients that were used in preparing the submitted batches of drug product (e.g., clinical, biobatch, production, primary stability).

Adequate DMFs with appropriate authorization should be submitted to the agency for major (e.g., propellant, carriers) and noncompendial excipients. A full description of the acceptance criteria and the test methods used to ensure the identity, assay, purity, functionality, and quality of each excipient should be submitted. If these materials are accepted based upon certificates of analysis from the manufacturers with a specific identification test, the applicant should also develop validated methods to all of the manufacturer's analytical and other test methods to allow the applicant to verify the reliability of the test results at appropriate intervals.

The suitability of excipients to be administered by the inhalation route should be thoroughly investigated and documented in terms of the physicochemical properties. Toxicological qualification of these excipients may be important under various circumstances including (1) increased concentration of an excipient above that previously used in inhalation drug products, (2) excipients used previously in humans but not by the inhalation route, and (3) novel excipients not previously used in humans.

C. Specifications for Finished Drug Product

The following test parameters are recommended for DPI drug products. appropriate acceptance criteria and validated test methods should be established for each test parameter.

1. Appearance and Color
2. Identification
3. Microbial Limits
4. Water or Moisture Content
5. Net Content (Fill) Weight (Device-metered)
6. Drug Content (Assay)
7. Impurities and Degradation Products
8. Dose Content Uniformity
9. Dose Content Uniformity Through Container Life (device-metered)
10. Particle Size Distribution of Emitted Dose
11. Microscopic Evaluation

D. Device

The clinical efficacy of a DPI drug product may be directly dependent on the design, performance and reproducibility of the container and closure system. The container and closure system consists of the overall device with all primary and protective packaging (e.g., overwrap). The design, composition, and quality control of the individual components of the container and the closure are key to maintaining the chemical and physical stability of the formulation and ensuring that the performance characteristics of the drug product are reproducible and in accord with label claim.

During development and before initiating critical clinical studies, the performance characteristics of the device and its compatibility with the formulation

should be thoroughly investigated. A properly performing DPI should deliver accurate, small doses of the drug substance in the desired physical form through the life of the device. From a clinical perspective, it is also recommended that a mechanism that would prevent unintentional multiple dosing be included. If used, these mechanisms should be described in the application.

The drug application should include the following specific information for device components:

1. Source(s) and fabricator(s) of the overall device
2. Source(s) and fabricator(s) for each part of the container and closure system
3. Item number(s) for each component
4. Schematic engineering drawings
5. Dimensional measurements
6. Composition and quality of materials
7. Device flow resistance
8. Controlled extraction studies
9. Toxicological evaluation of extractables

E. Drug Product Characterization Studies

1. Determination of Appropriate Storage Conditions
2. Stability of Primary (Unprotected) Package
3. Effect of Varying Flow Rates
4. Effect of Storage on the Particle Size Distribution
5. Dose Buildup and Flow Resistance
6. Effect of Orientation
7. In Vitro Dose Proportionality
8. Effect of Patient Use
9. Effect of Moisture
10. Photostability
11. Profiling of Doses Near Device Exhaustion
12. Priming
13. Fill Weight
14. Device Ruggedness
15. Cleaning Instructions

Besides the above mentioned guidance, stability and labeling considerations also need to be considered while submitting an application for DPI products.
(*Source: Guidance For Industry; USFDA, CDER 1998*).

2.4 Marketed DPIs:

A large number of dry powder inhalation products have got US FDA approval since 1997. Glaxo Smithkline and Novartis pharmaceuticals are the major players in US. Most of these products have application in respiratory disorders like asthma, COPD and respiratory distress. Dry powder formulations can be administered by the use of inhalation devices and thus all DPI formulations are marketed alongwith the delivery device. India has a market of around more than 250 DPI products majorly of respiratory disorders. Front-runner in the pulmonary field are Unique Pharma with approval of Ifiral (chromolyn sodium) in 1976 and leaders with many DPI products in market are Cipla, Ranbaxy, Dr. Reddy's, Ajanta Pharma, Glen mark, German Remedies and Lupin Pharma.

Table 2.11 Commercially available DPIs in US Market

(*Source: Newport Database*)

Tradename	Applicant	Active ingredient	Strength
Advair Diskus 100/50	Glaxosmithkline pharmaceuticals	Fluticasone propionate| salmeterol xinafoate	0.1mg/inh; eq 0.05mg base/inh
Advair Diskus 250/50	Glaxosmithkline pharmaceuticals	Fluticasone propionate| salmeterol xinafoate	0.25mg/inh;eq 0.05mg base/inh
Advair Diskus 500/50	Glaxosmithkline pharmaceuticals	Fluticasone propionate| salmeterol xinafoate	0.5mg/inh; eq 0.05mg base/inh
Arcapta Neohaler	Novartis pharmaceuticals	Indacaterol maleate	Eq 75mcg base
Aridol kit	Pharmaxis	Mannitol	5mg,10mg,20mg,40mg
Asmanex Twisthaler	Schering-plough corporation	Mometasone furoate	0.22mg/inh
Asmanex Twisthaler	Schering-plough corporation	Mometasone furoate	0.11mg/inh
Flovent Diskus 100	Glaxosmithkline pharmaceuticals	Fluticasone propionate	0.1mg/inh
Flovent Diskus 250	Glaxosmithkline pharmaceuticals	Fluticasone propionate	0.25mg/inh
Flovent Diskus 50	Glaxosmithkline pharmaceuticals	Fluticasone propionate	0.05mg/inh
Foradil	Novartis pharmaceuticals	Formoterol fumarate	0.012mg/inh
Pulmicort Flexhaler	Astrazeneca pharmaceuticals	Budesonide	0.08mg/inh
Pulmicort Flexhaler	Astrazeneca pharmaceuticals	Budesonide	0.16mg/inh
Relenza	Glaxosmithkline pharmaceuticals	Zanamivir	5mg
Serevent	Glaxosmithkline pharmaceuticals	Salmeterol xinafoate	Eq 0.046mg base/inh
Spiriva	Boehringer ingelheim pharmaceuticals inc	Tiotropium bromide	Eq 0.018 base/inh
Tudorza Pressair	Forest laboratories inc	Aclidinium bromide	0.375mg/inh

Table 2.12: Commercially Available DPIs in India market

(*Source: Newport Database*)

Marketed by	Active ingredient	Trade name	Strength
Ajanta pharma	Formoterol fumarate| mometasone furoate	Velcera	Caps 200µg /6µg 30
Ajanta pharma	Formoterol fumarate| mometasone furoate	Velcera	Caps 400µg/6µg 30
Blue cross	Camphor| eucalyptus oil| menthol| chlorothymol	Kolo	Caps inhal 10
Cadila pharma	Camphor|eucalyptus oil|menthol|turpentine|chlorothym	Genvol plus	Caps 10
Cipla	Formoterol fumarate|ciclesonide	Simplyone	100mg/6µg
Cipla	Formoterol fumarate|ciclesonide	Simplyone	200mg/6µg
Cipla	Cromolyn sodium	Cromal	20mg
Cipla	Formoterol fumarate|ciclesonide	Simplyone	400mg/6µg
Cipla	Zanamivir	Virenza	5mg
Cipla	Terpineol (alpha)| camphor|eucalyptus	Easibreathe	Caps 10
Cipla	Budesonide|formoterol fumarate	Foracort	Respicaps ft 400µg /12µg 30
Cipla	Beclomethasone dipropionate	Beclate	Rotac+rotaha 100µg 30
Cipla	Beclomethasone dipropionate|albuterol sulfate	Aerocort-old	Rotac+rotaha 200µg /100 30
Cipla	Albuterol sulfate	Asthalin	Rotac+rotaha 200µg 30
Cipla	Beclomethasone dipropionate	Beclate	Rotac+rotaha 200µg 30
Cipla	Albuterol sulfate	Asthalin	Rotac+rotaha 400µg 30
Cipla	Ipratropium bromide	Ipravent	Rotac+rotaha 40µg 30

Table 2.12: Commercially Available DPIs in India market (continued….)

Marketed by	Active ingredient	Trade name	Strength
Cipla	Salmeterol xinafoate	Serobid	Rotac+rotaha 50µg 30
Cipla	Beclomethasone dipropionate|levalbuterol	Aerocort	Rotacaps 100µg /100 30
Cipla	Ipratropium bromide|levalbuterol hydrochloride	Combolin-dp	Rotacaps 100µg /40µg 10
Cipla	Ipratropium bromide|levalbuterol hydrochloride	Duoset	Rotacaps 100µg /40µg 10
Cipla	Ipratropium bromide|levalbuterol sulfate	Duolin	Rotacaps 100µg /40µg 30
Cipla	Budesonide|formoterol fumarate	Foracort	Rotacaps 100µg /6µg 30
Cipla	Fluticasone propionate|formoterol fumarate	Maxiflo	Rotacaps 100µg /6µg 30
Cipla	Beclomethasone dipropionate	Beclate	Rotacaps 100µg 30
Cipla	Budesonide	Budecort	Rotacaps 100µg 30
Cipla	Fluticasone propionate	Flohale	Rotacaps 100µg 30
Cipla	Levalbuterol sulfate	Levolin	Rotacaps 100µg 30
Cipla	Formoterol fumarate	Foratec-old	Rotacaps 12µg 30
Cipla	Formoterol fumarate|ciclesonide|tiotropium	Triohale	Rotacaps 15
Cipla	Formoterol fumarate|tiotropium bromide	Duova	Rotacaps 18µg /12µg 15
Cipla	Tiotropium bromide	Tiova	Rotacaps 18µg 15
Cipla	Albuterol|beclomethasone dipropionate	Aerovent dp	Rotacaps 200µg /100 10
Cipla	Beclomethasone dipropionate|albuterol sulfate	Aerocort-old	Rotacaps 200µg /100 30
Cipla	Ipratropium bromide|levalbuterol hydrochloride	Duoset	Rotacaps 200µg /40µg 30
Cipla	Beclomethasone dipropionate|formoterol fumarate	Bekform	Rotacaps 200µg /6µg 10
Cipla	Formoterol fumarate|mometasone furoate	Evocort	Rotacaps 200µg /6µg 30

Table 2.12: Commercially Available DPIs in India market (continued….)

Marketed by	Active ingredient	Trade name	Strength
Cipla	Budesonide|formoterol fumarate	Foracort	Rotacaps 200µg /6µg 30
Cipla	Albuterol	Asthavent dp	Rotacaps 200µg 10
Cipla	Albuterol sulfate	Asthalin	Rotacaps 200µg 30
Cipla	Beclomethasone dipropionate	Beclate	Rotacaps 200µg 30
Cipla	Budesonide	Budecort	Rotacaps 200µg 30
Cipla	Ciclesonide	Ciclohale	Rotacaps 200µg 30
Cipla	Fluticasone propionate|formoterol fumarate	Maxiflo	Rotacaps 250µg /6µg 30
Cipla	Fluticasone propionate	Flohale	Rotacaps 250µg 30
Cipla	Beclomethasone dipropionate|albuterol sulfate	Aerocort-old	Rotacaps 400µg /200 30
Cipla	Beclomethasone dipropionate|formoterol fumarate	Bekform	Rotacaps 400µg /6µg 10
Cipla	Formoterol fumarate|mometasone furoate	Evocort	Rotacaps 400µg /6µg 30
Cipla	Budesonide|formoterol fumarate	Foracort	Rotacaps 400µg /6µg 30
Cipla	Beclomethasone dipropionate|formoterol fumarate	Fullform	Rotacaps 400µg /6µg 30
Cipla	Albuterol sulfate	Asthalin	Rotacaps 400µg 30
Cipla	Beclomethasone dipropionate	Beclate	Rotacaps 400µg 30
Cipla	Budesonide	Budecort	Rotacaps 400µg 30
Cipla	Ciclesonide	Ciclohale	Rotacaps 400µg 30
Cipla	Ipratropium bromide|albuterol sulfate	Duolin-old	Rotacaps 40µg /200 30
Cipla	Fluticasone propionate|salmeterol xinafoate	Seroflo	Rotacaps 50µg /100 30
Cipla	Fluticasone propionate|salmeterol xinafoate	Seroflo	Rotacaps 50µg /250 30

Table 2.12: Commercially Available DPIs in India market (continued….)

Marketed by	Active ingredient	Trade name	Strength
Cipla	Fluticasone propionate|salmeterol xinafoate	Seroflo	Rotacaps 50µg /500 30
Cipla	Fluticasone propionate	Flohale	Rotacaps 50µg 30
Cipla	Beclomethasone dipropionate|levalbuterol	Aerocort	Rotacaps ft 200µg /200 30
Cipla	Fluticasone propionate|formoterol fumarate	Maxiflo	Rotacaps ft 500µg /12µg 30
Delvin formulation	Sodium bicarbonate|sodium chloride	Rhinosaline	2300mg/700
Ranbaxy	Albuterol sulfate	Rheolin	Caps 200µg 28
Ranbaxy	Salmeterol xinafoate|fluticasone	Rheoran-sf	Octacaps 100µg /50µg 30
Ranbaxy	Fluticasone propionate|formoterol fumarate	Avessa	Octacaps 100µg /6µg 30
Ranbaxy	Budesonide|formoterol fumarate	Symbiva	Octacaps 100µg /6µg 30
Ranbaxy	Budesonide|formoterol fumarate	Symbiva	Octacaps 200µg /6µg 30
Ranbaxy	Salmeterol xinafoate|fluticasone	Rheoran-sf	Octacaps 250µg /50µg 30
Ranbaxy	Fluticasone propionate|formoterol fumarate	Avessa	Octacaps 250µg /6µg 30
Ranbaxy	Budesonide|formoterol fumarate	Symbiva	Octacaps 400µg /6µg 30
Ranbaxy	Salmeterol xinafoate|fluticasone	Rheoran-sf	Octacaps 500µg /50µg 30
Ranbaxy	Fluticasone propionate|formoterol fumarate	Avessa	Octacaps 500µg /6µg 30
Ranbaxy	Fluticasone propionate|salmeterol xinafoate	Rheoran-sf	Respicaps 100µg /50µg 14
Ranbaxy	Fluticasone propionate|salmeterol xinafoate	Rheoran-sf	Respicaps 100µg /50µg 14 x2
Ranbaxy	Budesonide|formoterol fumarate	Symbiva	Respicaps 100µg /6µg 14
Ranbaxy	Fluticasone propionate	Rheoran-f	Respicaps 100µg 14
Ranbaxy	Formoterol fumarate|ciclesonide|tiotropium	Zovair	Respicaps 160µg 14

Table 2.12: Commercially Available DPIs in India market (continued….)

Marketed by	Active ingredient	Trade name	Strength
Ranbaxy	Ciclesonide	Osonide	Respicaps 160µg 28
Ranbaxy	Formoterol fumarate|tiotropium bromide	Tiomax	Respicaps 18µg /12µg 14
Ranbaxy	Tiotropium bromide	Siyo-pd	Respicaps 18µg 14
Ranbaxy	Fluticasone propionate|salmeterol xinafoate	Rheoran-sf	Respicaps 250µg /50µg 14
Ranbaxy	Fluticasone propionate|salmeterol xinafoate	Rheoran-sf	Respicaps 250µg /50µg 14 x2
Ranbaxy	Fluticasone propionate	Rheoran-f	Respicaps 250µg 14
Ranbaxy	Formoterol fumarate|ciclesonide|tiotropium	Zovair	Respicaps 320µg 14
Ranbaxy	Ciclesonide	Osonide	Respicaps 320µg 28
Ranbaxy	Budesonide|formoterol fumarate	Symbiva	Respicaps 400µg /6µg 14
Ranbaxy	Fluticasone propionate|salmeterol xinafoate	Rheoran-sf	Respicaps 500µg /50µg 14
Ranbaxy	Fluticasone propionate|salmeterol xinafoate	Rheoran-sf	Respicaps 500µg /50µg 14 x2
Ranbaxy	Fluticasone propionate|formoterol fumarate	Avessa	Rotacaps 100µg /6µg 14
Ranbaxy	Formoterol fumarate|ciclesonide	Osovair	Rotacaps 160µg /6µg 14
Ranbaxy	Formoterol fumarate|ciclesonide|tiotropium	Zovair	Rotacaps 160µg 30
Ranbaxy	Fluticasone propionate|formoterol fumarate	Avessa	Rotacaps 250µg /6µg 14
Ranbaxy	Formoterol fumarate|ciclesonide	Osovair	Rotacaps 320µg /12µg 14
Ranbaxy	Formoterol fumarate|ciclesonide|tiotropium	Zovair	Rotacaps 320µg 30
Ranbaxy	Fluticasone propionate|formoterol fumarate	Avessa	Rotacaps 500µg /6µg 14
Dr reddys labs	Budesonide	Solbihale-b	Caps 200µg 30
Dr reddys labs	Budesonide	Solbihale-b	Caps 400µg 30

Table 2.12: Commercially Available DPIs in India market (continued….)

Marketed by	Active ingredient	Trade name	Strength
Dr reddys labs	Budesonide|formoterol fumarate	Combihale-fb	Respicaps 100µg /6µg 15
Dr reddys labs	Fluticasone propionate|formoterol fumarate	Combihale-ff	Respicaps 100µg /6µg 15
Dr reddys labs	Budesonide|formoterol fumarate	Combihale-fb	Respicaps 100µg /6µg 30
Dr reddys labs	Fluticasone propionate|formoterol fumarate	Combihale-ff	Respicaps 100µg /6µg 30
Dr reddys labs	Budesonide|formoterol fumarate	Combihale-fb	Respicaps 200µg /6µg 15
Dr reddys labs	Budesonide|formoterol fumarate	Combihale-fb	Respicaps 200µg /6µg 30
Dr reddys labs	Fluticasone propionate|formoterol fumarate	Combihale-ff	Respicaps 250µg /6µg 15
Dr reddys labs	Fluticasone propionate|formoterol fumarate	Combihale-ff	Respicaps 250µg /6µg 30
Dr reddys labs	Budesonide|formoterol fumarate	Combihale-fb	Respicaps 400µg /6µg 15
Dr reddys labs	Budesonide|formoterol fumarate	Combihale-fb	Respicaps 400µg /6µg 30
Dr reddys labs	Fluticasone propionate|formoterol fumarate	Combihale-ff	Respicaps 500µg /6µg 15
Dr reddys labs	Fluticasone propionate|formoterol fumarate	Combihale-ff	Respicaps 500µg /6µg 30
Dr reddys labs	Tiotropium bromide	Solbihale-t	Rotacaps 18µg 15
Emcure	Terpineol (alpha)| camphor|chlorothymol	Vifex	Caps 10
Emcure	Fluticasone propionate|salmeterol xinafoate	Fluticure	Caps 100µg /50µg 30
Emcure	Budesonide|formoterol fumarate	Forsmart	Caps 200µg /6µg 30
Emcure	Budesonide|formoterol fumarate	Forsmart	Caps 200µg /6µg 60
Emcure	Fluticasone propionate|salmeterol xinafoate	Fluticure	Caps 250µg /50µg 30
Emcure	Budesonide|formoterol fumarate	Forsmart	Caps 400µg /6µg 30
Emcure	Budesonide|formoterol fumarate	Forsmart	Caps 400µg /6µg 60

Table 2.12: Commercially Available DPIs in India market (continued….)

Marketed by	Active ingredient	Trade name	Strength
Glenmark pharma	Fluticasone propionate|salmeterol xinafoate	Airtec-sf	Caps 100µg /50µg 30
Glenmark pharma	Budesonide|formoterol fumarate	Airtec-fb	Caps 100µg /6µg 30
Glenmark pharma	Budesonide|formoterol fumarate	Airtec-fb	Caps 200µg /6µg 30
Glenmark pharma	Fluticasone propionate|salmeterol xinafoate	Airtec-sf	Caps 250µg /50µg 30
Glenmark pharma	Budesonide|formoterol fumarate	Airtec-fb	Caps 400µg /6µg 30
Glenmark pharma	Camphor|peppermint oil	Sensur	Softules 10
Ind-swift	Terpineol (alpha-)|camphor|eucalyptus	Indol plus	Caps 10
Intas	Salmeterol xinafoate|fluticasone	Quikhale sf	Respicaps 100µg /50µg 40
Intas	Budesonide|formoterol fumarate	Quikhale fb	Respicaps 100µg /6µg 40
Intas	Fluticasone propionate|formoterol fumarate	Quikhale ff	Respicaps 100µg /6µg 40
Intas	Tiotropium bromide	Quikhale-t	Respicaps 18µg 15
Intas	Budesonide|formoterol fumarate	Quikhale fb	Respicaps 200µg /6µg 40
Intas	Salmeterol xinafoate|fluticasone	Quikhale sf	Respicaps 250µg /50µg 40
Intas	Fluticasone propionate|formoterol fumarate	Quikhale ff	Respicaps 250µg /6µg 40
Intas	Budesonide|formoterol fumarate	Quikhale fb	Respicaps 400µg /6µg 40
Intas	Salmeterol xinafoate|fluticasone	Quikhale sf	Respicaps 500µg /50µg 40
Unique pharm	Cromolyn sodium	Ifiral	20mg
Kopran	Budesonide	Budvent	Caps 100µg 20
Kopran	Budesonide	Budvent	Caps 100µg 30
Kopran	Beclomethasone dipropionate	Bevent	Caps 200µg 20

Table 2.12: Commercially Available DPIs in India market (continued….)

Marketed by	Active ingredient	Trade name	Strength
Kopran	Budesonide	Budvent	Caps 200µg 20
Kopran	Budesonide	Budvent	Caps 200µg 30
Kopran	Budesonide	Budvent	Caps 400µg 30
Kopran	Beclomethasone dipropionate	Bevent	Rotacaps 100µg 20
Kopran	Salmeterol xinafoate	Salvent	Rotacaps 50µg 30
Lupin limited	Ipratropium bromide|levalbuterol hydrochloride	Salbair-i	Respicaps 100µg /40µg 30
Lupin limited	Budesonide|formoterol fumarate	Budamate	Respicaps 100µg /6µg 30
Lupin limited	Fluticasone propionate|formoterol fumarate	Formoflo	Respicaps 100µg /6µg 30
Lupin limited	Budesonide	Budate	Respicaps 100µg 30
Lupin limited	Albuterol	Salbair	Respicaps 100µg 30
Lupin limited	Albuterol|beclomethasone dipropionate	Salbair-b	Respicaps 200µg /100 30
Lupin limited	Albuterol|ipratropium bromide	Salbair-i	Respicaps 200µg /40µg 30
Lupin limited	Beclomethasone dipropionate|formoterol fumarate	Duomate	Respicaps 200µg /6µg 20
Lupin limited	Budesonide|formoterol fumarate	Budamate	Respicaps 200µg /6µg 30
Lupin limited	Budesonide	Budate	Respicaps 200µg 30
Lupin limited	Albuterol	Salbair	Respicaps 200µg 30
Lupin limited	Fluticasone propionate|salmeterol xinafoate	Esiflo	Respicaps 250µg /50µg 30
Lupin limited	Fluticasone propionate|formoterol fumarate	Formoflo	Respicaps 250µg /6µg 30
Lupin limited	Budesonide|formoterol fumarate	Budamate	Respicaps 400µg /6µg 30
Lupin limited	Budesonide	Budate	Respicaps 400µg 30

Table 2.12: Commercially Available DPIs in India market (continued….)

Marketed by	Active ingredient	Trade name	Strength
Lupin limited	Fluticasone propionate|salmeterol xinafoate	Esiflo	Respicaps 500µg /50µg 30
Lupin limited	Albuterol|beclomethasone dipropionate	Salbair-b	Respicaps ft 200µg /400 30
Lupin limited	Beclomethasone dipropionate|formoterol fumarate	Duomate	Respicaps ft 400µg /6µg 20
Lupin limited	Formoterol fumarate|tiotropium bromide	Tiomate	Rotacaps 18µg /12µg 15
Lupin limited	Tiotropium bromide	Tiate	Rotacaps 18µg 15
Lupin limited	Formoterol fumarate|ciclesonide	Ciclomate	Transcaps 160µg /6µg 30
Lupin limited	Formoterol fumarate|ciclesonide	Ciclomate	Transcaps 320µg /6µg 30
Macleods	Fluticasone propionate|formoterol fumarate	Fluticort-f	Caps 250µg /6µg 30
Macleods	Formoterol fumarate|tiotropium bromide	Aerotrop-f	Caps inhal 15
Macleods	Fluticasone propionate|salmeterol xinafoate	Flutrol	Respicaps 100µg /50µg 30
Macleods	Budesonide|formoterol fumarate	Budetrol	Respicaps 100µg /6µg 30
Macleods	Levalbuterol hydrochloride	Aerozest	Respicaps 100µg 30
Macleods	Budesonide|formoterol fumarate	Budetrol	Respicaps 200µg /6µg 30
Macleods	Fluticasone propionate|salmeterol xinafoate	Flutrol	Respicaps 250µg /50µg 30
Macleods	Budesonide|formoterol fumarate	Budetrol	Respicaps 400µg /6µg 30
Macleods	Fluticasone propionate|salmeterol xinafoate	Flutrol	Respicaps 500µg /50µg 30
Macleods	Budesonide	Bunase	Rotacaps 100µg 30
Macleods	Tiotropium bromide	Aerotrop	Rotacaps 18µg 15
Macleods	Budesonide	Bunase	Rotacaps 200µg 30
Macleods	Budesonide	Bunase	Rotacaps 400µg 30

Table 2.12: Commercially Available DPIs in India market (continued….)

Marketed by	Active ingredient	Trade name	Strength
Maneesh healthcare	Camphor|eucalyptus oil|chlorothymol	Calypso	Caps inhal 10
Maneesh healthcare	Terpineol (alpha-)|camphor|eucalyptus	Retherma-c	Caps inhal 10
Merck limited	Albuterol sulfate	Vent	200mg
Merck limited	Salmeterol xinafoate|fluticasone	Vent-sf	Caps 100µg /200 20
Merck limited	Fluticasone propionate	Ventiflo	Caps 100µg 20
Merck limited	Beclomethasone dipropionate|albuterol sulfate	Vent plus	Caps 200µg /100 30
Merck limited	Beclomethasone dipropionate|albuterol sulfate	Vent plus	Caps 400µg /200 30
Merck limited	Salmeterol xinafoate|fluticasone	Vent-sf	Caps 50µg /100 20
Merck limited	Terpineol (alpha)| camphor|eucalyptus	Aerway	Caps inhal 10
Merck limited	Budesonide|formoterol fumarate	Vent-fb	Respicaps 100µg /6µg 30
Merck limited	Budesonide|formoterol fumarate	Vent-fb	Respicaps 200µg /6µg 30
Merck limited	Budesonide|formoterol fumarate	Vent-fb	Respicaps 400µg /6µg 30
Merck limited	Beclomethasone dipropionate|albuterol sulfate	Vent bec	Rotacaps 200µg /100 30
Merck limited	Ipratropium bromide|albuterol sulfate	Ventipra	Rotacaps 200µg /40µg 30
Merck limited	Salmeterol xinafoate|fluticasone	Vent-sf	Rotacaps 250µg /50 20
Merck limited	Fluticasone propionate	Ventiflo	Rotacaps 250µg 20
Merck limited	Beclomethasone dipropionate|albuterol sulfate	Vent bec	Rotacaps 400µg /200 30
Merck limited	Salmeterol xinafoate|fluticasone	Vent-sf	Rotacaps 500µg /50µg 30
Merck limited	Salmeterol xinafoate|fluticasone	Vent-sf	Rotacaps 50µg /100 30
Merck limited	Salmeterol xinafoate|fluticasone	Vent-sf	Rotacaps 50µg /250 30

Table 2.12: Commercially Available DPIs in India market (continued….)

Marketed by	Active ingredient	Trade name	Strength
Micro labs	Terpineol (alpha-)|camphor|eucalyptus	Rhinosules	Caps 10
Novartis	Indacaterol	Onbrez	Respicaps 150µg 30
Novartis	Indacaterol	Onbrez	Respicaps 300µg 30
Wockhardt	Terpineol (alpha)| camphor|eucalyptus	Inhaline	Caps inhal 10
German remedies	Beclomethasone dipropionate|albuterol sulfate	Derisone	Respicaps 100µg /200 30
German remedies	Fluticasone propionate|salmeterol xinafoate	Forair	Respicaps 100µg /50µg 30
German remedies	Budesonide|formoterol fumarate	Formonide	Respicaps 100µg /6µg 30
German remedies	Fluticasone propionate|formoterol fumarate	Formosone	Respicaps 100µg /6µg 30
German remedies	Formoterol fumarate|mometasone furoate	Formost	Respicaps 100µg /6µg 30
German remedies	Formoterol fumarate	Deriform-old	Respicaps 12µg 30
German remedies	Formoterol fumarate|tiotropium bromide	Tioform	Respicaps 18µg /12µg 15
German remedies	Tiotropium bromide	Tiomist	Respicaps 18µg 15
German remedies	Beclomethasone dipropionate|albuterol sulfate	Derisone	Respicaps 200µg /400 30
German remedies	Ipratropium bromide|albuterol sulfate	Combimist	Respicaps 200µg /40µg 30
German remedies	Budesonide|formoterol fumarate	Formonide	Respicaps 200µg /6µg 30
German remedies	Formoterol fumarate|mometasone furoate	Formost	Respicaps 200µg /6µg 30
German remedies	Albuterol sulfate	Derihaler	Respicaps 200µg 30
German remedies	Budesonide	Derinide	Respicaps 200µg 30
German remedies	Fluticasone propionate|salmeterol xinafoate	Forair	Respicaps 250µg /50µg 30
German remedies	Fluticasone propionate|formoterol fumarate	Formosone	Respicaps 250µg /6µg 30

Table 2.12: Commercially Available DPIs in India market (continued….)

Marketed by	Active ingredient	Trade name	Strength
German remedies	Budesonide|formoterol fumarate	Formonide	Respicaps 400µg /6µg 30
German remedies	Formoterol fumarate|mometasone furoate	Formost	Respicaps 400µg /6µg 30
German remedies	Budesonide	Derinide	Respicaps 400µg 30
German remedies	Ipratropium bromide	Ipramist	Respicaps 40µg 30
German remedies	Fluticasone propionate|salmeterol xinafoate	Forair	Respicaps 500µg /50µg 30
German remedies	Budesonide|formoterol fumarate	Formonide	Respicaps ft 400µg /12µg 30

2.5 Patent review on inhalable formulation for pulmonary arterial hypertension

Inhalation therapy provides clinical efficacy by providing direct access to the lungs while minimizing the systemic side effects related to other routes of administration. Lot of studies have explored the therapeutic potential of drugs used for PAH through pulmonary route. Wayman *et al.*, have described compositions comprising diketopiperazine (DKP) salts of PDE5 inhibitors and DKP microparticles associated with PDE5 inhibitors to treat pulmonary hypertension and sexual dysfunction. Sterile solution of sildenafil mesylate was prepared by Ghazwan *et al.*, for nebulization to treat PAH. Beume *et al.*, prepared Roflumilast and its salts, administered as aerosol for the preventive or curative treatment of PH. In case of prostanoids Imtiaz chaudry prepared a solution or suspension of epoprostenol, suitable for administration via nebulization. Curtis Ruegg developed microparticles containing iloprost for the treatment of PAH.

Olschewski *et al.*, have performed open label study to determine safety, tolerability and hemodynamic effects of inhaled treprostinil. David *et al.* demonstrated a method of delivering nitric oxide to a mammal. Mazhari *et al.* invented the methods of treating, preventing or delaying the onset or development of PH using hydroxyl donors or pharmaceutically acceptable salts. Recently, interest has also been shown in combination Therapy due to complex pathophysiology of PAH**.** Patients, who do not respond to monotherapy, may be consequently treated with

combination therapeutics. Various combination therapies like fasudil and sildenafil dry powder formulation; inhaled iloprost with oral sildenafil; and prostacyclin with at least one additional agent from the category of an endothelin receptor antagonist, a PDE inhibitor, and a calcium channel blocker have been explored. Modified Therapies like use of other drugs directed at molecular pathways of the disease is being explored. Etienne and Bettina invented the treatment or prevention of PAH by pulmonary administration of dehydroepiandrosterone (DHEA). Schwarz treated PH by inhibiting endothelial monocyte activating polypeptide II.

Gene Therapy was accessed by Stewart and was capable of reversing or preventing the progression of established PH by transfer of VEGF gene. White *et al.*, described method of treating PH by administering therapeutic agent that inhibits tissue factor activity. A summary of inhalable formulation patents for PAH is shown in **Table 2.13.**

Table 2.13: Inhalable formulation patents for Pulmonary Arterial Hypertension

S. No.	Patent No. / Publication No. (Assignee)	Expiry	Disclosure
1.	**US 5,554,610** (Beecham)	Sep. 10 2013	MoU- disorders associated with pulmonary hypertension or right heart failure in mammals by administering to the mammal a therapeutically effective amount of a vasodilator selected from the group consisting of ganglion blockers, sympathetic nerve blockers, and direct vasodilators, wherein said vasodilator is administered to said mammal by inhaling.
2.	**US 5,968,911** (Trustees of Columbia Uni.)	Oct. 19, 2016	A method of selectively decreasing pulmonary vascular resistance in a subject having a pulmonary condition by administering endotracheally or endobronchially to a subject an effective amount of a drug selected from the group consisting of cyclic nucleotides, phosphodiesterase inhibitors, nitric oxide precursors, donors and analogs in an aerosol form thereby selectively decreasing pulmonary vascular resistance.
3.	**US 7,550,133** (Alexza Pharma)	Nov. 03, 2023	A composition and a kit for delivery of a drug, the compostion comprising a condensation aerosol a) wherein the condensation aerosol is formed by heating a thin film of a drug composition to produce a vapor, and condensing the vapor to form a condensation aerosol comprising the drug, b) wherein the condensation aerosol comprises particles that are characterized by less than 10% drug degradation products by weight, c) wherein the condensation aerosol has an MMAD of less than 5 microns, and d) wherein the drug is selected from a list as claimed.
4.	**US20110223116** (Penn-Century)	Mar. 09, 2030	An apparatus comprising: a reservoir in fluid communication with an outlet channel; a low pressure pump inlet in fluid communication with the outlet channel; a high pressure pump in fluid communication with the low pressure pump inlet; a high pressure pump outlet in fluid communication with the high pressure pump; a switching valve in fluid communication with the high pressure pump outlet, said switching valve having a delivery outlet and a release outlet; an aerosolizer in fluid communication with the delivery outlet; and a restrictor in fluid communication with the release outlet.
5.	**US20060099269** (MannKind Corp)	Aug 23, 2025	A composition comprising a diketopiperazine salt of a phosphodiesterase type 5 (PDE5) inhibitor for the treatment of sexual dysfunction and pulmonary hypertension.
6.	**US20090239883** (Pfizer)	Oct 20, 2020	A method of treating or preventing pulmonary hypertension in a patient which comprises treating the patient with an effective amount of a PDE5 inhibitor.

Table 2.13: Inhalable formulation patents for Pulmonary Arterial Hypertension (continued…..)

S. No.	Patent No. / Publication No. (Assignee)	Expiry	Disclosure
7.	**US20090215836** (Nycomed)	Apr. 12, 2026	A composition comprising a first amount of a compound selected from the group consisting of Roflumilast or Roflumilast-N-Oxide or salt thereof, and a second amount of a PDE5 inhibitor, wherein the first amount and the second amount together comprise an effective amount for the preventive or curative treatment of pulmonary hypertension, and at least one pharmaceutically acceptable auxiliary/ MoU- preventive or curative treatment of pulmonary hypertension by administering an effective amount of Roflumilast or Roflumilast-N-Oxide or salt thereof.
8.	**US20040265238 & US20060104913** (Chaudry et al.)	Jun 27, 2023 Jun 18, 2024	An inhalable formulation for the treatment of pulmonary hypertension comprising a therapeutically effective amount of a hypertension reducing agent, wherein said pulmonary hypertension reducing agent is at least one of an ACEI, ARB, beta-blocker, calcium-channel blocker or vasodilator and wherein said formulation is suitable for administration via inhalation to a mammal in need thereof/ MoU- pulmonary hypertension/ Kit
9.	**US20060147520** (Ruegg et al.)	Jul 26, 2025	A composition comprising a solid dose delivery system comprising a vehicle and an effective amount of iloprost wherein the vehicle comprises a hydrophobic derivatized carbohydrate/ MoU- PH/ Inhalation device
10.	**US20080200449** (United Therapeutics Corp.)	May 14, 2027	MoU- pulmonary hypertension by administering to a subject in need thereof treprostinil or treprostinil derivative, or a pharmaceutically acceptable salt thereof by a metered dose inhaler.
11.	**US20080280986** (United Therapeutics Corp.)	Feb. 08, 2028	MoU- condition associated with pulmonary fibrosis, comprising administration to a subject in need thereof an effective amount of Treprostinil or its derivative, or a pharmaceutically acceptable salt thereof.
12.	**US 6,756,033** (United Therapeutics Corp.)	Mar. 15, 2020	A method of delivering to a mammal in need thereof a therapeutically effective amount of a benzindene prostaglandin comprising administering to the mammal by inhalation a formulation comprising droplets measuring less than 10 micrometers in diameter, wherein said droplets comprise a therapeutically effective amount of the benzindene prostaglandin.

Table 2.13: Inhalable formulation patents for Pulmonary Arterial Hypertension (continued…..)

S. No.	Patent No. / Publication No. (Assignee)	Expiry	Disclosure
13.	**EP1429829A1** (Cyterra Corp)	Aug. 28, 2022	An apparatus and method for delivering a therapeutic gas including nitric oxide.
14.	**US20110160200** (Cardioxyl Pharma)	Nov. 22, 2030	MoU- pulmonary hypertension by administering an effective amount of a nitroxyl donor.
15.	**US 7,025,869** (CyTerra Corp.)	Aug. 28, 2022	A method of delivering nitric oxide to a mammal comprising disposing a cathode and an anode in a solution of a nitric oxide precursor that includes a precursor salt; applying a voltage across the cathode and anode to generate nitric oxide substantially devoid of nitrogen dioxide; contacting a transport gas with the solution of nitric oxide precursor to form a therapeutic gas; and transporting the therapeutic gas to a mammal.
16.	**US20090196930** (Aires Pharma)	Dec. 24, 2028	A nitrite compound formulation composition for pulmonary delivery, comprising a nitrite compound aqueous solution with pH greater than 7.0, but less than 9.0; containing the nitrite compound at a concentration of from about 0.667 mg NO_2^-/mL to about 100 mg NO_2^-/mL; a taste-masking agent; and a pH buffering agent/ MoU- treatment of pulmonary arterial hypertension, intra-nasal or pulmonary bacterial infections, or to treat or prevent ischemic reperfusion injury of the heart, brain and organs involved in transplantation.
17.	**US 5,958,427** (Salzman et al.)	Expired	A method of locally dilating blood vessels in a mammal, comprising the step of administering a non-polymeric mucosally impermeant nitric oxide donor compound to a specific site of an apical surface of a mucosa of said mammal in an amount effective to locally dilate said blood vessels at said site, said non-polymeric mucosally impermeant nitric oxide donor compound selected form the group consisting of a tertiary amino aliphatic nitric oxide donor compound and a quaternary amino aliphatic nitric oxide donor compound.
18.	**US20040028753** (Hedenstierna et al.)	Nov. 15,2021	Use of inhalable nitric oxide (NO), in the form of gaseous nitric oxide or a nitric oxide donor, in combination with a cyclooxygenase inhibitor for the manufacture of a medicament for treating pulmonary vasoconstriction or airway constriction in a mammal, especially man, in order to counteract a hypo- or non-response to treatment with gaseous nitric oxide or nitric oxide donor only and/or to counteract a rebound response in the case of withdrawal of treatment with gaseous nitric oxide or nitric oxide donor only

Table 2.13: Inhalable formulation patents for Pulmonary Arterial Hypertension (continued…..)

S. No.	Patent No. / Publication No. (Assignee)	Expiry	Disclosure
19.	**US20120003325** (Bayer Schering)	Oct. 20, 2029	A composition comprising a helium-oxygen gas mixture and one or more drugs selected from the group consisting of kinase inhibitors, tyrosine kinase inhibitors, sorafenib, imatinib, gefitinib, or erlotinib; nitric oxide (NO); NO-independent, but heme-dependent, stimulators of soluble guanylate cyclase; NO- and heme-independent activators of soluble guanylate cyclase; prostacyclin analogs, iloprost, beraprost, treprostinil, or epoprostenol; endothelin receptor antagonists, bosentan, darusentan, ambrisentan, or sitaxsentan; compounds which inhibit the degradation of cyclic guanosine monophosphate (cGMP) and/or cyclic adenosine monophosphate (cAMP), inhibitors of phosphodiesterases (PDE) 1, 2, 3, 4, and/or 5, sildenafil, vardenafil, or tadalafil; antibiotics, glycoside antibiotics, gyrase inhibitors, or penicillins; antiviral substances, aspirin; antiproliferative substances in the treatment of tumors; and general active ingredients which can develop an extrapulmonary (systemic) effect in the manner mentioned above/ MoU- pulmonary hypertension
20.	**US20110171139** (Asahi Kasei Pharma)	Oct. 25, 2026	A therapeutic combination, comprising an effective amount of fasudil and sildenafil.
21.	**US20070197544** (CoTherix)	Sep. 20, 2024	A therapeutic combination for the treatment of PAH, comprising a prostacyclin and at least one additional agent, selected from the group consisting of an endothelin receptor antagonist, a PDE inhibitor, and a calcium channel blocker, wherein the prostacyclin and the at least one additional agent are provided at dosages sufficient to ameliorate at least one symptom associated with PAH.
22.	**US20060204450** (Boehringer Ingelheim)	Mar. 07, 2026	A pharmaceutical composition comprising one or more anticholinergics and one or more PDE 5 inhibitors.
23.	**US20090036465** (United Therapeutics Corp.)	Oct. 17,2027	A method of treating pulmonary hypertension comprising administering to a subject in need thereof (A) a first amount of Treprostinil or its derivative, or a pharmaceutically acceptable salt thereof, and (B) a second amount of a Rho Kinase inhibitor or its derivative or apharmaceutically acceptable salt thereof,wherein the first amount and the second amount together comprise an amount effective for treatment of pulmonary hypertension.

Table 2.13: Inhalable formulation patents for Pulmonary Arterial Hypertension (continued…..)

S. No.	Patent No. / Publication No. (Assignee)	Expiry	Disclosure
24.	**US20110224236** (United Therapeutics Corp.)	Mar. 14, 2031	MoU- pulmonary hypertension comprising co-administering to a subject in need thereof a pharmaceutically effective amount of an oral therapeutic agent for treating pulmonary hypertension and a pharmaceutically effective amount of an inhaled therapeutic agent for treating pulmonary hypertension.
25.	**US20070232575** (Baulieu et al.)	Feb. 03,2025	A method for reducing pulmonary arterial pressure (PAP), comprising introducing an effective amount of dehydroepiandrosterone (DHEA), DHEAS, DHEA analog, or DHEA derivative into the pulmonary airways of a mammal.
26.	**US 7,264,803** (Childrens Hospital Los Angeles)	Expired	A pharmaceutical formulation useful for treating pulmonary hypertension comprising a mammalian antibody that specifically binds to endothelial-monocyte activating polypeptide II (EMAP II); and a supplemental compound selected from the group consisting of a vasodilator, a calcium channel blocker, an anticoagulant, prostacycline, nitroprusside, hydralazine, nitrous oxide, L-arginine, and digoxin.
27.	**WO12003220** (Gilead Sciences)	Jun. 29, 2031	A method of treating pulmonary hypertension in a patient in need thereof, said method comprising administering to the patient a therapeutically effective amount of an A_{2B} adenosine receptor antagonist.
28.	**US 6,482,406** (Stewart et al.)	Mar. 26, 2019	A process of inhibiting progression of a pulmonary disorder treatable by an angiogenic or vasodilator factor in a mammalian patient suffering from said pulmonary disorder by conducting gene therapy which comprises administration to the lung by injection into the venous side of the circulatory system of the mammalian patient of viable, transfected autologous mammalian cells, wherein said transfected mammalian cells express at least one angiogenic or vasodilator transgene.
29.	**US20080267969**	Oct. 06, 2025	A method of preventing or treating neointimal or plexiform lesion formation in lung vascular tissue, the method comprising: providing a therapeutic agent that inhibits tissue factor activity or a tissue factor-mediated downstream signaling pathway; andcontacting (i) vascular cells prior to neointimal or plexiform lesion development or (ii) a neointimal or plexiform lesion formation in lung vascular tissue, with the therapeutic agent, wherein said contacting inhibits development of a neointimal or plexiform lesion formation or reduces the size of the existing neointimal or plexiform lesion in lung vascular tissue.

Table 2.13: Inhalable formulation patents for Pulmonary Arterial Hypertension (continued…..)

S. No.	Patent No. / Publication No. (Assignee)	Expiry	Disclosure
30.	**US20090124697** (United Therapeutics Corp.)	Dec. 16, 2024	An inhalation formulation comprising a pharmaceutically effective amount of treprostinil sodium and a carrier suitable for administration with a nebulizer, wherein the formulation is in a solution form.
31.	**US20110265786** (Chaudry et al.)	Jun. 27, 2023	An inhalable formulation for the treatment of pulmonary hypertension, said formulation comprising about 0.001 mg/ml to about 20 mg/ml of a hypertension reducing agent, wherein said pulmonary hypertension reducing agent comprises at least one ACE inhibitor and wherein said formulation is suitable for administration via inhalation to a mammal in need thereof.
32.	**US 7,345,037** (Nitromed)	Mar. 19, 2017	An oral inhalation, nasal inhalation, intranasal mucosal composition of generically claimed compounds with pharmaceutically acceptable carrier or excipient for the treatment of respiratory disorders.
33.	**US20100166869** (Desai et al.)	May 05, 2028	A unit dosage form for treatment of pulmonary hypertension comprising (a) nanoparticles that comprise a carrier protein and rapamycin or taxane or a derivative thereof, wherein the amount of the rapamycin or derivative thereof in the unit dosage form is in the range of about 5 mg to about 500 mg, and (b) a pharmaceutically acceptable carrier.
34.	**EP0579260A1** (Beecham)	Withdrawn	A pharmaceutical inhalation composition, for use in the treatment and/or prophylaxis of disorders associated with pulmonary hypertension and/or disorders associated with right heart failure, which comprises a vasodilator and, if required, a pharmaceutically acceptable carrier therefor.
35.	**US20100076083** (United Therapeutics Corporation)	May 14, 2027	A method of treating pulmonary hypertension comprising administering by inhalation to a subject in need thereof a formulation comprising treprostinil or a pharmaceutically acceptable salt thereof at a frequency of 20 breaths or less per day.
36.	**US20100130500** (Biomarin Pharma)	Dec. 08, 2025	A method for treating an infant having below normal arterial oxygen pressure (PaO_2) comprising administering to said subject a composition comprising tetrahydrobiopterin (BH4) or a precursor or derivative thereof, wherein the administration of BH4 is administered in an amount effective to increase PaO_2 of said infant as compared to said PaO_2 in the absence of said administration of BH4.

2.6 Future prospects in pulmonary delivery

Market for advanced drug delivery technologies is growing at a fast track and that too of global pulmonary drug delivery technologies was $19.6 billion in 2010 and was predicted to be about $22.5 billion by 2011. This market is further anticipated to scope early $44 billion by 2016 at a compound annual growth rate (CAGR) of 14.3%. Among the pulmonary delivery techniques, metered dose inhalers (MDIs) grasped global market of $12.5 billion in 2010 and will touch $14.4 billion by the end of 2011. BCC estimates this market to grow by $29.8 billion in 2016 at a CAGR of 15.7%. However, the market for dry powder inhalers (DPIs) which was just $6.6 billion in 2010 is expected to increase to $7.5 billion in 2011 and reach $13.4 billion by 2016 at a CAGR of 12.3%.

A wide variety of agents has been administered to the lung via oral inhalation, for the treatment of diverse disease states. Controlled release formulations are widely used in oral or parenteral formulations but have not been established for pulmonary applications, which would be the promising step towards the pulmonary drug delivery instead of traditional drug delivery systems. Also, it would be an alternative to parenteral drug delivery, most notably for the delivery of inhaled insulin and also for peptide and protein therapeutics. Such drug delivery system will also provide bypass way for hepatic first pass metabolism of many potent drugs and reduce drug induced toxicity or adverse drug reactions.

From a commercial standpoint, validation of the importance of DPIs has come from the success of three of the most recent additions to the available aerosol therapies in the United States. In the last few years, Advair/Seretide (salmeterol/fluticasone, GlaxoSmithKline), Foradil (formoterol, Novartis) and Spiriva (tiotropium, Boehringer Ingelheim) were introduced to the United States market. They represent a range of inhaler technologies, both old and new drugs, and therapies for 2 diseases, asthma and chronic obstructive pulmonary disease. However, it is clear that, for the foreseeable future, the market for DPIs will continue to increase. It is likely that as market equilibrium is approached, nebulizers and pMDIs will represent some portion of overall sales, as there are applications and demographic groups for which these devices offer important therapeutic advantages.

Thus, it concludes that further research is necessary for pulmonary drug delivery in the treatment of life threatening disorders; as most promising, advanced and attractive economic drug delivery system.

Chapter: 3

Research Envisioned

3. RESEARCH ENVISIONED:

Pharmaceutical research and development is focussed on areas of new drug discovery, formulation research and development and currently more directed towards expansion of new drug delivery systems. Developing a new molecule is always associated with extensive utilization of workforce, overheads, extended time phase till approval and above all the time testing of unwanted effects of new chemical entity. Thus presently, the emphasis is more towards the improvement in already prevailing pharmaceutical active agent (API) with known and established side effects over a period of its custom. Known API can be moulded in many ways to extract its maximum efficacy while minimizing its untoward effects by reincarnation with a better drug delivery system and technology.

Many organizations are exclusively dedicated towards expansion, patenting and commercialization of drug delivery systems, technologies and products thereof. Novel or innovative drug delivery technologies should be intellectual enough to navigate the therapeutic molecule to the desired site and facilitate difficult-to-deliver compounds as well as offer better-quality, efficacy, safety, and patient compliance to existing drugs. The main aim is to achieve the desired therapeutic levels at the preferred site of action and maintain these levels for longer period of time at that location.

Ultimate goal of the any therapy is to treat the disease while justifying the patient needs regarding compliance and complete therapeutic benefit. In case of respiratory and other lung related diseases, pulmonary delivery to route drug directly into the lungs is the best option to achieve this therapeutic goal (Carlotta, M. *et al*. 2011). Perspective of this route compared to others like oral and intravenous are rapid drug deposition in the target organ for rapid onset of action with minimum systemic exposure due to lower dose as well as local effect.

3.1 Identification of problem:

Due to amassed frequency of pulmonary diseases with high mortality and debility, pulmonary drug delivery is evolving as a non-invasive and smart approach for the treatment of several pathologies. One of the rare but progressive and fatal

disorders of lungs is pulmonary arterial hypertension (PAH) characterized by high blood pressure (hypertension) of the main artery of the lungs (pulmonary artery) for no apparent reason. The projected sufferers across the US, EU and Japan reckoning 146,000 predominantly affecting women between 20-50 years of age with an estimate of 75% still not recognized and treated (Peacock, A. *et al*. 2009; Stakeholder Perspectives. 2006). Years may go without a diagnosis, because their symptoms are mild, nonspecific, or may be existent only during demanding exercise. However, it is important to treat PAH because without treatment high blood pressure causes right heart to work much harder and make right ventricular muscles weaker and ultimately may lead to heart failure.

The progressive nature of this disease articulates that initially the patient may experience only mild symptoms, but will finally require treatment and therapeutic attention to maintain a normal lifestyle. The poor prognosis bases a likely median life expectancy of 2.8 years from the time of diagnosis. The field of pulmonary hypertension has evolved considerably over the past era. Recently, The Asociación Nacional de Hipertensión Pulmonar (ANHP) has created the First World Pulmonary Hypertension Day on 5th May, 2012 to raise pulmonary hypertension awareness on a global scale (Gerald Fischer, 2012). Research in PAH is majorly activated towards refining the means of diagnosis, improving clinical treatment, and better understanding the mechanisms behind the development of the disease. Current knowledge of pulmonary hypertension emerges from more than a century of research, discovery and development. Novel revolutions in the field of pulmonary hypertension are created on a heritage established by originators of the field who promoted our knowledge about pathophysiology of the disease, diagnosis and new treatment strategies. Front-runners and their contributions collectively mount optimism towards cure of pulmonary hypertension in the future.

New treatment guidelines and increasing awareness of PAH is now attracting R&D investment to enter this compact but profitable market due to the high unmet needs and high treatment values per patient. Until 2001, there was no drug molecule approved for PAH treatment. In 2001, FDA approved Tracleer® (Actelion Ltd.) and then Remodulin®, Ventavis®, and Revatio® in 2002, 2004 and 2005 respectively accounting only a 24% market share to 2005 that has propagated at a Compound

Annual Growth Rate (CAGR) of 39.2% reaching $2,653m returns in 2009. Global market is projected to increase at a CAGR of 5% annually to triumph revenues worth $3,569m by 2015, due to competition among existing products, the release of new and enhanced treatment options, combination therapies such as epoprostenol plus bosentan and epoprostenol plus sildenafil and the development of safer and more efficacious therapies.

Commercially available treatments like Flolan® (Epoprostenol sodium) and Remodulin® (Treprostinil) as a continuous intravenous infusion; Tracleer® (Bosentan, twice a day), Revatio® (Sildenfil, thrice a day) as tablet dosage form are associated with limitatons of frequent dosing and patient incomplaince (Minai OA *et al*. 2007; Eells PL 2004; Benza RL *et al*. 2008; Dhillon S *et al*. 2009). Further, once a day available treatments like Thelin® (Sitaxsentan), Letairis® (Ambrisentan) and Adcirca® (Tadalafil) are associated with non-specific vasodilation and other toxic effects in whole vasculature instead of reducing pulmonary vascular resistance. Ventavis® (Iloprost sterile solution) and Tyvaso® (Treprostinil sterile solution) are the only commercially available inhalations, but with a drawback of high inconvenience to patient due to very frequent dosing of 6-9 times and four times (3 inhalations per treatment) per day respectively.

Table 3.1: Commercially available drug treatments for PAH

Drug	Trade name	Company	Class	Dosage form/Route	Dosing frequency
Epoprostenol sodium	Flolan®	Glaxo Smithkline	Prostacyclin analogue	Sterile solution/ IV infusion	Continuous iv infusion through a central venous catheter
Treprostinil	Remodulin®	United Therapeutics CORPORATION	Prostacyclin analogue	1, 2.5, 5 and 10mg/mL solution for sc or iv infusion	only as a continuous infusion
	Tyvaso®	United Therapeutics CORPORATION	Prostacyclin analogue	Sterile solution for oral inhalation	Four times (3 inhalations per

					treatment) a day using Tyvaso Inhalation System
Iloprost	Ventavis®	Schering Health care	Prostacyclin analogue	Clear, colorless, sterile solution for Inhalation via nebulizer	6-9 times per day
Bosentan	Tracleer®	Actelion Pharmaceuticals US, Inc.	Non-selective Endothelin receptor antagonist	62.5 mg and 125 mg film-coated tablet/ Oral	Twice a day
Sitaxsentan	Thelin®	Pfizer	ET(A) selective Endothelin receptor antagonist	100mg film coated tablet/ Oral	Once a day
Ambrisentan	Letairis®	Gilead Sciences, Inc.	ET(A) selective Endothelin receptor antagonist	5mg and 10mg film coated tablet/ Oral	Once a day
Sildenafil	Revatio®	Pfizer	PDE-5 inhibitor	1. White film coated 20mg tablet/ Oral. 2. 10mg (12.5mL) IV bolus injection. 3. Powder for oral suspension 10 mg sildenafil/mL	Thrice a day
Tadalafil	Adcirca®	Eli Lilly and Company	PDE-5 inhibitor	20mg film coated tablet/ Oral	Two 20mg tablets once a day

Due to the enormous physical debility and emotional stress associated with the PAH, the unwanted systemic side effects of oral and parenteral therapy and high inconvenience of administering available inhalation treatment adds up in worsening the quality of patients' life. As many treatment options are already available for PAH,

there comes a great need to improve upon the delivery of these agents to the desired site by designing a formulation with reduced frequency and more convenient method of administration.

3.2 Rationale for pulmonary delivery of sustained release dry powder formulations of sildenafil citrate :

For respiratory conditions, non-invasive pulmonary route of delivery is preferred to deliver the drug for instant and enhanced local (pulmonary arteries) action while reducing the acquaintance of drug to the systemic circulation and hence potentially minimizing adverse effects. Targeting to the pulmonary arteries can be achieved by making a dry powder formulation that can be inhaled for direct delivery to the lungs and hence thereby minimizing the exposure of the drug to the other vasculature. Recently, the application filed by United Therapeutics for oral treprostenil has been rejected by FDA due to higher systemic side effects, though its inhalation formulation is already a marketed product. By designing biocompatible, non-immunogenic lipid based dry powder inhaler formulations like liposomes, lipid composites and lipid particles, limitations of frequent administration can be largely circumvented due to its ability to act as pulmonary sustained release reservoir (Suggy SC *et al*. 2002; Franz H *et al*. 2008). Furthermore, the key challenge with the pulmonary delivery of dry powders is the high dispersibility of the powder for reproducible and higher deposition at the required site. Large porous particle technology which has been shown to improve the dispersibility of the powders and hence reproducible delivery of the drugs to the patients via lungs can be explored to attain further remunerations (Jennifer F *et al*. 2003; David AE *et al*. 1998).

Sildenafil citrate, a potent and selective PDE5 inhibitor successfully used for the treatment of erectile dysfunction was approved to improve PAH in 2005 (Nazzareno G *et al*. 2005). It is available as tablet and injection dosage forms and is associated with non-specific vasodilation and other toxic effects. 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). Revatio® is indicated for the treatment of PAH (WHO Group I) in adults to improve exercise ability and delay clinical worsening. Further, in pulmonary circulation, cGMP plays a

major role on pulmonary vascular resistance. PDE5, the enzyme that specifically hydrolyzes cGMP, is abundantly expressed in the whole lung and predominates in pulmonary artery smooth muscle cells and both the activity and the expression of PDE5 are increased in pulmonary arteries with PAH. Thus, Sildenafil citrate was chosen as the model drug to incorporate into suitable drug delivery systems for the treatment of pulmonary arterial hypertension. According to American College of Chest Physicians (ACCP), the recommended dose of Revatio® is 20 mg three times a day (TID) taken 4-6 hours apart. In clinical trials using the 20 mg three times daily dose, the most common side effects include nosebleed, headache, hypotension and extra-pulmonary vasorelaxation, visual disturbances due to its action on PDE6 enzyme of retinal photoreceptors, upset stomach, getting red or hot in the face (flushing), and trouble sleeping.

To address all the above arguments, our study was thus focused on to develop, characterize and evaluate the sustained release lipid based dry powder inhalation formulations of Sildenafil citrate to target the better deposition to the pulmonary arteries for the treatment of PAH. It is hypothesized that the pulmonary delivery of selective and powerful phosphodiesterase 5 (PDE5) inhibitors like Sildenafil citrate specific to cyclic guanosine monophosphate (cGMP), that are abundant in the lungs will provide effective targeted delivery at reduced dose for vasodilatation in pulmonary arteries only, avoiding side effects and vasorelaxation of other vasculature (Lepore *et al*., 2002; Prasad *et al*., 2000; Shekerdemian *et al*., 2002). Moreover, it also possesses antiremodeling potency through increased cAMP and cGMP levels (Clapp *et al*., 2002; Liu *et al*., 2007).

Further the pulmonary delivery of such agents by incorporation in biocompatible lipid based formulations can provide efficient local and long lasting effect resulting in reduced dose frequency leading to improved patient compliance. Moreover, the synergistic combination of anti-inflammatory effect of sildenafil citrate itself and reduced macrophage uptake of large porous liposphere due to desired aerodynamic diameter can provide better lung deposition and longer stay in lungs. These formulations are anticipated to be extremely beneficial since these will not only reduce the frequency of administration but also improve the clinical efficacy of sildenafil citrate therapy.

3.3 Plan of work:

Aim: Treatment of pulmonary arterial hypertension by pulmonary delivery.

Drug: Sildenafil citrate

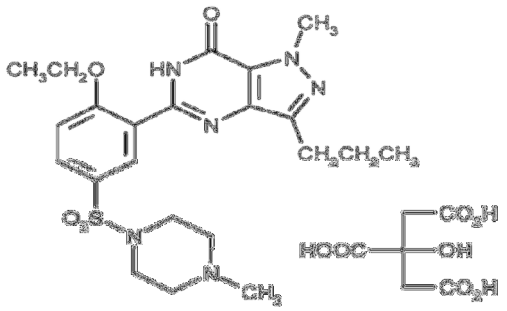
Category: Phosphodiesterase-5 (PDE-5) inhibitor

- **Literature survey**
- **Preformulation studies:**
  - Physicochemical characterization of drug
    - ✓ Solubility study
    - ✓ Drug distribution study
  - Establishment of analytical procedure
- **Selection of materials:** Various lipids and other excipients were selected for the present study-
  - Hydrogenated soya phosphatidylcholine (HSPC)
  - Dipalmitoyl phosphatidylcholine (DPPC)
  - Cholesterol
  - Solvents methanol and chloroform for HPLC
  - Protective/ diluents
    - ✓ Lactohale 100 and 200
    - ✓ Pharmatose 350 and 450
    - ✓ Mannitol
    - ✓ Trehalose
- **Preparation of sildenafil citrate dry powder formulations**
  - Formulation of sildenafil citrate Conventional DPI.
  - Formulation and optimization of spray drying process of sugar co-spray dried sildenafil citrate composites.
  - Formulation and optimization of spray drying process of sildenafil citrate loaded liposomal DPI.
  - Formulation and optimization of spray drying process of sildenafil citrate-lipid composites.
  - Formulation and optimization of spray drying process of sildenafil citrate loaded large porous lipospheres.

- **Characterization of dry powder formulations:**
  - Determination of percent yield
  - Determination of assay and/ or percent Encapsulation efficiency.
  - Transmission Electron Microscopy (TEM)
  - Scanning Electron Microscopy (SEM)
  - Geometric Particle size and zeta potential analysis
  - Differential scanning calorimetry (DSC)
  - X-Ray Diffraction studies
  - Moisture content
  - Residual solvent analysis
  - Flow properties like angle of repose, bulk and tapped density, Carr's index and Hausner's ratio
  - Aerosolization performance of dry powder formulations
    - Delivered Dose Uniformity
    - Aerodynamic particle size using Andersen cascade impactor (ACI)
  - Aerosolization performance of dry powder formulations
  - In vitro drug release studies
- **Macrophage uptake study**
- **Stability studies**
- ***In-vivo* evaluation:** by pulmonary delivery of sustained release dry powder formulations of sildenafil citrate to evaluate its prolonged local efficacy in monocrotaline-induced pulmonary hypertensive rats

3.4 Drug Profile:

Sildenafil citrate is a lipophilic compound, neural at physiological pH and has the chemical name 1-[4-ethoxy-3-(6,7-dihydro-1-methyl-7-oxo-3-propyl-1*H*pyrazolo [4,3-d]pyrimidin-5-yl)phenylsulphonyl]-4-methylpiperazine citrate, and the structural formula is the following:



Sildenafil citrate is a crystalline, white to off white monomorphic solid. The solubility of sildenafil citrate in water has been determined as being 3.5 mg/mL, at 23°C. To improve the solubility of the drug, polar substituents were added which gave compounds with a lower lipophilicity. This was found to also increase the enzyme affinity. Sildenafil citrate gave an excellent combination of enzyme inhibitory potency, selectivity, solubility and *in-vivo* characteristics. The Physicochemical characteristics of Sildenafil citrate drug substance as reported in the literature are outlined in the following page in **Table 3.2.**

3.4.1 Indication and uses:

REVATIO is indicated for the treatment of WHO Group I pulmonary arterial hypertension in adults to improve exercise ability and delay clinical worsening. This effect was also experienced when REVATIO was added to contextual epoprostenol therapy. Effectiveness has been established short-term for 12 to 16 weeks with mainly patients with New York Heart Association (NYHA) Functional Class II-III symptoms and idiopathic etiology (71%) or associated with connective tissue disease (CTD) (25%).

REVATIO efficacy in the treatment of pulmonary arterial hypertension (PAH) has not been adequately evaluated in patients taking bosentan. Sildenafil is used to treat high blood pressure in the lungs by blocking enzyme PDE5 in the body. It works by relaxing the blood vessels in the lungs and the rest of the body. Decreasing blood pressure in the lungs allows the heart and lungs to work better and improves ability to exercise.

Table 3.2: Physicochemical properties of sildenafil citrate

Parameters	**Description**
Generic Name of the drug	Sildenafil citrate
Therapeutic category	Sildenafil citrate is a phosphodiesterase 5 (PDE5) inhibitor indicated for a. Treatment of pulmonary arterial hypertension (WHO Group I) to improve exercise ability and delay clinical worsening, and b. Erectile dysfunction; and
Chemical name	IUPAC NAME: 1-[4-ethoxy-3-(6,7-dihydro-1-methyl-7-oxo-3-propyl-1*H*-pyrazolo[4,3-*d*]pyrimidin-5-yl)phenylsulfonyl]-4-methylpiperazine citrate
Molecular formula	$C_{22}H_3N_6O_4S$-$C_6H_8O_7$
Molecular weight	Sildenafil citrate: 666.7 Siladenafil base: 474.6 g/mol
CAS No.	[139755-83-2]
Pharmacopoeial Status	API is official in Indian Pharmacopoeia
Description	A white to Off white crystalline solids.
Melting point	Sildenafil citrate: 189.4°C Sildenafil base: 251.9°C
Solubility (at 23°C)	3.5mg/mL in water , 5.8 mg/mL in 1M HCl, 42.3 mg/mL in 1M NaOH
Density	Sildenafil citrate: 1.59g/cm^3 Sildenafil base: 1.17 g/cm^3
pH	0.3g/100mL aqueous solution gives pH= 3.7
pka	Protonation of tertiary amine 6.53 Deprotonation of pyrimidirone moiety 9.17
Partition coefficient	2.7 (Octanol/ water)
LogP	Sildenafil citrate: 0.8, Sildenafil base: 1.9

3.4.2 Mechanism of action:

Endothelium-derived nitric oxide (NO) activates soluble guanylate cyclase, increasing cyclic guanosine monophosphate (cGMP) production, which opens potassium channels via cGMP kinase causing pulmonary vasorelaxation. Phosphodiesterase 5 (PDE5) enzyme is responsible for degradation of cGMP. Sildenafil is an inhibitor of cyclic cGMP enzyme PDE5 resulting in vasodilatation through the NO/cGMP pathway at sites expressing this enzyme. Since the pulmonary epithelium contains substantial levels of PDE5, sildenafil has the potential clinical benefit in the treatment of PAH. Sildenafil promotes selective smooth muscle relaxation in lung vasculature.

3.4.3 Pharmacokinetics:

3.4.3.1 Absorption and distribution

REVATIO is rapidly absorbed after oral administration, with a mean absolute bioavailability of 40%. Maximum reductions in the relevant pulmonary parameters like mean PVR and mean PAP appeared to be reached at plasma sildenafil concentrations of 100 ng/mL at peak plasma time of 30-120 min. The mean steady state volume of distribution (Vss) for sildenafil is 105 L. Sildenafil and its major circulating N-desmethyl metabolite are both approximately 96% bound to plasma proteins. Protein binding is independent of total drug concentrations.

3.4.3.2. Metabolism

Metabolized by hepatic P450 enzyme CYP3A4 & minor amounts metabolized by hepatic P450 enzyme CYP2C9. Active metabolite N-desmethyl metabolite has 50% of the phosphodiesterase type 5 inhibitory activity of sildenafil.

3.4.3.3. Elimination

Elimination half-life of sildenafil (parent drug) is 3-4 h and 10-70 min for N-desmeth- yl active metabolite. After either oral or intravenous administration, sildenafil is excreted as metabolites predominantly in the feces (approximately 80% of the administered oral dose) and to a lesser extent in the urine (approximately 13% of the administered oral dose).

3.4.4 Warnings and Precautions

1. Cardiovascular effects: Carefully consider whether patients with certain underlying conditions (e.g., resting hypotension, fluid depletion) could be

adversely affected by vasodilatory effects of REVATIO. Not recommended in patients with pulmonary venoocclusive disease.

2. Use with alpha-blockers: Note additive blood pressure-lowering effects.
3. Bleeding: In patients with PAH secondary to CTD, higher rates of epistaxis with REVATIO than placebo, including with concomitant oral vitamin K antagonists.
4. Priapism: Advise patients to seek emergency treatment if an erection lasts > 4 hours. Use REVATIO with caution in patients predisposed to priapism.
5. Use with PDE5 inhibitors: Avoid use with VIAGRA or other PDE5 inhibitors.
6. Use with ritonavir and other potent CYP3A inhibitors: Coadministration not recommended.
7. Effects on the eye: Consider discontinuing REVATIO if sudden loss of vision occurs, which could be non-arteritic ischemic optic neuropathy (NAION).
8. Hearing impairment: Discontinue REVATIO if sudden decrease or loss of hearing occurs.

3.4.5 Market dosage forms:

3.4.5.1 REVATIO® Tablets: REVATIO is formulated as white, film-coated round tablets with 20 mg of sildenafil for oral administration. In addition to the active ingredient, sildenafil citrate, each tablet contains the following inactive ingredients: microcrystalline cellulose, anhydrous dibasic calcium phosphate, croscarmellose sodium, magnesium stearate, hypromellose, titanium dioxide, lactose monohydrate, and triacetin. The recommended dose of REVATIO is 20 mg three times a day (TID). Administer REVATIO doses 4-6 hours apart.

3.4.5.2 REVATIO® Injection: REVATIO is supplied as a clear, colorless, sterile, ready to use solution containing 10 mg (12.5 mL) of sildenafil. Each mL of solution contains 1.124 mg sildenafil citrate, 50.5 mg dextrose and water for injection. The recommended dose is 10 mg (12.5 mL) administered as an intravenous bolus injection three times a day (TID). The dose of REVATIO injection does not need to

be adjusted for body weight. REVATIO injection is supplied as a single use vial containing 10 mg (12.5 mL) of sildenafil.

3.4.5.3 REVATIO® for Oral Suspension: REVATIO is supplied in an amber glass bottle as a white to off-white powder providing a white to off-white grape flavored oral suspension when constituted. Bottles containing 32.27 g powder for oral suspension are intended for constitution with 90 mL water to produce an oral suspension containing 10 mg/mL sildenafil.

In addition to the bottle, a press-in bottle adapter and an oral dosing syringe (2 mL) are provided. The inactive ingredients include sorbitol, citric acid anhydrous, sucralose, sodium citrate dihydrate, xanthan gum, titanium dioxide, sodium benzoate, colloidal silicon dioxide anhydrous and grape flavor. Each bottle contains 32.27 g of powder for oral suspension. Following constitution, the volume of the oral suspension is 112 mL (10 mg sildenafil/mL). A 2 mL oral dosing syringe and a press-in bottle adaptor are also provided (Nahata MC, 2006; Pfizer Labs, 2012).

Chapter: 4

Materials And Methods

4. MATERIALS AND METHODS

4.1 Materials:

The materials used in the present study are enlisted below (**Table 4.1**):

Table 4.1: List of materials

Chemicals/Materials	Source/Manufacturer
Sildenafil citrate	Shilpa Medicare Limited/ (Gift sample from Alembic research Centre)
Hydrogenated soya phosphatidylcholine (HSPC)	Lipoid, GmbH, UK
Dipalmitoyl phosphatidylcholine (DPPC)	Genzyme, Switzerland
Cholesterol	Sigma, U.S.A.
Pharmatose 325/450	DMV, Int.
Lactohale 100/200	DMV, Int.
Sucrose	International Lab., U.S.A.
Sorbitol	International Lab., U.S.A.
D (+) Trehalose, dihydrate	HiMedia, Mumbai, India
Mannitol	International Lab., U.S.A.
Potassium dihydrogen phosphate	S.D. Fine Chemicals Pvt. Ltd, India
Disodium hydrogen phosphate	S.D. Fine Chemicals Pvt. Ltd, India
Disodium hydrogen phosphate (anhydrous)	S.D. Fine Chemicals Pvt. Ltd, India
Citric acid monohydrate	S.D. Fine Chemicals Pvt. Ltd, India
Hypromellose Quali V® capsules	Zydus Research Centre, Gujarat, India (Gift sample)
Chloroform for HPLC	Merck Ltd, Mumbai, India
Acetonitrile for HPLC	Merck Ltd, Mumbai, India
Methanol for HPLC	Merck Ltd, Mumbai, India
Orthophosphoric acid	Merck Ltd, Mumbai, India
Hydrochloric acid	S.D. Fine Chemicals Pvt. Ltd, India
Formaldehyde	S.D. Fine Chemicals Pvt. Ltd, India

Table 4.1: List of materials (continued….)

Chemicals/Materials	Source/Manufacturer
Latex beads, carboxylate-modified polystyrene, fluorescent yellow green	Sigma Life Sciences
Fluorescein (free acid)	Sigma Aldrich, Inc
Monocrotaline	Sigma Aldrich, Co.
Anket® Ketamine injection I.P. 50mg/mL	Neon Laboratories Ltd.
Xylazine injection USP 20mg/mL	G. Loucatos and Co.
Heparin sodium injection (Beparine, 25000 I.U. in 5mL)	Biological E. limited, Hyderabad
Sodium chloride	S.D. Fine Chemicals Pvt. Ltd, India
Picric acid	S.D. Fine Chemicals Pvt. Ltd, India
cGMP complete EIA kit	(Enzo life sciences)
Deionized water	Milli-Q ultrapure water system (Millipore, Elix5 and Milli Q)

4.2 Equipments

The equipments used in the present study are enlisted below (**Table 4.2**):

Table 4.2: List of equipments

Equipment	Source/Company/Manufacturer
Electronic balance	Sartorius Philippenes
Incubator shaker	Orbitek
UV-Visible Spectrophotometer	Shimadzu UV-1601, Japan
Rotary evaporator with thermostatically controlled water bath	Superfit Equipments, India
Vacuum pump	Bharath Vacuum pumps, Bangalore
Cyclomixer	SPINIX, Mumbai
pH meter	Labindia
Ultracentrifuge	Hermle, Z323

Table 4.2: List of equipments (continued….)

Equipment	Source/Company/Manufacturer
Zeta Sizer	Malvern Instruments Ltd., UK
Particle Size Analyser	Master Sizer 2000, Malvern Instruments Ltd., UK
High speed Mechanical Stirrer	Remi Motors, Mumbai, India
Vacuum pump	Toshniwal high vacuum pump, Tovac equipments, Chennai, India
Mechanical Shaker	Cetromat, India
Particle Size Analyser	Master Sizer 2000, Malvern Instruments Ltd., UK
Differential Scanning Calorimetry	Metter TA 4000 system
Laboratory Micro Centrifuge	Remi Motors, Mumbai, India
Scanning Electron Microscope	JSM 6100 JEOL, Japan
Nikon Eclipse E600 microscope mounted with camera	Nikon, Japan
Fluorescent Microscope with imaging software: DP controller/ DP manager	Olympus microscope
Microtips	Tarsons
Finnetip, 5mL syringe barrel with plunger	Tarsons
Fibre optic lamp with two flexible arms	HGY3 Fibre Optic Illuminator
Power LAB® Software setup with PowerLab systems, ML785 PowerLab/8SP	AD Instruments, Colorado Springs, CO.
Optical density Plate reader with Soft max Pro software	(Spectra Max Plus, Molecular Devices)
Tissue Tearor (Model 985370)	Biospec Products Inc.
5µ C-18 (250X4.6mm column)	Fortis
Agilent 1100 series Chem station for HPLC	Agilent

4.3 Preformulation studies of sildenafil citrate

Preformulation is the preliminary step in pharmaceutical product development. Study of few physicochemical parameters of a drug can assist in designing an optimum drug delivery system. Characterization of drug molecule is a very important step at the preformulation phase of product development. Sildenafil citrate was characterized with respect to its solubility in different solvents, pH dependent solubility, drug distribution to find distribution coefficient between organic and aqueous phase like water and buffer of pH 7. Sildenafil citrate can be analyzed by various analytical techniques like UV spectroscopy, colorimetry, thin layer chromatography and HPLC (Mahmoudian M, 2005). For this project analytical method based on UV spectroscopy and HPLC technique were developed.

4.3.1 Establishment of analytical technique:

4.3.1.1 Spectrophotometric standard plots of sildenafil citrate

Sildenafil citrate is slightly soluble in water and methanol. It also shows pH dependent solubility, thus standard plot in methanol, water and at various pH (2.0, 2.5, 3.6, 4.0, 5.0, 6.8 and 7.4) were prepared. λmax of sildenafil citrate in water, methanol and different buffers were determined by UV scan (at various wavelengths). Concentration range within 2-50 µg/mL of sildenafil (in triplicate) was selected and prepared in each solvent. Absorbance of each solution was taken at the respective λmax and plotted against the corresponding concentration. From the Beer Lambert's graph, equation of the line was obtained and was used for further calculations and selection of hydration medium and solvent for analysis.

4.3.1.1.1 Standard plot of Sildenafil citrate in water and methanol:

To determine the percent drug content or percent entrapment efficiency in the sildenafil citrate, analytical technique was established in suitable mediums i.e. water and methanol. An accurately weighed amount of the sildenafil citrate (14mg equivalent to 10mg of Sildenafil) was dissolved in 100mL of water and in methanol separately to prepare stock solutions of 100 µg/mL. Suitable dilutions were made to obtain the concentration range of 2-50 µg/mL (in triplicate) for both the mediums and absorbance was determined at 292 nm.

4.3.1.1.2 Standard plot of Sildenafil citrate in different buffers:

To determine the solubility of sildenafil citrate at different pH, its calibration curves in different pH buffers were prepared. UV scan (at various wavelengths) for buffers of different pH was taken to determine the respective λmax. Concentration range within 1-400 µg/mL of sildenafil (in triplicate) was prepared in buffer of each pH. Absorbance of each solution was determined at respective λmax and plotted against corresponding concentration.

4.3.1.2 HPLC method for estimation of Sildenafil citrate in lung homogenates

4.3.1.2.1 Standard stock solution and working standards

Drug equivalent to 1mg of sildenafil was dissolved in 1ml of Milli-Q water. Hundred µL of this solution (1000µg/ml) was diluted to 1ml with Milli-Q water to make stock solution of 100µg/ml. The calibration curve standards were prepared by adding known amounts of drug to supernatant of rat lung homogenate (proteins denatured by the addition of 0.1N HCl) and linearity was made in the range of 780ng/mL to 200 µg/mL.

4.3.1.2.2 Mobile Phase

Orthophosphoric acid (0.05M): Acetonitrile (65:35) was used as mobile phase.

4.3.1.2.3 Flow rate

The flow rate was 1mL/min.

4.3.1.2.4 Instrumentation

Agilent 1100 Series HPLC station having quaternary pump, vacuum degasser with HP1100 series photodiode array detector (Germany), HP 1100 series autosampler was used to perform HPLC analysis. Sixty µL of each sample injection were analysed using Fortis 5µ C18 column (250 x 4.6mm) at a mobile phase flow rate of 1mL/min. The column was operated at a temperature of 40°C. The wavelength of detection was set at 240 nm.

4.3.1.2.5 Precision

Precision studies were carried out as follows:

- System precision

  Six samples of the same concentration (100µg/mL) were injected in HPLC column and analysed by proposed method.

- Intra-day precision

Three different concentrations were injected into column at different time points in a day to evaluate for intra-day reproducibility.

- Inter-day precision

Three different concentrations were injected into column for six consecutive days to evaluate for inter-day reproducibility.

4.3.1.2.6 Accuracy/recovery studies

Accuracy was tested by fortifying a mixture of degraded sample solution with known three different concentration of drug and determining the recovery of added drug by HPLC.

4.3.1.2.7 Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components that may be expected to be present in the sample matrix (USP 2004). Specificity of the method for sildenafil citrate was demonstrated by determining the purity of drug peak in lung homogenate samples form control and toxin treated rats, i.e. any interference at the resolution time of the drug peak was determined.

4.3.2 Solubility study

Solubility studies of sildenafil citrate in water, methanol and buffers of various pH (2.0, 2.5, 3.6, 4.0, 5.0, 6.8 and 7.4) were carried out to select the suitable solvent/buffer for further studies. Following steps were used for solubility study (Gerry S *et al*. 2009):

1) Buffers of various pH (2.0, 2.5, 3.6, 4.0, 5.0, 6.8 and 7.4) were prepared and 5 ml of each were taken in different flasks (in triplicate)
2) Excess of drug was added in each flask
3) All the flasks were kept in incubator shaker for 24 h at 37ºC.
4) The samples were then filtered through 0.22 µ filter and after suitable dilution with respective buffers, analyzed spectrophotometrically at respective λ_{max} and the
5) Amount of drug was determined using $E^{1\%}_{1cm}$ in respective buffer.

4.3.3 Drug distribution study

Distribution of sildenafil citrate was determined in

(a) Octanol and water,

(b) Octanol and buffer system,

(c) Chloroform and water and

(d) Chloroform and buffer system

by calculating log D values in respective systems.

1) Saturated solution of sildenafil citrate was prepared in phosphate buffer pH 7.4 and water, and initial drug content in buffer (24mcg/mL) and water (2.85mg/mL; pH of the solution 3.7) were determined spectrophotometrically.
2) Then, in duplicate, 4 ml each of octanol and Chloroform was taken in three separate 10 ml volumetric flask (i.e. for each organic phase studies were planned in triplicate).
3) Then to one set of each above flask, 2 mL of saturated solution of drug in phosphate buffer pH 7.4 was added and to the another set of flask 2mL of saturated solution of drug in distilled water was added.
4) Each flask was kept in incubator shaker for 24 h at 37ºC.
5) Content of each flask were centrifuged at 3000 rpm for 15-30 min. and aqueous layer was separated.
6) After suitable dilution, drug content remaining in aqueous phase was determined spectrophotometrically.
7) Log D values and percent drug distributed in each organic phase was determined using following relationship (Gerry S *et al*. 2009; Wilkinson AM, 1997).

$$D = \frac{C_t - C_a}{C_a} x \frac{V_o}{V_a}$$

D= Distribution coefficient

C_t = Total concentration of drug added to the aqueous phase initially

C_a = Concentration remaining after partitioning in aqueous phase

C_{t-} C_a = Concentration in organic phase

V_o = Volume of organic phase, 4 ml

V_a = Volume of aqueous phase, 2 ml

4.4. Formulation of conventional dry powder formulations for inhalation (CDPI) of sildenafil citrate

Conventional dry powder formulations are composed of micronized drug alone or in combination with the carriers on surface of which it gets adsorbed. Dry powder formulations must illustrate consistent dose uniformity to ensure that all doses from the device deliver the active pharmaceutical agent in the right amount. It is important that the dose released by the DPI device is accurately the same every time, regardless of a patient's inhalation ability. Micronized drugs (<5µm) have agglomerates due to highly cohesive particles that hinder the accurate dose delivery. Dose-consistent delivery may be achieved by using a lactose carrier with the appropriate properties in the formulation.

Inhalation formulations are usually composed of lower doses of the drug and carriers are required to dilute the drug. Thus a non-toxic and physiologically acceptable carrier that can improve the ability of powder to flow and drug detachment on inhalation for better lung deposition, must be selected. Drug particles must be disengaged from the carrier surface during inhalation to penetrate into the respiratory airways. This happens when the forces exhibited by inhalation surpass the interparticle forces between drug and carrier particles. Strong adhesion forces can lead to poor detachment, resulting in lower respirable fractions of the drug. Various physicochemical properties of both drug and carrier particles must be considered while formulating into dry powder for inhalation. α-lactose monohydrate is a well-known inert, safe and widely used carrier for DPI applications. Various inhalable grades of lactose are available that are marketed under the brand name of Pharmatose, Sorbolac, Inhalac, Flowlac, Respitose and Lactohale and can be used to design the dry powder formulations for inhalation.

4.4.1 Preparation technique for CDPI:

CDPI formulations of Sildenafil citrate were prepared by mixing different ratios of lactose carriers of different average particle sizes with micronized drug. Two fine grades of lactose i.e. Lactohale® 200 (LH200, 80±0.15µ); Pharmatose® 450M (P450M, 20±3.2µ) and two coarse grades Lactohale® 100 (LH100, 140±0.24µ) and Pharmatose® 350M (P350M, 100±0.35 µ) were blended alone with drug and also in various combinations of 60:40 and 70:30 ratio. Sildenafil citrate was co-sieved with the fine grade through 100# sieve and then mixed well for 10 min in a polybag. Then

this mixture was co-sieved with the coarse lactose in geometric proportions through 80# sieve and mixed well in polybag for 20minutes. Final sieving of this mixture is done through 80# again to get homogenous mixture. Ten samples of 20mg were taken randomly for content uniformity test.

The final mixture contained the drug equivalent to 600 µg of sildenafil in 9.24mg of the formulation. All formulations were filled in size 3 hypromellose Quali V® capsules manually in a way that each capsule contained 9.24 ± 0.1 mg of the formulation. Different CDPI formulations of sildenafil citrate were prepared to evaluate the effect of different lactose grades, its fraction and particle size on aerosolization behavior with two different devices (Rotahaler® and Handihaler®). Content uniformity test, aerodynamic particle size and fine powder fraction of the delivered formulation were used to decide the lactose of suitable grade.

4.5. Formulation of Drug –sugar composites (DS) of sildenafil citrate:

Particles to be inhaled need to be within the 1 to 5µm aerodynamic diameter range in order to reach the airways and particle size distribution range should be minimum to achieve better respirable fraction. Micronization is the conventional method for the preparation of inhalation dry powders [5]. It can reduce the size of crystalline material into fine aerosol particles. But, only slight control is attained over particle size, shape and surface morphology, and thus the powders generated are highly cohesive and show poor aerosolization behaviour. In 1990s, spray-drying was established as an alternative method to conventional micronization.

Spray drying is a simple, one-step, rapid, reproducible, economic and easy to scale-up production process (J. Broadhead *et al.* 1992). It has been intensively studied for pharmaceuticals and excipients for pulmonary drug delivery in dry powder inhalation system to treat several diseases including asthma, tuberculosis, diabetes and bacterial infection in the lung (Zijlstra GS *et al.* 2007; Corrigan DO *et al.* 2003; Joshi M *et al.* 2001). It has the potential to generate highly dispersible powders for inhalation in the range from 1 to 5 µm size with a particle morphology that can more easily be predisposed compared to micronization and milling (Heng PWS *et al.* 2000). Sugar alcohols have been used as diluent and as protective sugars for spray drying the drugs (Orla N´ı O´ ga´in *et al*, 2010). Thus, it was planned to formulate sugar composites (DS) of sildenafil citrate in order to achieve the desired aerodynamic size for inhalation while reducing the processing steps and limitation of drug micronization.

4.5.1 Preparation technique for drug-sugar composites (DS):

Sildenafil citrate was co-spray dried (using LD-48 JISL spray dryer) with different sugars like lactose, sucrose, mannitol, sorbitol and trehalose to formulate its sugar composites. Placebo batches were first spray dried to determine the optimum concentration of sugar with good powder flow properties and better yield. Maximum yield and excellent free flowing properties were obtained using mannitol as a spray drying adjunct. Thus, mannitol was chosen for the formulation of spray dried composites using different ratios of mannitol with SDC and percentage in spray drying solution. Then effect of various spray drying parameters on formulation characteristics like percent drug content, percent yield, moisture content and aerosolization behaviour was statistically analyzed using Stat-Ease software (Design-Expert® 8). Final formulation contained 0.4% drug and 4.0% of mannitol in spray drying solution.

Table 4.3: Design of experiments to optimize spray drying process for drug-sugar composites of sildenafil citrate using fractional factorial design

	Factor 1	**Factor 2**	**Factor 3**	**Factor 4**
Run	**A:Feed rate**	**B:Air pressure**	**C:Vacuum**	**D:Inlet temperature**
	mL/min	Bar	mm of WC	°C
1	1	3	-160	80
2	3	3	-200	80
3	1	2	-200	80
4	1	2	-200	100
5	3	2	-160	80
6	3	3	-200	100
7	3	3	-160	80
8	1	3	-200	100
9	3	2	-160	100
10	3	2	-200	80
11	1	3	-200	80
12	3	2	-200	100
13	1	2	-160	100
14	1	3	-160	100
15	1	2	-160	80
16	3	3	-160	100

4.6 Formulation of liposomal dry powder for inhalation of sildenafil citrate:

Pulmonary delivery of drug has become a well-established method in the treatment of localised disease states within the lung. However, most of these pharmaceutical agents require inhalation daily at least 3-4 times due to relatively short duration of action. Same is true in case of PAH treatment, the only available inhalation formulations, Ventavis® and Tyvaso® sterile solutions, require six to nine inhalations per day. Spray dried drug sugar composites can improve the aerosolization behaviour and can make the product as immediate release dosage form.

To impart the sustained release characteristics to the drug formulations in the lung many approaches have been studied including prodrug approach, conjugation of drugs to macromolecules, reducing aqueous solubility of drug or by incorporating the drugs in relatively insoluble materials like biodegradable polymers or lipids/ phospholipids. Microspheres prepared using biodegradable polymers have shown a good potential to sustain the release in the drug but are associated with a major limitation of very slow clearance from the lungs that lead to excessive deposition in the lung airways.

Liposomes are prepared using phospholipids endogenous to the lung as surfactants and have shown the potential to produce controlled delivery to the lung. Use of spray drying to formulate liposomal dispersion into dry powder formulation can further provide a more stable product with better aerosolization behaviour.

4.6.1 Preparation technique for liposomal dry powder for inhalation:

Sildenafil citrate, Hydrogenated soya phosphatidylcholine and/or Dipalmitoyl phosphatidylcholine and cholesterol in different molar ratios were dissolved in different volumes and ratios of mixture of methanol and chloroform and subjected to dry thin film formation in Rotavapor system. The liposomes thus obtained were hydrated with water or phosphate buffer pH 7.4. This liposomal dispersion was passed for 2-4 cycles through high pressure homogenizer (GEA NIRO Soavi S.P.A., Italy) to obtain desired particle size. Then liposomal dispersion was kept for annealing for 30-90 min to form stable dispersion.

Liposomes were prepared by varying various formulation parameters like drug lipid ratio from 1:4 to 1:10 with HSPC or DPPC alone and with both HSPC and DPPC, lipid: cholesterol ratio of 8:2 and 9:1, composition and volume of solvent system (chloroform and methanol, 0.5-1.5mL in 2:1, 1:1 and 1:2 ratio), hydration medium and its volume (1-2mL), time of hydration. Thin film hydration process

parameters like time of solvent evaporation (15-90min.) and hydration time (1-2.5h), speed of rotation during film formation (100-130) and hydration (50-80), vacuum, number of homogenization cycles (2-4) for particle size reduction and annealing time (30-90min).

The prepared liposomal dispersion was subjected to centrifugation to facilitate separation of unentrapped drug from liposomes. Liposomes hence obtained were spray dried by dispersing in aqueous solution of mannitol to obtain dry liposomal powder. The spray drying process to prepare dry powder liposomes for inhalation was optimized using fractional factorial design. Process parameters like feed rate (mL/min), compressed air pressure (bars), vacuum (mm of WC) and inlet temperature (°C) were varied to achieve the product with desired characteristics for pulmonary delivery. The effect of spray drying process parameters on percent drug retained, percent yield, aerodynamic diameter and moisture content was statistically analyzed using Stat-Ease software (Design-Expert® 8).

Table 4.4: Design of experiments to optimize spray drying process to prepare liposomal dry powder for inhalation of sildenafil citrate using fractional factorial design

	Factor 1	**Factor 2**	**Factor 3**	**Factor 4**
Run	**A:Feed rate**	**B:Air pressure**	**C:Vacuum**	**D:Inlet temperature**
	mL/min	Bar	mm of WC	°C
1	1	3	-100	80
2	3	3	-200	80
3	1	2	-200	80
4	1	2	-200	100
5	3	2	-100	80
6	3	3	-200	100
7	3	3	-100	80
8	1	3	-200	100
9	3	2	-100	100
10	3	2	-200	80
11	1	3	-200	80
12	3	2	-200	100
13	1	2	-100	100
14	1	3	-100	100
15	1	2	-100	80
16	3	3	-100	100

4.7 Formulation of Drug lipid composites of sildenafil citrate:

4.7.1 Preparation technique for Drug-lipid composites:

Keeping in mind the easy scalability of the manufacturing process, it was planned to simplify the large no. of steps involved in liposome formulation by using single step spray drying of the drug lipid mixtures to formulate lipid microspheres or drug-lipid composites of SDC. Sildenafil citrate and lipids (HSPC: DPPC: Cholesterol) were dissolved in methanolic solution containing sugar alcohol, trehalose as protective carrier [Kim *et al* 2001]. The effect of various formulation parameters like lipid:carrier ratio, %w/v of total solid content and %v/v of water in methanol was studied on the percent drug content, percent yield and flow characteristics of the formulation. The prepared solution was introduced into the hot air stream of drying chamber of LD-48 JISL spray dryer. The spray drying process parameters like feed rate (mL/min), compressed air pressure (bars), vacuum (mm of WC) and inlet temperature (°C) were varied according to fractional factorial design to get the optimum formulation with respect to percent drug content, percent yield, moisture content and aerodynamic diameter.

Table 4.5: Design of experiments to optimize spray drying process for drug-lipid composites of sildenafil citrate using fractional factorial design

	Factor 1	**Factor 2**	**Factor 3**	**Factor 4**
Run	**A:Feed rate**	**B:Air pressure**	**C:Vacuum**	**D:Inlet temperature**
	mL/min	Bar	mm of WC	°C
1	1	3	-160	50
2	3	3	-200	50
3	1	2	-200	50
4	1	2	-200	70
5	3	2	-160	50
6	3	3	-200	70
7	3	3	-160	50
8	1	3	-200	70
9	3	2	-160	70
10	3	2	-200	50
11	1	3	-200	50
12	3	2	-200	70
13	1	2	-160	70
14	1	3	-160	70
15	1	2	-160	50
16	3	3	-160	70

4.8 Formulation of large porous liposphères of sildenafil citrate:

4.8.1 Preparation technique for large porous liposphères:

To improve the dispersibility and respirable fraction of lipid based dry powder for pulmonary delivery, large porous liposphères were prepared by spray drying an emulsion based feed stock. A submicron emulsion is prepared by combining lipid phase (containing HSPC and drug dissolved in methanol and 1-2% chloroform) with aqueous phase (containing DPPC with or without mannitol) using high pressure homogenization. Emulsion is stabilized by the monolayer of long chain phospholipids, DPPC. The inclusion of chloroform acted as a blowing agent during spray drying (Naikwade SR et al 2009). Effect of drug:lipid ratio, surfactant concentration (%w/v), Blowing agent (% v/v) and Protective sugar (% w/v) was studied on the percent drug content, percent yield, moisture content and flow characteristics of the formulation. The spray drying process parameters like feed rate (mL/min), compressed air pressure (bars), vacuum (mm of WC) and inlet temperature (°C) were varied according to fractional factorial design to get the optimum formulation with respect to percent drug content, percent yield, moisture content and aerodynamic diameter. Inclusion of a solvent with lower boiling point like chloroform (61.2°C) act as blowing agent while spray drying the emulsion. Due to higher air stream temperature at the droplet surface than within the droplet, the solvent at the surface got evaporated causing the solvent inside the droplet to diffuse through the surface creating pores and hollow particles while passing through the drying chamber (Hirst PH et al 2002; Ungaro F et al 2006).

Table 4.6: Design of experiments to optimize spray drying process for dry powder large porous liposphere of sildenafil citrate using fractional factorial design

	Factor 1	Factor 2	Factor 3	Factor 4
Run	**A:Feed rate**	**B:Air pressure**	**C:Vacuum**	**D:Inlet temperature**
	mL/min	Bar	mm of WC	°C
1	1	3	-100	50
2	3	3	-200	50
3	1	2	-200	50
4	1	2	-200	90
5	3	2	-100	50
6	3	3	-200	90
7	3	3	-100	50
8	1	3	-200	90
9	3	2	-100	90
10	3	2	-200	50
11	1	3	-200	50
12	3	2	-200	90
13	1	2	-100	90
14	1	3	-100	90
15	1	2	-100	50
16	3	3	-100	90

4.9 Characterization and In-vitro evaluation of dry powder formulations of sildenafil citrate

4.9.1 Geometric Particle size and Zeta potential:

Particle size and zeta potential analysis of preformed liposomal dispersion before spray drying and emulsion for preparing large porous liposheres was carried using Malvern Zetasizer ver. 6.12 (Malvern Instruments Ltd., UK). The paticle size of dry powder formulations was determined by dispersing in propane-2-ol to attain obstruction of 10-20% at 1750rpm, using Dynamic light scattering principle (DLS) instrument, Mastersizer 2000 ver. 5.12 (at Zydus Research Centre, Ahmedabad, Gujarat, India). This method of particle size determination gives information about the geometric particle size which is different from the aerodynamic particle size. Mean median aerodynamic diameter of dry powder formulations was determined using Andersen Cascade Impactor.

4.9.2 Transmission Electron Microscopy (TEM)

The morphology of the liposomal dispersion and emulsion feed stock to prepare large porous lipospheres before spray drying was examined by transmission electron microscopy with CCD camera (Philips Tecnai 20, equipped with accelerating voltage of 200KV, twin objective lens for high point resolution 0.27nm or better and line resolution 2.0nm or better) using a negative staining technique employing a 2% uranyl acetate solution.

4.9.3 Scanning Electron Microscopy (SEM)

Scanning Electron Microscopy (SEM) is one of the standard techniques for surface characterization of lipid and polymer based powders with a much higher resolution. Investigation of surface texture and morphology of fractured or sectioned surfaces can be performed with this technique. The modern SEM has a resolution of 3 nm and provides magnifications ranging from less than 30-fold to 300,000-fold. Remarkable adaptability of this method is its major benefit over other practices. SEM is helpful to examine shape and surface topography of micro and nanoparticles.

The surface morphology of dry powders was viewed and photographed by scanning electron microscopy. The powder samples were gold coated by sprinkling on carbon tape affixed on aluminum stubs. The aluminum stubs were placed in the

vacuum chamber of a scanning electron microscope JFC 1100, JEOL Japan. Then the samples were examined with SEM (JEOL JSM 6100, Tokyo, Japan).

4.9.4 Drug content and Entrapment Efficiency (%EE)

To determine %EE in liposomes and large porous lipospheres, assay of the Sildenafil citrate in lipid dispersion was determined by taking the sample from the dispersion immediately after homogenization (to ensure the uniform sample representative of the whole dispersion). In case of drug lipid composites, the known amount of the dry powder was dispersed well in water using spinix mixer. Then the free and entrapped drug was measured. The free Sildenafil citrate (unentrapped) in the dispersion was separated by ultracentrifugation at 15000 rpm for 30min at 4°C and the supernatant was removed without disturbing the pellet and analyzed for free drug content in buffer (Fb). Then, pellet was redispersed with known aliquots of water and the centrifugation cycle was repeated twice. The entrapped Sildenafil citrate (E) was determined (using UV spectroscopy) by dispersing the pellets in methanol. Mass balance and percent recovery was determined by comparing the assay with sum of entrapped and total free drug. In case of conventional DPI and drug-sugar composites, assay was determined by dissolving the known amount of dry powder in water and analyzing at 292nm using UV spectroscopy. Drug solution in water and methanol showed good linearity (R^2 = 0.99 for both) at concentration range within 2-50 mcg/mL of sildenafil citrate.

Entrapment efficiency (%) = (Drug in pellet / Initial assay of the drug) X 100

For mass balance:

Recovery (%) = {[Total free drug + Entrapped drug (E)]/ Assay} X 100

4.9.5 Determination of Angle of Repose

A pile of powder was carefully built up by dropping the powders through a funnel tip from a height of 2 cm. The angle of repose was calculated by inverting tangentially the ratio of height and radius of the formed pile.

4.9.6 Bulk density and Tapped Density

Bulk density (ρ_b) was determined by adding 500mg of sample into a 10mL graduated measuring cylinder. After observing the initial volume, the cylinder was

mechanically tapped more than 500 times to get the closest packed densities or tapped density (ρ_t).

4.9.7 Compressibility index and Hausner Ratio

Compressibility index (CI) and Hausner ratio of powder as a measure of flow and dispersibility were measured by methods as described in United States Pharmacopoeia, (2001). The compressibility index was calculated by the following formula:

$CI = 100 (\rho_t - \rho_b)/ \rho_t$

Hausner Ratio = ρ_t / ρ_b

Where ρ_b is the bulk density of the weighed sample &

ρ_t is the tapped density of the sample after 500 taps.

4.9.8 Moisture content

Moisture content of dry powder formulations is very important parameter to impart better aerosolization characteristics. Ideally, it should be less than 4% w/w. The moisture content of prepared formulations (1g) was determined by Karl-Fischer titration. Commercially available pyridine free reagent was used for analysis. The reagent was standardized with addition and determination of known quantity of water (250mg). Firstly, 40mL of methanol was added into the titration vessel and then titrated with the reagent to determine the amount of water present in the samples.

4.9.9 Residual solvent

Residual solvents in pharmaceuticals are volatile organic chemicals that are used in and are produced during the synthesis of drug substances or can be used in production of drug formulations. Many of these volatile organic chemicals generally cannot be completely removed by standard manufacturing procedures and are left behind, preferably at low levels. High levels of residual organic solvents represent a risk to human health because of their toxicity. Residual solvents can create odor problems and color changes in final product. Residual solvents are classified in three categories on the basis of their toxicity level and the degree to which they can be considered an environmental hazard. Class I solvents are the most toxic and should be avoided, for example, benzene, carbon tetrachloride etc. Class II solvents are considered at lesser risk but they should be limited in pharmaceutical products

because of their inherent toxicity, for example, acetonitrile, dichloromethane, chloroform and methanol etc. Class III solvents are at the lowest risk category, for example, acetic acid, pentane etc. The concentration limits for class I solvents is generally between 2-8 ppm except for 1,1,1-trichloroethane for which the limit is 1500 ppm. For Class II solvents limits vary from 50-4000 ppm. Class III solvents require only GMP based testing and are limited to 5000 ppm or 0.5% w/w.

In order to gain US FDA approval for any microparticulate formulation, it is necessary to consider regulatory requirements of residual solvents content in it. Virtually all nano or microparticulate fabrication processes require the use of an organic solvent such as chloroform, methanol, dichloromethane, ethyl acetate for lipid or polymer dissolution. These solvents may pose significant health risks on long term exposure. Regulatory agencies often require that formulator should attempt to reduce residual solvents as much as possible. FDA limits based on recommendations of ICH and Pharmacopoeial (USP) limits of dichloromethane (class II) are 600 ppm. Additionally, the presence of residual solvents at high levels may have direct impact on encapsulated drug stability. High residual methanol levels may reduce the glass transition temperature of polymer causing microspheres to agglomerate or clump under storage and may result in poor dispersibility.

Residual solvent testing can be conducted by a number of analytic techniques. The most popular and most appropriate, specific residual solvent analysis is done by gas chromatography (GC). GC has the ability to separate component solvents, thus identifying them, and is capable of low detection limits when the appropriate detector is used. FID (flame ionization detector) is the most widely used detector for GC because of its low detection limits, wide linear dynamic range, and general reliability and utility, especially for trace organic compounds.

Chloroform and methanol were used in formulation of liposomal dry powder and large porous lipospheres and methanol for drug-lipid composites. Residual solvent limit for chloroform is 60ppm and that for methanol is 3000ppm. Thus, residual solvent content was determined in liposomal dry powder, drug-lipid composites and large porous liposphere dry powder formulations using gas chromatography.

4.9.10 Differential Scanning Calorimetric studies

DSC is the most widely used calorimetric techniques for the determination of various thermal parameters, which allow a better understanding of drug-lipid interactions, drug excipient interactions and thermal denaturation of lipids. DSC measures the heat capacity of the system as a function of temperature. Following the change in heat capacity of the sample as a function of temperature allows for the detection of phase transitions of various orders.

DSC studies of drug, lipids, excipients and microspheres were carried out in an attempt to define physical state of drug in these carriers and possibility of interaction between the drug and excipients within the vesicles, network or reservoirs of lipids in formulations. Small amount of solid samples were placed in hermetically sealed aluminium pans and heated from 30°C to 300°C at a heat flow rate of 10°C/min under nitrogen spurge using DSC and TGA apparatus (Perkin Elmer, Model Pyris 1) at Zydus Research Centre, Ahmedabad. The glass transition temperature (Tg °C) and melting point of samples was recorded as endotherms.

4.9.11 X-ray Diffraction studies (XRD):

X-ray Diffraction (XRD) studies were conducted for API, optimum batches of the different formulations along with respective placebo formulations using X-Ray Diffraction instrument (Rigaku, Model Multiflex) Zydus Research Centre, Ahmedabad. The main objective of this study was to determine possible changes in crystallinity of drug after processing.

4.9.12 Aerosolization performance of the formulations:

Aerosolization behaviour of the developed formulations was determined based on delivered dose uniformity and Mean median aerodynamic particle size using Dosage unit sampling apparatus (DUSA) for DPI and Andersen cascade impactor (ACI) respectively of Copley Scientific.

4.9.12.1 Delivered Dose Uniformity:

Dosage Unit Sampling Apparatus (DUSA by Copley Scientific) for DPIs was used to perform the tests specified by the Pharmacopoeias that relate to content uniformity namely “Delivered Dose Uniformity”. The sample collection tube was fitted with a 47 mm glass fibre filter for dosage collection. The two doses were discharged one by one from the inhaler (Rotahaler) fitted to the mouthpiece, into the

collection tube by activating the timer (adjusted 5sec to get 4KPa to give an inspiration volume of 4L) on the Critical Flow Controller controlling the two way solenoid valve. The sample was collected by adding solvent (water for CDPI and DS/ methanol in case of DL, DPL and LPL) to the collection tube and shaking vigorously by applying rinsing caps before assaying the contents. The procedure was repeated ten times. The test is passed if not less than nine out of ten doses should are between 75-125% of average dose calculated and none is outside the range of 65-135% of average dose calculated as per USP.

4.9.12.2 Aerodynamic particle size using Andersen cascade impactor (ACI):

Andersen cascade impactor (ACI) is the instrument of choice as per pharmacopoeia (USP apparatus 3 for DPIs) as well as regulatory bodies to determine aerodynamic particle size distribution of inhalation formulations. It operates on the principle of inertial impaction on each stage of impactor comprising series of jets through which sample laden air is drawn, with the help of vacuum, from the inhaler device. Stages are arranged in a stack in decreasing order of particle size. Particle mass below 5 μm (range 1-5 μm) is usually described as the respirable fraction or fine powder fraction that actually settles in the lung and bigger particles get impacted in oropharynx and particles below 1 μm are exhaled as such without lung deposition.
ACI with well dried silicone coated stages was assembled and inhaler device (Rotahaler) was primed by connecting to the mouthpiece adapter for air-tight connection with induction port.

Flow meter was attached to the induction port, pump was switched on and two way solenoid valve was opened to adjust the flow control valve to the desired flow rate. ACI was operated at 28.3L/min flow rate to determine the aerodynamic particle size of the dry powder formulations. This was achieved by activating the timer (adjusted 5sec to get 4KPa to give an inspiration volume of 4L) on the Critical Flow Controller controlling the two way solenoid valve. Flow rate stability was ensured throughout the operation by measuring the absolute pressure at a point on either side of flow control valve. Ratio of pressures on either side should be ≤ 0.5 to ensure stable flow. Inhaler device was loaded with the capsule, containing the dry powder formulation, to induction port via mouthpiece adapter.

Ten capsules were discharged into the apparatus one by one from Rotahaler® device at appropriate flow rate by activating the timer to give a pressure drop of 4kPa.

At the end, samples were collected by rinsing with suitable solvents by rinsing the walls of respective stages.

Analysis of the amount of drug deposited on each stage was done to determine recovered dose (RD), emitted dose (ED), fine powder fraction (FPF), Mass Median Aerodynamic Diameter (MMAD) and Geometric Standard Deviation (GSD).

Recovered dose (RD) was determined as the total amount of sildenafil citrate recovered from the inhaler, capsule shell and the apparatus and was expressed as the percentage of the average assay amount.

The emitted dose (ED) was calculated as the amount emitted from the inhalation device and capsule into the apparatus.

Fine particle dose (FPD) was considered as the amount of sildenafil citrate found below effective cut-off diameter < 4.7µ.

Fine particle fraction (FPF) was the ratio of FPD to RD, expressed as percentage, while dispersibility was expressed as the percentage of FPD to ED.

Mass Median Aerodynamic Diameter (MMAD) is defined as the diameter at which 50% of the particles by mass are larger and 50% are smaller. USP <601> calls for determining the MMAD by plotting, on log probability paper, the percentages of mass less than the stated aerodynamic diameters versus the aerodynamic diameters. Weight of the particles is determined on each stage by calculating the difference in initial weight of each stage and weight of that satge after sample loading. Then cumulative percent fraction containing less than each size range is determined. The MMAD is taken as the intersection of the line with the 50% cumulative percent. Computational methods can also be applied.

Geometric Standard Deviation (GSD) is a measure of the spread of an aerodynamic particle size distribution. Typically calculated as follows:

$GSD = (d_{84}/d_{16})^{1/2}$

where d_{84} and d_{16} represent the diameters at which 84% and 16% of the aerosol mass are contained, respectively, in diameters less than these diameters.

4.9.13 *In-vitro* Release study:

The release from the formulation is dependent both on diffusion through the lipid matrix and on lipid degradation. The possible mechanisms of drug release include; initial release from surface, release through the pores dependent on particulate structure, diffusion through the intact lipid barrier, which is dependent on

intrinsic properties of the delivery system and core solubility, diffusion through a water swollen barrier dependent on hydrophilicity, which in turn may depend on glass transition temperature of the lipid, its erosion and bulk degradation, release may be affected by the rate of erosion and hydrolysis of lipids, leading to pore formation in matrix.

An *in vitro* release study of the sildenafil citrate dry powder formulations was performed by using dialysis bag method in rotating bottle dissolution apparatus (Electrolab). The dissolution medium was 10 mL deionized water and the temperature of the dissolution medium was kept at 37 °C. During the study, samples were withdrawn at predetermined time intervals. Release medium was replaced with an equal amount after each withdrawl. The concentrations of sildenafil citrate were determined by the UV spectrophotometer UV–Visible system (Shimadzu UV-1601, Japan) at 292 nm. According to the determined concentrations of released sildenafil citrate, cumulative percent release (Q) was determined at each time point (t). Different graphs Q vs t for Zero order, log Q vs t for First order, Q vs √ t for Higuchi model, cube root of Q vs t for Hixson- Crowell and log Q vs log t for Korsmeyer-Peppas were plotted to evaluate the best fit release kinetic model for various SDC dry powder formulations.

4.9.14 Stability studies:

Stability testing is an important issue in order to demonstrate that clinical effect, patient safety and quality of the drug is maintained during its maximal time of storage and intended use. The purpose of stability testing is to provide evidence on how the quality of a drug substance or drug product varied with time under the influence of temperature, humidity and light in order to establish recommended storage conditions and shelf-life period. Importance of the stability can be adjudged from the fact that in USA (In 1999), 10% of the NDA'S were delayed due to inadequate stability data, (In 2001); more than 25% of the recalls were related to stability failures and more than 36% warning letters cited stability problems.

In general, a shelf life of at least one year is a minimum prerequisite for a commercial product. Lipid based formulations are more susceptible to degradation, hydrolysis and drug leakage, especially as liquid dosage form. Though, dry powder formulations impart stability and resistance to leakage and degradation of lipid based formulations, its stability evaluation is important to establish the quality of the

product with respect to its assay amount. In case of dry powder inhalation formulations, particle size, moisture content and percent respirable fraction (%FPF) are other important features need to be considered during stability evaluation as these parameters affect the lung deposition and hence therapeutic efficacy of the product.
All the dry powder formulations containing sildenafil citrate equivalent to 600µg of sildenafil were filled in size 3 hypromellose Quali V® capsules and then kept in HDPE bottles for conducting storage stability.

Optimized batches of conventional DPI, drug-sugar composites, spray dried liposomes, large porous lipospheres and drug-lipid composites (along with respective placebos) were kept under various temperature conditions of 30°C/65%RH, 25°C/60%RH, 2-8°C and -20°C. Conventional DPI and drug-sugar composites were kept at 40°C/75%RH, 30°C/65%RH and 25°C/60%RH. These formulations were evaluated for appearance, assay and/or percent drug retained, moisture content and/or aerodynamic size. Samples on -20°C and 25°C/60%RH were kept as control and should be analyzed only if the samples failed at accelerated stability or long term stability conditions in case of lipid based formulations.

4.9.15 Macrophage uptake study:

4.9.15.1 Introduction

Alveolar macrophages are an important first line defense system against inhaled viable and nonviable particles. Alveolar macrophages are regarded to be very tough phagocytic cells (P. Camner *et al*. 2002). Lung denotes a potential site for treatment of pulmonary arterial hypertension and many other lung diseases. Identification of factors that affect localization and retention of lipid based formulations in the lungs is needed for its efficient and sustained delivery to the lungs (Geiser M. 2002; 2010). One such factor is the particle size of the formulation. Generally the favourable size for macrophage uptake is less than 5micron. Thus lipid based dry powder formulations prepared to have higher geometric size i.e. greater than 5µm were tested for the rate and extent of uptake by alveolar macrophages.

4.9.15.2 The Fluorescence Process

Fluorescence is the result of a three-stage process of excitation, excitation life-time and fluorescence emission that occurs in certain molecules called fluorophores or fluorescent dyes. Fluorescein (free acid, CAS Number 2321-07-5) is a fluorophore, which has absorption maxima at 493.5 and 460 nm. Fluorescein has empirical formula $C_{20}H_{12}O_5$ and Molecular weight 332.31. It shows the excitation wavelength of 475-490 nm and emission wavelength of 510-520 nm with peak fluorescence at pH 7.4. Fluorescein is soluble in methanol. It shows good aqueous solubility in buffers of pH>7.5 and constant results due to lack of interaction with surfaces beyond this pH (Sjoback R *et al* 1995). Thus, fluorescein was incorporated into the lipid based dry powder formulations to compare the macrophage uptake of these formulations with standard Latex beads (carboxylate-modified polystyrene, fluorescent yellow green) of 2μ size.

Fluorescence intensity is quantitatively dependent on the same parameters as absorbance defined by the Beer-Lambert law as the product of the molar extinction coefficient, optical path length and solute concentration as well as on the fluorescence quantum yield of the dye and the excitation source intensity and fluorescence collection efficiency of the instrument. In dilute solutions or suspensions, fluorescence intensity is linearly proportional to these parameters. Fluorescence instruments are primarily of four types, eliciting different information:

1. Spectrofluorometers and microplate readers measure the *average* properties of bulk (μL to mL) samples.
2. Fluorescence microscopes resolve fluorescence as a function of spatial coordinates in two or three dimensions for microscopic objects (less than ~0.1 mm diameter).
3. Fluorescence scanners, including microarray readers, resolve fluorescence as a function of spatial coordinates in two dimensions for macroscopic objects such as electrophoresis gels, blots and chromatograms.
4. Flow cytometers measure fluorescence per cell in a flowing stream, allowing subpopulations within a large sample to be identified and quantitated.

To quantify the fluorescence in our samples depicting macrophage uptake of different formulations at different times, we used spectrofluorometer with microplate reader and Fluorescence microscopes for imaging the macrophage uptake, at different time points, in the wells. Since, fluorescein shows photobleaching, all the preparations and macrophage uptake study was done under subdued light, using amber coloured glass apparatus, black plates and by covering the apparatus well with aluminium foil.

4.9.15.3 Preparation of lipid based dry powder formulations with fluorescein:

4.9.15.3.1 Preparation of fluorescein loaded liposomal dry powder for inhalation:

Sildenafil citrate, Hydrogenated soya phosphatidylcholine and/or Dipalmitoyl phosphatidylcholine and cholesterol in optimized molar ratios were dissolved in mixture of methanol and chloroform and subjected to dry thin film formation in Rotavapor system. Sildenafil citrate showed similar percent entrapment efficiency at pH 7.4 and pH 8.0, thus already established method can be used by dissolving fluorescein in aqueous phase of phosphate buffer, pH 8.0. The liposomes thus obtained were hydrated with phosphate buffer pH 8.0 containing fluorescein. The formulation contained fluorescein: sildenafil citrate: HSPC: DPPC: cholesterol in molar ratio of 1:5:18:4.5:2.5. This liposomal dispersion was passed for 2-4 cycles through high pressure homogenizer (GEA NIRO Soavi S.P.A., Italy) to obtain desired particle size. Then liposomal dispersion was kept for annealing for 30-90 min to form stable dispersion.

The prepared liposomal dispersion was subjected to centrifugation to facilitate separation of unentrapped drug or fluorescein from liposomes. Supernatant was decanted and pellette was washed again by repeating the dispersion, vortexing and centrifugation process, twice with phosphate buffer pH 8.0 to remove any unentrapped fluorescein and twice in water to remove any unentrapped drug. Liposomes hence obtained were spray dried by dispersing in aqueous solution of mannitol to obtain dry liposomal powder labeled with fluorescein. The spray drying process was performed under the optimized process conditions for liposomal dry powder for inhalation.

4.9.15.3.2 Preparation of fluorescein loaded drug-lipid composites: Sildenafil citrate, lipids (HSPC: DPPC: Cholesterol) and fluorescein were dissolved in methanolic solution with trehalose as protective carrier. The prepared solution was

introduced into the hot air stream of drying chamber of LD-48 JISL spray dryer, under optimized spray drying conditions for drug-lipid composites, to get dry powder of fluorescein labeled sildenafil citrate-lipid composites.

4.9.15.3.3 Preparation of fluorescein loaded large porous lipospheres:

A submicron emulsion is prepared by combining lipid phase (containing HSPC, drug and fluorescein dissolved in methanol-chloroform solvent system) with aqueous phase (phosphate buffer pH 7.4 containing DPPC and mannitol dispersed at 80°C) using high pressure homogenization. Emulsion is stabilized by the monolayer of long chain phospholipids, DPPC.

4.9.15.4 Calibration curves, determination of percent entrapment and macrophage uptake:

A calibration curve of fluorescein (free acid) in methanol and Dulbecco's Modified Eagle Medium (DMEM) was generated using a concentration range from 0 to 200 nM. Stock solutions of 200nM (6.646mg/ 100mL in methanol and separately in tris buffer pH 8) were prepared and then suitable dilutions were done with respective medium to prepare a concentration range between 0 to 200nM. Fluorescein RFU of the labeled plates was read on Fluorescence Microplate reader (FL_x 800, Biotec Instruments Inc. with Tungsten Halogen light source and a sensitivity of Fluorescein 5 pM typical, 1 fmol/well 96-well plate) using filters Ex: 485/20 and Em: 575/15. The same plates were covered well with aluminium foil and read again after 1day to overrule any possibility of photobleaching or change in RFU with time. The prepared formulations were assayed for drug and fluorescein using UV spectroscopy and fluorescence plate reader respectively.

Lipid based formulations were dissolved in methanol to determine the percent entrapment of drug and fluorescein in the sample. For macrophage uptake study, the formulations were diluted by dispersing in DMEM to achieve the desired particle count. Dilutions of standard beads (2µm polystyrene fluorescent beads), liposomal dry powder with HSPC alone, liposomal dry powder with HSPC and DPPC, drug-lipid composites and large porous lipospheres were prepared in DMEM to achieve $7.5x10^4$ beads or particles per mL (counted on hemacytometer) for each formulation. Thus, calibration curve in DMEM was prepared to quantify the macrophage uptake in

terms of relative fluorescence units of fluorescein detected in each sample at particular time point. After recording RFU in the samples, the plates were also imaged using inverted Olympus microscope with Camera and DP controller software.

4.9.15.5 Isolation of alveolar macrophages

Raw macrophage cell line in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen) with 10% foetal bovine serum (FBS) was obtained from the cell culture department of Zydus Research Centre. Live cells were adhered to the surface of the flask and the dead cells in media suspensions were discarded. Trypsinization was done by incubating for 5minutes in 2mL of solution containing 25% trypsin and 0.5% ethylenediaminetetraacetate (EDTA) to remove FBS that hinders the removal of cells from the surface. This process removes the adhered cells from the surface of the flask. Remove the cells with pipette from the flask into the centrifuge tube. Washings of flask with DMEM were also added to it to retrieve maximum number of cells. Spin for 2min at 5000rpm to get a pellet and clear supernatant. Supernatant was discarded and pellet was suspended in DMEM with 10% FBS. Ten µL of medium containing macrophages was added to hemacytometer to get the mean cell count per mL.

4.9.15.6 Macrophage uptake study of different lipid based dry powder formulations: Raw macrophages were seeded at $1.0x10^5$ cells/well into 24-well plates containing Dulbecco's Modified Eagle Medium (DMEM; Invitrogen) with 15% FBS (foetal bovine serum) and incubated at 37°C for 24h in Stericycle CO_2 incubator (Thermo electron Corporation). Then DMEM was removed carefully and cells were washed with phosphate buffer saline (pH 7.4). 20µL of the prepared dilutions of standard beads (2µm polystyrene fluorescent beads), liposomal dry powder with HSPC alone, liposomal dry powder with HSPC and DPPC, drug-lipid composites and large porous lipospheres (containing $1.5x10^3$ beads or particles/well) were added to the wells labeled according to the type of formulation and different time points. A row of wells was labeled as control containing adhered macrophage cells and DMEM without any beads or particles. Initial RFU of the labeled plates was read on Fluorescence Microplate reader.

The plates were then kept in incubator at 37°C and reaction was stopped at each time point (30min, 1h, 2h, 4h, 12h and 24h) by making three washings with phosphate buffer saline (pH 7.4) and incubating for 15min with 500 µL of 4%

paraformaldehyde (PFA solution) to fix the cells. Washing with phosphate buffer saline (pH 7.4) was done again to remove any residual PFA. Relative fluorescence unit for different formulations in the labeled plates were recorded finally using Fluorescence Microplate reader for quantitative determination of the particle uptake by macrophages. Then, imaging was done using Olympus microscope under UV light filter for yellow green fluorescence and image software (DP controller/ DP manager) at magnification of 4x and 10x.

4.9.16. *In-vivo* pharmacological evaluation of sildenafil citrate dry powder formulations for inhalation

PAH is a complex, devastating, incompletely understood pathophysiological condition predominantly affecting the small pulmonary arteries. It is characterized by vasoconstriction, right ventricular hypertrophy, intimal lesions, medial hypertrophy, and adventitial thickening of the precapillary pulmonary arteries. The progressive pulmonary hypertension leads to right heart failure and death due to increased afterload on right heart. Increased vasomotor tone and chronic remodeling of the precapillary resistance vessels, including marked vascular smooth muscle cell growth are assumed to be underlying pathogenetic mechanisms.

Current treatments for PAH target the constriction of the arteries, to suppress or reverse the underlying vascular remodeling. Cyclic guanosine monophosphate (cGMP) is an intracellular second messenger that has vasodilator and antiproliferative actions. Phosphodiesterase-5 (PDE-5) metabolizes cyclic GMP, decreasing intracellular levels, and limiting vasodilator effects. Many studies showed that the cGMP-specific PDE 5 is highly expressed in lung tissue (Giordano *et al*). Moreover, further upregulation of PDE5 may occur under conditions of pulmonary hypertension, thereby contributing to increased pulmonary vascular resistance (Murray *et al*). Sildenafil citrate (SDC), a selective inhibitor of PDE-5 enzyme slows down the metabolism of cyclic GMP and hence intensifies the pulmonary vasodilator actions in patients suffering from pulmonary hypertension. (Prasad *et al* 2000; Lepore *et al* 2002; Shekerdemian *et al* 2002).

Beyond acute pulmonary vasodilation, PDE-5 inhibitors may also possess antiremodeling potency through increased cAMP and cGMP levels (Clapp *et al*). Sildenafil citrate is available in the market as Revatio® tablets (20mg thrice a day) and Revatio® injection (12.5mL thrice a day) for the treatment of PAH. Current therapy is associated with inconvenience of frequent administration and moreover, the parenteral

and peroral administration of the drugs to treat PAH is accompanied with non-specific systemic vasodilation leading to many side effects like postural hypotension, headache, flushing and visual disturances. Thus the potential of these agents to improve PAH can be further remunerated by offering direct delivery of these agents to the lungs for beneficial long-term administration.

A number of animal models have been used to study pulmonary hypertension, most commonly employing hypoxia or monocrotaline. Monocrotaline is a toxin that causes endothelial injury leading to medial hypertrophy in the pulmonary arterioles. Also, pneumonectomized rats injected with monocrotaline have exhibited neointimal overgrowth in addition to medial hypertrophy (Nishimura *et al.* 2003). No animal model, however, reproduces the full spectrum of changes seen in lung specimens from patients.

In the current study, we addressed this issue in a model of chronic pulmonary hypertension, employing the alkaloid monocrotaline (MCT). This toxin causes pulmonary arterial endothelial cell injury and subsequent pulmonary artery smooth muscle hypertrophy with persistent severe pulmonary hypertension after one injection in rats (Rosenberg *et al.* 1988). We investigated the *in-vivo* efficacy of lipid based sustained release sildenafil citrate dry powder formulations and its conventional and immediate release dry powder formulations to prevent and treat PAH after pulmonary administration using endotracheal intubation technique.

4.9.16.1 MATERIALS:

Monocrotaline (Sigma Aldrich, Co.); Anket® Ketamine injection I.P. 50mg/mL (Neon Laboratories Ltd.) and Xylazine injection USP 20mg/mL (G. Loucatos and Co.); Portex Fine Bore Polyethene tubing 30m (0.76mm ID and 1.22mm OD), Microtips (Tarsons), Finnetip (Tarsons), Adjustable metal plate, Fibre optic lamp with two flexible optical arms, metal laryngoscope to lift the lower jaw, Rubber bands to suspend the rat at ~ 45° angle by the two front upper teeth, Liquid nitrogen in a cryocan to flash freeze the lungs, Nitrogen gas cylinder to purge into labelled tubes containing frozen lungs before storing at -80ºC, PE 50 tubing for canulation, Normal saline and Heparin sodium injection (Beparine, 25000 I.U. in 5mL; Biological E. limited, Hyderabad) to prepare heparinized saline, cGMP complete EIA kit (Enzo life sciences), Labelled bottles containing 10% formalin for fixing lungs for histopathology.

4.9.16.2 METHODS:

4.9.16.2.1 MCT Treatment:

Monocrotaline (Sigma Aldrich, Co.) was dissolved in 0.1N HCl and pH was adjusted to 7.4 with 0.1N NaOH. This solution was given as a single subcutaneous injection (40mg/Kg) to 7-8 week old male wistar rats having weight between 180-220g. The rats were divided into different groups **(Table 4.7)** of **1)** MCT treatment only, Only-MCT_{14} (till 14 days) and Only-MCT_{28} (till the end, 28^{th} day of study) **2)** Group A as Preventive study group of formulations 1-5 (1A, 2A, 3A, 4A and 5A) for 14 days; and **3)** Group B as therapeutic group to study the effect of formulations 1-5 (1B, 2B, 3B, 4B, and 5B and respective placebos) till 28 days. Group B had extra animals to study the sustained effect of the formulations at different time points after first administration. Control rats received an equal volume of normal saline. Each group included 6 rats for each time point.

Table 4.7: Description of the groups and formulations used for in-vivo study

Formulation code*	Group	Description
F1	A for preventive study B for therapeutic study	Conventional dry powder for inhalation (CDPI) of sildenafil citrate with lactose carriers
F2	A for preventive study B for therapeutic study	Drug-sugar composites of sildenafil citrate with mannitol prepared by spray drying technique
F3†	A for preventive study B for therapeutic study	Liposomal dry powder for inhalation (LDPI) prepared by thin film hydration and spray drying
F4†	A for preventive study B for therapeutic study	Drug-lipid composites of sildenafil citrate prepared by spray drying technique
F5†	A for preventive study B for therapeutic study	Large porous liposphères prepared by emulsification and spray drying technique
Control	For preventive and therapeutic study	Animals received a single subcutaneous injection of normal saline
Only-MCT_{14}	For observations on 14^{th} day of study as final for preventive and initial for therapeutic study	Single subcutaneous injection (40mg/Kg) of monocrotaline
Only-MCT_{28}	For final observations on 28^{th} day of study	Single subcutaneous injection (40mg/Kg) of monocrotaline

*Respective placebos of formulations F1 to F5 were also prepared without drug to see any effect on the study parameters. † Lipid based formulations

4.9.16.2.2 Experimental design:

All the prepared formulations were evaluated for preventive and therapeutic potential in PAH.

4.9.16.2.2.1 Preventive study (Protocol 1):

After 24h of MCT treatment, each animal of group A was administered respective formulation daily for 2weeks. All the formulations were administered via intratracheal route using endotracheal intubation technique (Robert H. Brown *et al*., 1999). Animals were anaesthetized using ketamine/ Xylazine solution 0.22mL/ 200g (intramuscular injection at a dose of 50-60mg/Kg of ketamine and 5-10mg/kg of xylazine) that was sufficient to keep the animals anaesthetized for around 30 min. Then each rat was suspended one by one at an angle of ~45º by two front upper teeth by a rubber-band attached to a metal plate. A halogen light source with two flexible fiber-optic arms was positioned (one under the lower jaw and another arm adjusted above the mouth) for each rat for easy visualization of the trachea just below the vocal cords.

A metal laryngoscope was used to open the mouth from lower jaw and move the tongue little out on one side to maximize oropharyngeal exposure and to have a clear view of tracheal opening. Then a 4cm long PE 30m catheter attached to a microtip (2-200µ) containing weighed dry powder formulation, was carefully inserted ~8mm-1cm into the trachea. We could see the up-down movement of the powder synchronous with the breathing pattern of the rat confirming the correct placement of the catheter. The dry powder was then slowly pushed, by attaching a finnetip syringe barrel on to the microtip attached with catheter, at a speed of ~1.5cm/sec during inspiration by rat. Few rats could inhale the dry powder formulations without any barrel push. The endotracheal tube was removed carefully and immediately after the inhalation. After 24 hours of the final administration on 14^{th} day, the animals of each subgroup of Group A were anaesthetized by intraperitoneal injection of urethane (1.5g/kg) for various recordings and evaluation of parameters.

4.9.16.2.2.2 Therapeutic study (Protocol 2):

After 2weeks of MCT treatment (on 14^{th} day), each animal of group B (F1B-F5B) was administered respective formulation. Various hemodynamic parameters and cGMP levels were recorded after 2h, 8h, 24h and 48 h and SDC levels in lungs at 0h, 2h, 4h, 8h, 12h, 24h and 48 h of the first administration of formulations and compared

with that of only MCT treated and control group to evaluate the sustained release effect of the prepared formulations. Rest of the group B animals was administered different formulations and their respective placebo daily for 2weeks (till 28th day of the study).

All the formulations were administered via intratracheal route using endotracheal intubation technique as described above in the protocol 1. After 24 hours of the final administration on 28th day, the animals of each subgroup of Group B were anaesthetized by intraperitoneal injection of urethane (1.5g/kg) for various recordings and evaluation of parameters.

4.9.16.2.3 Study parameters:

PAH is characterized by increase in right ventricular systolic pressure due to pulmonary arterial vasoconstriction, right ventricular hypertrophy and vascular remodeling including intimal lesions and medial hypertrophy. Thus these parameters were recorded to mark the disease development and effectiveness of the treatment.
Hemodynamic measurements: A right heart catheter (PE 50 tubing), attached with heparinized saline filled syringe, was inserted through right jugular vein into the right ventricles for measurement of mean right ventricular systolic pressure. The left carotid artery was cannulated using another catheter for monitoring mean systemic arterial pressure.

All pressure measurements were made through reusable BP transducer using chart 5 of Power LAB® Software setup with PowerLab systems (ML785 PowerLab/8SP, AD Instruments, Colorado Springs, CO). After exsanginations, the left lung was fixed in 10% neutral-buffered formalin for histopathology study and the right lung was immediately frozen in liquid nitrogen and stored in labelled tubes at -80 ºC after nitrogen purging for cGMP and SDC analysis.

4.9.16.2.3.1 Right ventricular hypertrophy measurement:

The right ventricle (RV) was dissected from the left ventricle (LV) and septum (S) after removal of the atria. The ventricles were blotted dry well and weighed accurately. Total right ventricular weight (g), total weight of left ventricles and septum (LV+ S, in g), and ratio of RV to LV plus S [RV/ (LV + S)] were calculated as an index of right ventricular hypertrophy and was expressed as percentage.

4.9.16.2.3.2 Measurement of cGMP levels in lung homogenates:

Frozen lung tissue was fine powdered under liquid nitrogen in a stainless steel mortar. Powdered tissue was weighed immediately after evaporation of liquid nitrogen and homogenized in 10 volumes of 0.1M HCl using Tissue Tearor (Model 985370, Biospec Products Inc.). Then the samples were centrifuged at 3000g at 4 ºC for 10min, to pellet the debris. The supernatant was analyzed for cGMP levels and protein content. cGMP levels (pmoles/mL) were measured using cGMP complete EIA kit (Enzo Life Sciences®). The protein content was estimated using Lowry's method. The cGMP levels were expressed as pmol/ mg of protein content. Optical density of all the samples was determined using plate reader (Spectra Max Plus, Molecular Devices) with Soft max Pro software.

4.9.16.2.3.3 Measurement of drug levels in lung homogenates:

Lung tissue samples of control, MCT treated and different formulation groups were weighed and powdered using liquid nitrogen and homogenized in 0.1M HCl as described above. Then the denatured protein from the samples was separated by centrifugation at 10,000rpm for 15 min at 4 ºC. Then 200µL of supernatant was taken into HPLC vials and analyzed using Agilent 1100 series Chem station for HPLC with Fortis 5µ C-18 (250X4.6mm column). All samples were analyzed using Orthophosphoric acid, 0.05M and acetonitrile (65:35) as mobile phase and detection wavelength of 240nm.

4.9.16.2.3.4 Histopathology: Fixed left lobes of rat lungs were cut in blocks from different regions including anterior, posterior and the hilus part for paraffin embedding. Then 5µ sections were cut using microtome (MICROM). These cut sections were taken on the slides and stained for Hematoxylin-Eosin and Elastica staining using regular histopathological technique. Microscopic evaluation and photography was performed using NIS-Elements software with Nikon Eclipse E600 microscope mounted with camera (Nikon, Japan). The slides were characterized for inflammatory reaction, muscularization of arteries and medial thickening of peribronchial arteries.

4.9.16.2.4 Analysis of data:

Comparison between the groups at the end of preventive and therapeutic study was assessed using one way ANOVA and Student-Newman-Keuls post-hoc test for multiple comparisons. Two way ANOVA and Bonferroni post-test for multiple comparisons was used to evaluate the data at different time points for different groups to evaluate the sustained effect of different formulations. Pulmonary pharmacokinetics with respect to drug levels in the lungs was determined by non-compartmental modeling using software Win NonlinPro^{TM} Professional Edition, ver. 2.1.

Chapter: 5

Results and Discussion

5. RESULTS AND DISCUSSION:

5.1 UV Spectrophotometric calibration curves of sildenafil citrate

5.1.1 Spectrophotometric standard plots of sildenafil citrate in water and methanol

Standard plot of sildenafil citrate in water and methanol exhibited good correlation coefficient. Value of r^2 was found to be 0.9984 and 0.9989 in case of water and methanol respectively **(Fig. 5.1 and 5.2)**. $E^{1\%}_{1cm}$ in case of water was 312 and 320 in case of methanol. λ_{max} was 292 nm in both cases, which was in accordance with reported values.

5.1.2 Spectrophotometric standard plots of sildenafil citrate in buffers of different pH

Standard plot of sildenafil citrate in phosphate buffers of different pH (2.0-7.4) prepared at respective λ_{max} **(Table 5.4)** and showed good correlation coefficient. Value of r^2 was found to be close to 0.99 in all the cases **(Fig. 5.3-5.9)**. These calibrations were used to calculate the solubility of sildenafil citrate at different pH.

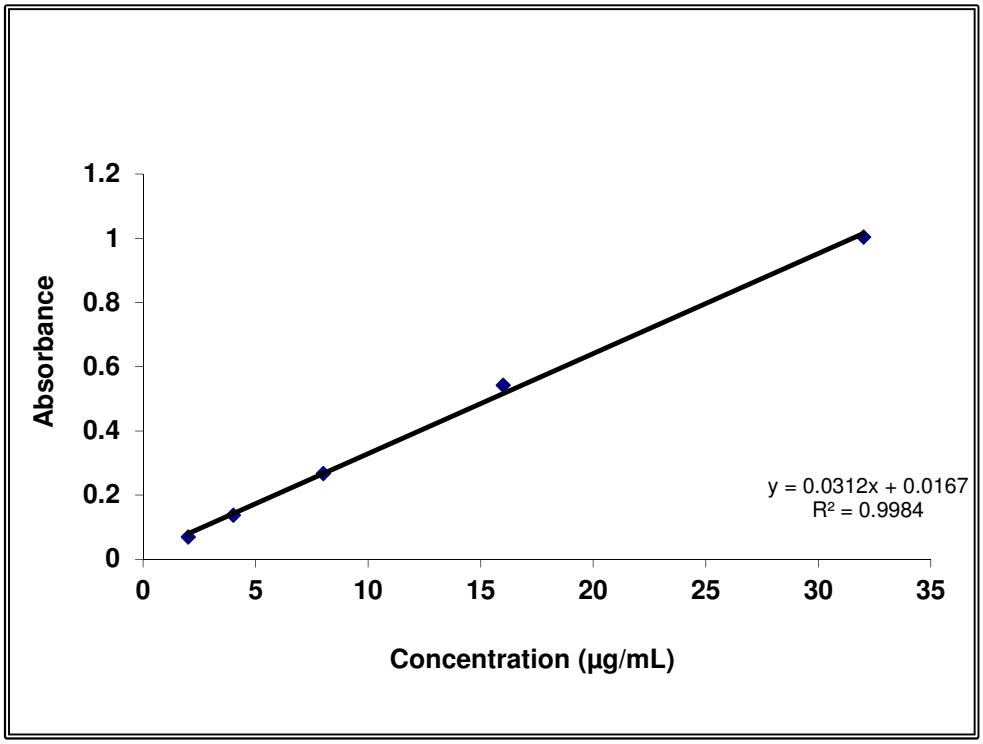


Fig. 5.1: Standard plot of sildenafil citrate in water

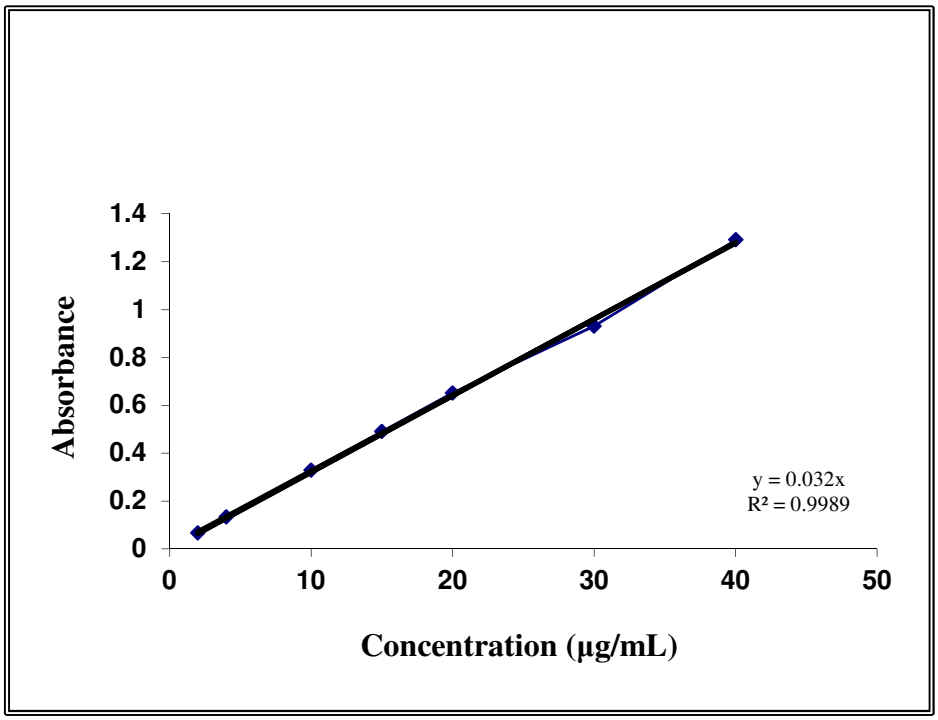


Fig. 5.2: Standard plot of sildenafil citrate in methanol

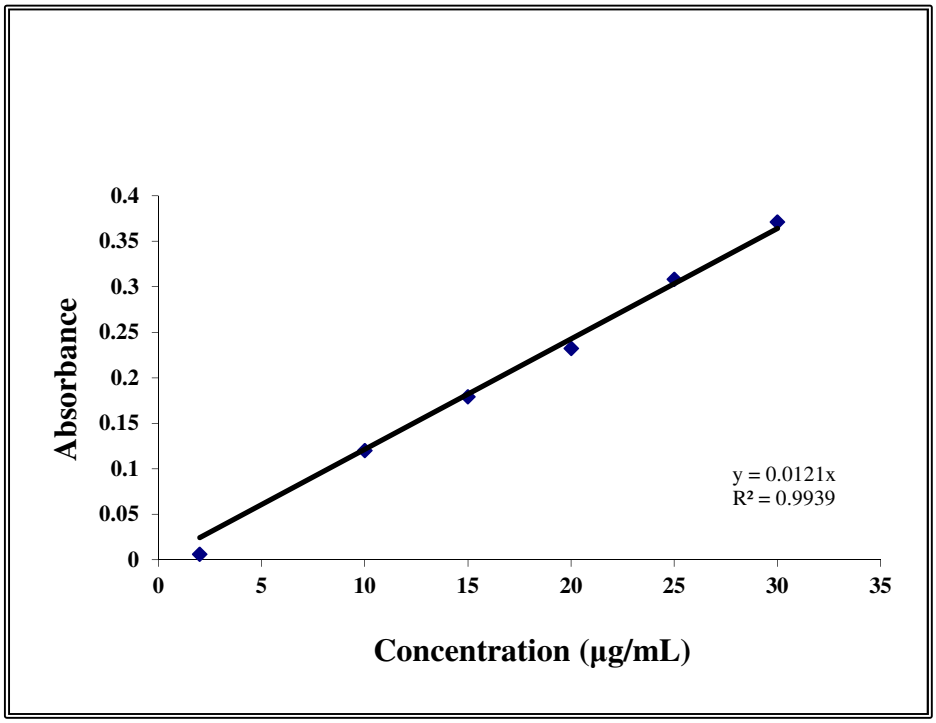


Fig. 5.3: Standard plot of sildenafil citrate in phosphate buffer pH 2.0

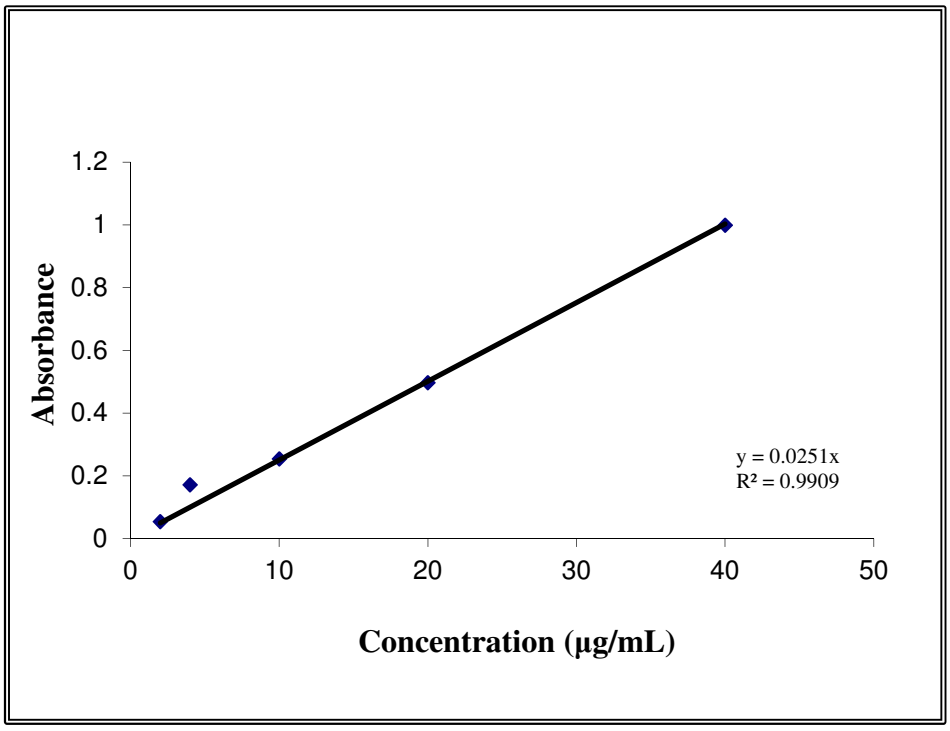


Fig. 5.4: Standard plot of sildenafil citrate in phosphate buffer pH 2.5

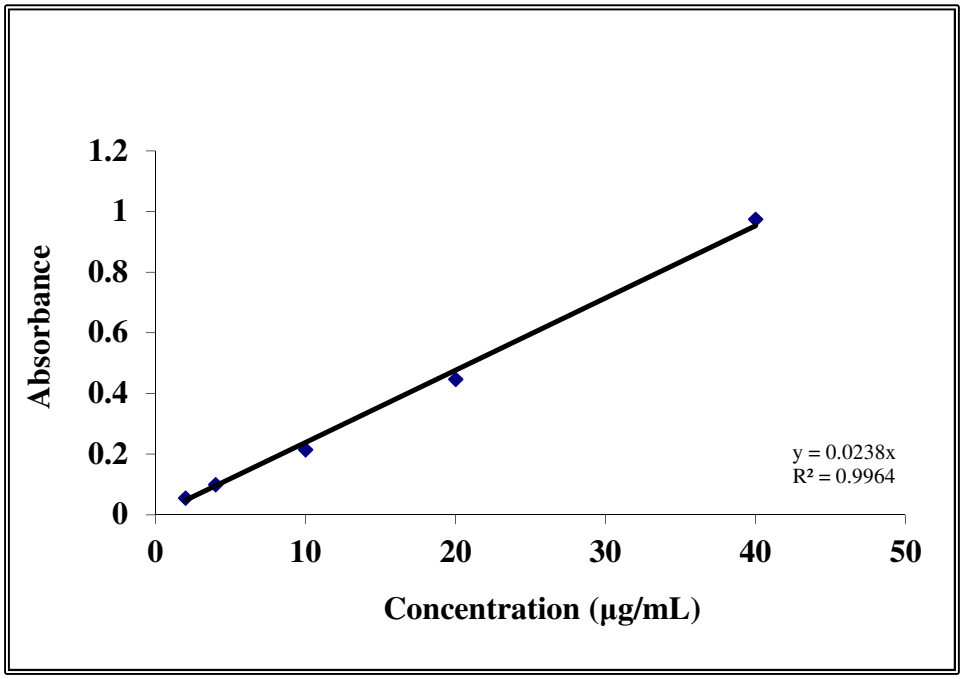


Fig. 5.5: Standard plot of sildenafil citrate in phosphate buffer pH 3.6

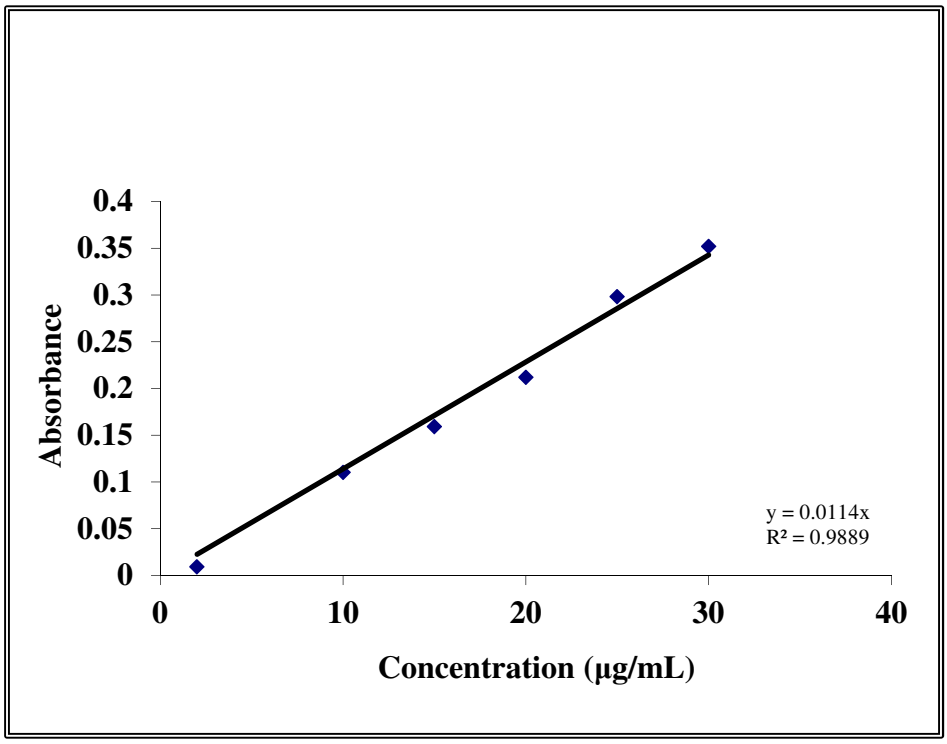


Fig. 5.6: Standard plot of sildenafil citrate in phosphate buffer pH 4.0

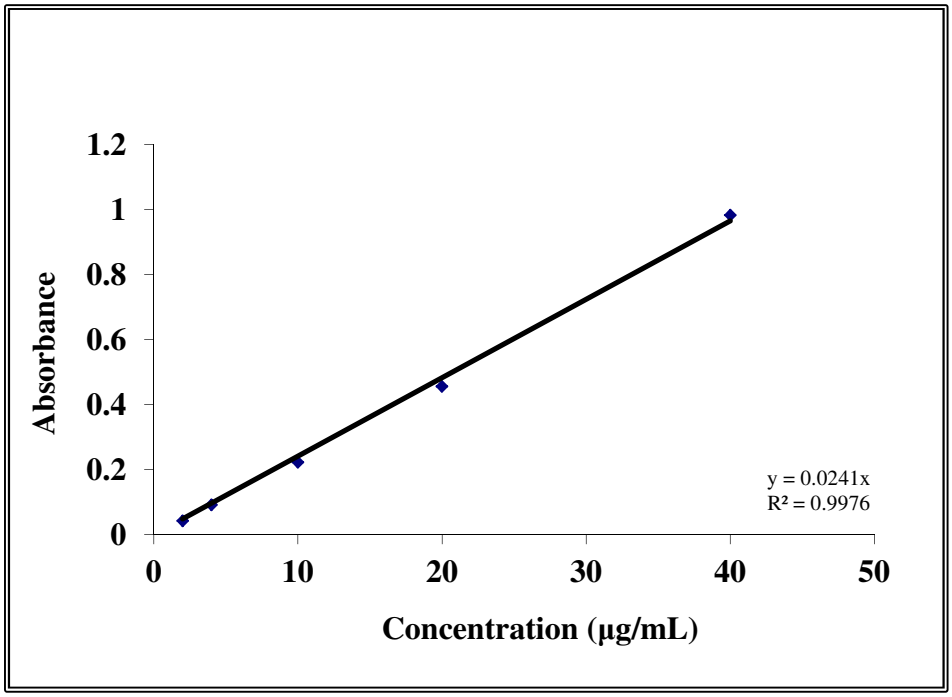


Fig. 5.7: Standard plot of sildenafil citrate in phosphate buffer pH 5.0

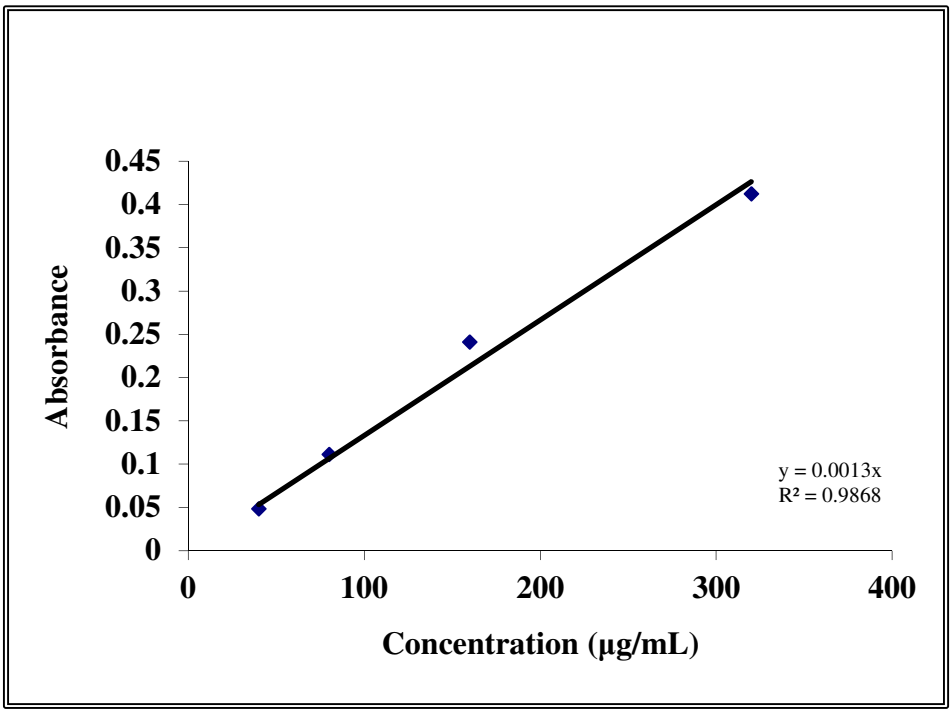


Fig. 5.8: Standard plot of sildenafil citrate in phosphate buffer pH 6.8

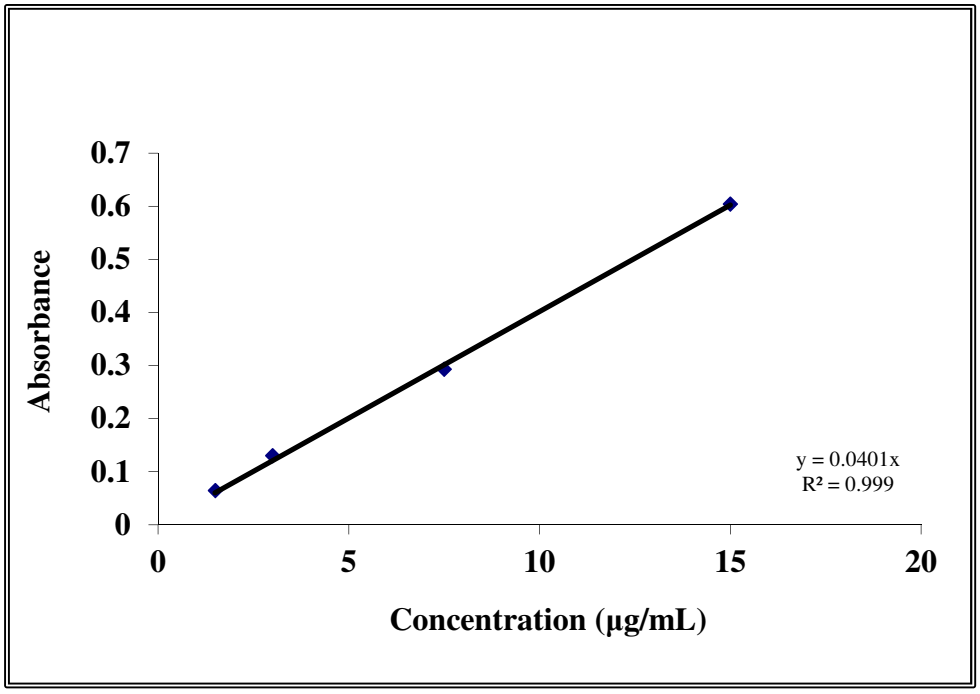


Fig. 5.9: Standard plot of sildenafil citrate in phosphate buffer pH 7.4

5.2 HPLC method for estimation of sildenafil citrate in *in vitro* samples

5.2.1 Calibration curve

The standard plot curve exhibited good linearity over the range of 780 ng/mL to200 µg/mL (Fig. 5.10 and 5.11). The value of correlation coefficient was 0.999 with R.S.D. less than 5%.

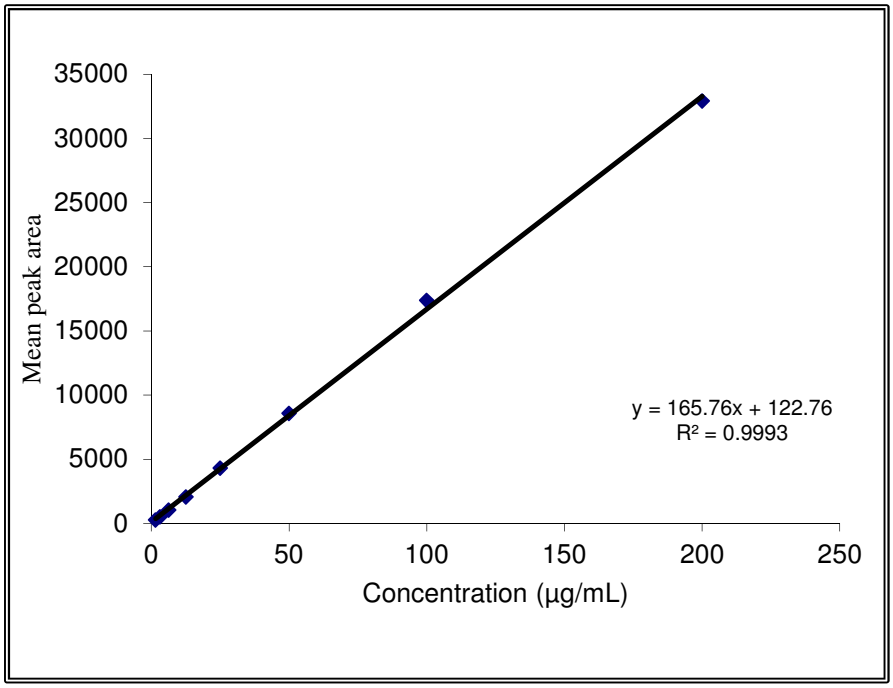


Fig. 5.10: Calibration curve of sildenafil citrate in rat lung homogenate

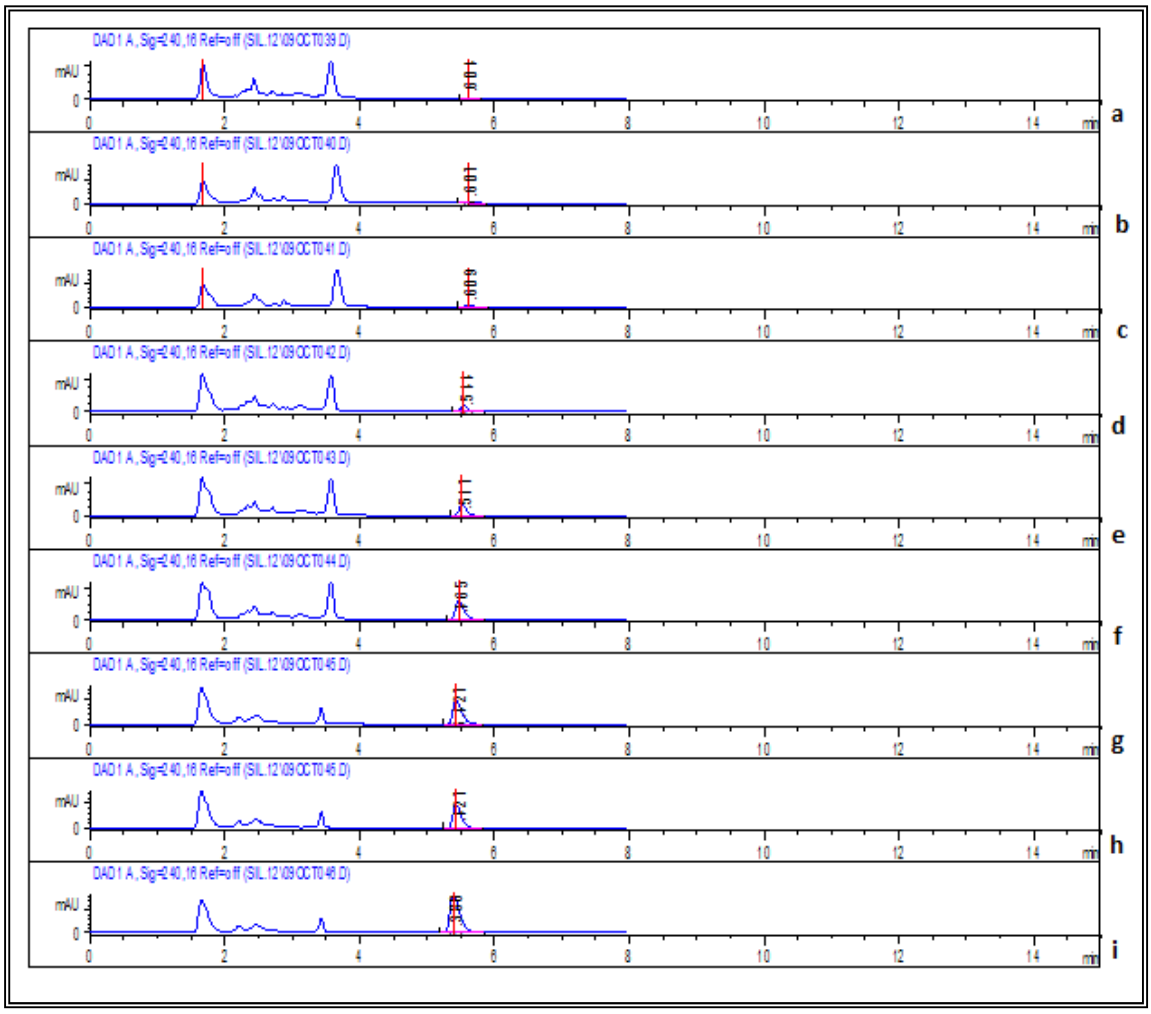


Fig. 5.11: HPLC linearity chromatogram of sildenafil citrate in rat lung homogenates in concentration range 780 ng/mL to 200 µg/mL (a to i)

5.2.2 Precision Study

The results of system precision **(Table 5.1)** revealed excellent precision with R.S.D. less than 0.2%. The results of intra-day and inter-day precision indicated excellent within day and between day reproducibility of the method (R.S.D. less than 0.2%) **(Table 5.2).**

Table 5.1. System Precision study

Sample number	Area
1	17376
2	17381
3	17333
4	17350
5	17322
6	17328
R.S.D.	**0.14**

Table 5.2. Intra-day and Inter-day precision study

Intra-day precision				**Inter-day precision**		
Concentration (μg/ml)	**Mean Area**	**S.D.**	**R.S.D (%)**	**Mean Area**	**S.D.**	**R.S.D (%)**
10	2072	1.23	0.06	2085	3.35	0.16
20	4298	3.31	0.08	4301	3.21	0.07
50	8570	2.45	0.03	8611	2.24	0.03

5.2.3 Recovery studies

The recovery of sildenafil citrate was found to be 102.09% with R.S.D. less than 2% and is depicted in **Table 5.3.**

Table 5.3. Recovery studies

Sample Number	% Recovery
1	104.00
2	102.09
3	100.20
Mean	102.09
R.S.D.	1.86

5.2.4 Specificity

Chromatograms of 10µg/mL of sildenafil citrate and the lung homogenate samples (from control and toxin treated animals) were taken. Samples did not interfere with the drug peak and thus, the method is specific for sildenafil citrate.

5.3 Solubility study

Sildenafil citrate was found to be slightly soluble in water having solubility of 3.8mg/mL. Solubility in methanol was higher (6.0mg/mL) as compared to water. Sildenafil citrate showed pH dependent solubility with highest of 15.74mg/mL at pH 2.0. The trend was of decrease in solubility with increase of pH except a solubilty of 13.51mg/mL at pH 4.0. Phosphate buffer of pH 6.8 exhibited a solubility of only 400 µg/mL and minimum solubility of 24 µg/mL was found at pH 7.4 **(Table 5.4)**. On the basis of these results,

- Water was chosen as solvent to determine the drug content in conventional dry powder formulations and drug-sugar composites.
- Water was chosen as dissolution medium for *in-vitro* release study of various sildenafil citrate dry powder formulations containing sildenafil equivalent to 600µg each, as sink conditions can be well maintained at a solubility of 3.8mg/mL.
- However, methanol was used for drug analysis in lipid based formulations as both lipids and drug could be dissolved well in this solvent.
- Phosphate buffer of pH 7.4 was used for hydration step of liposomes and aqueous phase to prepare large porous lipospheres in order to increase the percent drug entrapment.

Table 5.4: Solubility (mg/mL) of sildenafil citrate in different buffers/solvents

S. No.	Solvents/Buffer pH	λ_{max}	Saturated solubility mg or mcg of sildenafil /mL of the solvent
1	Water	292.0	3.8mg/mL
2	Methanol	292.0	6.0mg/mL
3	2.0	291.4	15.74 mg/mL
4	2.5	292.0	6.13 mg/mL
5	3.6	291.4	3.2 mg/mL
6	4.0	270.4, 306.2	13.51 mg/mL
7	5.0	294.9	2.88 mg/mL
8	6.8	292	400 mcg/mL
9	7.4	293.8	24 mcg/mL

5.4 Drug distribution study:

Drug distribution study showed the highest logD value in chloroform and buffer system. Thus, the distribution study reflects the distribution or partitioning of the drug in different phases of the liposomes and lipid emulsion to prepare lipid based dry powder formulations. Drug was found to be best partitioned into the system with chloroform and buffer system **(Table 5.5)**. Phosphate buffer of pH 7.4 shifted the partitioning of the sildenafil citrate towards the organic phase due to its lesser capacity to hold drug in its own domain.

Table 5.5. Values of D, logD and % drug distribution in organic phase

S. No.	System	D	Log D	% Drug distribution in organic phase
1	Octanol And water	6.32	0.8	75.95
2	Octanol And Buffer	17.27	1.24	89.6
3	Chloroform And water	2.9	0.5	59.29
4	Chloroform And	32.04	1.5	94.12

5.5. Preparation and characteristics of the various sildenafil citrate formulations

5.5.1 Conventional DPI

Pharmatose® 450M/350M and Lactohale® 200/300, α-lactose monohydrate with different particle sizes **(Table 5.6)**, were blended alone with drug and also in various combinations at different ratios (60:40 and 70:30). Fine carriers LH 200 (11.2 ± 1.39) and P 450M 15.2 ± 1.23 alone showed better %FPF than coarse carriers, however it was significantly lower for efficient lung deposition. Efficiency of a carrier for lung deposition depends on the aerodynamic particle size and surface morphology. It should form a bond strong enough to emit the drug from the device, but at the same time the bond should be loose enough to detach the drug for its deposition in deeper areas of the lungs. Thus, various lactose grades were combined in different ratios to alter the surface morphology of the drug carriers.

Combining the fine carriers with a combination of Pharmatose® 450M/350M at 70:30 ratio also showed a good flow with angle of repose 29.08 ± 2.01 **(Table 5.7)**. Formulations prepared with Lactohale 200 and Pharmatose 350M showed good flow characteristics at both 60:40 And 70:30 ratios (angle of repose value of 28.12 ± 1.23 and 27.05 ± 1.19 respectively). All the formulations passed the test for content uniformity. However, Reproducible delivery of the powder from the capsules was evaluated for all the prepared combination with two different devices (Rotahaler® and Handihaler®). Optimized batch was selected on the basis of highest %FPF. 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. 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) and therefore was used in further studies.

A balance between adhering to and detachment of the drug particles from the carrier surface is important for optimizing its delivery to the desired sites (Paul Wan Sia HENG, 1999). Though there was no significant difference in emitted fraction with any of the combinations prepared, overall the presence of Lactohale 200 in higher proportion showed better fine powder fraction. In coarse carriers, Pharmatose 350M (45 -150µm) had a narrow particle size distribution range as compared to Lactohale 100 (50 -230µm). Thus on actuation, the particles were emitted from the device with no significant difference, but the presence of Lactohale 100 with average size of >100

µm, which are too large and exhibit too high a degree of mass inertia to be entrained by the air led to lower %FPF values. Hence, Lactohale 200 combined with Pharmatose 350M, showed better FPF than in combination with Lactohale100.

There should be an optimum surface roughness of the carrier to balance the adherence and detachment of the drug particles which could be achieved with Lactohale 200 for sildenafil citrate. Agglomerates of micronized drug could break and get entrained on surface of lactohale 200 due to better bonding on its surface. It is possible that the higher fines proportion occupies the high energy surface sites of the lactose crystals and enables easier detachment of the micronized drug particles (J.N. Staniforth *et al*. 1995). Presence of fine lactose particles seemed to shift the fine particle fraction to higher values (Steckel H *et al*. 2004; Steckel H *et al*. 2002).

Few single-unit dose devices like Spinhaler®, Rotahaler® and Handihaler®, are supplied with individual single-dose gelatin capsules which must be introduced into the inhaler before use. In case of Handihaler®, after opening dust cap and mouth piece, the capsule is placed into a chamber located at the centre and then mouthpiece is closed and capsule is opened by pressing the side button.

While in the Rotahaler® the capsule is severed by a twisting action. Once the capsule has been broken, the patient inhales through the device triggering the propeller to turn and vibrate to disperse the powder into the inspired airstream. After use, the remains of the gelatin capsule must be removed from the inhaler before the next capsule can be placed in the device. The patient actuation was simulated by activating the timer (adjusted 5sec to get 4KPa to give an inspiration volume of 4L on the Critical Flow Controller controlling the two way solenoid valve DUSA apparatus.

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® **(Table 5.8)**.

Handihaler® exhibits higher air resistance, thus more drag force might be required to achieve turbulence of the air stream for deaggregation and detachment of drug particles from the surface of carrier for better %FPF. Therefore, the results obtained for FPF and dispersibility of fine sildenafil citrate particles from formulations which were aerosolised using Rotahaler® were significantly better as compared to Handihaler®. Handihaler® has been shown to be related with a significantly high and age-related error rate, because of the complexity of its operation (Dahl R, *et al*., 2003). In addition, the Handihaler™ has a high internal

resistance and many COPD patients have problems achieving an adequate inhalation rate to emit the required dose to be deposited in the airways (Tarsin W., 2003; Al-Fadhl SA., 2005; H Chrystyn, 2007). The results suggest that the lactose and delivery device need to be selected carefully for the efficient delivery of a drug dry powder from the inhaler.

Table 5.6 Particle size distributions and flow characteristics of different lactose grades using Rotahaler (mean ± SD, n = 3)

S.No.	Lactose Grades	Code	$d_{10\%}$ (µm)	$d_{50\%}$ (µm)	$d_{90\%}$ (µm)	Bulk density (g/cc)	Tapped density (g/cc)	%CI	Hausner's ratio	Recovered Dose (%)	Emitted dose (%)	% FPF	MMAD	GSD
1	Lactohale 200	A (Fine carrier)	4.5± 0.2	80± 0.15	145.8± 0.2	0.504 ± 0.25	0.984 ± 1.04	48.88	1.95	98.37 ± 0.47	64.98 ± 0.64	11.2 ± 1.39	10.68 ±2.41	3.55± 1.04
2	Lactohale 100	B (Coarse carrier)	50± 0.01	140± 0.24	230± 0.08	0.608 ± 1.2	0.721 ± 0.08	15.67	1.19	99.07 ±1.79	43.15 ± 0.63	5.3 ± 3.75	25.68 ± 2.23	3.92± 2.11
3	Pharmatose 350M	C (Coarse carrier)	45± 0.18	100± 0.35	150± 0.1	0.528 ± 1.32	0.635 ± 1.00	16.85	1.20	98.92± 0.82	54.98 ± 0.74	6.9 ± 2.37	12.68 ± 2.07	3.67± 1.36
4	Pharmatose 450M	D (Fine carrier)	2.5± 0.2	20± 3.2	62±2.8	0.463 ± 1.38	0.896 ± 1.07	48.3	1.93	98.63 ± 0.97	70.08 ± 0.49	15.2 ± 1.23	6.68 ± 1.51	3.95± 1.06

Table 5.7 Percent drug content and flow characteristics of conventional dry powder formulations (mean ± SD, n=3)

S.No.	Batch code	Combination of lactose grades	Ratio of lactose grades	Percent drug content ± SD	Bulk density	Tapped density	Angle of repose	%CI	Hausner's ratio	Content uniformity
1	CD2	A:B	(60:40)	102.3 ± 0.24	0.45 ± 0.26	0.594 ± 0.01	31.13 ± 0.55	24.24	1.32	97.98 ± 1.74
2	CD6	D:B		99.1 ± 1.53	0.536 ± 1.12	0.67 ± 0.8	31.09 ± 0.64	20.00	1.25	97.84 ± 1.03
3	CD4	A:C		100.3 ± 0.06	0.453 ± 0.48	0.571 ± 0.92	28.12 ± 1.23	20.67	1.26	98.20 ± 2.01
4	CD8	D:C		98.8 ± 0.27	0.409 ± 1.27	0.554 ± 1.46	31.21 ± 1.53	26.17	1.35	99.01 ± 1.39
5	CD1	A:B	(70:30)	107.5 ± 0.28	0.445 ± 1.29	0.581 ± 2.23	30.98 ± 1.46	23.41	1.31	98.48 ± 2.09
6	CD5	D:B		103.7 ± 0.29	0.468 ± 0.86	0.622 ± 0.99	30.07 ± 0.08	24.76	1.33	99.21 ± 1.66
7	CD3	A:C		102.15 ± 0.30	0.418 ± 0.25	0.511 ± 0.18	27.05 ± 1.19	18.20	1.22	98.80 ± 1.12
8	CD7	D:C		102.15 ± 0.31	0.413 ± 1.37	0.538 ± 2.03	29.08 ± 2.01	23.23	1.30	99.01 ± 1.39

Table 5.8: Aerosolization characteristics of conventional DPI formulations with two different devices using ACI (mean ± SD, n = 3)

Batch No.	Excipients	Excipient Ratio	Type of device	Recovered Dose (%)	Emitted dose (%)	%FPF	MMAD	GSD
CD1	A:B	(70:30)	Handihaler®	95.92± 0.82	73.37 ± 0.18	21.4 ± 0.35	5.22 ± 0.39	3.54±1.12
CD2	A:B	(60:40)		96.87 ± 0.74	72.7 ± 0.72	19.3 ± 0.75	5.68 ± 1.41	3.29±1.56
CD3	A:C	(70:30)		96.23 ± 0.72	72.22 ± 0.32	31.1 ± 0.64	4.01 ± 0.22	2.32±1.81
CD4	A:C	(60:40)		98.08 ± 1.02	73.05 ± 0.13	20.8 ± 1.1	6.16 ± 0.23	2.63±1.65
CD5	D:B	(70:30)		96.57 ± 0.36	70.88 ± 0.72	15.5 ± 0.71	5.02 ± 1.40	2.83±2.01
CD6	D:B	(60:40)		97.37 ± 0.17	71.02 ± 0.09	11.7 ± 0.32	5.23 ± 0.46	3.29±2.19
CD7	D:C	(70:30)		98.53 ± 0.91	73.10 ± 0.34	22.2 ± 0.53	4.25 ± 1.03	3.55±1.65
CD8	D:C	(60:40)		95.58 ± 0.8	70.95 ± 0.64	19.9 ± 0.38	6.39 ± 0.73	2.65±2.88
CD1	A:B	(70:30)	Rotahaler®	96.80 ± 0.49	75.03 ± 1.12	30.7 ± 0.04	2.86 ± 1.32	3.43±3.15
CD2	A:B	(60:40)		97.15 ± 0.13	73.65 ± 0.55	25.4 ± 0.08	4.88 ± 0.25	2.41±1.06
CD3	**A:C**	**(70:30)**		**99.30 ± 0.02**	**75.88 ± 0.43**	**36 ± 0.02**	**6.12± 2.23**	**2.26±1.92**
CD4	A:C	(60:40)		100.88 ± 0.33	75.32 ± 0.48	26.3 ± 0.09	4.92 ± 1.09	3.36±1.71
CD5	D:B	(70:30)		101.67 ± 0.59	76.25 ± 0.67	18.5 ± 0.54	3.98 ± 2.18	3.33±1.92
CD6	D:B	(60:40)		99.25 ± 0.02	74.28 ± 0.36	13.9 ± 0.06	6.36± 0.94	2.54±2.61
CD7	D:C	(70:30)		99.78 ± 0.5	75.12 ± 0.09	24.5 ± 0.98	4.37 ± 0.71	3.64±1.33
CD8	D:C	(60:40)		98.68 ± 0.64	74.98 ± 0.44	23.4 ± 0.12	6.25 ± 1.83	2.87±1.48

5.5.2 Preparation of Drug-sugar composites (DS) of sildenafil citrate:

Optimisation of the aerodynamic characteristics of dry powder inhaler formulations is critical to ensure optimum deposition of the formulation into the respiratory tract. This control can be better achieved by spray drying process rather than conventional micronization process to get dry powder formulations for pulmonary delivery. Sildenafil citrate was co-spray dried with different sugars like lactose, sucrose, mannitol, sorbitol and trehalose. Out of all, mannitol gave non-sticky product with excellent flow characteristics.

Mannitol has been shown as a potential carrier for spray drying drugs like budesonide and salbutamol with excellent aerosolization characteristics (Steckel H *et al*, 2004; P. Harjunen, *et al*, 2003). Various formulation parameters like drug and mannitol ratio and percent of mannitol in feed solution were optimized to get a product with good flow properties **(Table 5.9)**. Drug: Mannitol ratio was varied from 1:5 to 1:20. There was no effect on percent drug content at any ratio, but percent yield was increased by increasing mannitol till 1:10 (drug: mannitol) ratio. At this ratio, mannitol was sufficient to carry the drug efficiently and further increase in mannitol might have facilitated the influx of water inside the particles and hence reduced the percent yield due to uptake of moisture (Steckel H *et al*, 2004; P. Harjunen, *et al*, 2003).

Increase in solid content to 4.4% (drug + mannitol) could further increase the yield. It has been shown that the increase in solid content can lead to the increase porosity of the particles which is evident from the decrease in bulk density of the formulation from 0.224 ± 0.87 at 2.2% to 0.215 ± 1.15 at 4.4%. This made the product lighter with better aerodynamic behaviour and better flow with improved Hausner ratio from 1.24 to 1.16. 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. Batches from DS1 to DS4 were prepared at spray drying parameters of 2mL/min. feed rate, 2.5 bars compressed air pressure, inlet temperature of 90°C and vacuum at -150 mm of WC. Spray drying process parameters were further optimized for the above finalized formulation using fractional factorial design using Stat-Ease software (Design-Expert 8).

Table 5.9: Effect of formulation parameters on percent yield and flow characteristics of sildenafil citrate-sugar composites (mean ± SD, n = 3)

S.No.	Batch code	Drug: Sugar	Mannitol % w/w	% drug content ± SD	% yield ± SD	Bulk density	Tapped density	Angle of repose	% CI	Hausner's ratio
1	DS1	1:5	2	97.9 ± 0.44	45.2 ± 1.56	0.243 ± 1.12	0.294 ± 0.11	33.02 ± 0.28	18.61	1.2
2	DS2	1:10	2	98.2 ± 0.37	48.1 ± 2.13	0.224 ± 0.87	0.279 ± 2.02	30.12 ± 0.35	19.71	1.24
3	**DS3**	**1:10**	**4**	**99.4 ± 0.82**	**60.9 ± 0.05**	**0.215 ± 1.15**	**0.246 ± 0.63**	**27.03 ± 0.86**	**14.59**	**1.16**
4	DS4	1:20	8	98.3 ± 1.06	52.8 ± 0.86	0.233 ± 0.58	0.301 ± 1.29	32.12 ± 0.38	22.59	1.29

5.5.2.1 Optimization of spray drying process:

Final formulation containing 0.4% drug and 4.0% of mannitol was optimized for spray drying process parameters like feed rate (mL/min), compressed air pressure (bars), vacuum (mm of WC) and inlet temperature (°C). Parameters were varied according to fractional factorial design to get the optimum formulation with respect to percent drug content, percent yield, moisture content and aerodynamic diameter (**Table 5.10**). None of the parameters had significant effect on percent drug content. There was no impact of feed rate, air pressure and inlet temperature. As vacuum decreases, the % drug content increases. However, from a practical stand-point, this effect is negligible as the impact is insignificant. A minor interaction effect is seen between inlet temperature and feed rate. No other interaction effects are seen with respect to percent drug content. However, Vacuum has a significant impact on % yield. Although other factors are not influencing individually, the combination of feed rate-vacuum, feed rate-air pressures have significant impact on % yield.

1. An interaction is seen between air pressure and feed rate. At higher air pressure, yield increases as feed rate increases, whereas at lower air pressure, a decrease in yield is observed as feed rate increases. Higher air pressure can reduce the particle size and slower feed rates can lead to the formation of bigger droplets which could have been compensated at increased air pressure and hence forming smaller particles with easy evaporation of moisture from increased surface area and hence, the % yield (R. P. Patel *et al*.)
2. Another interaction is seen between vacuum and feed rate. At lower vacuum, yield increases as feed rate increases, whereas at higher vacuum, a decrease in yield is observed as feed rate increases. This might be due to generation of excessive fines which could be removed at increased vacuum leading to lower % yield values.

3. One more interaction is seen between inlet temperature and vacuum. At both higher and lower inlet temperature, yield increases as vacuum decreases. However this effect is more prominent at lower inlet temperature.

Feed rate, air pressure and a combination of both these factors significantly impacts aerodynamic particle size. Aerodynamic particle size decreases as both feed rate and air pressure increase.

1. An interaction is seen between air pressure and feed rate. At both higher and lower air pressures, particle size decreases as feed rate increases. Increased feed rate leads to formation of smaller droplets.
2. Another interaction is seen between vacuum and air pressure. At both higher and lower vacuum, particle size decreases as air pressure increases.
3. An interaction is seen between air pressure and inlet tempetaure. At both higher and lower inlet temperatures, particle size decreases as air pressure increases. Hence, overall, increase in air pressure leads to the reduction of particle size.

Inlet temperature and air pressure negatively influence moisture content. Vacuum and feed rate positively influence moisture content. Although, interactions are seen, they are not of a practical significance **(Fig. 5.12-5.26)**. Based on the overall analysis, optimized solutions were provided by Design-Expert® 8 as the predicted numbers for responses and recommended factor values for best results. Formulation DSS7 had no significant difference ($p \leq 0.05$) of factor and response values compared to solution no. 4 provided by numerical optimization **(Table 5.10-5.11)**. Hence, formulation DSS7 was chosen as the optimized formulation with respect to formulation and spray drying parameters. Thus, final formulation was spray dried at optimized parameters of 3mL/min feed rate, atomized at compressed air pressure of 3bars with -160mm of WC vacuum at 80°C inlet temperature.

Table 5.10: Effect of spray drying process parameters on formulation characteristics of sildenafil citrate-sugar composites

	Factor 1	Factor 2	Factor 3	Factor 4	Response 1	Response 2	Response 3	Response 4		
Run	A:Feed rate	B:Air pressure	C:Vacuum	D:Inlet temperature	Percent Drug Content	Percent Yield	Aerodynamic Particle Size	Moisture content	Bulk density (ρb)	Volume mean diameter
	mL/min	Bar	mm of WC	°C	%	%	(µm)	%	g/cm^3	(µm)
DSS1	1	3	-160	80	98.8 ± 1.41	45.2 ± 1.11	6.04	3.29 ± 0.98	0.323± 1.24	10.62± 3.00
DSS2	3	3	-200	80	80.1 ± 1.35	32.3 ± 1.62	3.25	2.53 ± 0.88	0.211± 2.54	7.075± 2.58
DSS3	1	2	-200	80	82.18 ± 1.64	41.2± 1.59	12.38	2.31 ± 0.35	0.248± 0.97	24.85± 1.87
DSS4	1	2	-200	100	76.32 ± 1.31	42.9 ± 0.88	13.31	2.19 ± 0.74	0.294± 1.11	24.54± 1.54
DSS5	3	2	-160	80	98.2 ± 2.15	55.3± 1.42	5.93	9.09 ± 1.18	0.244± 1.56	12± 0.99
DSS6	3	3	-200	100	88.2 ± 1.45	38.2 ± 1.05	3.56	2.01 ± 1.06	0.261± 0.84	6.96± 1.58
DSS7	**3**	**3**	**-160**	**80**	**99.6 ± 0.53**	**59.9 ± 0.07**	**2.96**	**3.07 ± 0.82**	**0.211± 2.03**	**6.44± 0.66**
DSS8	1	3	-200	100	85.1 ± 2.15	40.4 ± 0.95	6.86	2.08 ± 1.19	0.25± 1.65	13.72± 0.59
DSS9	3	2	-160	100	97.5 ± 0.83	44.7 ± 0.38	4.08	4.39 ± 0.93	0.224± 0.79	8.62± 1.56
DSS10	3	2	-200	80	86.22 ± 1.28	30.8 ± 1.61	6.84	3.11 ± 1.24	0.322± 2.73	12.05± 1.17
DSS11	1	3	-200	80	94.83 ± 1.85	29.3 ± 0.28	6.27	2.26 ± 0.57	0.311± 1.02	11.24± 1.19
DSS12	3	2	-200	100	88.2 ± 2.65	35.1 ± 1.49	7.16	2.21 ± 1.19	0.228± 2.13	14.99± 1.56
DSS13	1	2	-160	100	96.22 ± 1.21	48.8 ± 1.38	9.33	2.53 ± 2.23	0.254± 3.08	18.51± 2.56
DSS14	1	3	-160	100	91.2 ± 1.25	40.2 ± 0.83	6.56	2.13 ± 1.65	0.243± 1.98	13.31± 3.41
DSS15	1	2	-160	80	92.92 ± 1.16	49.2 ± 2.36	12.27	4.32 ± 1.82	0.335± 1.44	21.19± 1.51
DSS16	3	3	-160	100	90.9 ± 2.24	58.6 ± 0.05	3.56	2.05 ± 2.15	0.259± 2.18	6.99± 1.68

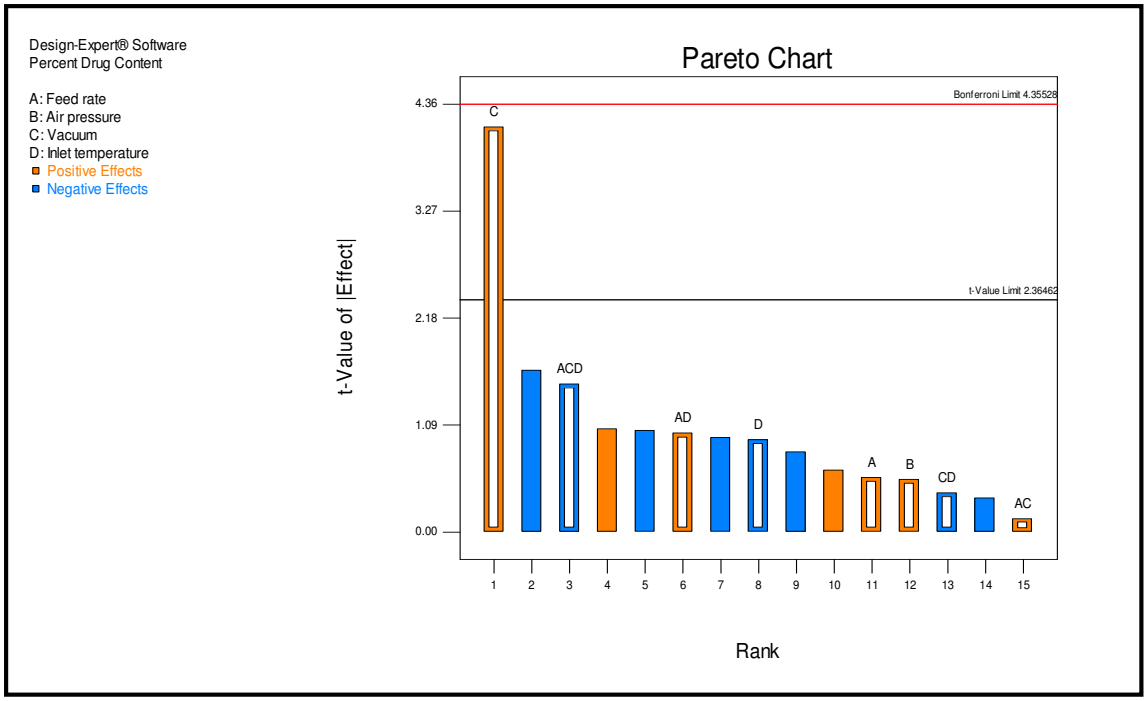


Fig. 5.12: Standardized effect of spray drying parameters on percent drug content of drug-sugar composites

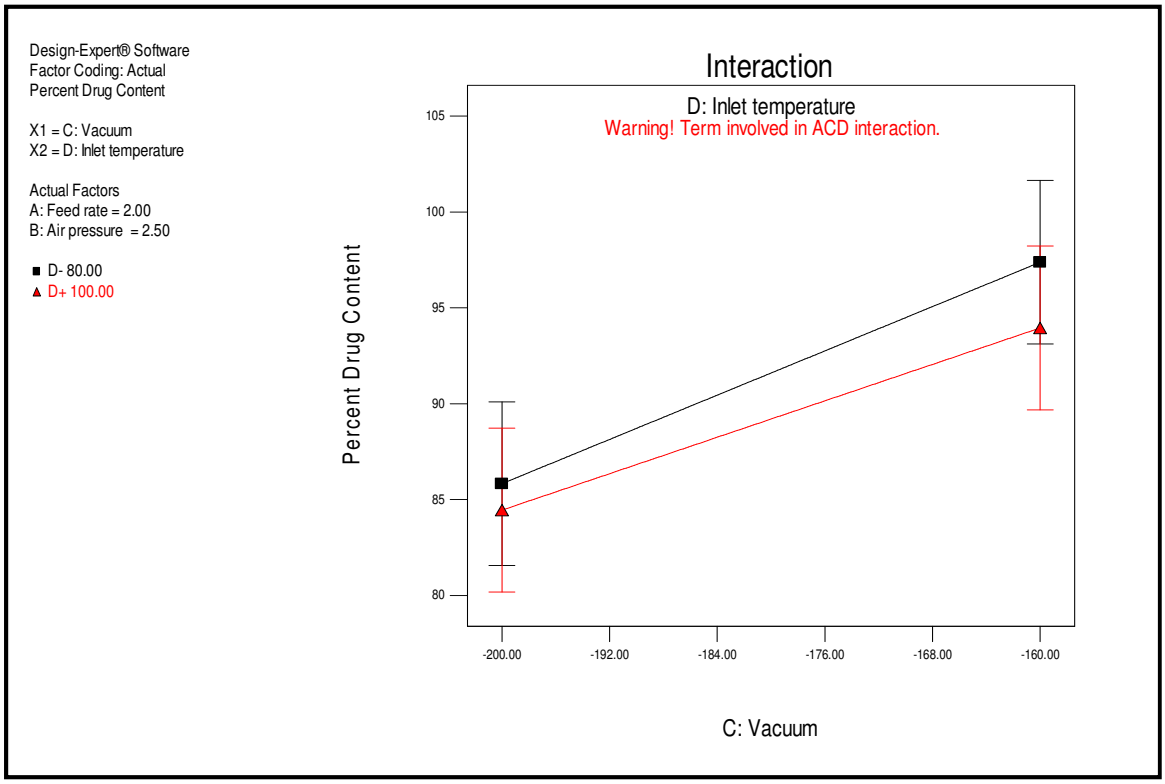


Fig. 5.13: Interaction of process variables affecting percent drug content of drug-sugar composites

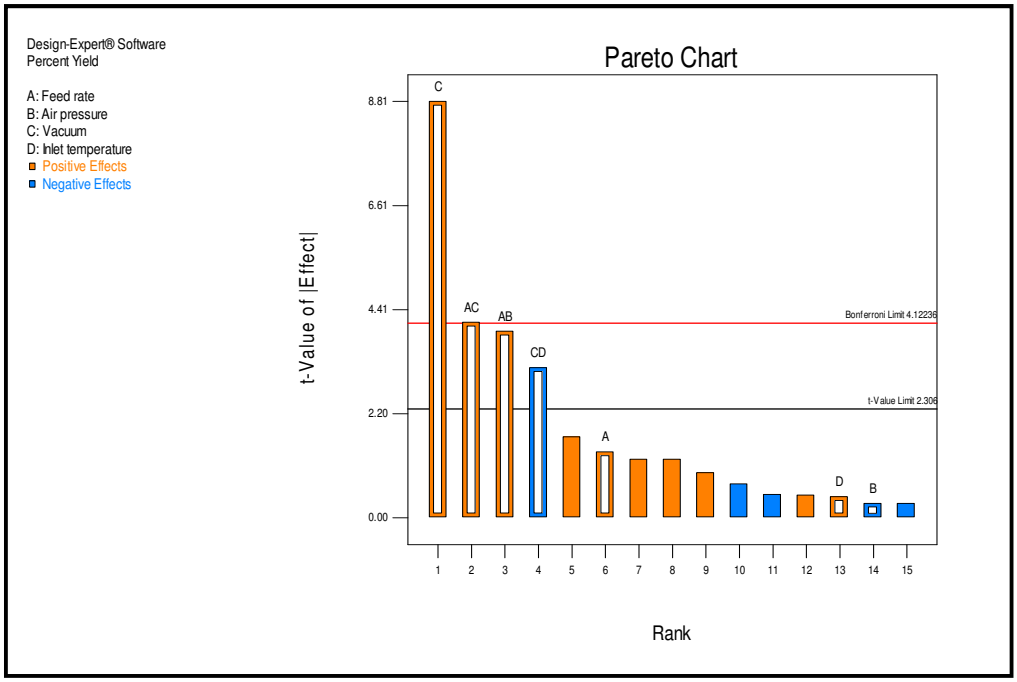


Fig. 5.14: Standardized effect of variables on percent yield of drug-sugar composites

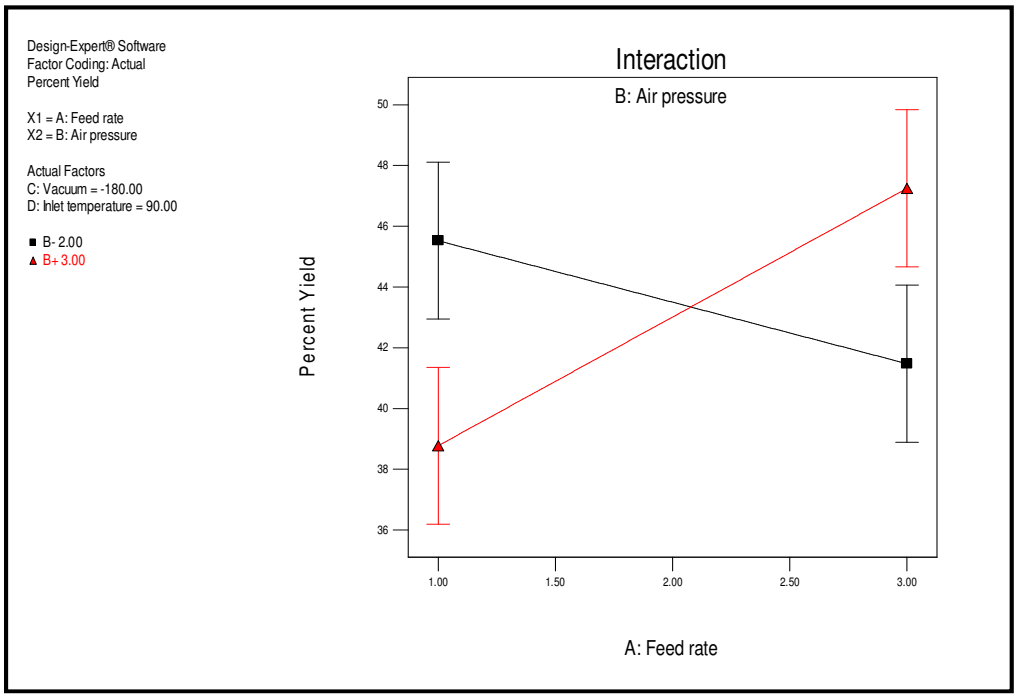


Fig. 5.15: Interaction between air pressure and feed rate to influence percent yield of drug-sugar composites

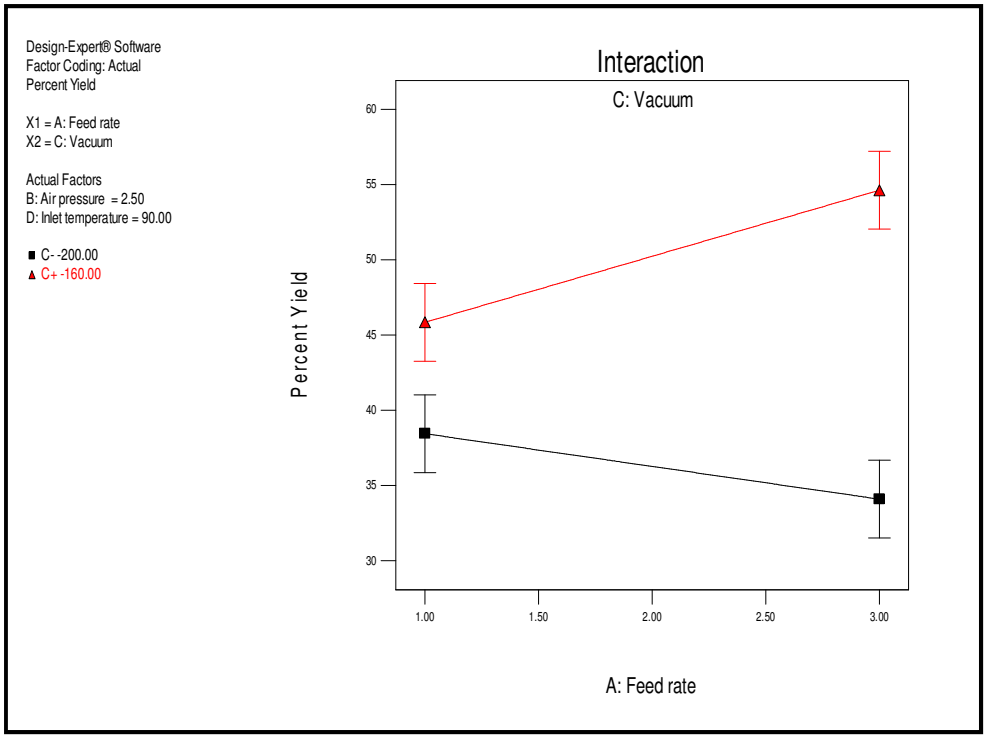


Fig. 5.16: Interaction between Vacuum and feed rate to influence percent yield of drug-sugar composites

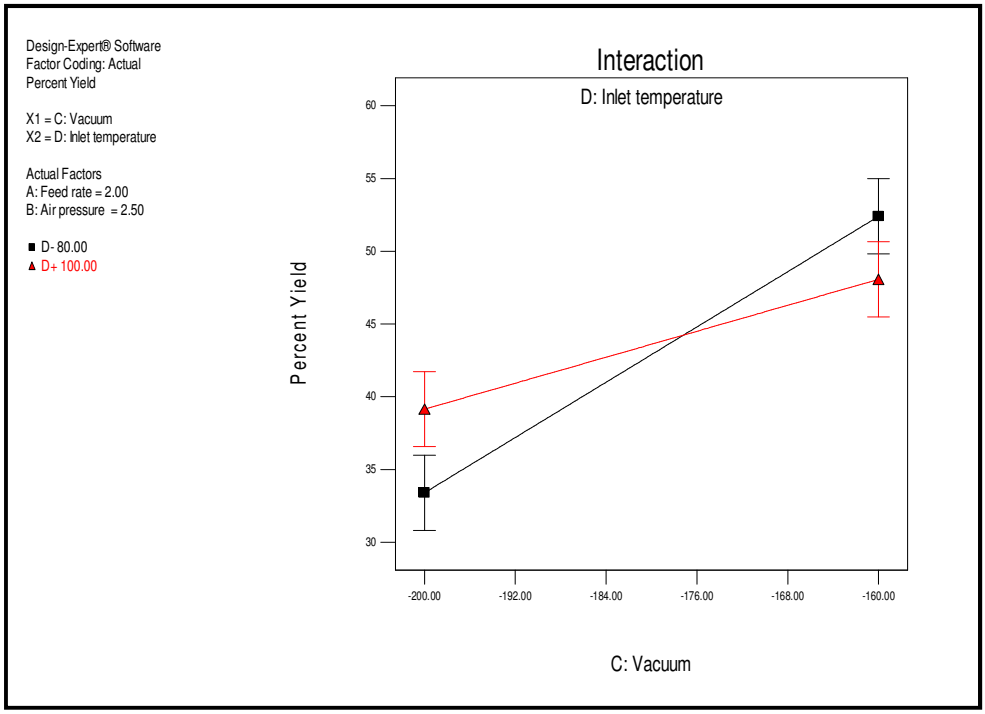


Fig. 5.17: Interaction between inlet temperature and vacuum to influence percent yield of drug-sugar composites

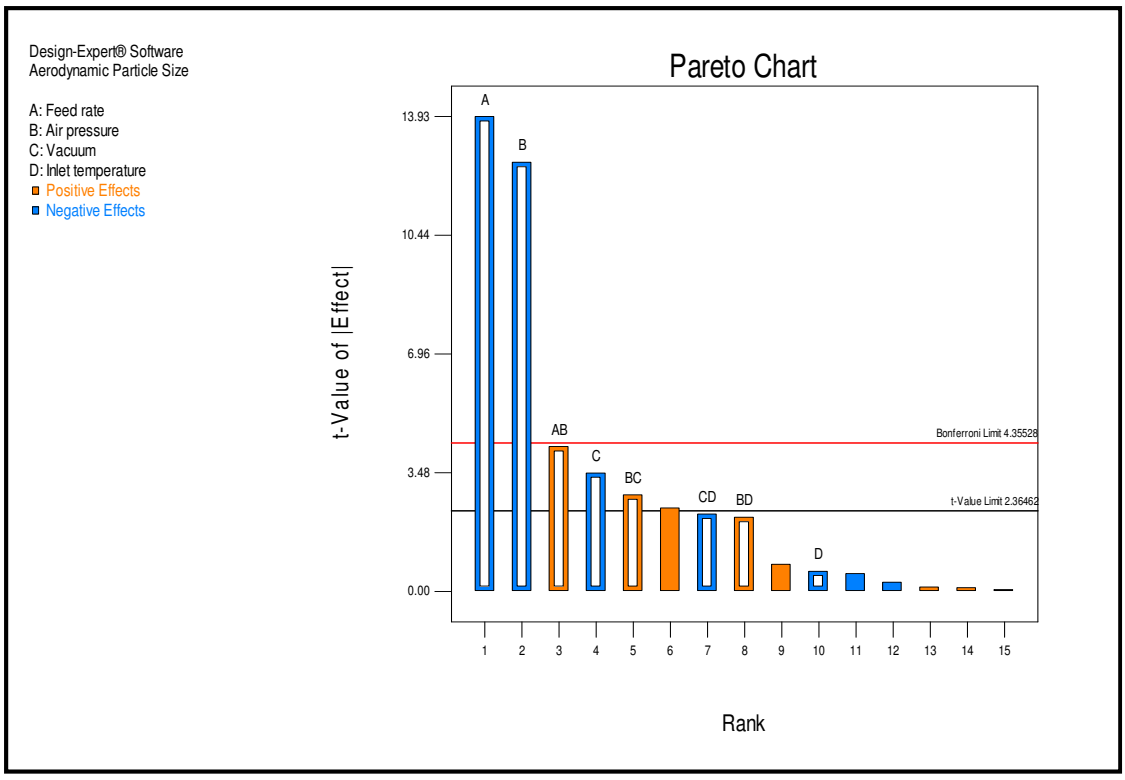


Fig. 5.18: Effect of Variables on Aerodynamic Particle Size of drug-sugar composites

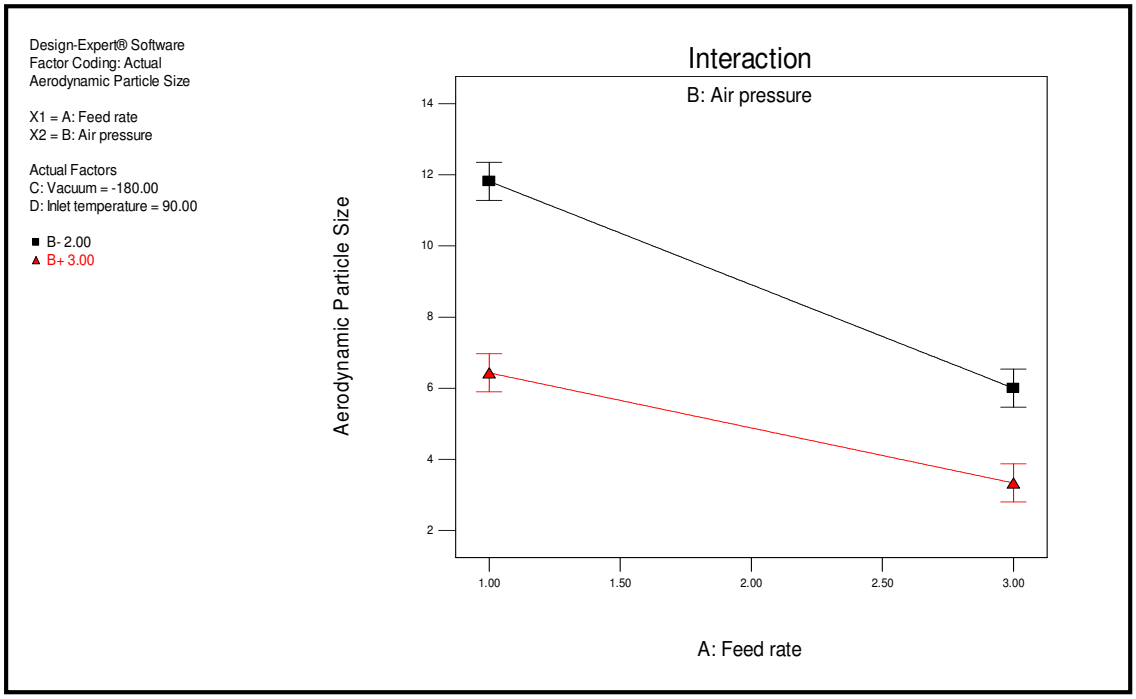


Fig. 5.19: Interaction between air pressure and feed rate to influence aerodynamic particle size of drug-sugar composites.

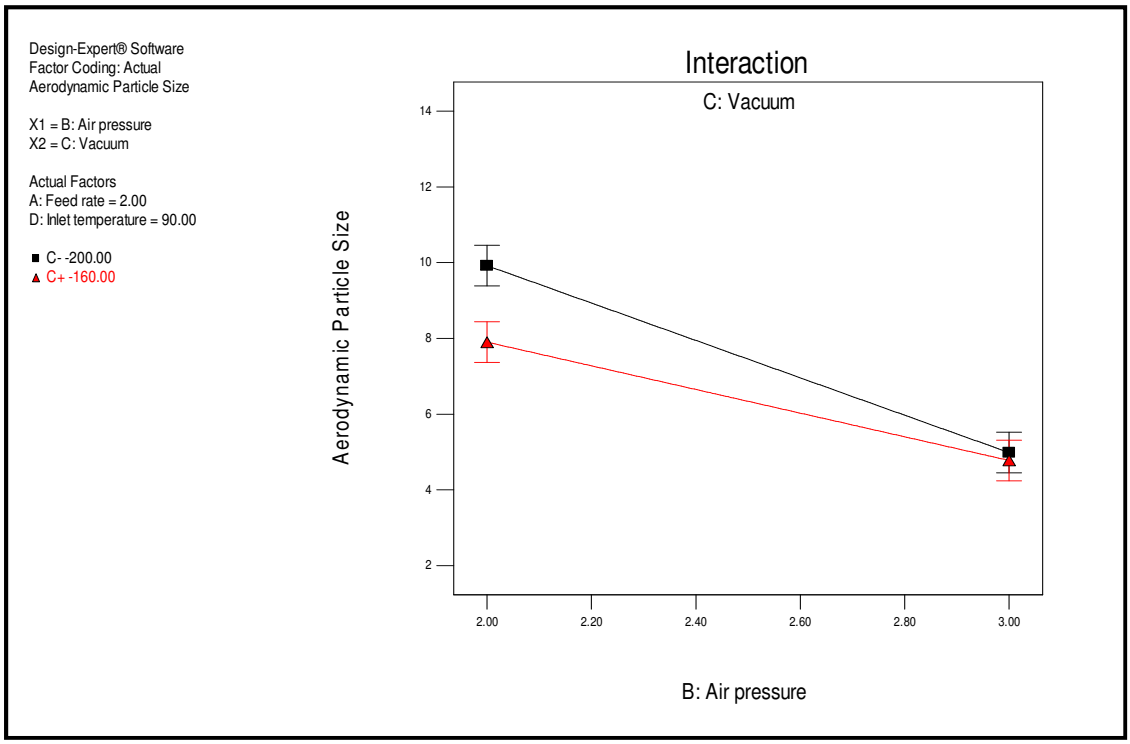


Fig. 5.20: Interaction between air pressure and vacuum to influence aerodynamic particle size of drug-sugar composites

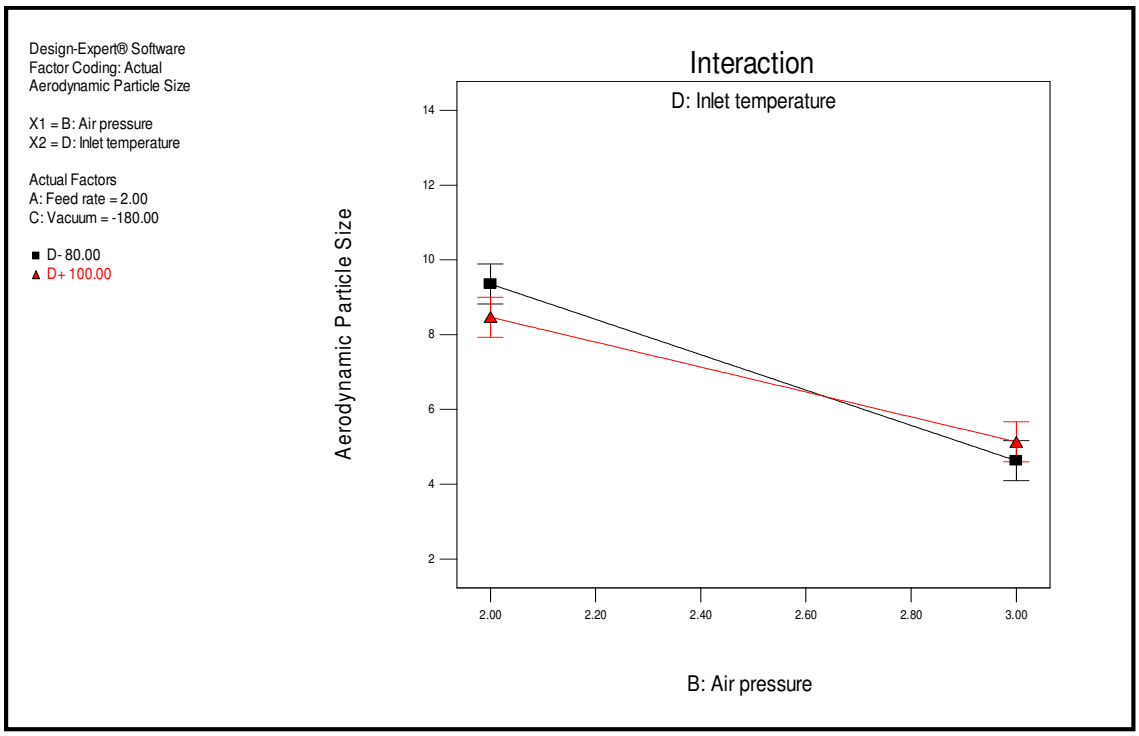


Fig. 5.21: Interactions of inlet temperature and air pressure to affect aerodynamic particle size of drug-sugar composites

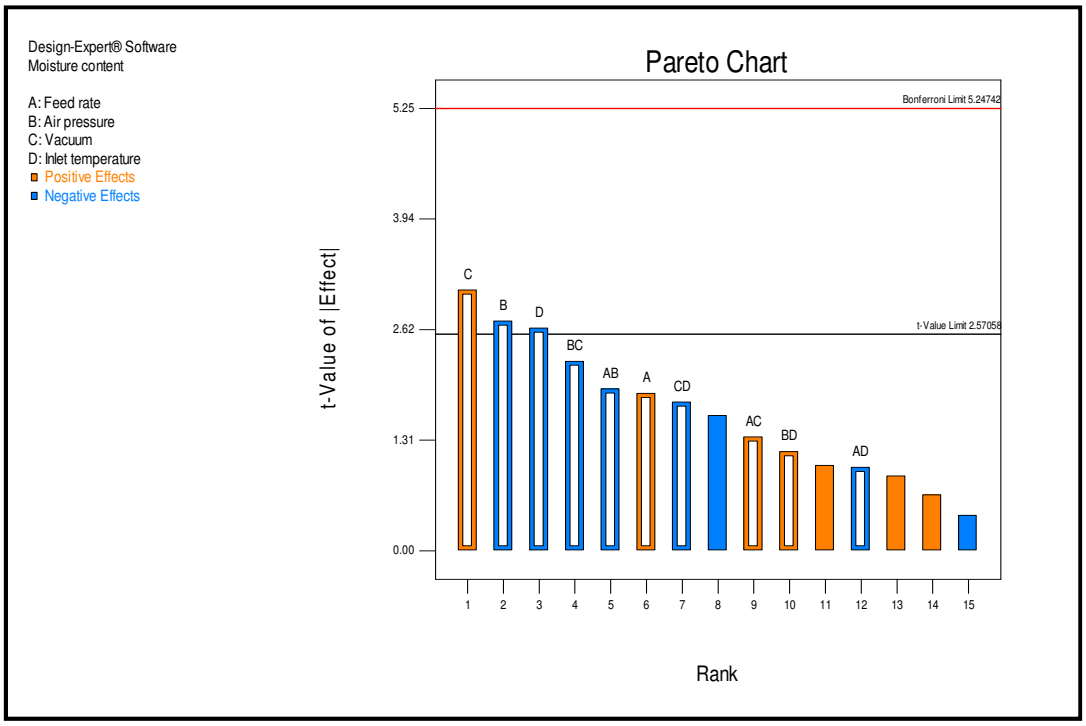


Fig. 5.22: Effect of Variables on Moisture Content of drug-sugar composites

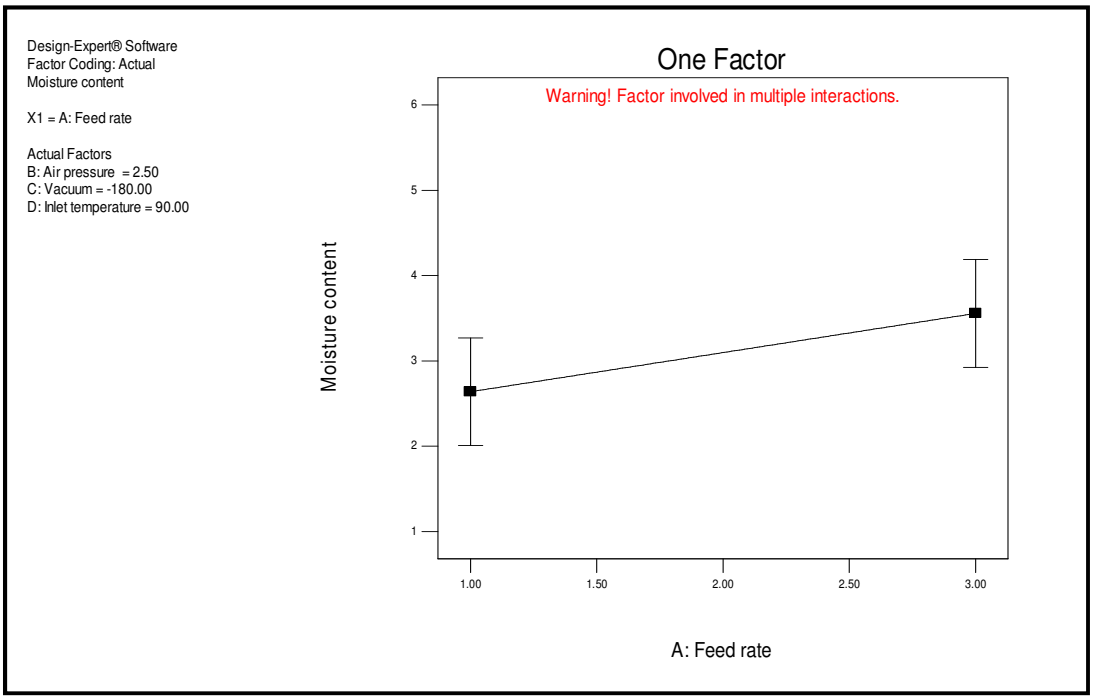


Fig. 5.23: Effect of feed rate on Moisture Content of drug-sugar composites

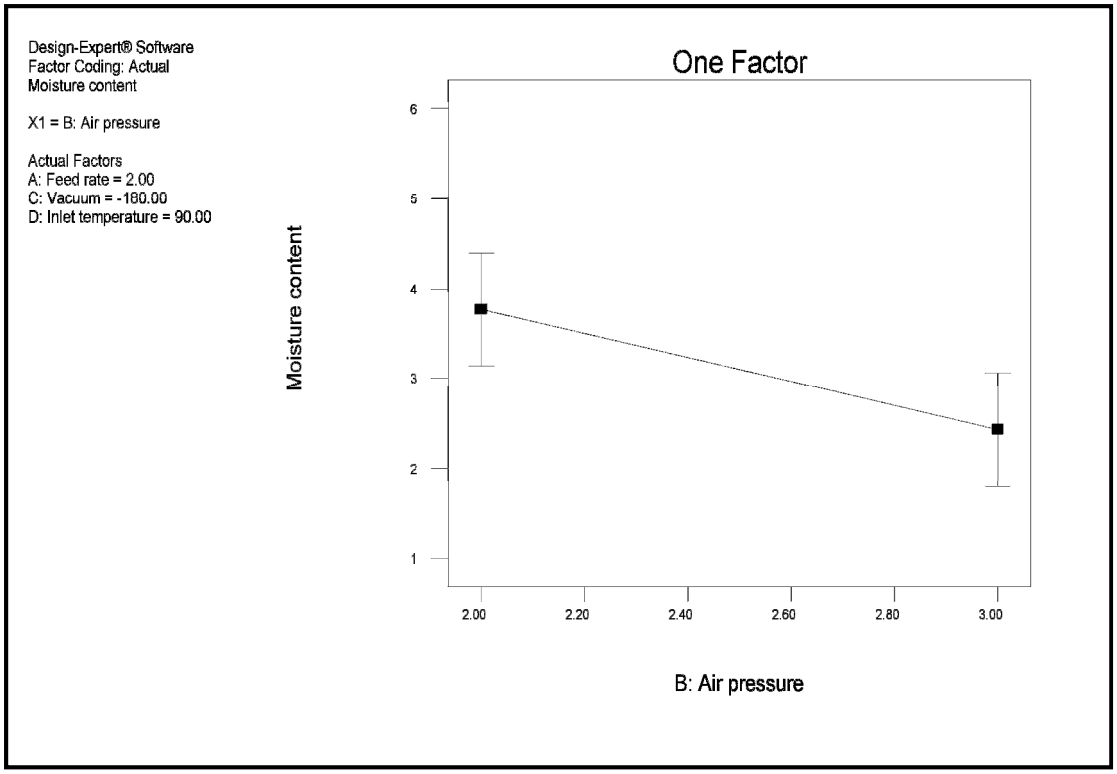


Fig. 5.24: Effect of air pressure on Moisture Content of drug-sugar composites

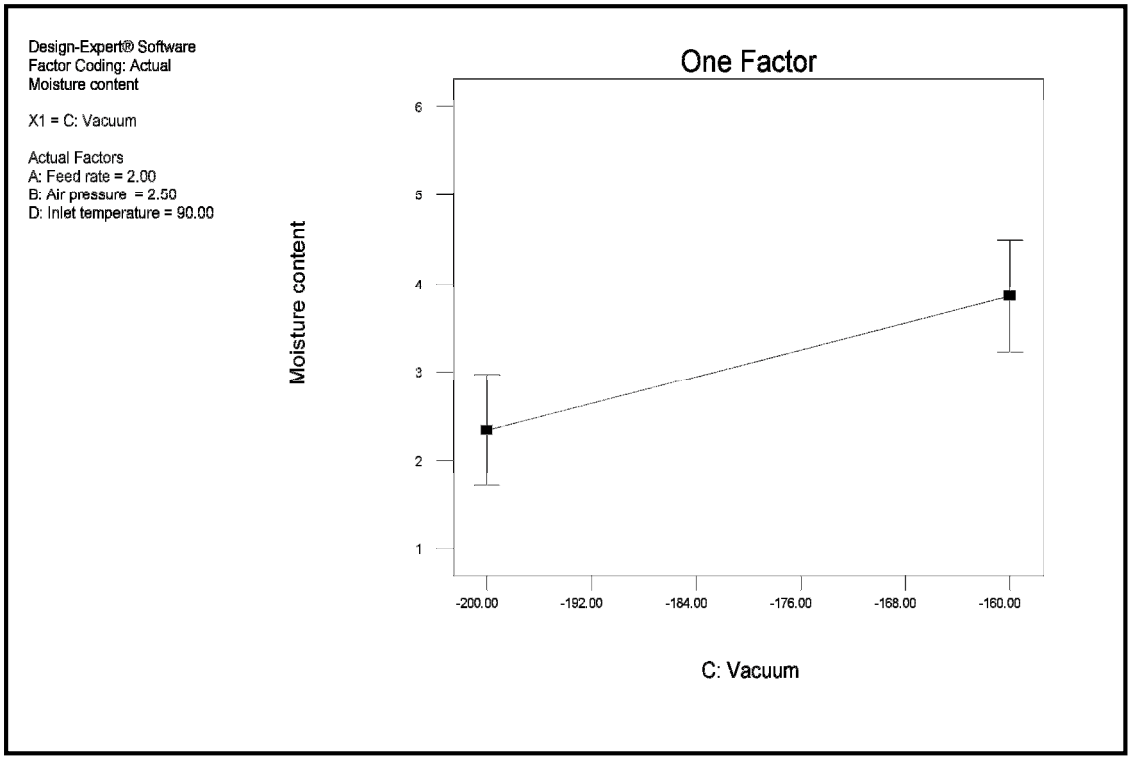


Fig. 5.25: Effect of vacuum on Moisture Content of drug-sugar composites

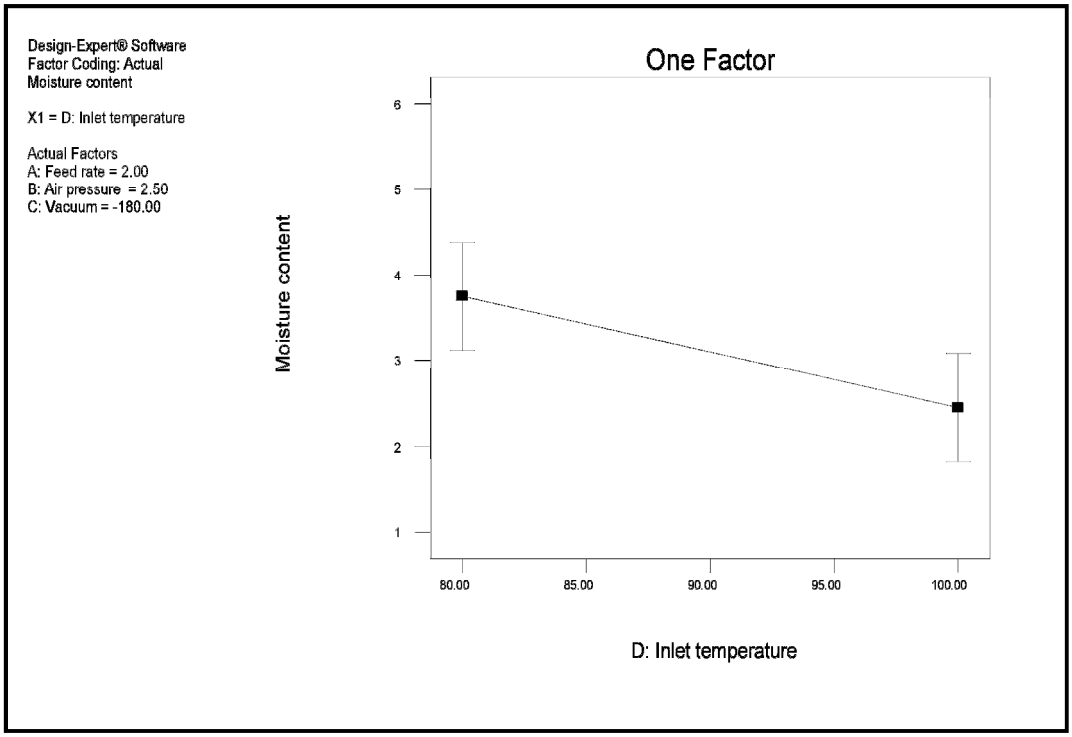


Fig. 5.26: Effect of inlet temperature on Moisture Content of drug-sugar composites

Table 5.11: Solutions provided after numerical optimization by Design-Expert® 8 for recommended factor values and predicted number for responses:

Solution no.	Feed rate	Air pressure	Vacuum	Inlet temperature	Percent Drug Content	Percent Yield	Aerodynamic Particle Size	Moisture content	Desirability
					maximize	maximize	in range (2-5 µm)	minimize	
1	3.00	3.00	-160.00	85.90	98.1989	58.3988	3.27387	3.37972	0.897
2	3.00	3.00	-160.00	86.20	98.1276	58.3331	3.27051	3.34817	0.897
3	3.00	3.00	-160.00	84.60	98.5003	58.653	3.29385	3.51311	0.896
4	**3.00**	**3.00**	**-160.00**	**82.08**	**99.0973**	**59.2253**	**3.31612**	**3.77631**	**0.896**
5	3.00	3.00	-160.00	90.16	97.1961	57.4748	3.22693	2.93751	0.895
6	3.00	3.00	-160.00	90.83	97.0415	57.3334	3.21963	2.86901	0.895
7	3.00	3.00	-160.00	80.94	99.3642	59.471	3.32859	3.89421	0.895

5.5.3 Preparation of liposomal dry powder for inhalation of sildenafil citrate:

Liposomes were prepared with sildenafil citrate, HSPC and or DPPC and cholesterol (Chol). HSPC has a potential to sustain the drug release for longer time as compared to DPPC and hence was chosen to prepare liposomes. Liposomes were also prepared using DPPC alone which is the major component of lung surfactant, but drug loading with DPPC alone was significantly low as compared to HSPC alone. Since the presence of DPPC in some proportion can lead to the formation of better liposomes with better integrating potential with alveolar membrane for longer stay in the lungs, it was thought to replace little HSPC with DPPC. Maximum drug loading could be achieved with formulation at drug: HSPC: DPPC: Cholesterol at 5:18:4.5:2.5 molar ratio under optimized process conditions **(Table 5.12)**. At this ratio, 85% to 88.02% entrapment could be achieved and its 3%w/v solution finally spray dried at inlet/outlet temperature of 80°C/52-56°C to get dry powder formulation using mannitol (Lipid: mannitol; 1:3) as carrier.

Best thin film formation was realized by evaporating solvent (1mL of 1:2 Chloroform : methanol) at 55°C for 60 min. while rotating in round bottom flask at 120rpm between 15" Hg to 25" Hg of vacuum **(Table 5.13)**. Hydration with water yielded product with only 50% of entrapment efficiency. Thus, phosphate buffer pH 7.4 was used to push more of the drugs in lipid compartments by creating pH gradient effect. Sildenafil citrate has shown more % drug distribution towards organic phase when aqueous phase of phosphate buffer pH 7.4 was used during drug distribution study. Hence, were the results for % entrapment efficiency that showed 72% increase as compared with water **(Table 5.14)**. Size reduction using pressure homogenizer was efficient method to obtain particles with low poly dispersity index (pdi). Three homogenization cycles at 1500 bars at 35°C were sufficient to achieve required particle size 419.4µm with low pdi of 0.462 **(Table 5.15)**. Liposomes prepared with inclusion of both HSPC and DPPC at 4:1 molar ratio (SHD4) showed the highest drug loading of 86.38% **(Table 5.12)**. 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 and its 3%w/v solution finally spray dried at inlet/outlet temperature of 80°C/52-56°C to get dry powder formulation using mannitol (Lipid: mannitol; 1:3) as carrier. Thus, this formulation

was considered for spray drying and further optimized with respect to spray drying process parameters as per the fractional factorial design using Design-Expert® 8.

Table 5.12: Effect of different lipids and drug lipid ratios on entrapment efficiency (%) of Sildenafil citrate in liposomes (mean ± SD, n = 3)

S. No.	Batch No.	Drug: Lipid	Lipid: Cholesterol	Entrapment efficiency (%)	Drug loading (%)	Assay (%)	Recovery (%)
A.	**Batches with HSPC**						
	SH1	1:4	4:0	50.17±0.38	7.40±0.84	96.81±0.21	95.45±1.21
	SH2	**1:5**	**5:0**	**76.33±1.02**	**9.80±0.65**	**98.26±0.58**	**97.47±0.97**
	SH3	1:8	8:0	79.99±1.14	5.96±0.99	97.55±0.47	92.40±2.31
	SH4	1:10	10:0	88.14±0.52	5.30±1.65	95.18±0.39	98.70±0.46
B.	**Batches with HSPC and cholesterol**						
	SH5	**1:5**	**9:1**	**85.61±0.56**	**10.28±1.8**	**98.02±0.67**	**98.53±0.86**
	SH6	1:5	8:2	70.56±0.84	9.03±2.06	96.53±0.99	93.25±1.25
C.	**Batches with DPPC**						
	SD1	1:4	4:0	45.45±1.58	6.90±1.11	92.01±1.02	93.75±1.14
	SD2	**1:5**	**5:0**	**68.43±1.24**	**8.22±1.03**	**99.81±0.83**	**86.19±1.09**
	SD3	1:8	8:0	71.21±1.36	5.56±0.69	98.74±1.83	97.23±1.08
	SD4	1:10	10:0	79.29±0.99	4.99±0.38	97.17±1.53	96.10±1.77
E.	**Batches with DPPC and cholesterol**						
	SD5	**1:5**	**9:1**	**74.9±1.46**	**9.43±1.11**	**95.58±0.53**	**88.06±1.45**
	SD6	1:5	8:2	61.1±1.82	8.18±1.79	99.82±.61	99.38±1.23
F.	**Batches with HSPC, DPPC and cholesterol**						
	SHD4	**1:5 (HSPC: DPPC= 4:1)**	**9:1**	**86.38±1.22**	**10.40± 1.73**	**98.66±0.29**	**98.10±0.15**

Table 5.13: Effect of various film formation process and formulation parameters of thin film hydration on liposomal formulation

S. No.	Variables	Inference			
		Observations	**(% EE)**	**(%) Drug loading**	**Assay (%)**
A.	**Composition of solvent system (Chloroform:Methanol)**				
	2:1	Drug was not dissolved properly	-	-	-
	1:1	Whitish thin film and lower EE	76.68± 2.11	9.39±1.06	98.02±1.48
	1:2	**Thin translucent and uniform film with improved EE**	**86.38±1.22**	**10.40±1.73**	**98.66±0.29**
B.	**Volume (mL) of solvent system (Chloroform: Methanol; 1:2)**				
	0.5	Insufficient to dissolve the contents of the formulation	-	-	-
	1.0	**Thin translucent and uniform film with improved EE**	**86.38±1.22**	**10.40±1.73**	**98.66±0.29**
	1.5	No further improvement of EE	86.03±1.12	10.39±1.06	96.64±1.03
C.	**Time of solvent evaporation (min)**				
	15	Film starts appearing	-	-	-
	30	Dry film could be seen	-	-	-
	60	Suitable to ensure complete solvent removal	**86.38±1.22**	**10.40±1.73**	**98.66±0.29**
	90	No further improvement	84.29±0.98	10.21±0.46	98.02±1.12
D.	**Vacuum**				
	15” of Hg	Initial application of 15” Hg vacuum till the appearance of dry film and then increasing the vacuum to 25” of Hg (to ensure complete removal of the solvent) gave thin, transparent to translucent uniform film. Keeping the vacuum of any level constant throughout the film formation produced broken film at high vacuum level and excessive drying time at lower vacuum level.			
	20” of Hg				
	25” of Hg				
E.	**Speed of rotation during film formation**				
	100 rpm	Non-uniform thick film	-	-	-
	120 rpm	Uniform thin film	**86.38±1.22**	**10.40±1.73**	**98.66±0.29**
	130 rpm	Splashing of the contents that created non uniformity in the film	80.97±0.49	9.80±1.02	92.66±0.67

Table 5.14: Effect of various hydration process and formulation parameters by thin film hydration on liposomal formulation

S. No.	Variables	Inference			
		Observations	**(%) Entrapment efficiency (EE)**	**(%) Drug loading**	**Assay (%)**
A.	**Speed of rotation during hydration**				
	50 rpm	Hydration time was longer than 2.0h	79.99±1.23	9.54±1.11	95.36±1.67
	65 rpm	Complete hydration	**86.38±1.22**	**10.40±1.73**	**98.66±0.29**
	80 rpm	Incomplete hydration at 2.0h	68.12±1.02	8.18±0.96	90.01±1.12
B.	**Type of hydration medium**				
	Water	Lower EE	50±1.39	5.98±0.83	90.53±0.67
	Phosphate buffer saline pH=7.4	Suitable. EE was improved as the drug is having negligible solubility in this medium	**86.38±1.22**	**10.40±1.73**	**98.66±0.29**
	Phosphate buffer pH=6.8	Not suitable as it became turbid on contacting methanol while determining EE	-	-	-
C.	**Volume of hydration medium (mL)**				
	1.0	Incomplete hydration	78.93±0.35	9.37±0.52	90.08±0.51
	1.5	Sufficient for complete hydration	**86.38±1.22**	**10.40±1.73**	**98.66±0.29**
	2.0	**No further improvement**	85.79±0.59	9.81±0.69	90.43±0.62
D.	**Hydration time (h)**				
	1	Milky white suspension with few bigger particles	66.68±0.32	8.01±1.28	91.11±1.15
	1.5	Milky white suspension	74.38±0.55	8.99±1.02	94.37±1.67
	2.0	**Translucent suspension with bluish tint and improved EE**	**86.38±1.22**	**10.40±1.73**	**98.66±0.29**
	2.5	**No further improvement**	87.09±0.96	9.31±2.16	98.80±0.93

Table 5.15: Effect of method of size reduction and annealing time on liposomal characteristics

S. No.	Variables	Observation				
		(%) Entrapment efficiency (EE)	**(%) Drug loading**	**Assay (%)**	**Recovery (%)**	**Particle size distribution (nm)**
A.	**Method of size reduction**					
	Pressure homogenization at 35°C and 1500 bars					
	2 cycles	86.22±0.35	10.38±0.96	98.74±1.06	96.78±2.05	752.3 ± 0.98 (Pdi=0.980)
	3 cycles	**86.38±1.22**	**10.40±1.73**	**98.66±0.29**	**98.10±0.15**	**419.4 ± 0.54 (Pdi=0.462)**
	4 cycles	87.39±0.94	10.52±1.65	97.94±1.48	99.83±1.08	108.0 ± 0.67 (Pdi=0.542)
B.	**Annealing time (min) after size reduction**					
	30	79.94±0.69	9.72±1.06	98.54±1.39	92.88±2.05	132.4 ± 0.22 (Pdi=0.665)
	60	**86.38±1.22**	**10.40±1.73**	**98.66±0.29**	**98.10±0.15**	198.4 ± 0.54 (Pdi=0.328)
	90	85.97±0.88	10.39±1.25	97.81±1.14	98.73±2.31	209.6 ± 0.97 (Pdi=0.350)

5.5.3.1. Optimization of spray drying process parameters for sildenafil citrate-liposomal dry powder for inhalation

Total of 16 experiments were run as per fractional factorial design to achieve maximum percent drug retained, maximum percent yield, aerodynamic particle size between 2-5µ and minimum moisture content **(Table 5.16)**.

The effect of various spray drying parameters are depicted in **Fig. 5.27-5.38**. None of the factors individually affected the percent drug retained (or content) and percent yield. However, combination of feed rate and vacuum had significant impact on % drug content. At higher vacuum, drug content decreased as feed rate was increased. At lower vacuum, drug content increased as feed rate was increased. This might be due to suction of fine droplets at excessive vacuum pressure which led to the leakage of the drug from the surface of the droplets before complete drying in hot air stream.

At lower vacuum, percent yield of liposomal dry powder was increased as feed rate was increased. This might be due to the loss at higher vacuum of excessive fines generated at higher feed rate. At lower temperature, percent yield increased as vacuum deceased and at higher temperature, yield decreased as vacuum was decreased. At higher temperature the droplets dry faster and hence should be immediately removed from the drying chamber into the cyclone for better percent yield.

There was a positive impact of feed rate on aerodynamic particle size however inlet temperature and air pressure had no significant impact. Further there was a significant impact of combination of vacuum and inlet temperature on moisture content. At higher temperature, moisture increased as air pressure was increased while at lower temperature, moisture deceased as air pressure was increased. Increased air pressure tend to increase the surface area of the droplets by reducing its size and hence leading to better evaporation of the moisture. At higher temperature, moisture increased on decreasing vacuum which might be due to the inefficient removal of the water at lower vacuum. Feed rate had positive impact on moisture content but was not of practical significance and also no influence of air pressure alone was found on moisture content.

Thus, no single process factor can yield the product with required particle characteristics. There must be a balance of air pressure and vacuum at a selected feed rate and inlet temperature. Numerical optimization provided solutions **(Table 5.17)** of recommended factor values for response factors. Formulation DPL7 prepared according to the design showed response values closest to the goal with maximum percent drug retained (98.79 ± 1.42%) at maximum yield (72.15 ± 0.69%) and lower moisture content (1.16 ± 2.24% w/w) and aerodynamic particle size in range (4.03µm) for better lung deposition and higher geometric size (8.99± 1.26 µm) for reduced uptake by alveolar macrophages. Hence, formulation DPL7 was chosen as the optimized formulation with respect to formulation and spray drying parameters. Thus, final formulation was spray dried at optimized parameters of 3mL/min feed rate, atomized at compressed air pressure of 3bars with -160mm of WC vacuum at 80°C inlet temperature. Formulation with HSPC and DPPC both (SHD4), only HSPC (SH5) were spray dried at optimized spray drying conditions to consider for studies like macrophage uptake.

Table 5.16: Effect of spray drying process parameters on formulation characteristics of sildenafil citrate-liposomal dry powder for inhalation

	Factor 1	Factor 2	Factor 3	Factor 4	Response 1	Response 2	Response 3	Response 4		
Run	A:Feed rate	B:Air pressure	C:Vacuum	D:Inlet temperature	Percent Drug	Percent Yield	Aerodynamic Particle Size	Moisture content	Bulk density (ρb)	Volume mean diameter
	mL/min	Bar	mm of	°C	%	%	(µm)	%	g/cm^3	(µm)
DPL1	1	3	-160	80	95.28 ±	45.36 ± 1.08	7.73	5.24 ± 1.57	0.316± 1.23	13.75± 0.88
DPL2	3	3	-200	80	95.95 ±	32.72 ± 2.34	5.89	4.42 ± 1.19	0.307± 2.15	10.63± 1.06
DPL3	1	2	-200	80	87.84 ±	42.31 ± 0.85	10.17	3.35 ± 0.65	0.309± 0.92	18.30± 2.57
DPL4	1	2	-200	100	81.39 ±	54.27 ± 1.36	9.59	1.27 ± 2.38	0.32± 2.46	16.95± 2.64
DPL5	3	2	-160	80	88.45 ±	52.76 ± 1.33	6.12	5.42 ± 1.79	0.265± 1.02	11.89± 1.99
DPL6	3	3	-200	100	96.52 ±	44.97 ± 0.33	3.59	1.01 ± 2.08	0.225± 1.17	7.57± 0.79
DPL7	**3**	**3**	**-160**	**80**	**98.79 ±**	**72.15 ± 0.69**	**4.03**	**1.16 ± 2.24**	**0.201± 2.05**	**8.99± 1.26**
DPL8	1	3	-200	100	92.35 ±	36.75 ± 1.02	4.55	2.76 ± 1.53	0.233± 3.51	9.43± 0.67
DPL9	3	2	-160	100	86.52 ±	64.97 ± 0.33	3.59	1.01 ± 2.08	0.251± 1.09	7.17± 2.88
DPL10	3	2	-200	80	85.72 ±	48.99 ± 1.26	7.01	3.42 ± 1.75	0.222± 1.39	14.88± 3.01
DPL11	1	3	-200	80	93.57 ±	34.11 ± 1.67	6.55	1.72 ± 0.45	0.326± 2.03	11.47± 2.93
DPL12	3	2	-200	100	91.16 ±	47.07 ± 1.96	6.27	3.12 ± 0.79	0.319± 1.14	11.10±1.84
DPL13	1	2	-160	100	88.27 ±	57.06 ± 1.83	7.81	5.88 ± 1.01	0.323± 2.73	13.74± 1.26
DPL14	1	3	-160	100	90.99 ±	51.63 ± 0.77	6.38	0.97 ± 0.75	0.324± 0.89	11.21± 0.92
DPL15	1	2	-160	80	94.01 ±	62.29 ± 1.63	9.53	7.53 ± 2.09	0.326± 1.00	16.69± 0.34
DPL16	3	3	-160	100	98.27 ±	62.06 ± 1.72	4.22	1.06 ± 1.12	0.251± 1.09	8.42± 1.13

Table 5.17: Solutions provided by Design-Expert® 8 for recommended factor values and predicted number for responses:

Solution no.	Feed rate	Air pressure	Vacuum	Inlet temperature	Percent Drug Content	Percent Yield	Aerodynamic Particle Size	Moisture content	Desirability
					maximize	maximize	in range (2-5 μm)	minimize	
1	1.00	2.00	-200.00	91.93	98.2966	67.1014	3.59001	1.2372	0.927
2	1.00	2.00	-199.77	91.96	98.2769	67.0662	3.61295	1.2616	0.922
3	1.00	2.01	-200.00	91.89	97.2112	66.9426	3.59003	1.22952	0.921
4	**3.00**	**2.00**	**-160.00**	**91.85**	**98.2964**	**67.0965**	**3.65744**	**1.31392**	**0.910**
5	2.97	2.00	-200.00	91.74	97.2723	67.0576	3.67176	1.3282	0.897

Table 5.18: Effect of formulation parameters on formulation characteristics of liposomes during spray drying

S.No.	Batch code	Lipids composition	Lipid : Carrier	Solid content %w/v	Percent drug retained ± SD	Percent yield ± SD	Bulk density	Tapped density	Angle of repose	%CI	Hausner's ratio
1	DPL17	HSPC, DPPC and cholesterol	(1:2)	3	78.33 ± 0.84	65.82 ± 0.88	0.229	0.306	35.01 ± 1.33	25.16	1.330
2	DPL7	HSPC, DPPC and cholesterol	(1:3)	3	98.79 ± 1.42	72.15 ± 0.69	0.201	0.223	28.06 ± 0.4	9.87	1.109
3	DPL18	HSPC, DPPC and cholesterol	(1:4)	3	98.66 ± 0.96	73.99 ± 0.81	0.217	0.262	30.65 ± 1.52	17.18	1.21
4	DPL19	HSPC, DPPC and cholesterol	(1:3)	1.5	97.25 ± 0.63	69.98 ± 1.78	0.233	0.311	34.87 ± 1.61	25.08	1.334
5	DPL20	HSPC, DPPC and cholesterol	(1:3)	4.5	92.27 ± 1.22	70.43 ± 1.19	0.183	0.202	26.18 ± 1.44	9.41	1.104
6	DPL21	HSPC and cholesterol	(1:3)	3	98.12 ± 1.22	67.68 ± 1.59	0.235	0.301	31.93 ± 0.97	21.93	1.28
7	DPL22	DPPC and cholesterol	(1:3)	3	82.35 ± 1.15	52.36 ± 1.05	0.212	0.275	40.01 ± 1.82	22.90	1.29

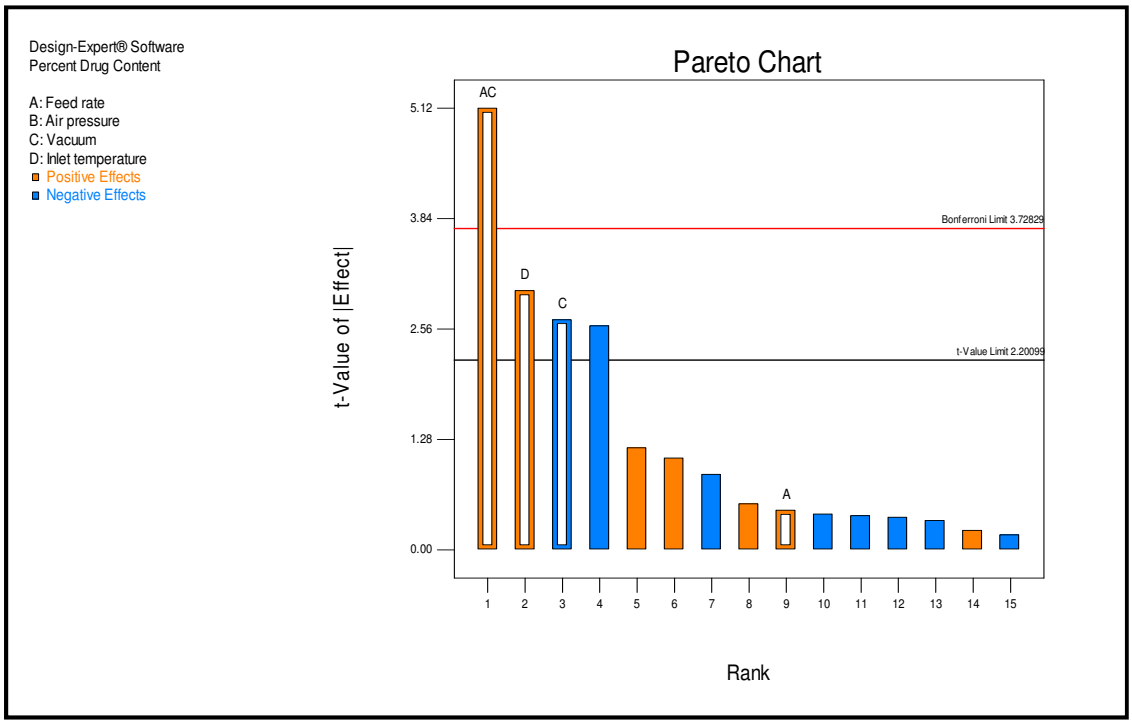


Fig. 5.27: Standardized effects of Variables on percent drug retained in liposomal dry powder for inhalation

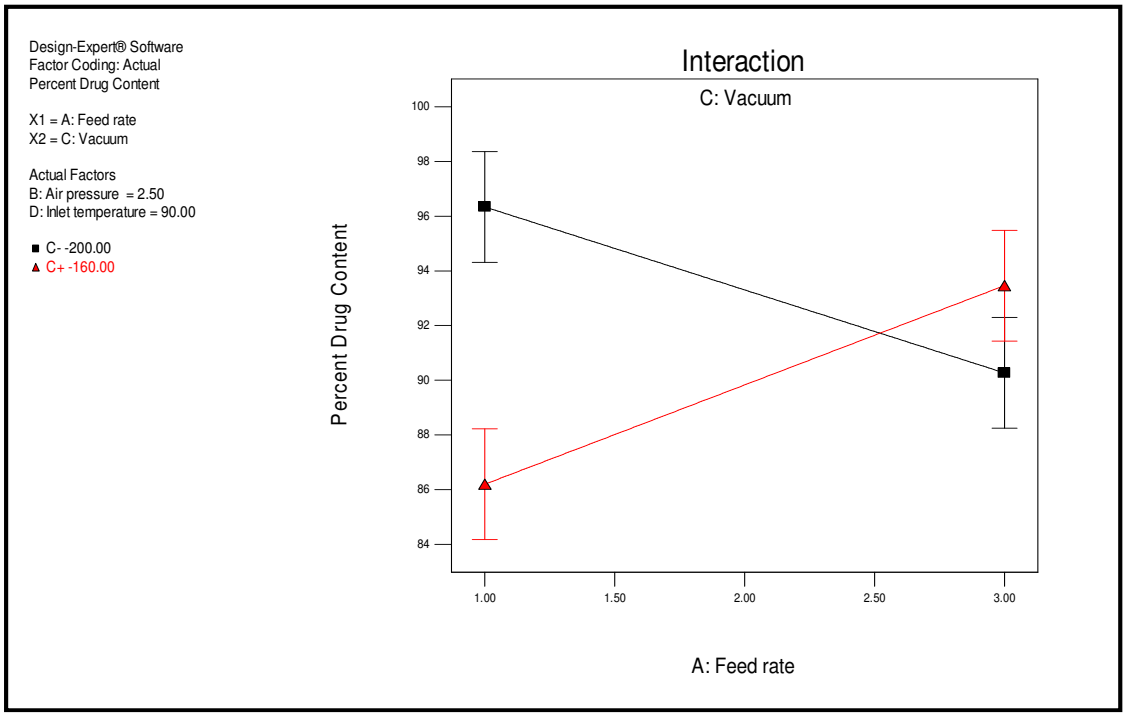


Fig. 5.28: Interaction of feed rate and vacuum to influence Percent Drug retained in liposomal dry powder for inhalation

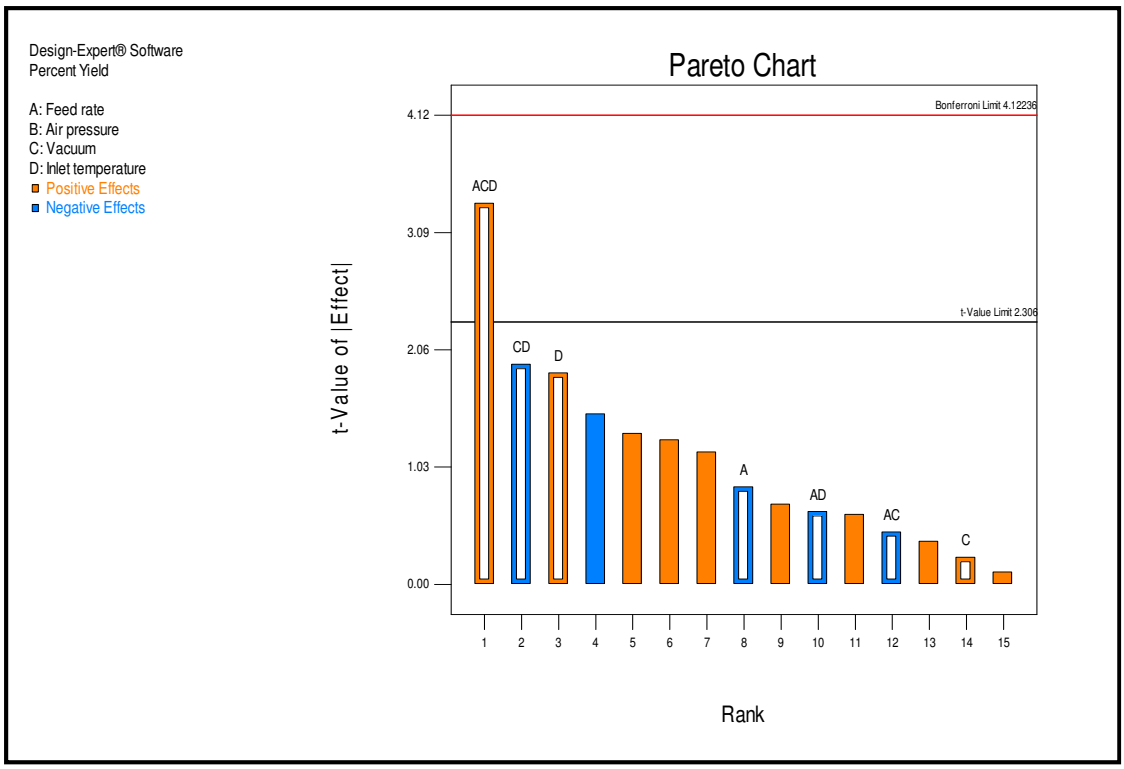


Fig. 5.29: Standardized effects of variables on percent yield of liposomal dry powder for inhalation

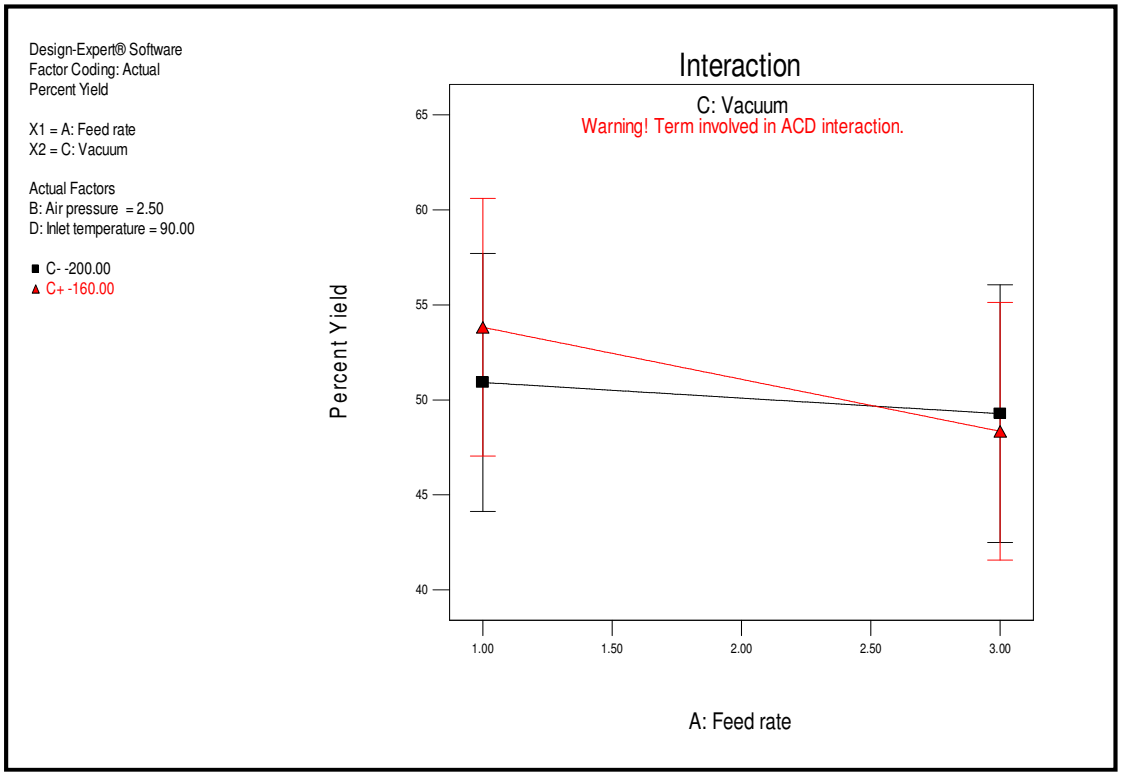


Fig. 5.30: Interaction of feed rate and vacuum to influence percent yield of liposomal dry powder for inhalation

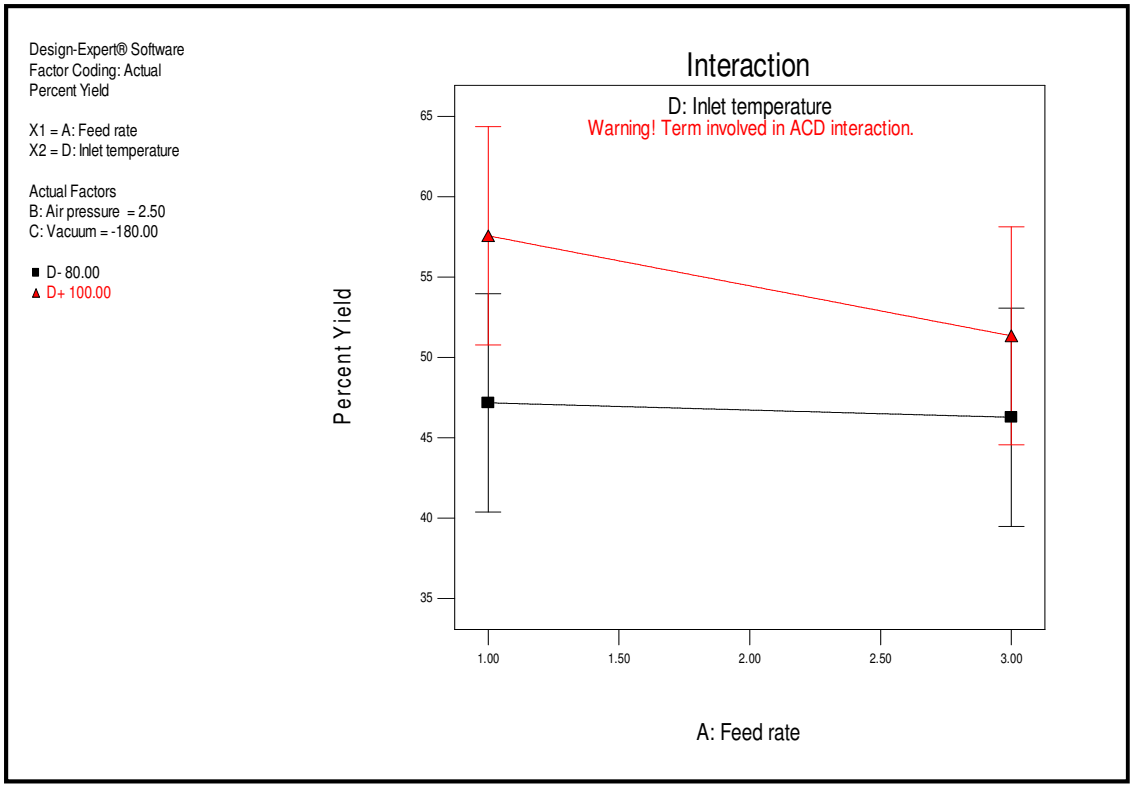


Fig. 5.31: Interaction of feed rate and inlet temperature to influence Percent yield of liposomal dry powder for inhalation

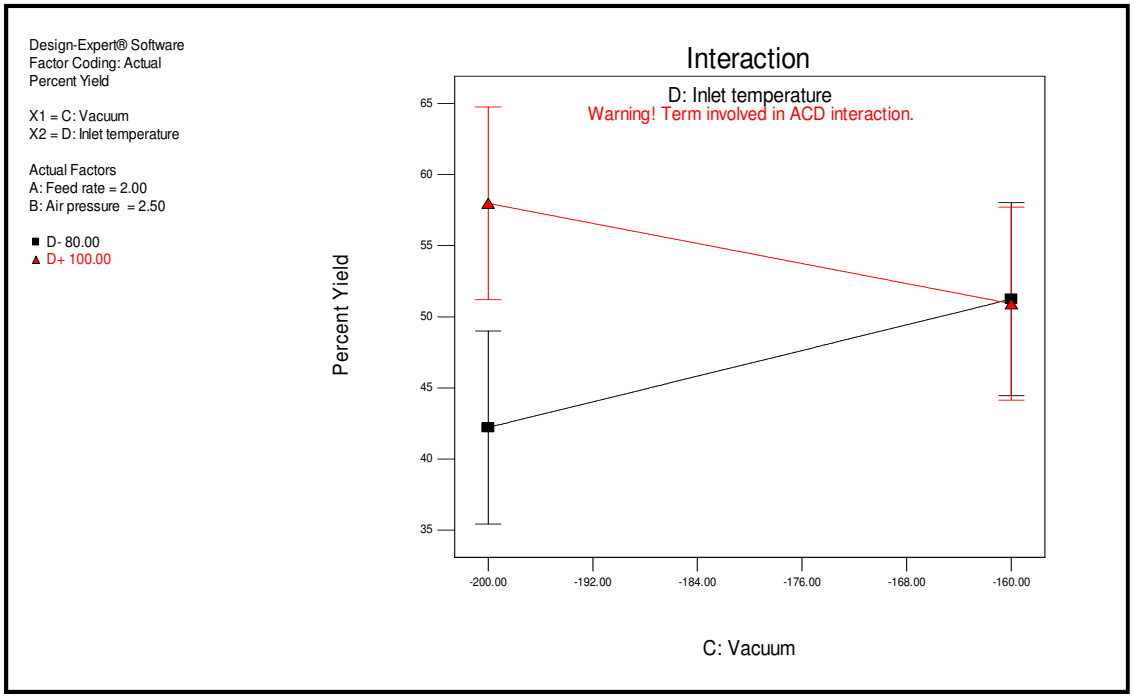


Fig. 5.32: Interaction of vacuum and inlet temperature to influence percent yield of liposomal dry powder for inhalation

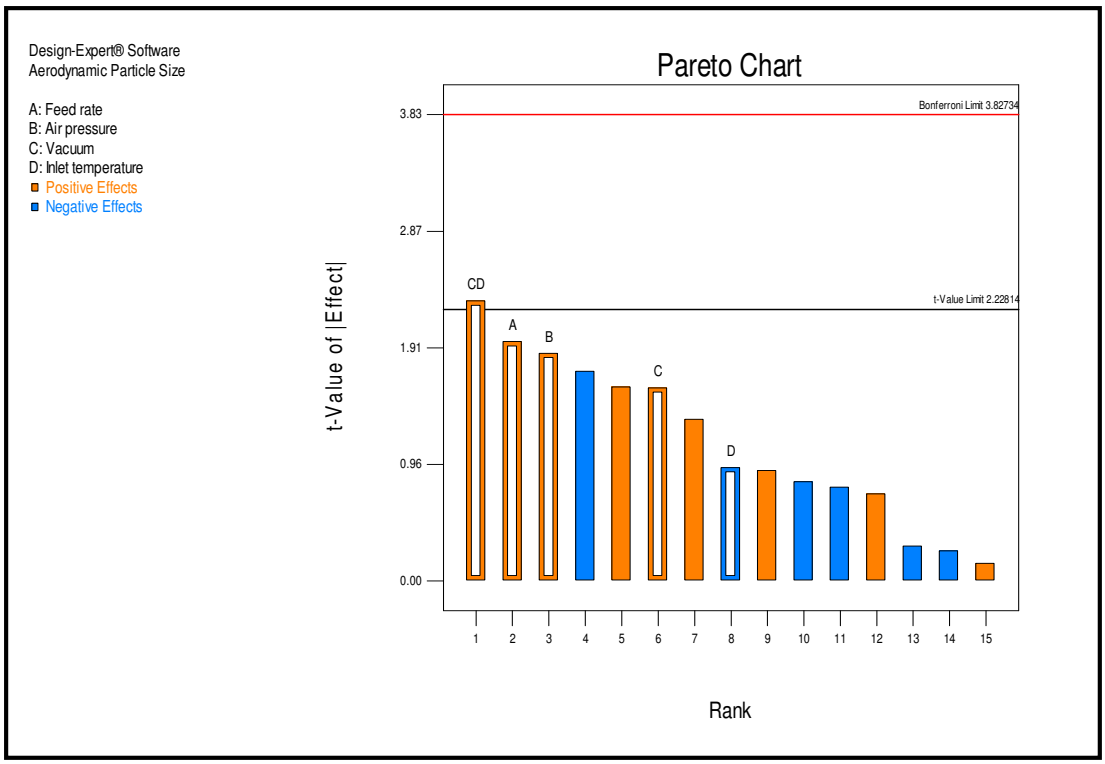


Fig. 5.33: Effect of Variables on aerodynamic particle size of liposomal dry powder for inhalation

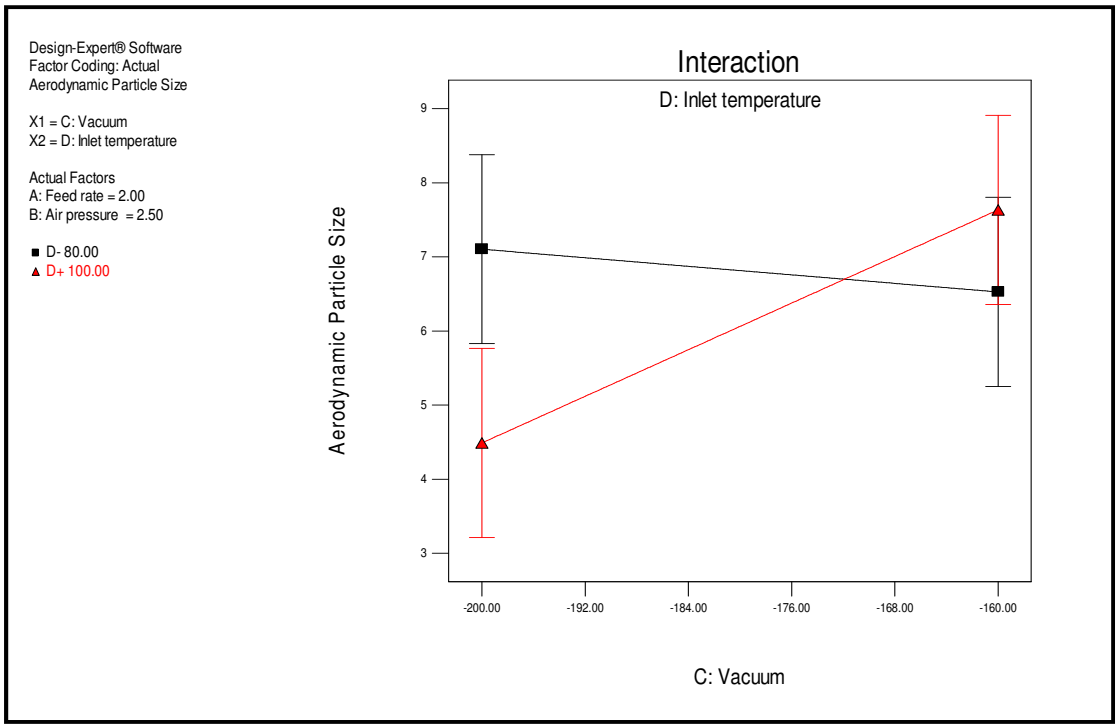


Fig. 5.34: Interaction vacuum and inlet temperature to influence aerodynamic particle size of liposomal dry powder for inhalation

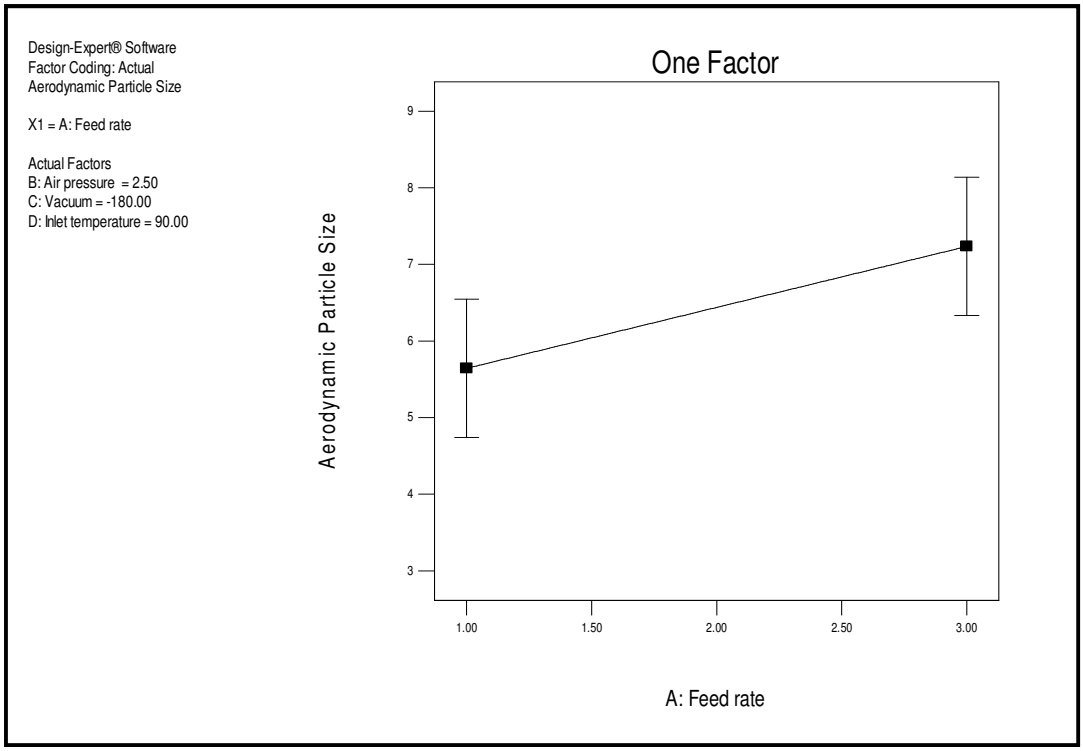


Fig. 5.35: Positive impact of feed rate on aerodynamic particle size of liposomal dry powder for inhalation

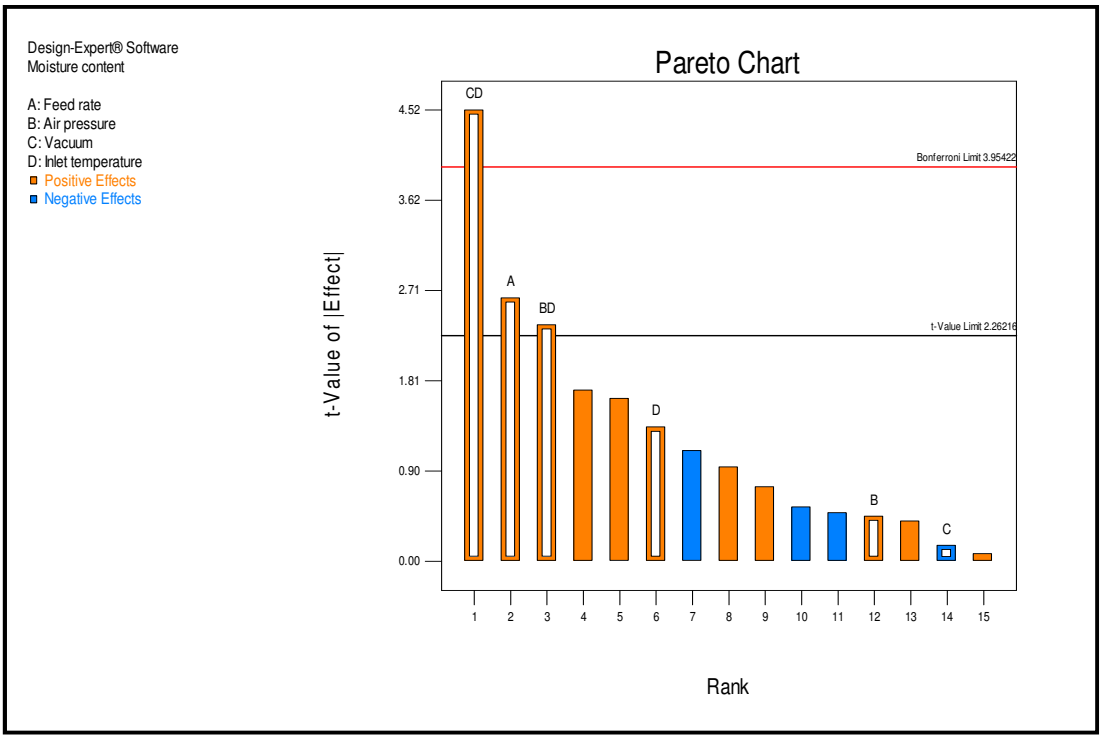


Fig. 5.36: Standardized effect of Variables on moisture content of liposomal dry powder for inhalation

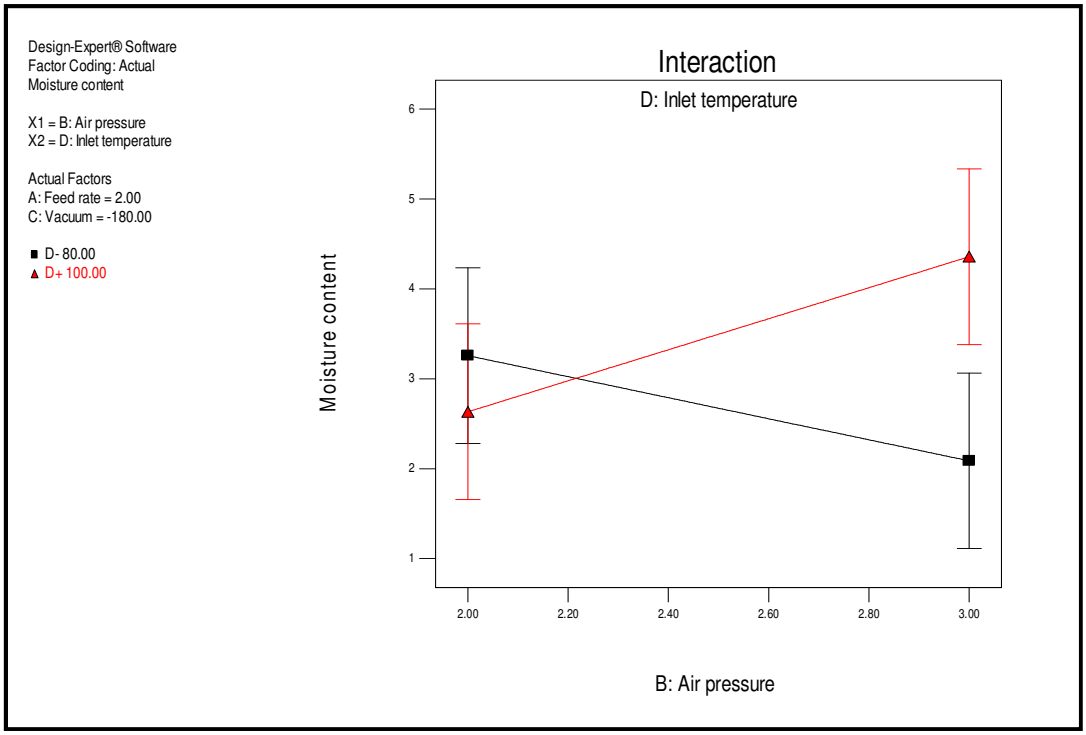


Fig. 5.37: Interaction of air pressure and inlet temperature on moisture content of liposomal dry powder for inhalation

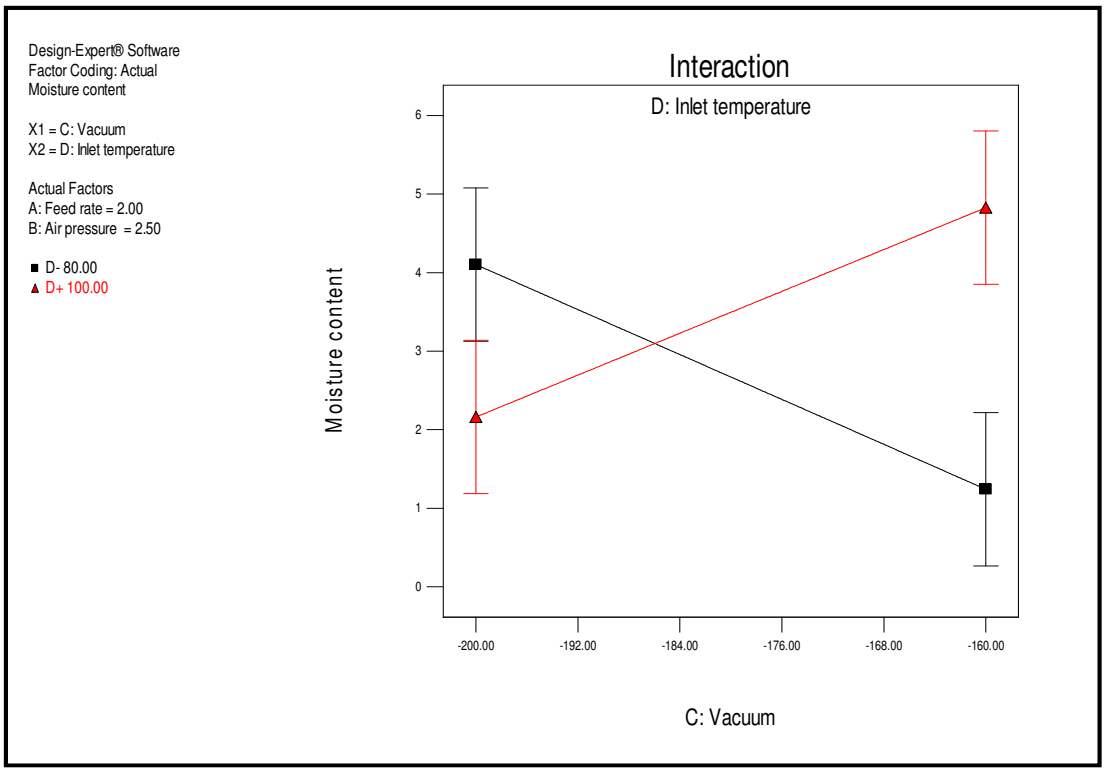


Fig. 5.38: Interaction of vacuum and inlet temperature on moisture content of liposomal dry powder for inhalation

5.5.4 Preparation of drug-lipid composites of sildenafil citrate:

Drug and lipids in optimized molar ratios for liposomes were dissolved in solvent (methanol alone or methanol with 10-20% of water) for single step spray drying to achieve lipid composites or microspheres of sildenafil citrate. 1.18% of Drug and lipids in the above optimized molar ratio were spray dried using trehalose carrier at 1:0.5 to 1:1.5 lipid: trehalose ratio in methanol solvent to get drug-lipid composites in single step process. Trehalose has been shown as stabilizing sugar for proteins and lipid membranes during processing (Steckel H, 2004). Mannitol used for drug-sugar composites and liposomes could not be used here due to its comparatively lower solubility in methanol. Inclusion of water led to the formation of non-homogenous products with very high interbatch variations.

Optimized product showed 96-98% percent drug content at 1.18% solid content in 100% methanol as solvent and 1:1 lipid:trehalose ratio and was further optimized with respect to spray drying process parameters. Decrease in trehalose content significantly lowered the percent drug content. This might be due to the lack of sufficient trehalose required to accommodate added amount of drug. Free drug must be present as very fine particle that could have been lost at the applied vacuum. Moreover, on increasing lipid: trehalose ratio (from 1:1 to 1: 1.5) hygroscopic product was formed leading to lower percent yield **(Table 5.18)**. Batches from DL1 to DL7 were prepared at spray drying parameters of 2mL/min. feed rate, 2.5 bars compressed air pressure, inlet temperature of 60°C and vacuum at -180 mm of WC. Spray drying process parameters were further optimized for the above finalized formulation using fractional factorial design using Stat-Ease software (Design-Expert 8).

5.5.4.1 Optimization of spray drying process parameters for sildenafil citrate-lipid composites

Analysis of responses obtained after experimentation as per fractional factorial design for optimizing spray drying process **(Table 5.19)**, following outcomes were observed.

None of the variables had significant effect on % drug content. Combined impact of various parameters was seen on percent yield:

1. Interaction was seen between air pressure and feed rate. At higher air pressure, yield increases as feed rate increases, whereas at lower air pressure, a decrease in yield was observed as feed rate increased. At higher air pressure, higher feed rate must have balanced the time required for solvent evaporation. At lower air pressure hygroscopicity of the product was higher which must be the reason for lower % yield even at higher feed rate.
2. Interaction was seen between vacuum and feed rate too. At higher vacuum, yield increased as feed rate was increased, whereas at lower vacuum, a decrease in yield was observed as feed rate increased. Higher vacuum pull most probably had protected the product from being hygroscopic by pulling it faster from the hot air stream.
3. Interaction was seen between inlet temperature and feed rate. At higher inlet temperature, yield increased as feed rate increased, whereas at lower inlet temperature, a decrease in yield was observed as feed rate increased.
4. Interaction was seen between vacuum and air pressure. At higher vacuum, yield decreased as air pressure increased, whereas at lower vacuum, there was no effect.

Inlet temperature and air pressure individually had a significant impact on yield. As air pressure increased, % yield decreases. As inlet temperature was increased, % yield was decreased.

Aerodynamic particle size was increased at both increased inlet temperature and increased air pressure. Air pressure and a combination of feed rate and vacuum significantly impacts aerodynamic particle size.

1. An interaction was seen between vacuum and feed rate. At higher vacuum, particle size decreased as feed rate increased. At lower vacuum, particle size increased as feed rate was increased.
2. An interaction was seen between inlet temperature and feed rate. At higher inlet temperature, particle size decreased as feed rate increased. At lower inlet temperature, particle size increased as feed rate increased.

A combination of feed rate, air pressure and vacuum significantly influenced the moisture content.

1. At higher air pressure, moisture decreased with increase in feed rate.

2. At higher vacuum, moisture decreased with increase in feed rate and at lower vacuum, moisture increased with increase in feed rate
3. At higher temperature, moisture decreased with increase in vacuum and at lower temperature, moisture increased with increase in vacuum.

All the above said effects are depicted in **Fig. 5.45-5.51.** Numerical optimization provided solutions **(Table 5.20)** of recommended factor values for response factors. Formulation DLS7 prepared according to the design showed response values closest to the goal with maximum percent drug retained (98.9 ± 0.06%) at maximum yield (68.8 ± 0.86%) and lower moisture content (2.69 ± 0.09% w/w) and is also significantly closer to the numerical optimized solution no. 3. Hence, formulation DLS7 was chosen as the optimized formulation with respect to formulation and spray drying parameters. Thus, final formulation was spray dried at optimized parameters of 3mL/min feed rate, atomized at compressed air pressure of 3bars with -160mm of WC vacuum at 50°C inlet temperature.

Table 5.18: Effect of formulation variables on percent drug content, percent yield and flow characteristics of sildenafil citrate-lipid composites (mean ± SD, n = 3)

S.No.	Batch code	Lipid : Carrier	%v/v of Methanol	Solid content %w/v	Percent drug content ± SD	Percent yield ± SD	Bulk density	Tapped density	Angle of repose	% CI	Hausner ratio
1	DL1	(1:1)	80	1.18	54.1 ± 3.38	30.8 ± 4.87	Batches were non homogenous. Interbatch variation was also high.				
2	DL2	(1:1)	90	1.18	60.6 ± 2.15	32.9 ± 5.32					
3	**DL3**	**(1:1)**	**100**	**1.18**	**98.0 ± 0.89**	**67.9 ± 1.06**	**0.211± 0.75**	**0.232 ± 0.02**	**29.43± 1.00**	**10.77**	**1.09**
4	DL4	(1:0.5)	100	1.18	86.4 ± 1.12	65.3 ± 1.67	0.218 ± 0.89	0.251 ± 0.35	32.52 ± 0.65	13.14	1.15
5	DL5	(1:1.5)	100	1.18	94.5 ± 0.26	43.1 ± 2.16	0.313 ± 1.08	0.435 ± 1.14	47.32 ± 1.49	28.04	1.34
6	DL6	(1:1)	100	0.78	98.5 ± 1.05	63.2 ± 1.59	0.243 ± 1.04	0.303 ± 2.01	33.50 ± 0.82	19.8	1.25
7	DL7	(1:1)	100	2.36	87.1 ± 1.18	66.6 ± 0.94	0.195 ± 1.18	0.321 ± 1.53	43.41 ± 0.57	39.25	1.64

Table 5.19: Effect of spray drying process parameters on % yield, % drug content, moisture content and aerodynamic particle size of sildenafil citrate-lipid composites

	Factor 1	Factor 2	Factor 3	Factor 4	Response 1	Response 2	Response 3	Response 4		
Run	**A:Feed rate**	**B:Air pressure**	**C:Vacuum**	**D:Inlet temp**	**Percent Drug Content**	**Percent Yield**	**Aero-dynamic Particle Size**	**Moisture content**	**Bulk density (ρb)**	**Volume mean diameter**
	mL/min	Bar	mm of WC	°C	%	%	Micron	%	g/cm^3	(µm)
DLS1	1	3	-160	50	82.4 ± 0.64	62.6 ±	1.80	2.07 ±	0.328±	3.14± 1.45
DLS2	3	3	-200	50	93.2 ± 0.53	37.2 ±	2.70	1.56 ±	0.211±	5.88± 0.79
DLS3	1	2	-200	50	98.5 ± 0.33	36.5 ±	13.21	1.89 ±	0.289±	24.57± 0.83
DLS4	1	2	-200	70	33.2 ± 2.56	20.5 ±	11.32	3.15 ±	0.269±	21.83± 1.10
DLS5	3	2	-160	50	30.56 ± 0.91	62.9 ±	17.42	2.93 ±	0.325±	30.56± 1.24
DLS6	3	3	-200	70	38.5 ± 1.01	22.9 ±	5.62	4.01± 0.67	0.246±	11.33± 1.07
DLS7	**3**	**3**	**-160**	**50**	**98.9 ± 0.06**	**68.8 ±**	**2.33**	**2.69 ±**	**0.207±**	**5.12± 0.91**
DLS8	1	3	-200	70	26.66 ± 1.19	18.76 ±	6.35	4.21 ±	0.253±	12.62± 0.56
DLS9	3	2	-160	70	78.2 ± 0.14	62.9 ±	5.33	4.13 ±	0.244±	10.79± 0.88
DLS10	3	2	-200	50	80.7 ± 0.86	31.4 ±	18.22	2.42 ±	0.337±	31.39± 2.10
DLS11	1	3	-200	50	76.4 ± 1.14	38.2 ±	2.20	2.01 ±	0.224±	4.65± 2.51
DLS12	3	2	-200	70	30.2 ± 1.96	18.5 ±	18.32	3.63 ±	0.339±	31.46± 2.06
DLS13	1	2	-160	70	38.9 ± 1.17	16.9 ±	12.23	3.83 ±	0.328±	21.35± 0.99
DLS14	1	3	-160	70	24.0± 1.19	20.4 ±	9.82	3.88 ±	0.339±	16.87± 1.19
DLS15	1	2	-160	50	98.5 ± 0.33	45.9 ±	11.21	2.99 ±	0.276±	21.34± 1.62
DLS16	3	3	-160	70	22.3 ± 1.19	17.1 ±	13.78	3.14 ±	0.301±	25.12± 0.94

Table 5.20: Solutions provided by Design-Expert® 8 for recommended factor values and predicted number for responses:

Solution no.	Feed rate	Air pressure	Vacuum	Inlet temperature	Percent Drug Content	Percent Yield	Aerodynamic Particle Size	Moisture content	Desirability
					maximize	maximize	in range (2-5 μm)	minimize	
1	3.00	2.00	-160.00	57.00	98.14	56.29	3.81	0.63	0.926
2	3.00	2.00	-200.00	58.70	98.24	56.44	3.85	0.63	0.928
3	**3.03**	**2.85**	**-160.00**	**52.21**	**97.63**	**55.82**	**3.77**	**0.64**	**0.891**
4	3.00	2.89	-200.00	52.29	97.29	55.61	3.79	0.65	0.825
5	3.00	2.45	-200.00	52.59	98.89	55.61	4.12	0.64	0.816

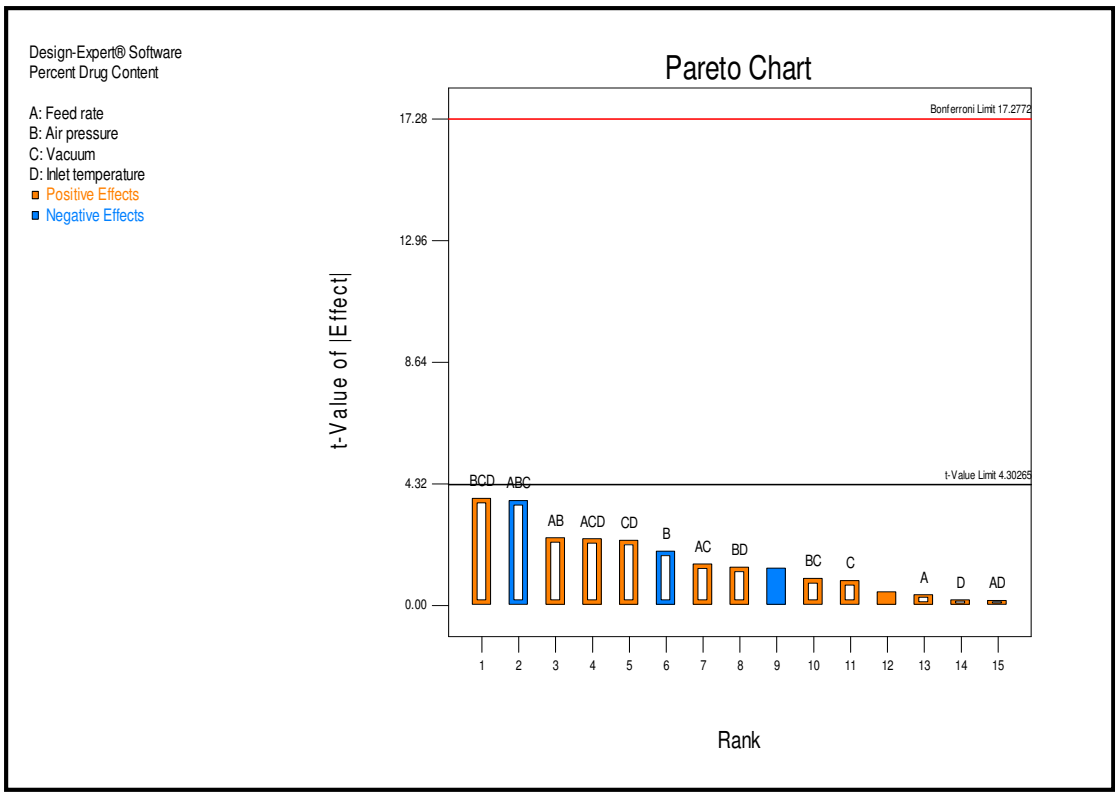


Fig. 5.39: Standardized effects of variables on percent Drug Content of sildenafil-citrate lipid composites

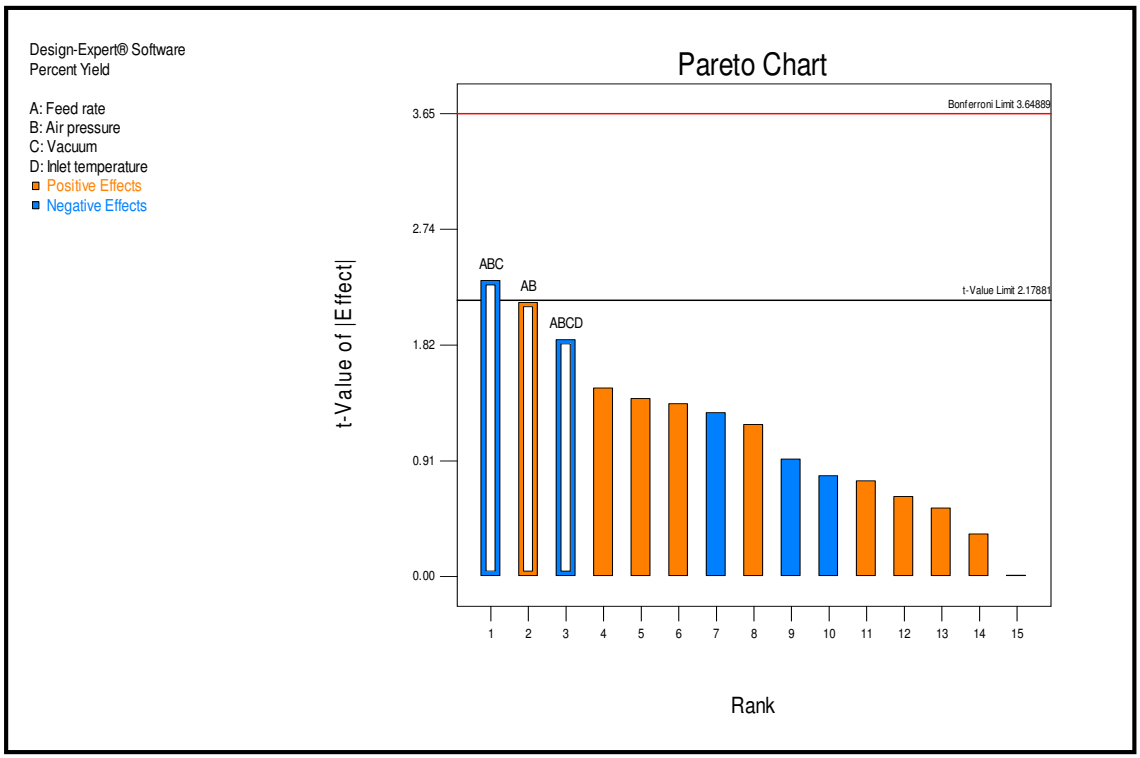


Fig. 5.40: Standardized effects of Variables on percent yield of sildenafil-lipid composites

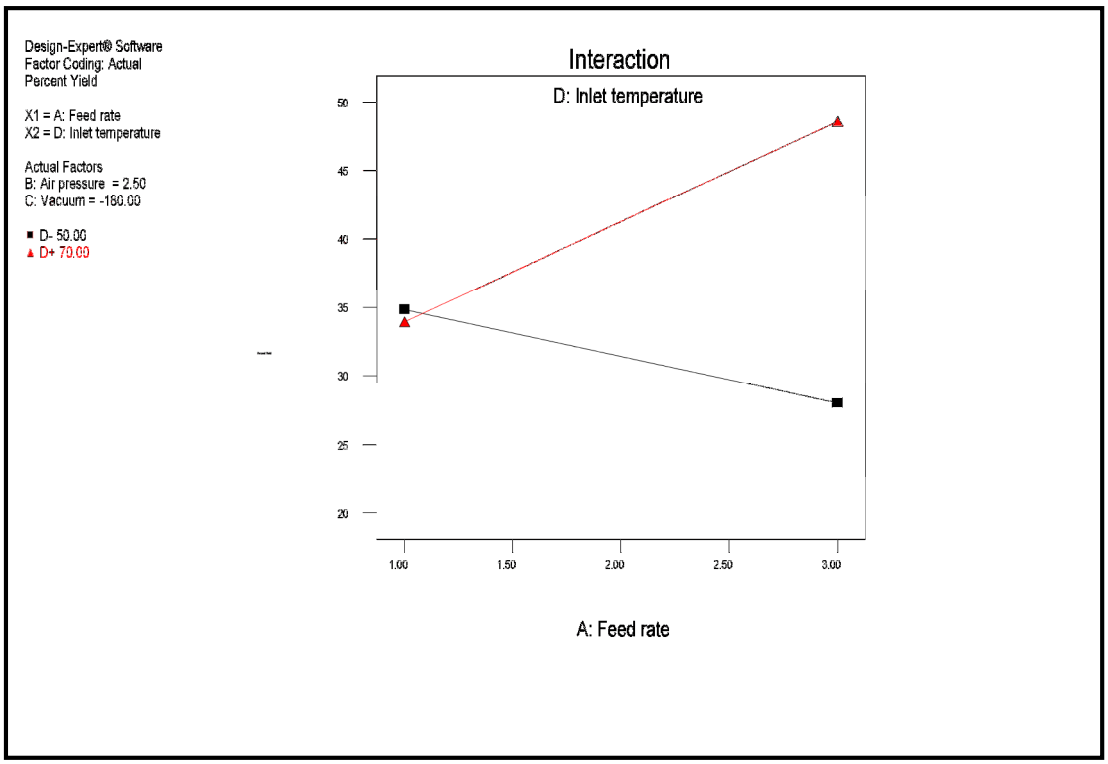


Fig. 5.41: Impact of interaction of variables on percent yield of sildenafil citrate-lipid composites

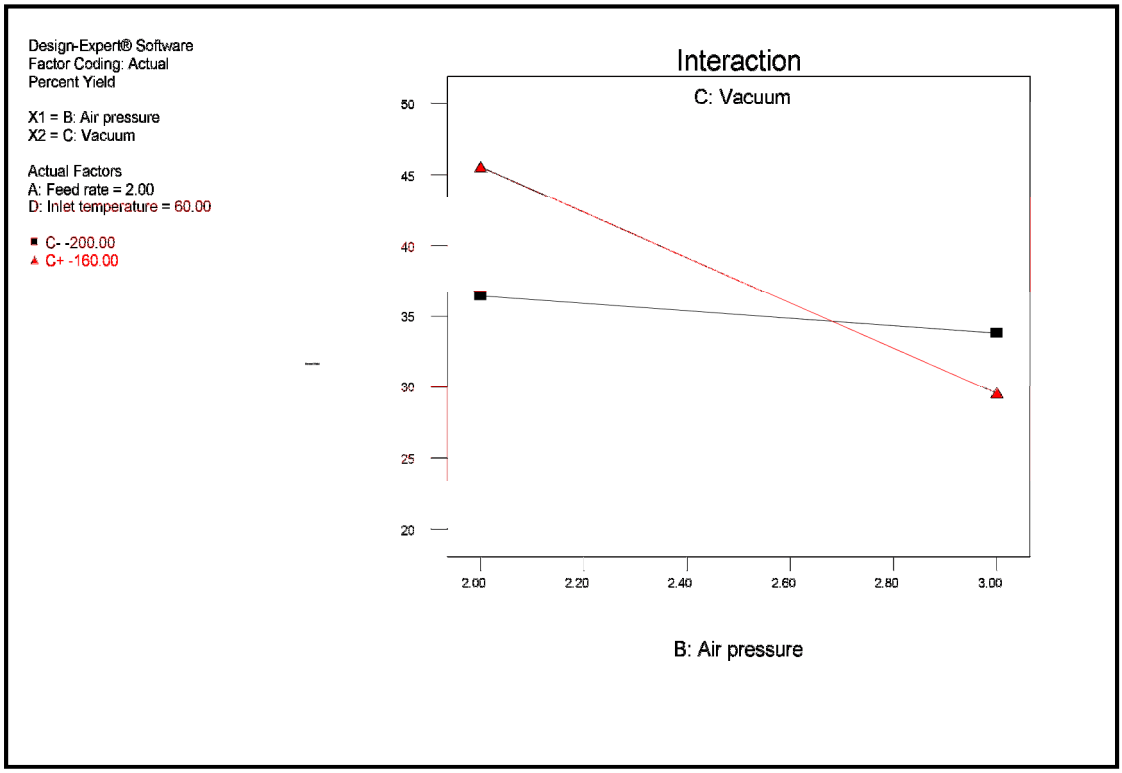


Fig. 5.42: Impact of interaction of variables on percent yield of sildenafil citrate-lipid composites

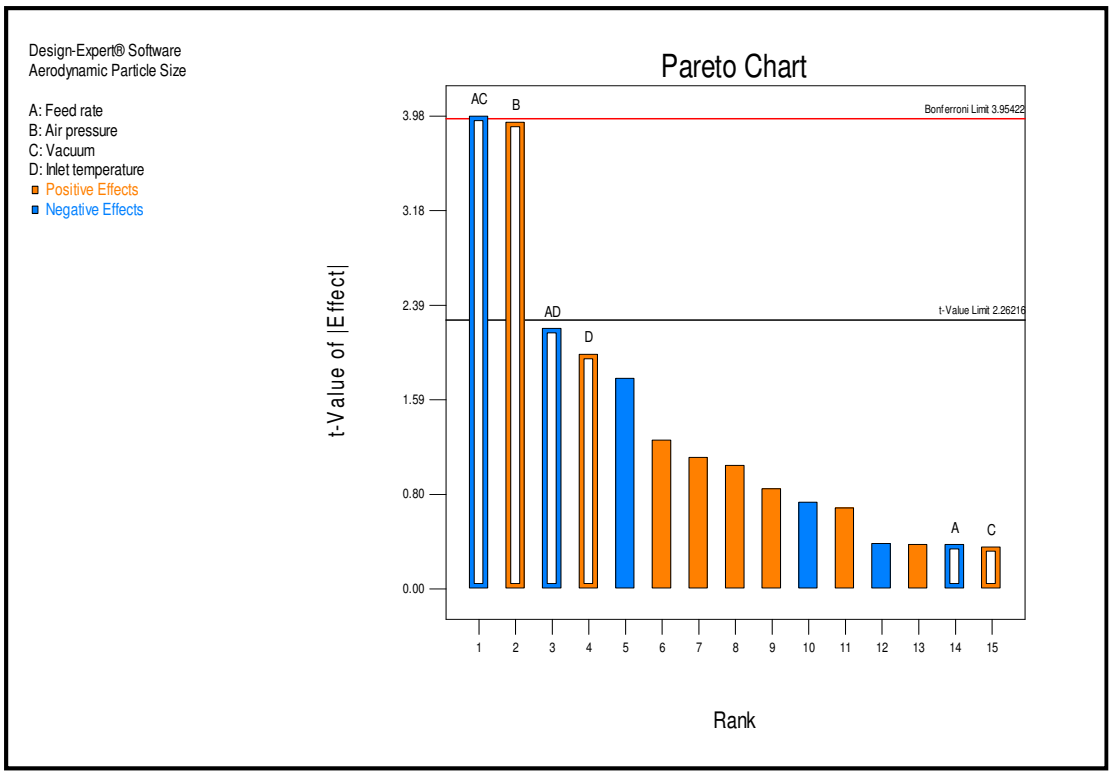


Fig. 5.43: Standardized effects of variables on aerodynamic particle size of sildenafil citrate-lipid composites

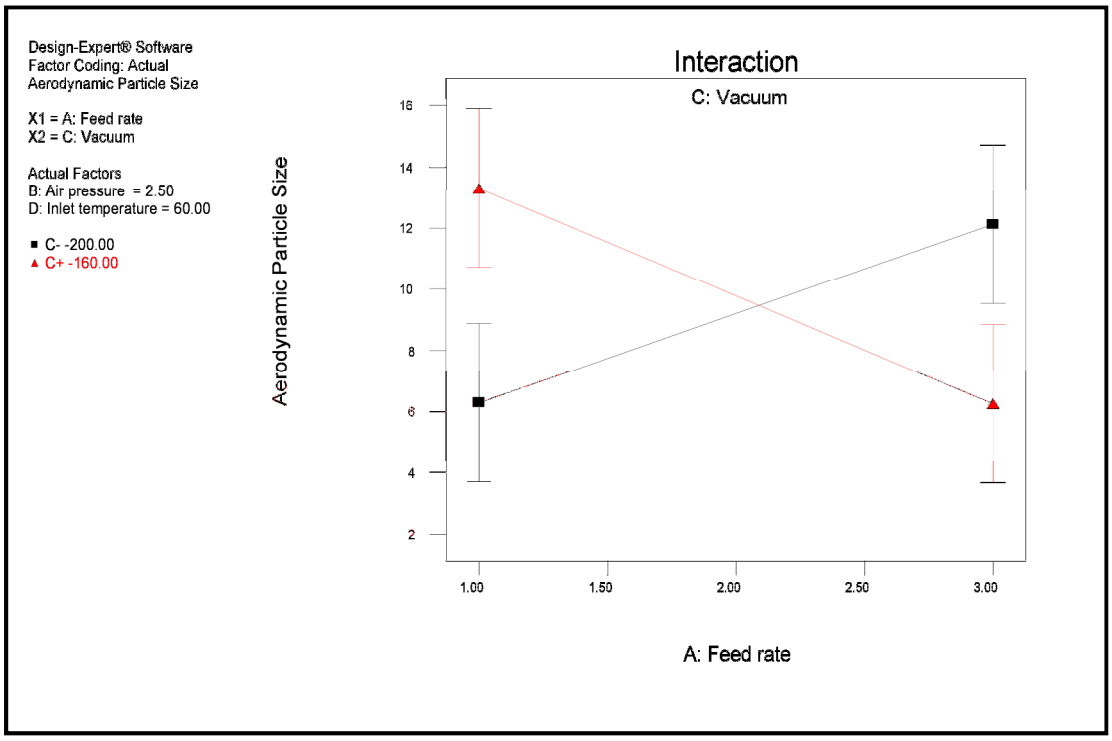


Fig. 5.44: Impact of interaction of variables on aerodynamic particle size of sildenafil citrate-lipid composites.

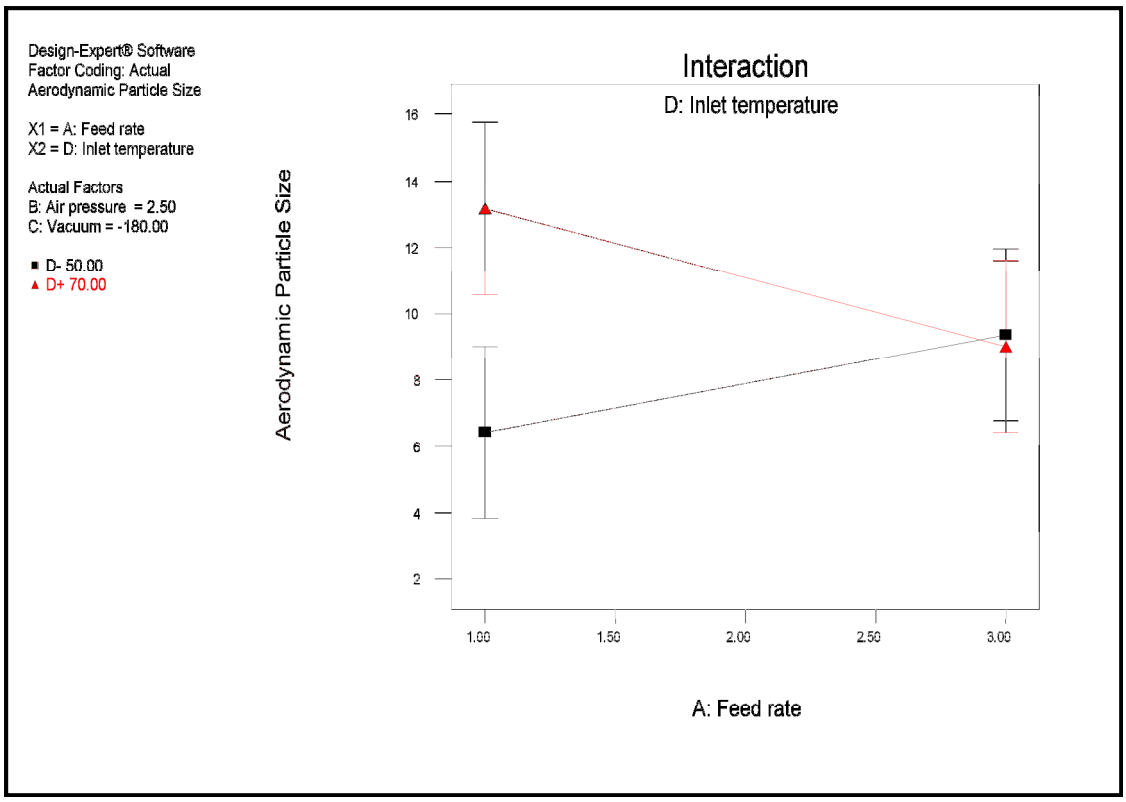


Fig. 5.45: Impact of interaction of variables on aerodynamic particle size of sildenafil citrate lipid composites

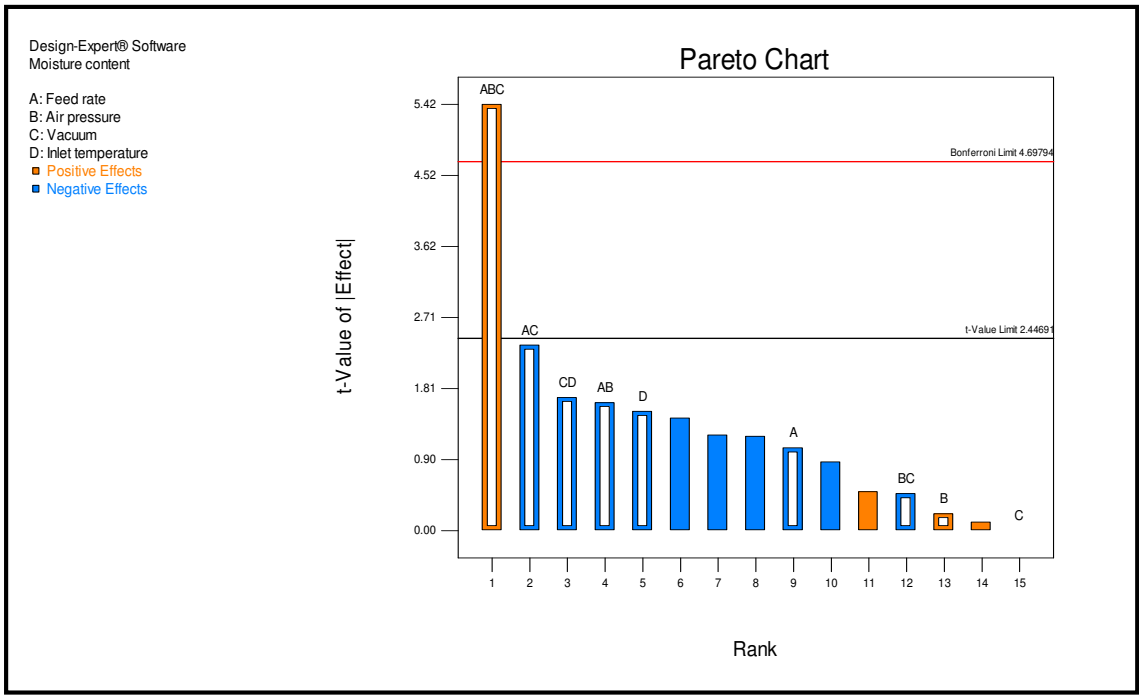


Fig. 5.46: Standardized effects of variables on moisture content of sildenafil citrate-lipid composites

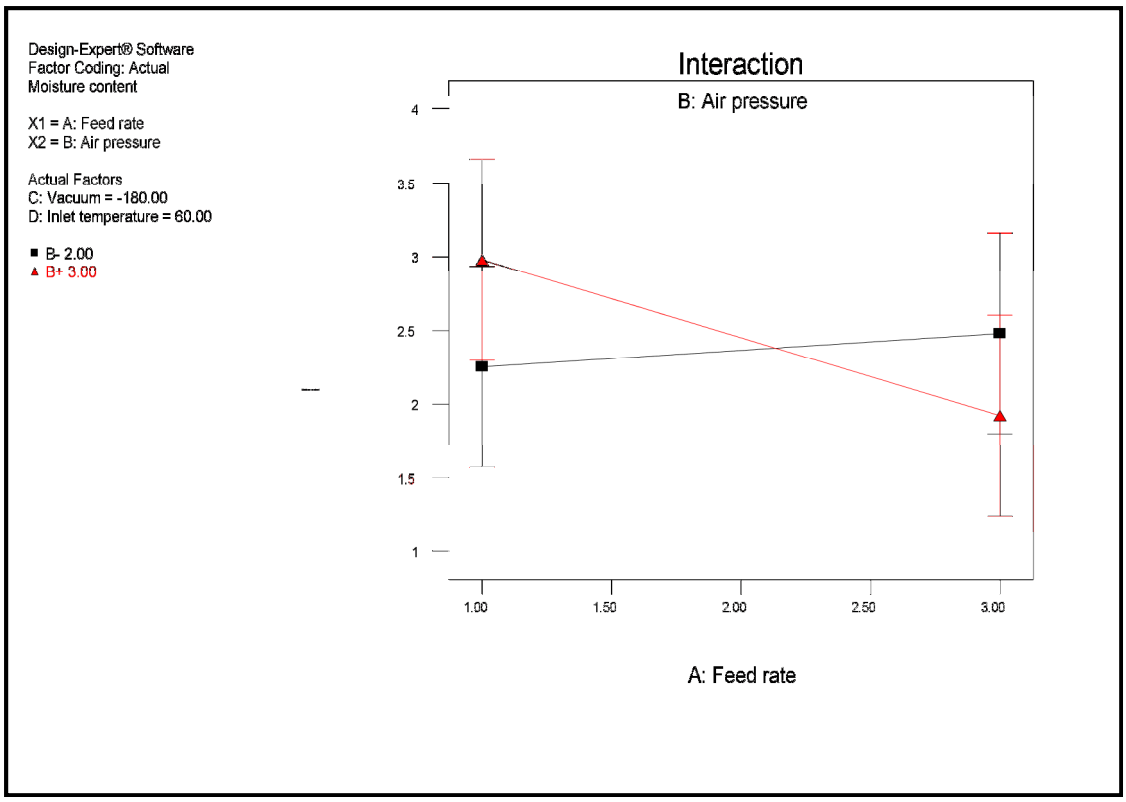


Fig. 5.47: Impact of interaction of variables on moisture content of sildenafil citrate-lipid composites

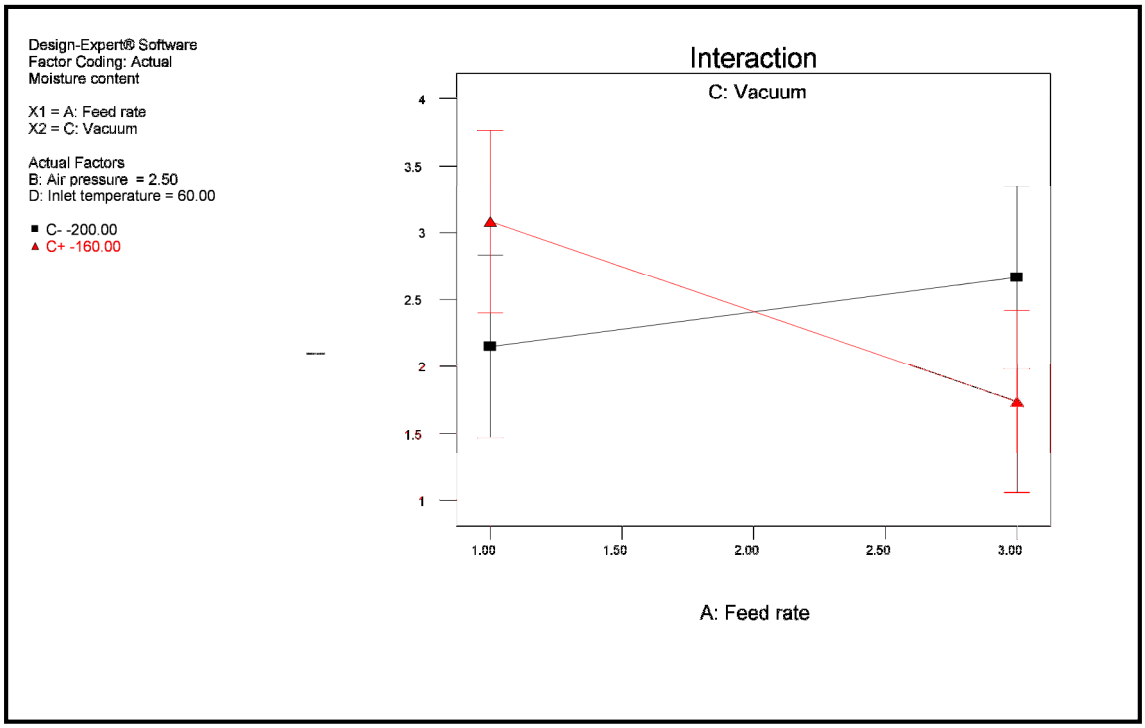


Fig. 5.48: Impact of interaction of variables on moisture content of sildenafil citrate-lipid composites

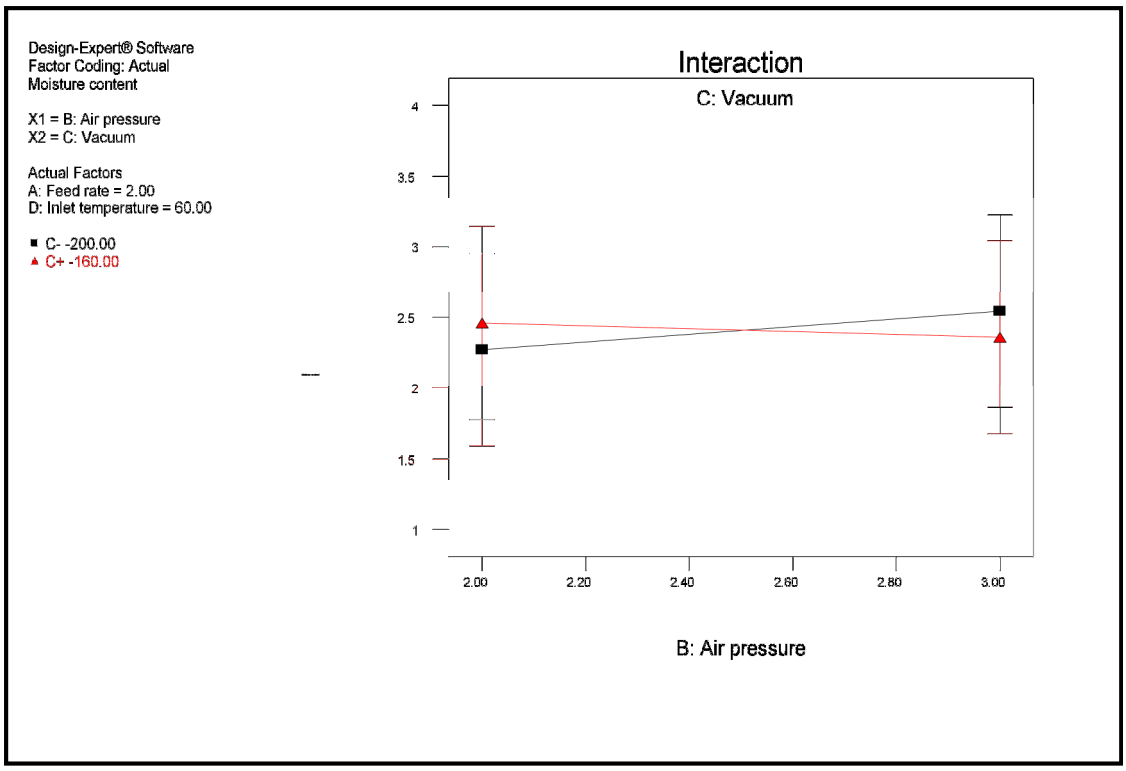


Fig. 5.49: Impact of interaction of variables on moisture content of sildenafil citrate-lipid composites

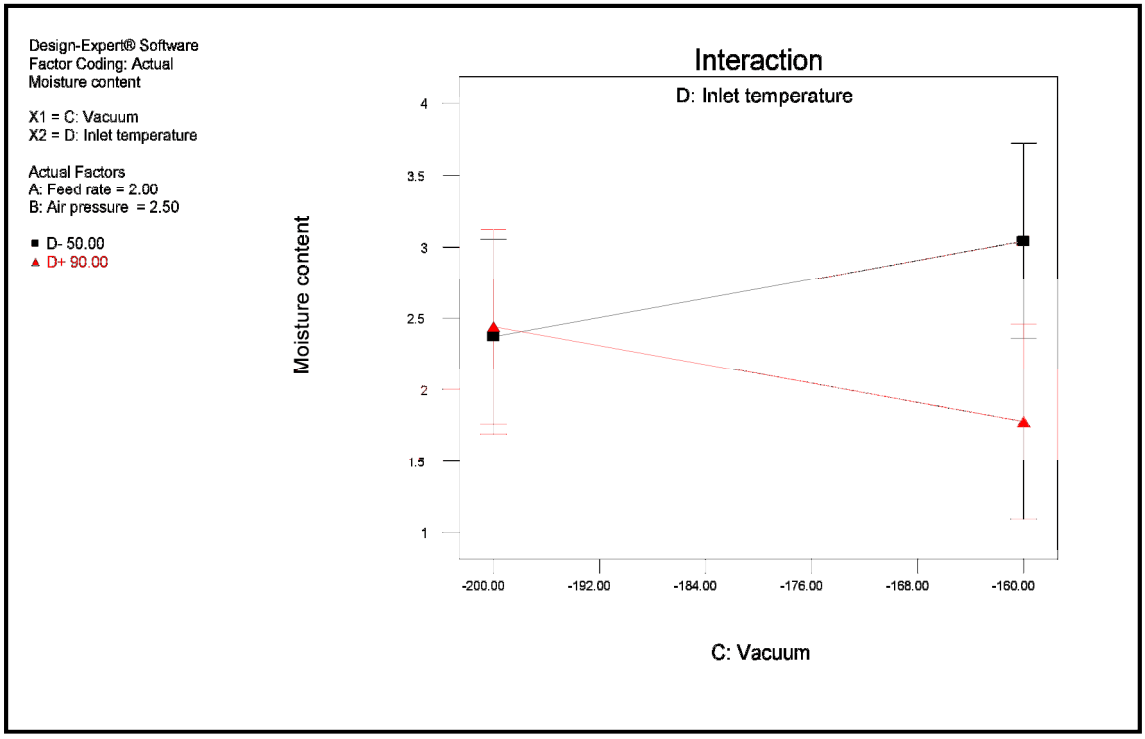


Fig. 5.50: Impact of interaction of variables on moisture content of sildenafil citrate-lipid composites

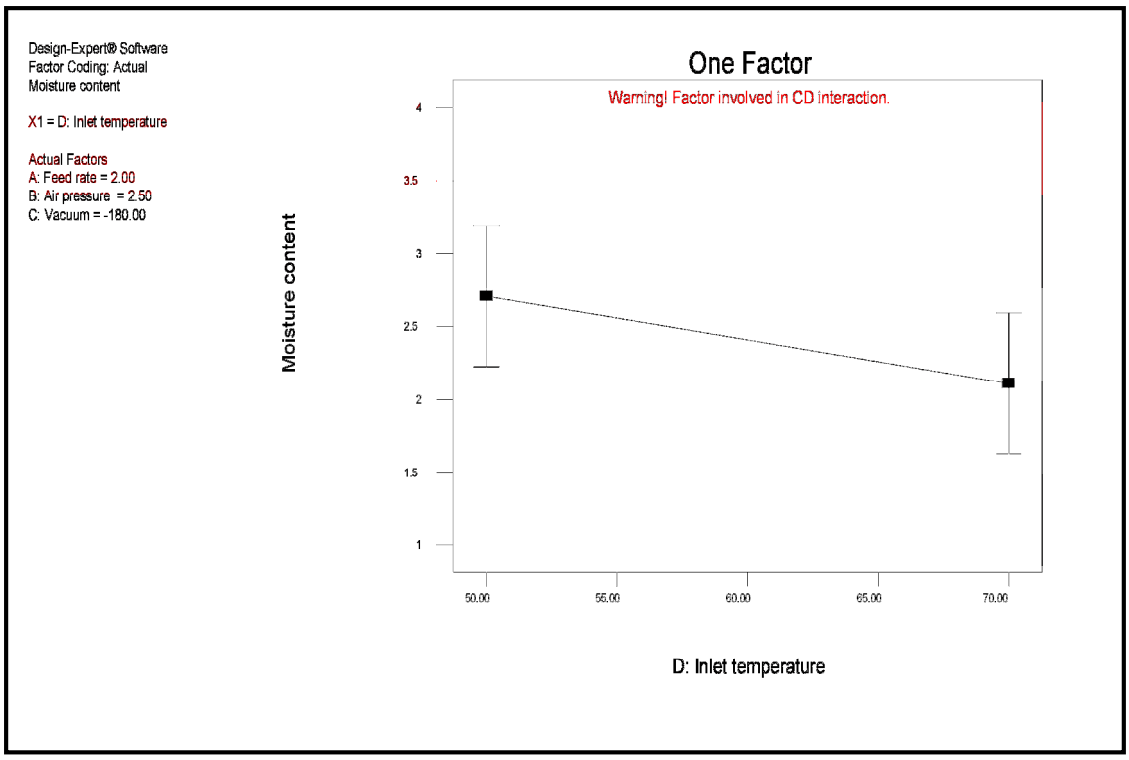


Fig. 5.51: Effect of inlet temperature on moisture content of sildenafil citrate-lipid composites.

5.5.5 Preparation of large porous liposphères of sildenafil citrate:

Being hygroscopic, the formulations prepared with trehalose tend to show relatively poor dispersibility and aerodynamic behaviour as compared to mannitol (Hardy J., 2002). Thus it was planned to improve the formulation parameters by adhering to the convenient single step spray drying process while changing the formulation composition from solution to emulsion based feed stock. Blowing agent was also included to formulate large porous liposphères which have the potential of better aerosolization and lower macrophage uptake due to large size. Increase of blowing agent from 1%v/v to 1.5%v/v significantly increased the percent yield from 62.16 ± 0.99% to 71.53 ± 0.36%. However, there was no further improvement on increasing its concentration to 2%v/v.

DPPC was used as co-surfactant to form monolayer mixed with HSPC to impart better flexibility alongwith rigidity to the lipid surface layer. It showed maximum percent entrapment efficiency (97.75 ± 1.18%) at 0.15%w/v concentration. At lesser concentration DPPC was not sufficient to provide the integrity to the lipid layer to entrap the drug and at higher concentration decrease of percent entrapment efficiency might be due to the dissolution of the drug in presence of excess of co-surfactant that led the drug to come out of the internal phase. Optimized large porous liposphères showed 95.08 to 97.86 % entrapment efficiency were prepared by spray drying emulsion of drug: HSPC (1:3) and 1.5% v/v blowing agent in aqueous solution containing 0.15% w/v DPPC as surfactant and 2.4% w/v mannitol **(Table 5.21)**. Batches from LPL1 to LPL9 were prepared at spray drying parameters of 2mL/min. feed rate, 2.5 bars compressed air pressure, inlet temperature of 70°C and vacuum at -150 mm of WC. Spray drying process parameters were further optimized at the above finalized formulation composition.

Table 5.21: Effect of formulation variables on percent drug content, percent yield and flow characteristics of the large porous liposphere formulations (mean ± SD, n = 3)

S.No.	Batch code	Drug: Lipid ratio	Surfactant conc. (% w/v)	Blowing agent (% v/v)	Protective sugar (% w/v)	% drug entrapment ± SD	% yield ± SD	Bulk density	Tapped density	Angle of repose	%CI	Hausner's ratio
1	LPL1	(1:2)	0.15	1.5	2.4	72.85 ± 1.67	68.32 ± 0.18	0.128 ± 0.02	0.162 ± 0.72	31.05 ± 1.05	20.99	1.27
2	**LPL2**	**(1:3)**	**0.15**	**1.5**	**2.4**	**97.75 ± 1.18**	**71.53 ± 0.36**	0.105 ± 1.72	0.117 ± 1.33	26.12 ± 0.77	13.68	1.16
3	LPL3	(1:6)	0.15	1.5	2.4	94.21 ± 0.65	62.38 ± 0.95	0.212 ± 0.38	0.272 ± 1.16	32.33 ± 1.88	22.06	1.28
4	LPL4	(1:3)	0.1	1.5	2.4	81.08 ± 1.67	65.88 ± 1.36	0.131± 1.19	0.205 ± 1.13	35.71 ± 0.64	36.10	1.56
5	LPL5	(1:3)	0.2	1.5	2.4	54.22 ± 0.58	63.55 ± 0.83	0.126 ± 2.23	0.211 ± 0.67	34.22 ± 1.71	40.28	1.67
6	LPL6	(1:3)	0.15	1	2.4	96.76 ± 2.16	62.16 ± 0.99	0.188 ± 0.49	0.276 ± 0.09	40.33 ± 0.89	31.88	1.47
7	LPL7	(1:3)	0.15	2	2.4	84.33 ± 0.58	61.74 ± 0.29	0.075 ± 1.14	0.115 ± 0.39	42.01 ± 0.83	34.78	1.53
8	LPL8	(1:3)	0.15	1.5	1.2	55.22 ± 1.55	50.12 ± 0.88	0.112 ± 2.36	0.144 ± 1.34	30.01 ± 0.86	22.22	1.29
9	LPL9	(1:3)	0.15	1.5	4.8	96.01± 0.24	62.36 ±1.38	0.224 ± 0.36	0.299 ± 0.28	33.45 ± 0.97	25.08	1.33

4.5.5.1 Optimization of spray drying process parameters for large porous lipospheres of sildenafil citrate:

Spray drying process was optimized based on fractional factorial design **(Table 5.22)** and all analysis was done at $p<0.05$. There was no significant impact of any variables individually or in combination with each other within the experimental range selected, on percent drug content of large porous lipospheres. However, combination of various variables influenced percent yield, aerodynamic diameter and moisture content.

1. At lower temperature, % yield increased as feed rate was increased. This might be due to the smaller droplets generated at higher feed rate providing the increased surface area for better moisture loss and hence less loss due to sticking in drying chamber.
2. At lower temperature, there was no impact of vacuum on percent yield, however at higher temperature, %yield decreased as vacuum was decreased which might be due to the slower removal of the dried particles from drying chamber to the collecting flask.
3. At higher vacuum, particle size was increased as feed rate increased which might be due to the lesser time for droplets in drying chamber for appropriate moisture removal, however at lower vacuum, smaller particles formed at increased feed rate could maintain the lower size due to sufficient moisture evaporation.
4. At lower vacuum, moisture increased as feed rate was increased, however, there was no impact at higher vacuum.
5. At higher temperature, moisture increased as the vacuum was decreased and at lower temperature, there was no significant impact on moisture on decrease of vacuum.

The effect of spray drying parameters and impact of interaction between variables are graphically represented in **Fig. 5.52-5.60.** Numerical optimization provided solutions **(Table 5.23)** of recommended factor values for desired response factors. Formulation LPL16 prepared according to the design showed response values closest to the goal with maximum percent drug retained (97.86 ± 1.42%) at maximum yield (70.24 ± 1.29%) and mimimum moisture content (0.97 ± 1.16% w/w) and aerodynamic particle size in range (4.22µm) for better lung deposition and higher

geometric size (13.28± 0.51 µm) for reduced uptake by alveolar macrophages. Hence, formulation LPL16 was chosen as the optimized formulation with respect to formulation and spray drying parameters. Thus, final formulation was spray dried at optimized parameters of 3mL/min feed rate, atomized at compressed air pressure of 3bars with -100mm of WC vacuum at 90°C inlet temperature.

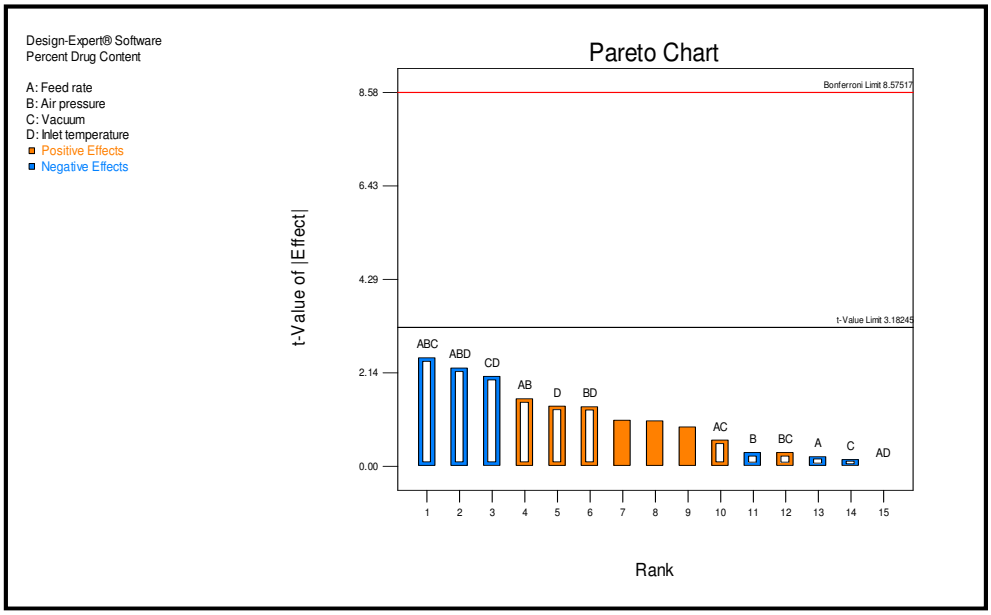


Fig. 5.52 A: Standardized effects of variables on percent drug content of sildenafil citrate loaded large porous lipospheres

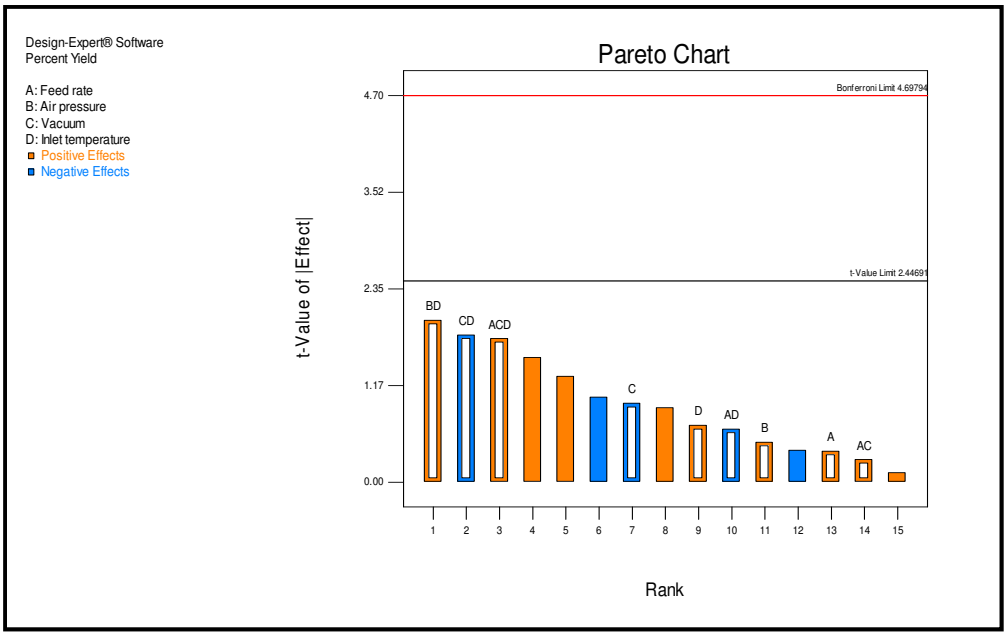


Fig. 5.52 B: Standardized effects of variables on percent yield of sildenafil citrate loaded large porous lipospheres

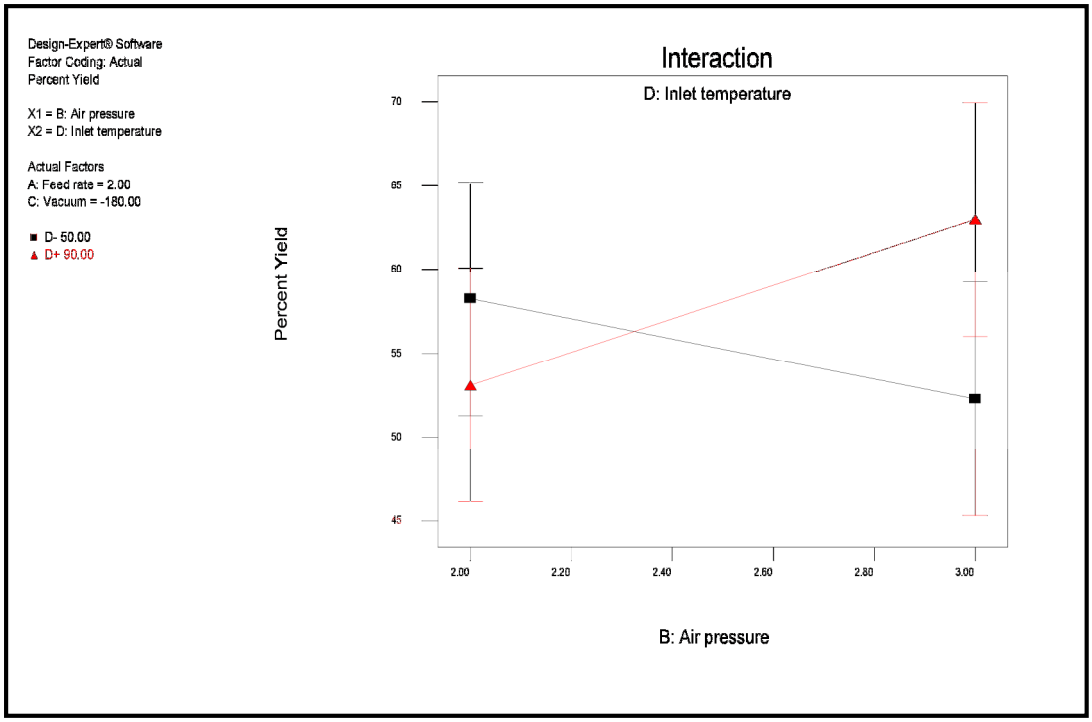


Fig. 5.53: Impact of interaction of inlet temperature and air pressure on percent yield of sildenafil citrate loaded large porous lipospheres

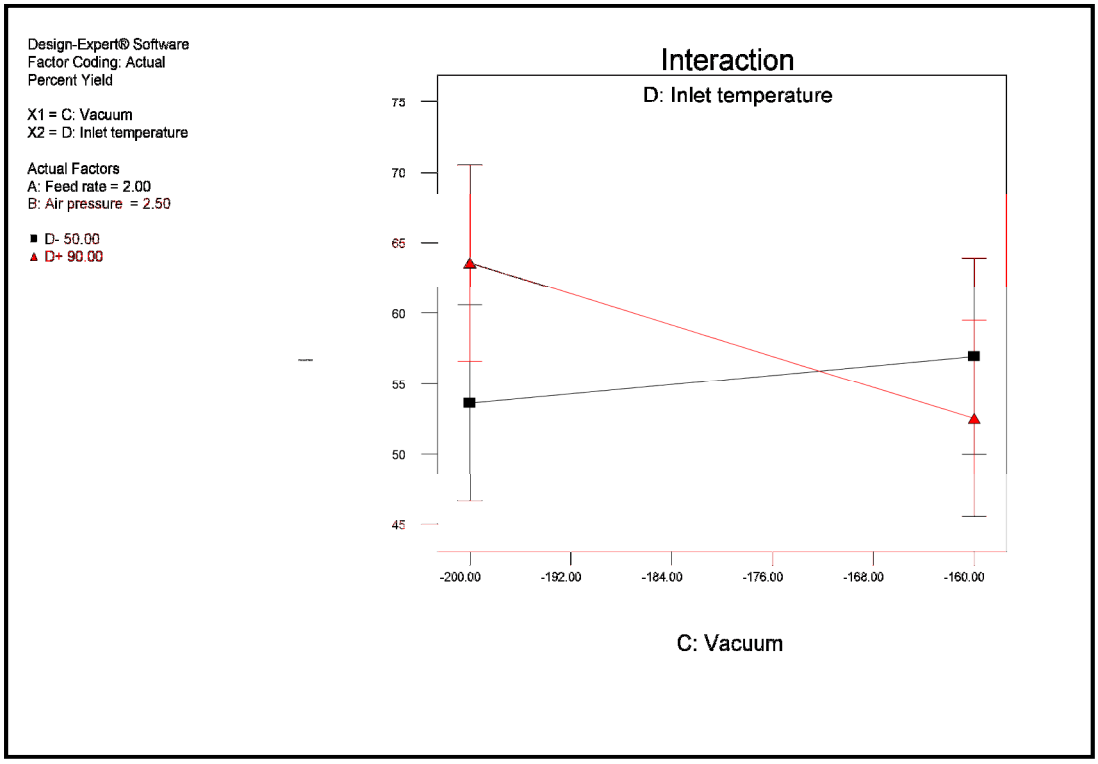


Fig. 5.54: Impact of interaction of inlet temperature and vacuum on percent yield of sildenafil citrate loaded large porous lipospheres

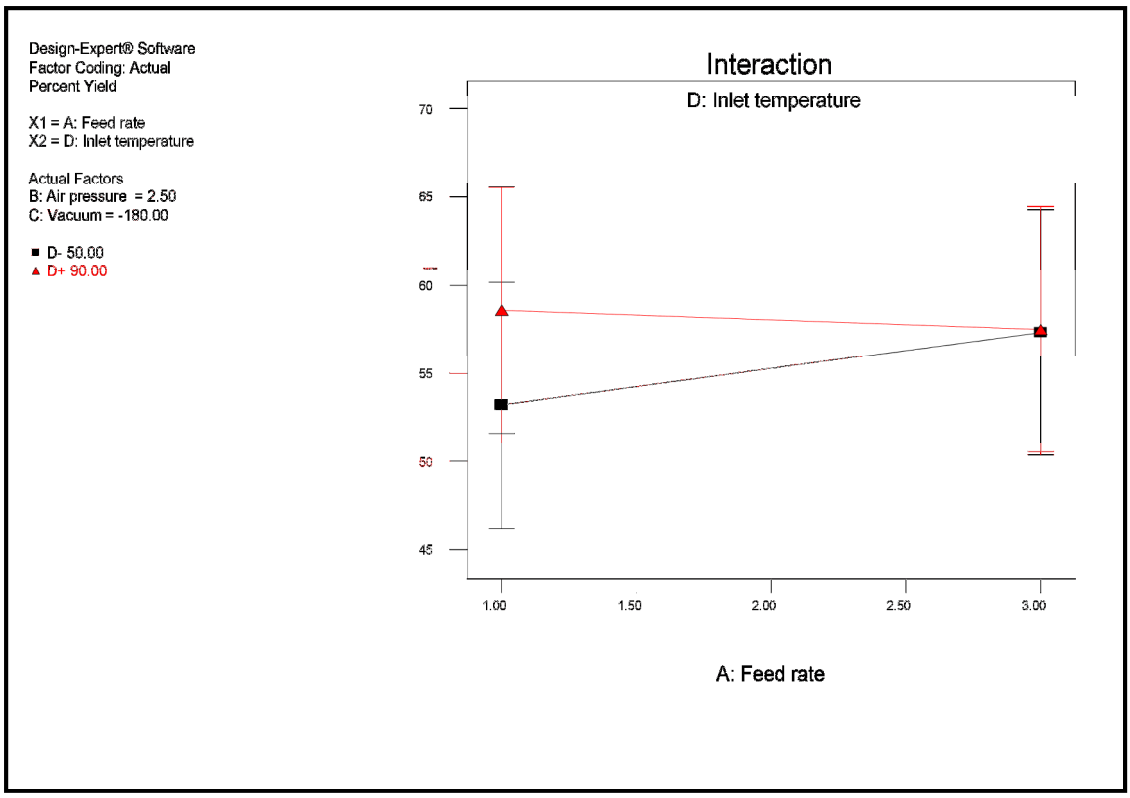


Fig. 5.55: Impact of interaction of inlet temperature and feed rate on percent yield of sildenafil citrate loaded large porous lipospheres

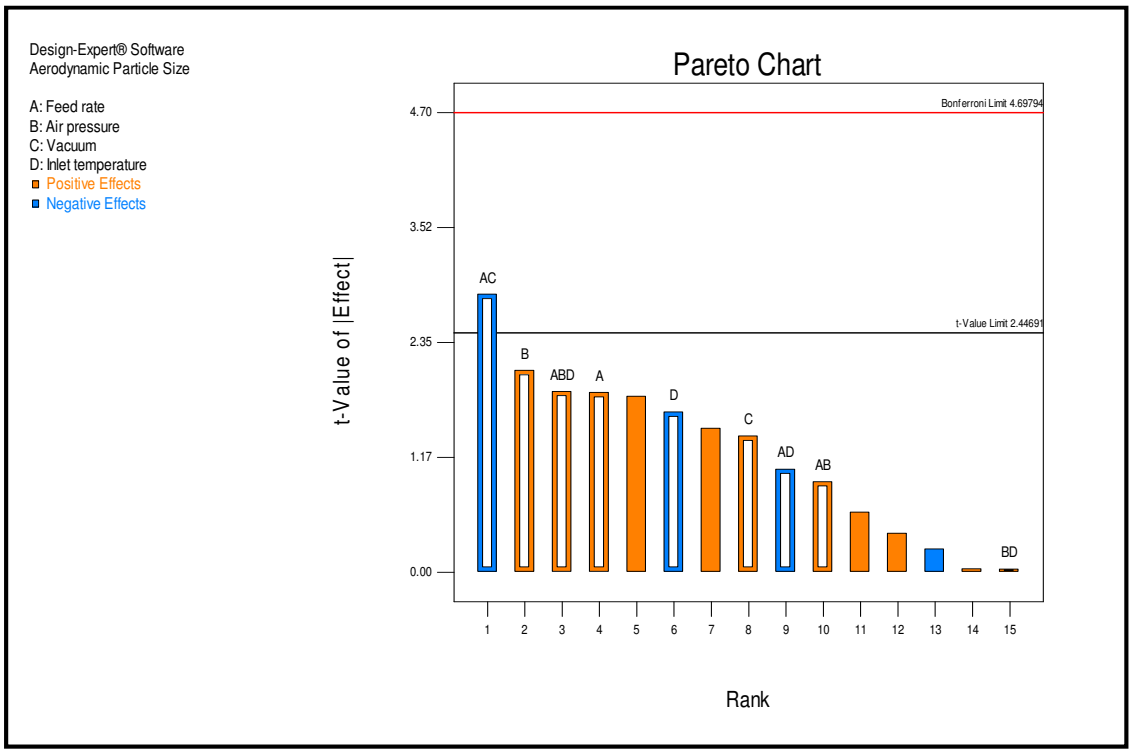


Fig. 5.56: Standardized effects of variables on aerodynamic particle size of sildenafil citrate loaded large porous lipospheres

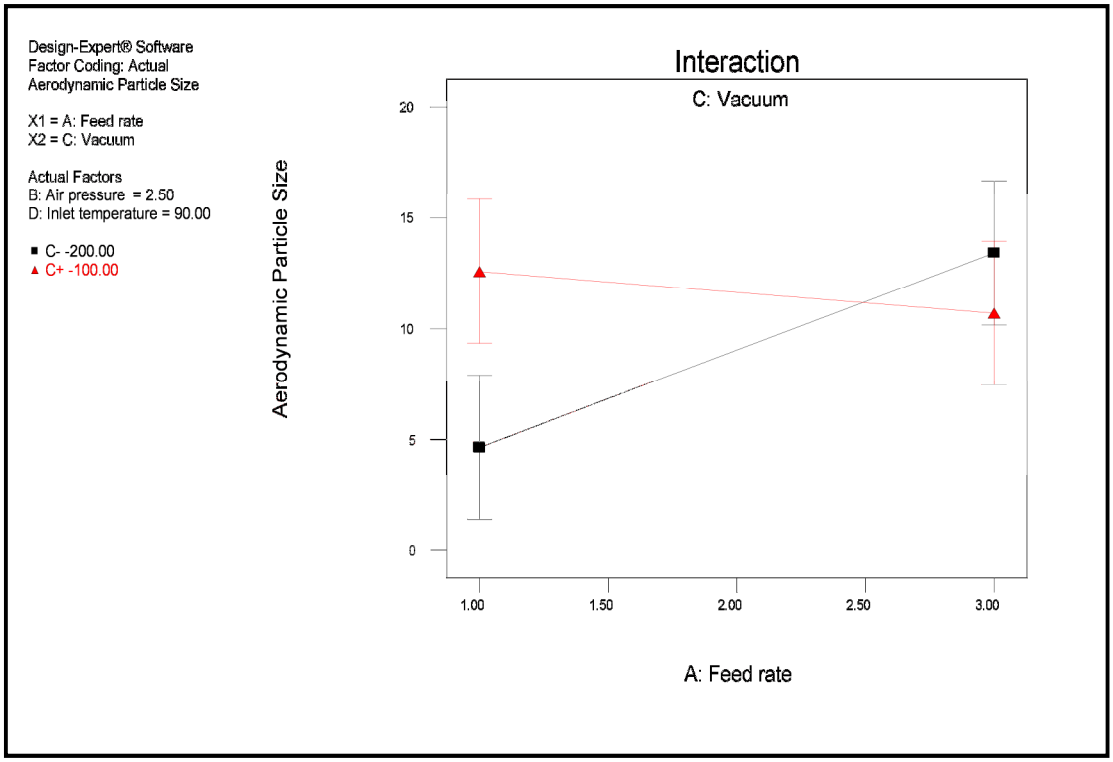


Fig. 5.57. Impact of interaction of vacuum and feed rate on aerodynamic particle size of sildenafil citrate loaded large porous lipospheres

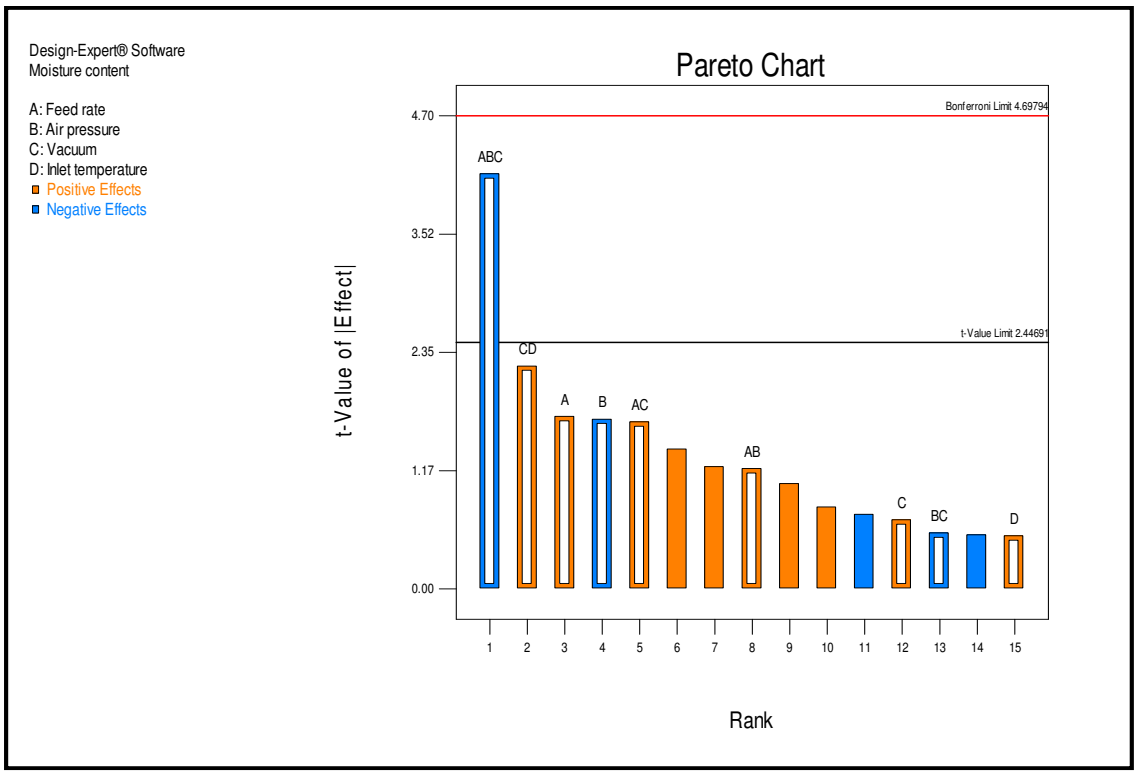


Fig. 5.58: Standardized effects of variables on moisture content of sildenafil citrate loaded large porous lipospheres

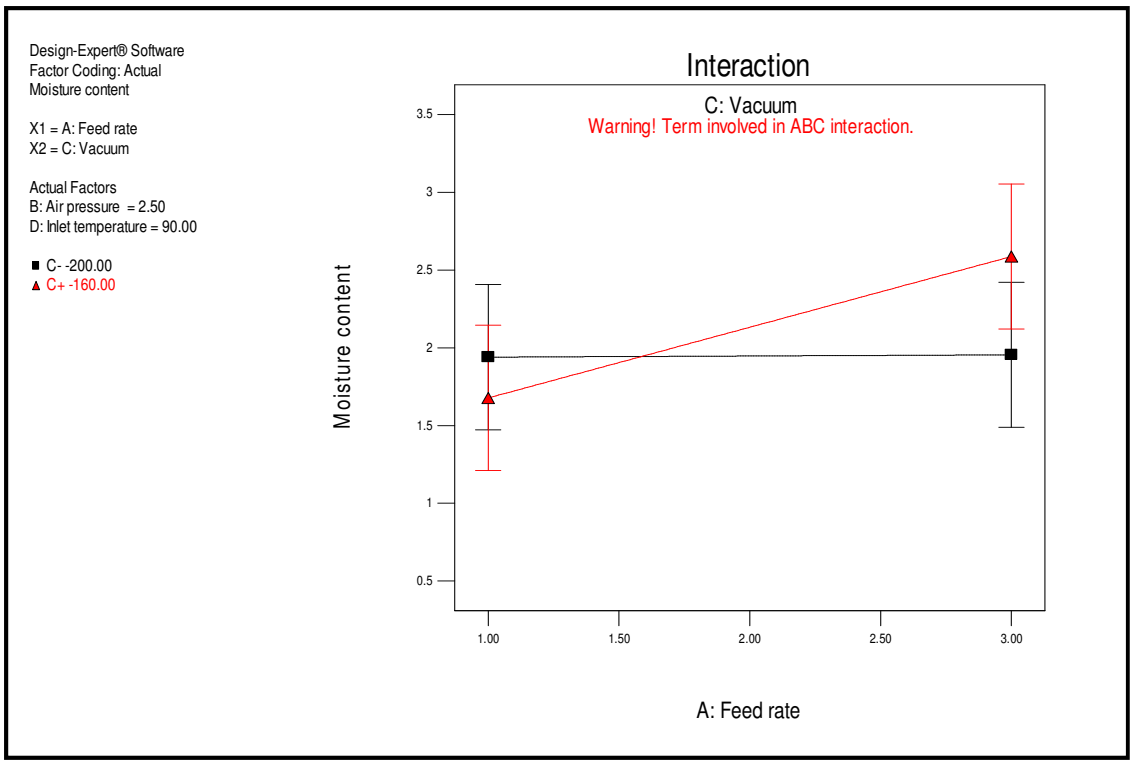


Fig. 5.59: Impact of interaction of vacuum and feed rate on moisture content of sildenafil citrate loaded large porous lipospheres

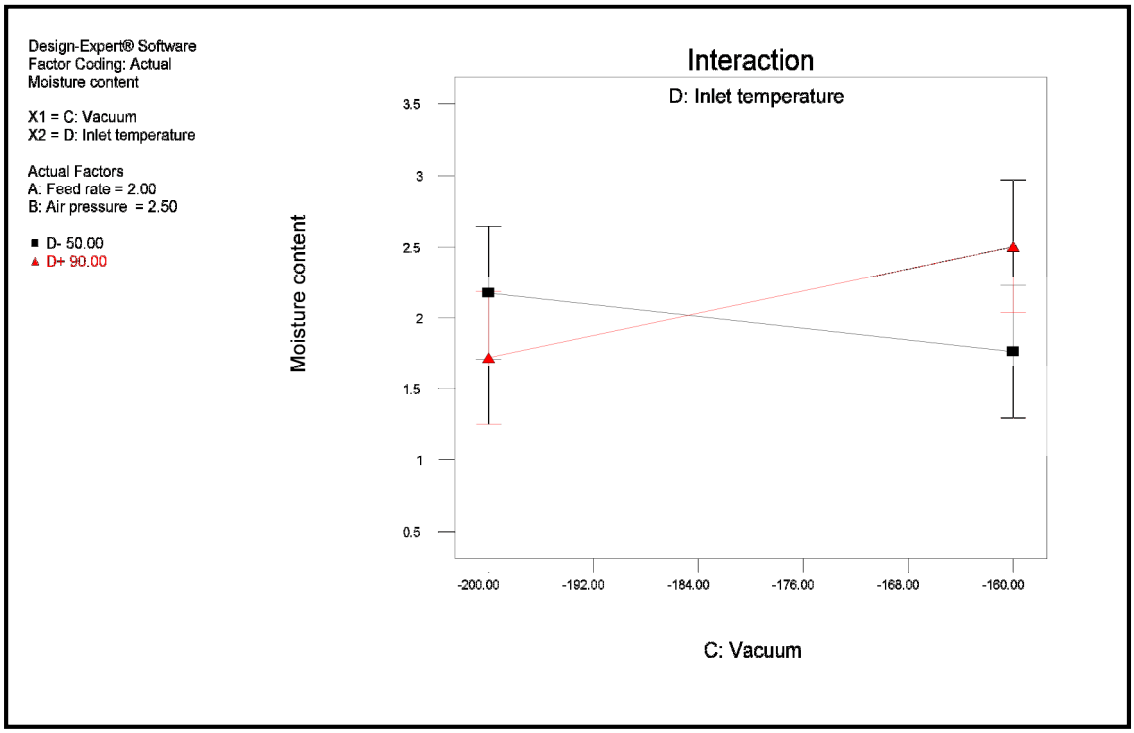


Fig. 5.60: Impact of interaction of vacuum and inlet temperature on aerodynamic particle size of sildenafil citrate loaded large porous lipospheres

Table 5.22: Effect of spray drying process parameters on % yield, % drug content, moisture content and aerodynamic particle size of sildenafil citrate loaded large porous liposphere

	Factor 1	Factor 2	Factor 3	Factor 4	Response 1	Response 2	Response 3	Response 4		
Run	A:Feed rate	B:Air pressure	C:Vacuum	D:Inlet temperature	Percent Drug Retained	Percent Yield	Aerodynamic Particle Size	Moisture content	Bulk density (ρb)	Volume mean diameter
	mL/min	Bar	mm of WC	°C	%	%	Micron	%	g/cm^3	(µm)
LPL1	1	3	-100	50	96.29 ± 1.12	46.28± 1.06	5.92	3.82 ± 1.91	0.213± 2.19	12.83± 1.48
LPL 2	3	3	-200	50	98.01 ± 1.77	48.21± 2.22	3.25	3.01 ± 0.78	0.134± 1.88	8.88± 0.85
LPL3	1	2	-200	50	97.42 ± 2.66	50.26± 1.15	20.01	2.83 ± 1.88	0.284± 1.23	37.55± 0.78
LPL4	1	2	-200	90	95.42 ± 0.86	49.97± 2.13	17.36	1.22 ± 1.66	0.224± 1.64	36.68± 1.83
LPL5	3	2	-100	50	97.05 ± 2.51	67.88 ± 1.83	11.64	2.21 ± 0.89	0.211± 2.16	25.34± 1.48
LPL6	3	3	-200	90	96.58 ± 0.92	50.04± 2.18	4.01	1.09 ± 1.34	0.108± 1.86	12.20± 0.38
LPL7	3	3	-100	50	98.85 ± 0.52	66.12 ± 1.08	4.58	2.61 ± 0.94	0.159± 0.93	11.49± 2.15
LPL8	1	3	-200	90	91.29 ± 2.88	48.96± 1.05	6.51	1.17± 1.61	0.183± 1.45	15.22± 0.68
LPL9	3	2	-100	90	97.77 ± 1.57	65.71 ± 1.05	9.53	1.99 ± 2.58	0.201± 2.72	21.26± 1.88
LPL10	3	2	-200	50	97.12 ±1.52	48.01 ± 1.13	10.64	2.32 ± 1.47	0.22± 1.38	22.68± 1.35
LPL11	1	3	-200	50	96.88 ± 1.28	52.11± 2.06	9.06	2.75 ± 0.99	0.216± 1.95	19.49± 0.78
LPL12	3	2	-200	90	93.22 ± 2.88	49.99 ± 0.68	12.83	1.18 ± 0.28	0.221± 0.97	27.29± 1.55
LPL13	1	2	-100	90	92.25 ± 0.88	66.97 ± 1.65	18.04	1.69 ± 1.57	0.264± 1.94	35.11± 1.06
LPL14	1	3	-100	90	95.22 ± 0.88	59.96 ± 2.51	11.25	1.09 ± 1.21	0.218± 1.08	24.09± 0.59
LPL15	1	2	-100	50	96.39 ± 0.48	65.81 ± 1.51	16.58	2.69 ± 1.07	0.222± 1.77	35.19± 0.46
LPL**16**	**3**	**3**	**-100**	**90**	**97.86 ± 1.42**	**70.24 ± 1.29**	**4.22**	**0.97 ± 1.16**	0.101± 1.43	13.28± 0.51

Table 5.23: Solutions provided by Design-Expert® 8 for recommended factor values and predicted number for responses:

Solution	Feed rate	Air pressure	Vacuum	Inlet temperature	Percent Drug Content	Percent Yield	Aerodynamic Particle Size	Moisture content	Desirability
1	3.00	2.00	-100.00	90.00	96.2025	54.0213	6.32625	1.1625	0.743
2	2.95	2.00	-199.99	50.00	96.2524	54.2341	6.2888	1.19589	0.739
3	3.00	2.02	-199.46	90.00	96.2517	54.1949	6.48705	1.21998	0.737
4	2.93	2.01	-200.00	90.00	96.295	54.4309	6.33112	1.22199	0.736
5	3.00	3.00	-100.00	90.00	96.4575	53.0537	4.2688	1.52	0.734

5.6 Transmission Electron Microscopy, Particle size and Zeta potential

Structural morphology of Liposomal dispersion and large porous lipospheres before spray drying was determined using Transmission electron microscopy. **Figure 5.61** illustrates the formation of liposomal dispersion by thin film hydration method. Liposomal vesicles were found to be spherical in shape. Globules in the dispersed phase of emulsion used to prepare large porous lipospheres could be seen in TEM photographs as shown in **Figure 5.62.** Dynamic light scattering technique of Malvern zeta sizer ver. 6.12 was used to further determine the size, polydispersity index and zeta potential of the formulations. Zeta potential of placebo liposomes was found to be -0.128mV± 2.62mV and that of sildenafil citrate loaded liposomal dispersion was 0.0943± 2.73mV **(Figure 5.64-5.65)**. Similarly, the placebo emulsion for preparing large porous lipospheres had a zeta potential of -0.087mV± 3.88mV and that of sildenafil citrate loaded emulsion for preparing large porous lipospheres had a zeta potential of 0.0822mV± 3.24mV **(Figure 5.66-5.67)**.

Thus, the formulations prepared using non-ionic lipids like HSPC and DPPC showed almost neutral charge with very little shift towards negative charge. Sildenafil citrate had an electrophoretic mobility of -0.174 mV± 4.68mV **(Table 5.24, Figure 5.63)**. Particle size distribution of placebo and sildenafil citrate loaded liposomal dispersion **(Figure 5.68-5.71)** and emulsion for large porous lipospheres before spray drying is shown in **Figure 5.66-5.73** and **Table 5.24** before and after size reduction using pressure homogenizer. Suitable particle size with narrow particle size distribution as evident from lower poly dispersity index values could be achieved after 3 cycles of homogenization for both the formulations at 35°C temperature under homogenization pressure of 1500 bars.

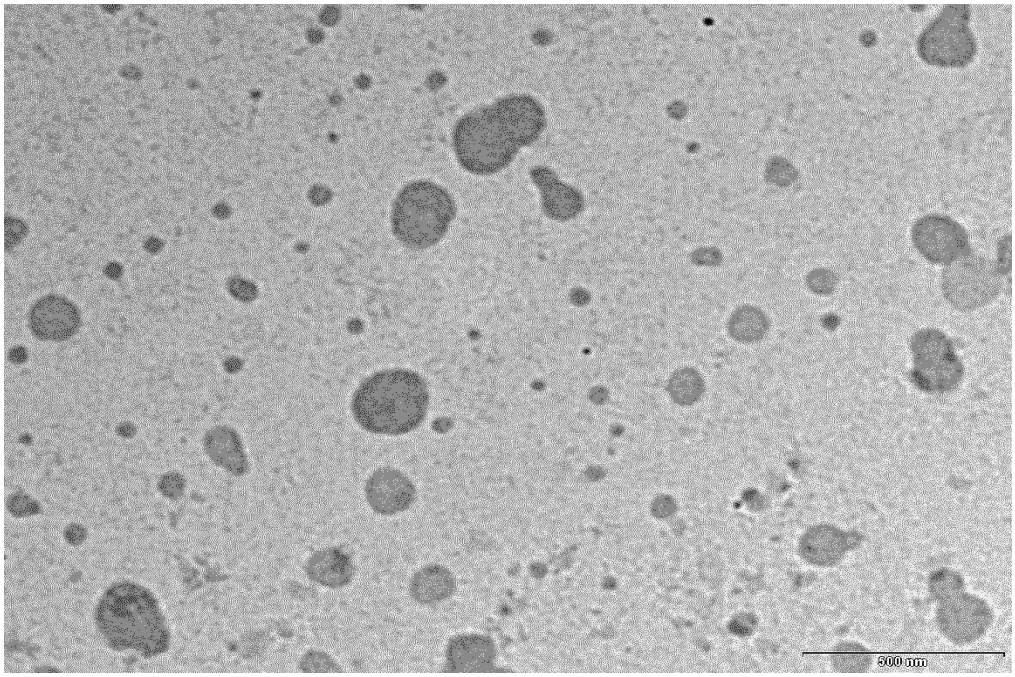


Figure 5.61: Transmission electron microscopic pictures of liposomal dispersion before spray drying (Magnification = 31,000 x)

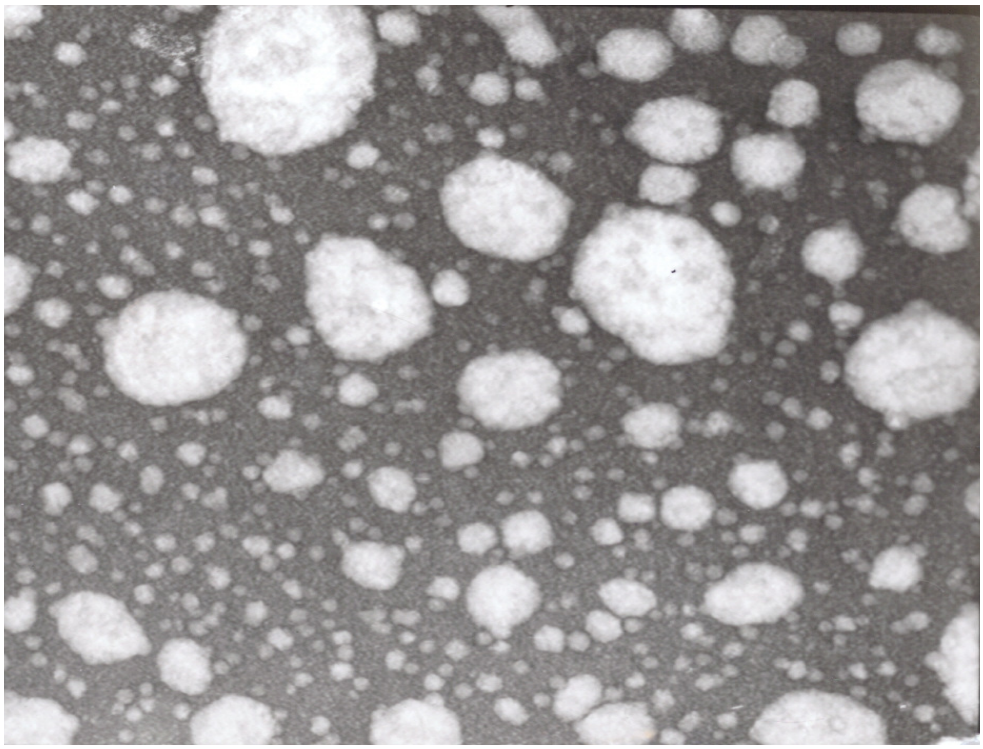


Figure 5.62: Transmission electron microscopic pictures of emulsion feed stock to prepare large porous lipospheres before spray drying (Magnification = 31,000 x)

Table 5.24: Zeta potential and Volume mean diameters of sildenafil citrate solution, its liposomal dispersion and emulsion to prepare large porous lipospheres

S.No.	Formulation (Batch code)	Zeta potential/ (Zeta Deviation) mV	Volume mean diameter in nm (Poly dispersity Index)	
			Before homogenization	After homogenization
1	Sildenafil citrate solution	-0.174 (4.68)		
Liposomal dispersion				
2	Placebo liposomal dispersion (SHD4)	-0.128 (2.62)	594.3 (0.911)	287.8 (0.374)
3	Sildenafil citrate loaded liposomal dispersion (SHD4)	0.0943 (2.73)	739.1 (0.843)	419.4 (0.462)
Emulsion to prepare large porous lipospheres			After 2 cycles	After 3 cycles
4	Placebo emulsion for large porous lipospheres	-0.087 (3.88)	739 (0.912)	504.8 (0.029)
5	Sildenafil citrate loaded emulsion for large porous lipospheres	0.0822 (3.24)	936.8 (0.504)	468.4 (0.504)

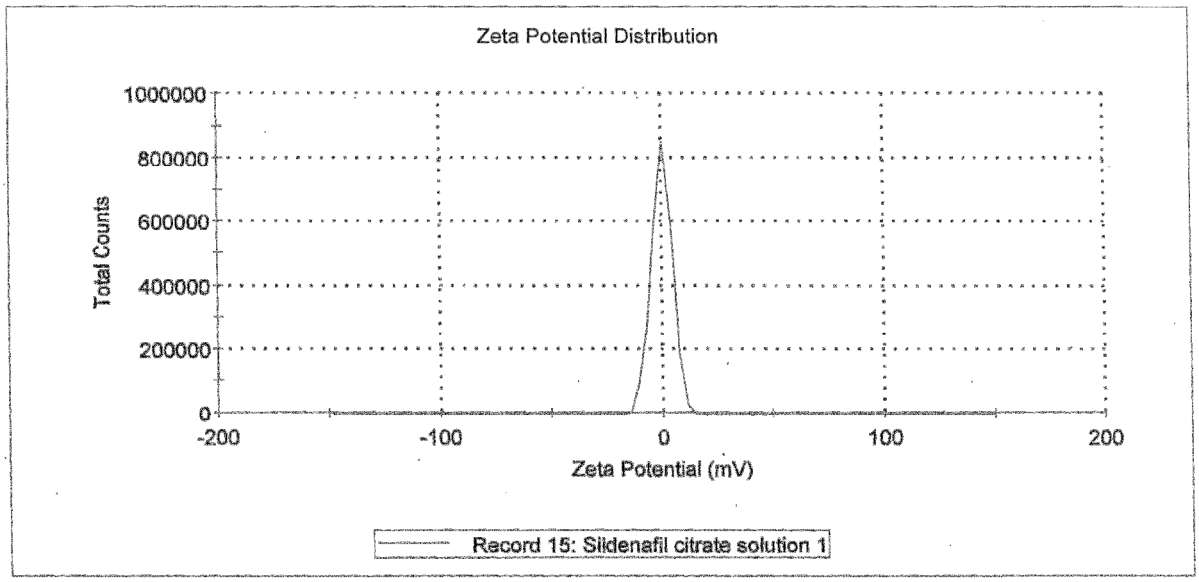


Figure 5.63: Zeta potential distribution of sildenafil citrate solution

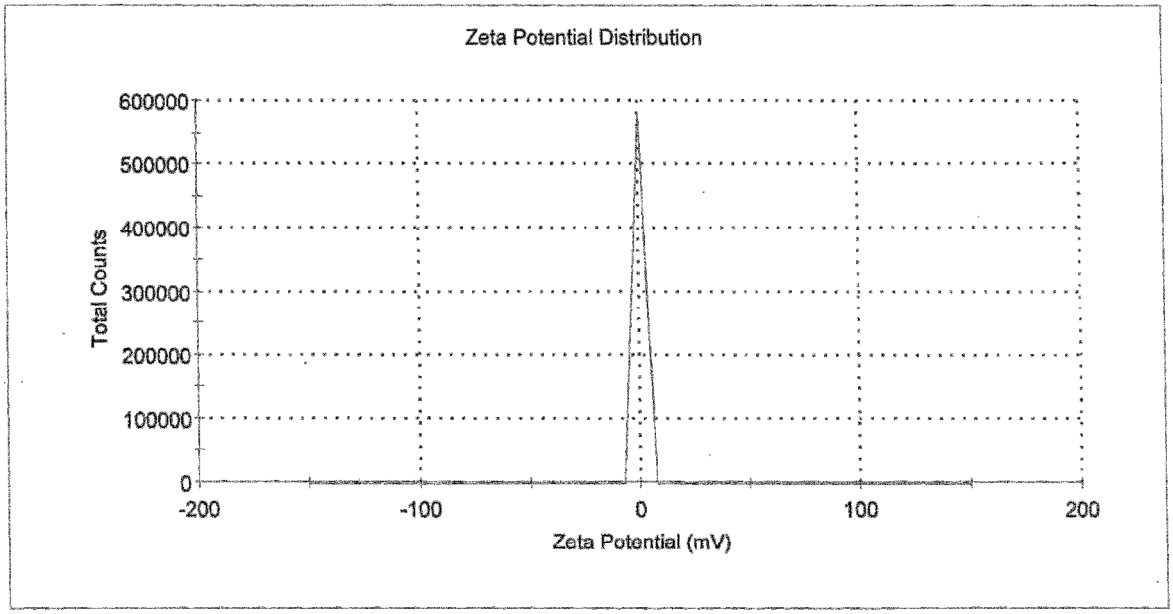


Figure 5.64: Zeta potential distribution of placebo liposomal dispersion.

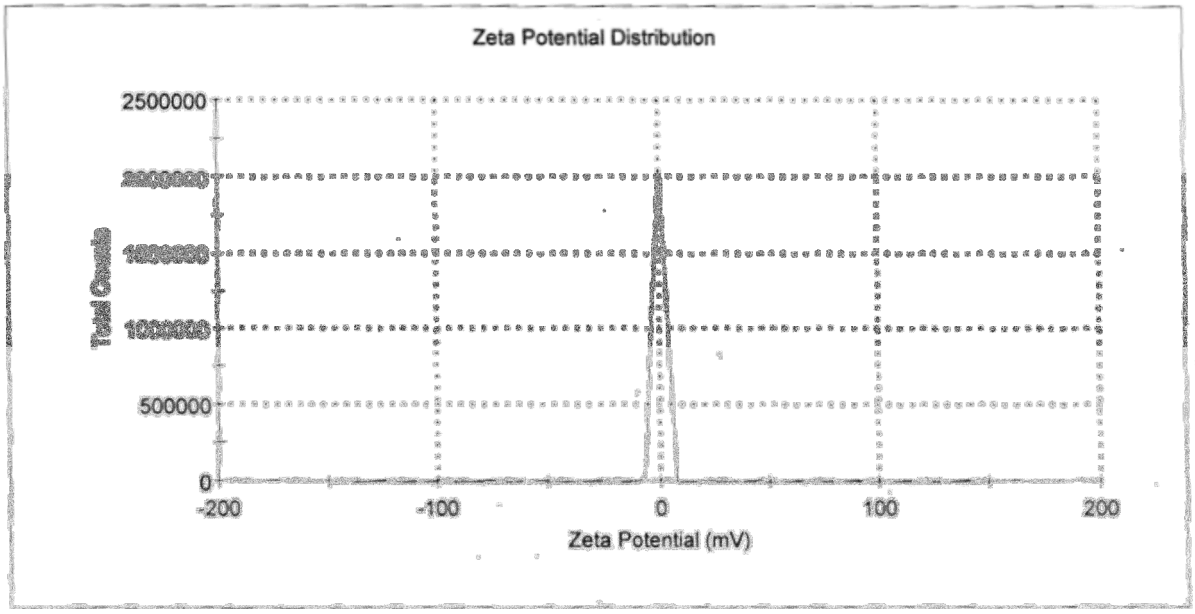


Figure 5.65: Zeta potential distribution of sildenafil citrate loaded liposomal dispersion.

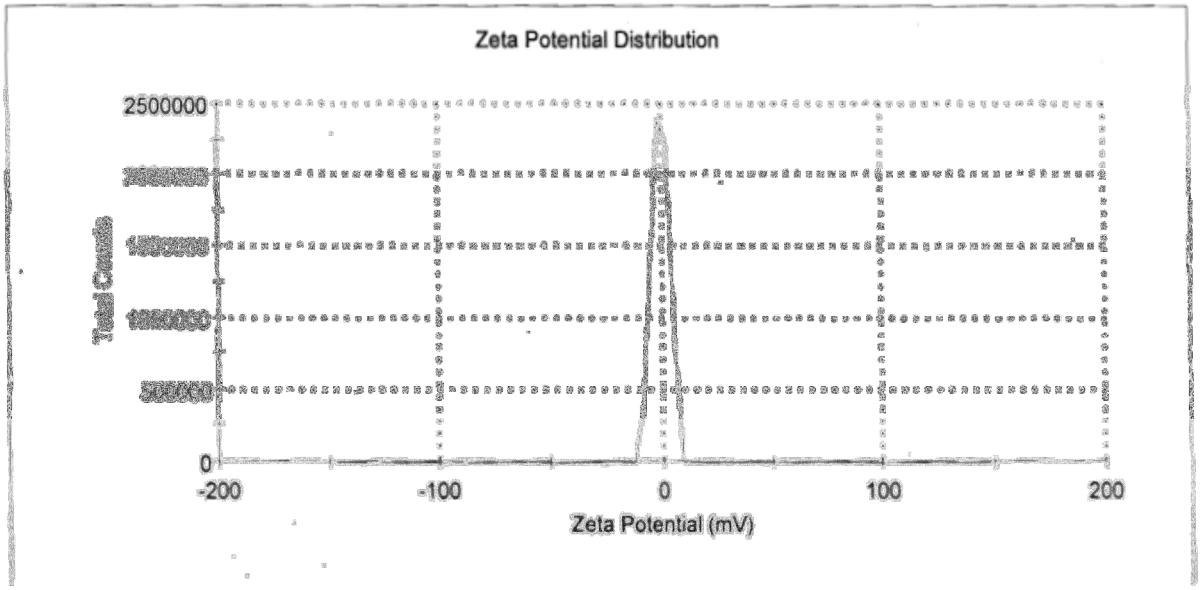


Figure 5.66: Zeta potential distribution of placebo emulsion for large porous lipospheres

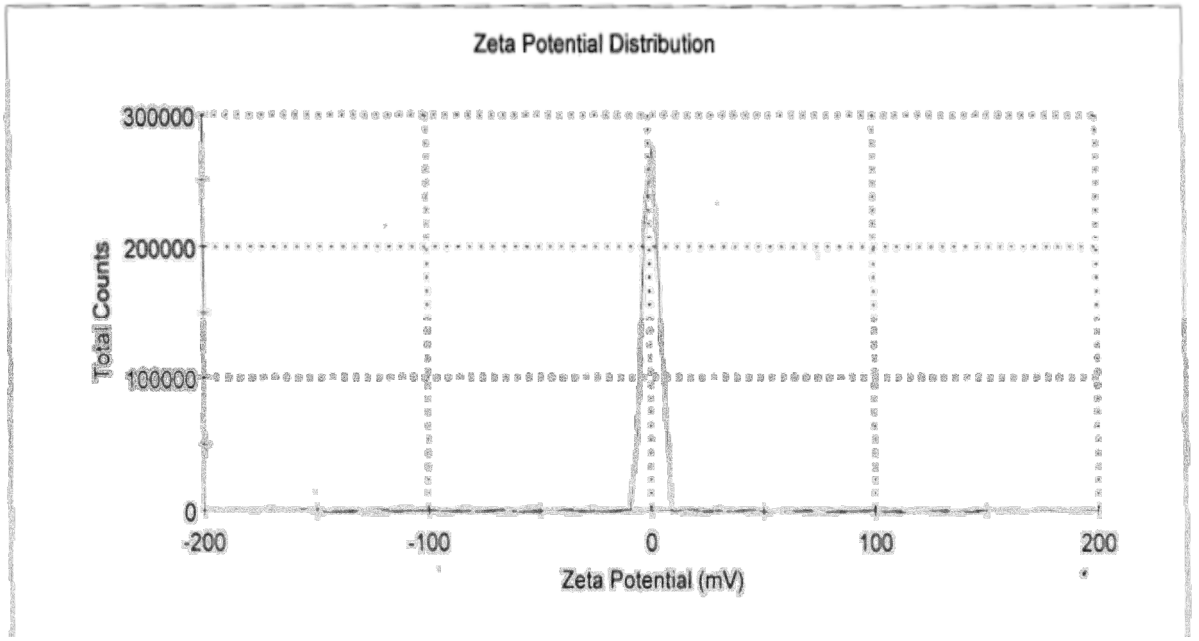


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Figure 5.67: Zeta potential distribution of sildenafil citrate loaded emulsion for large porous lipospheres

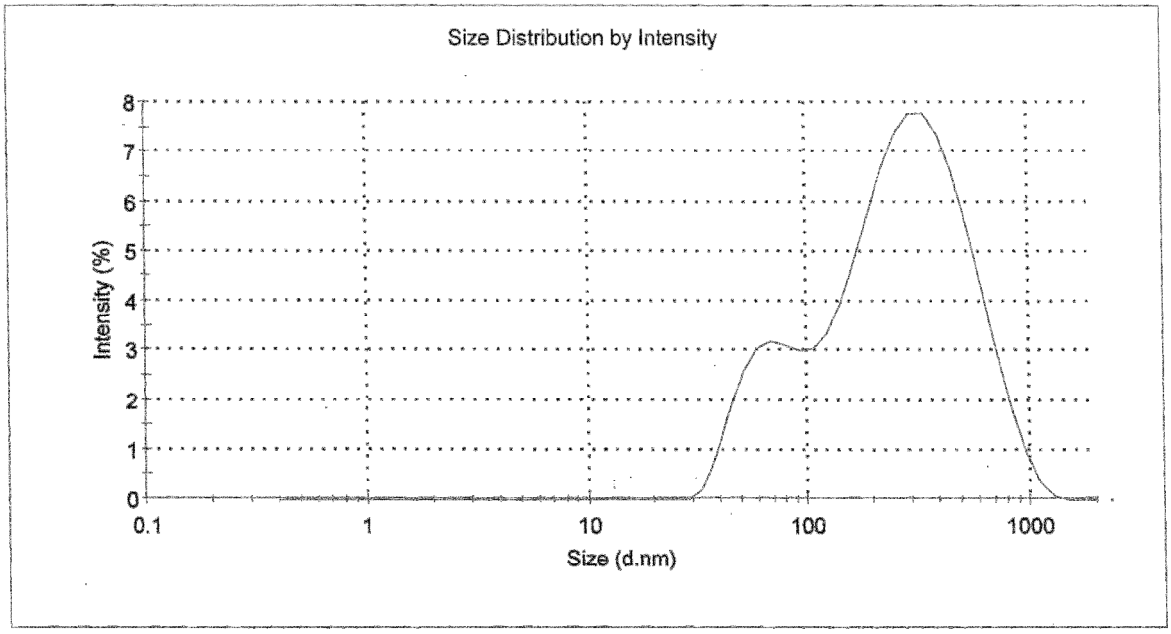

Figure 5.68: Particle size distribution of sildenafil citrate loaded liposomal dispersion before homogenization

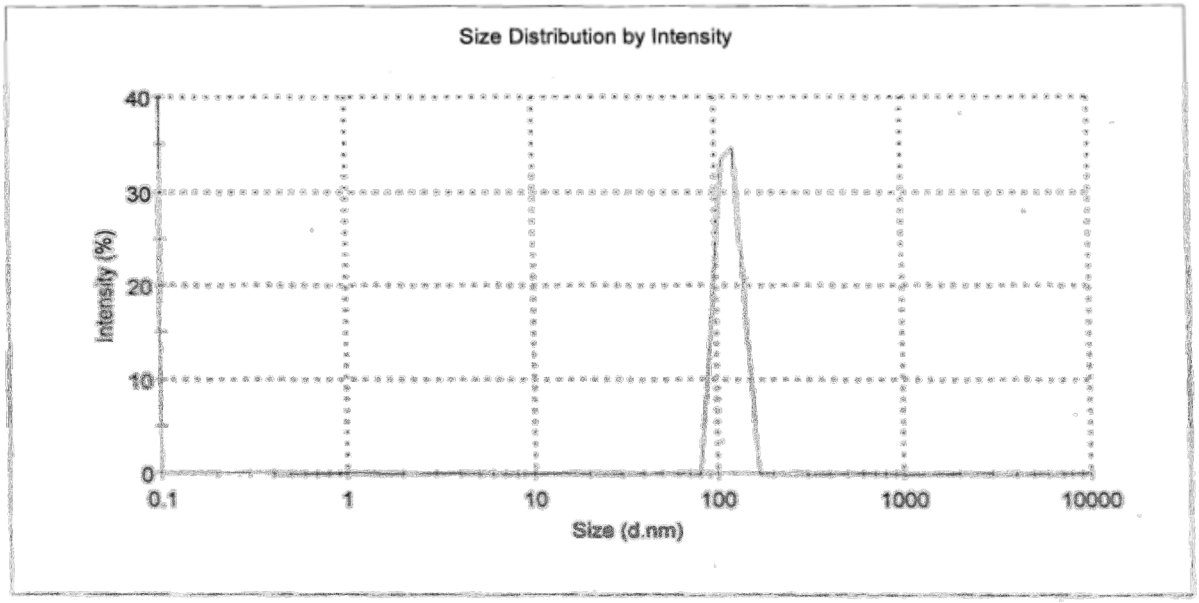

Figure 5.69: Particle size distribution of sildenafil citrate loaded liposomal dispersion after 3 cycles of homogenization

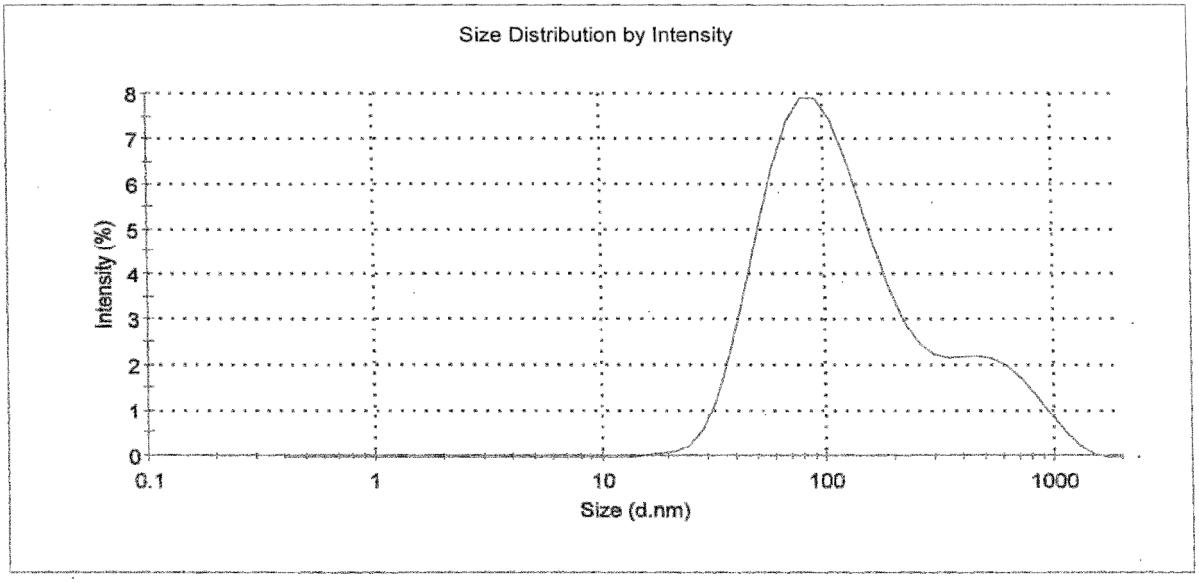

Figure 5.70: Particle size distribution of Placebo liposomal dispersion before homogenization

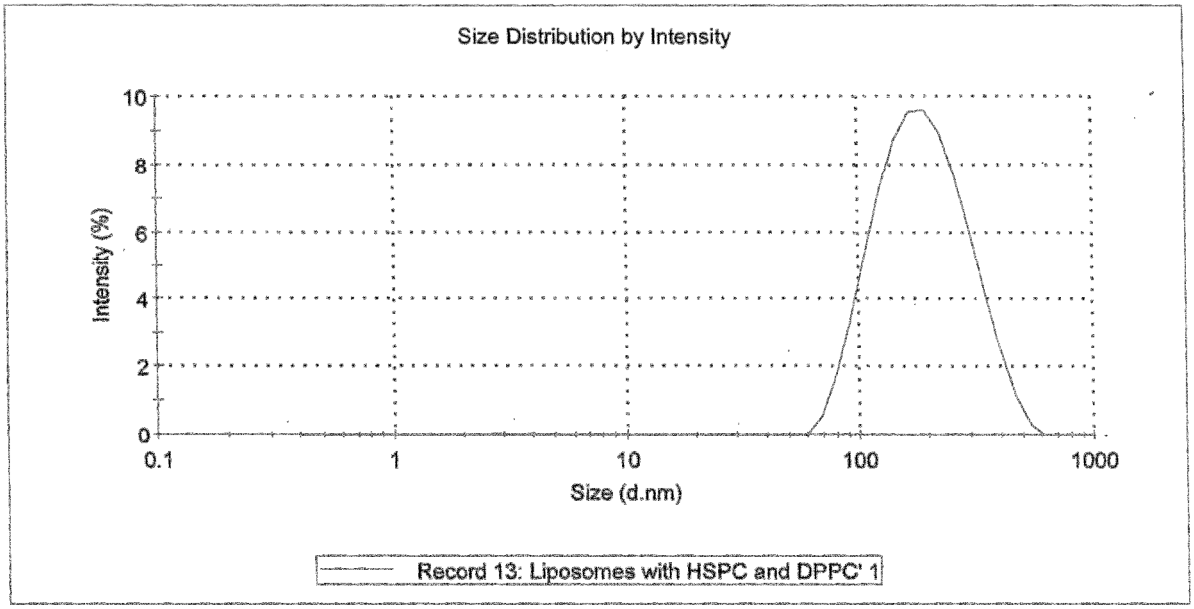

Figure 5.71: Particle size distribution of Placebo liposomal dispersion after homogenization

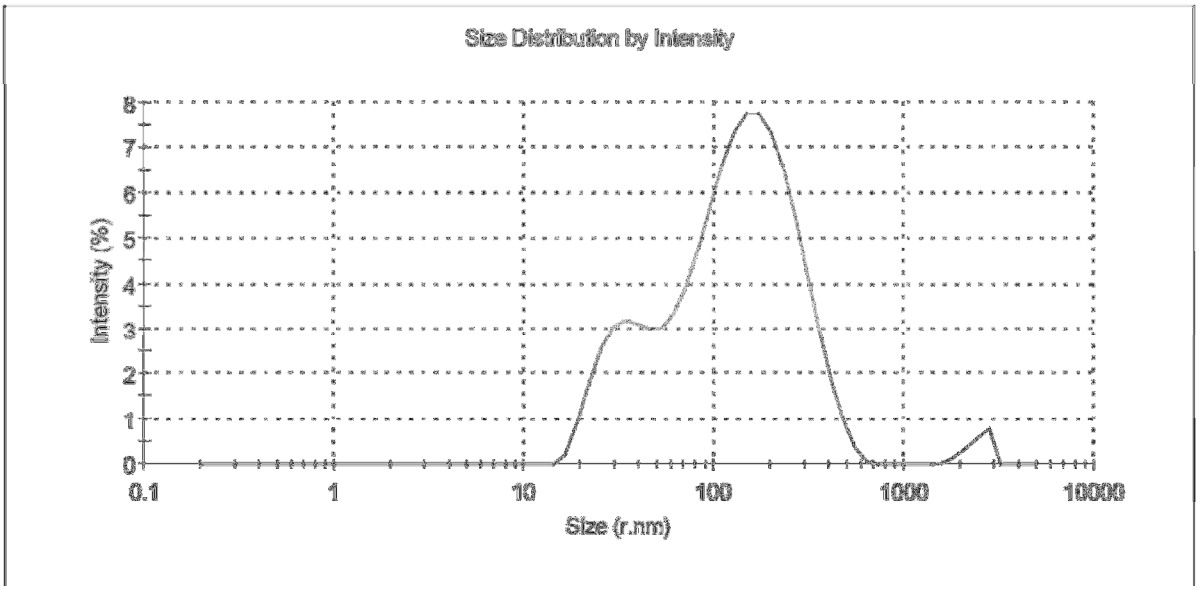

Figure 5.72: Particle size distribution of Placebo emulsion for large porous lipospheres after 2 cycles of homogenization

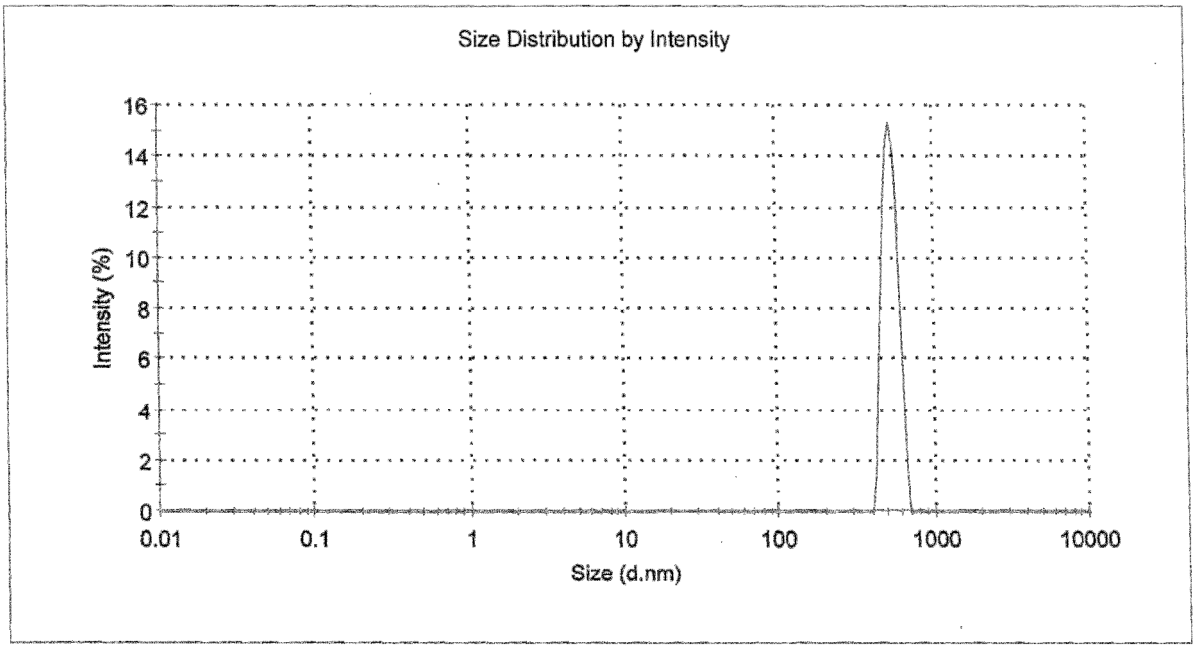

Figure 5.73: Particle size distribution of Placebo emulsion for large porous lipospheres after 3 cycles of homogenization

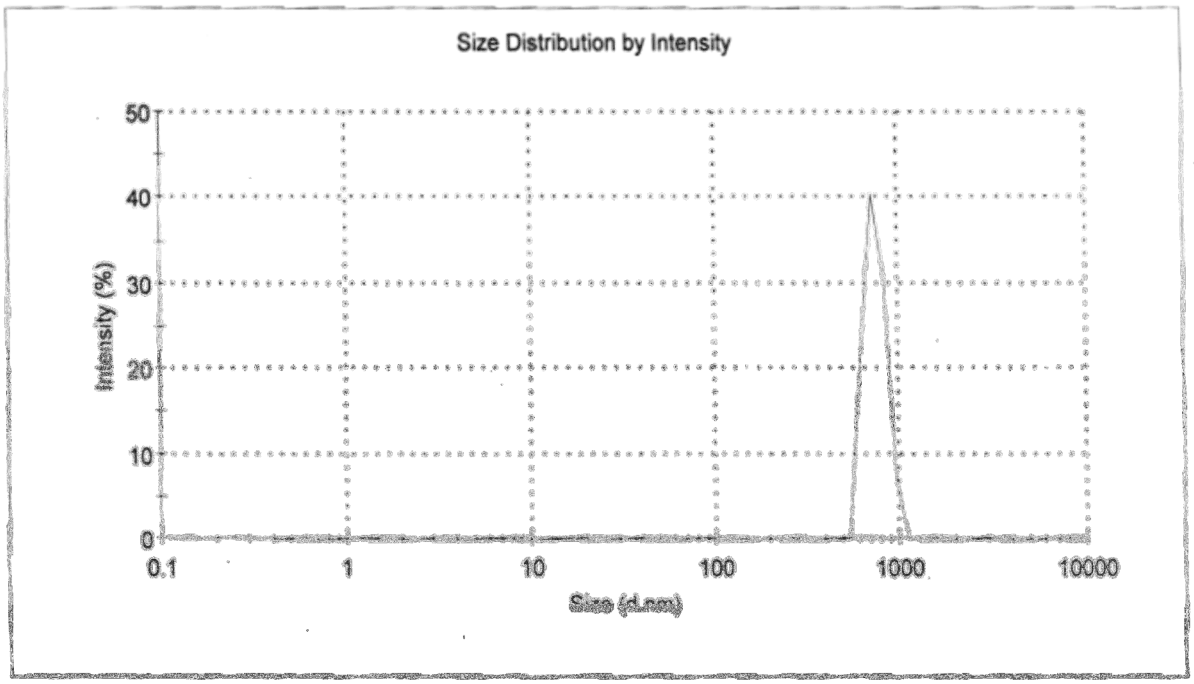

Figure 5.74: Particle size distribution of sildenafil citrate loaded emulsion for large porous lipospheres after 2 cycles of homogenization

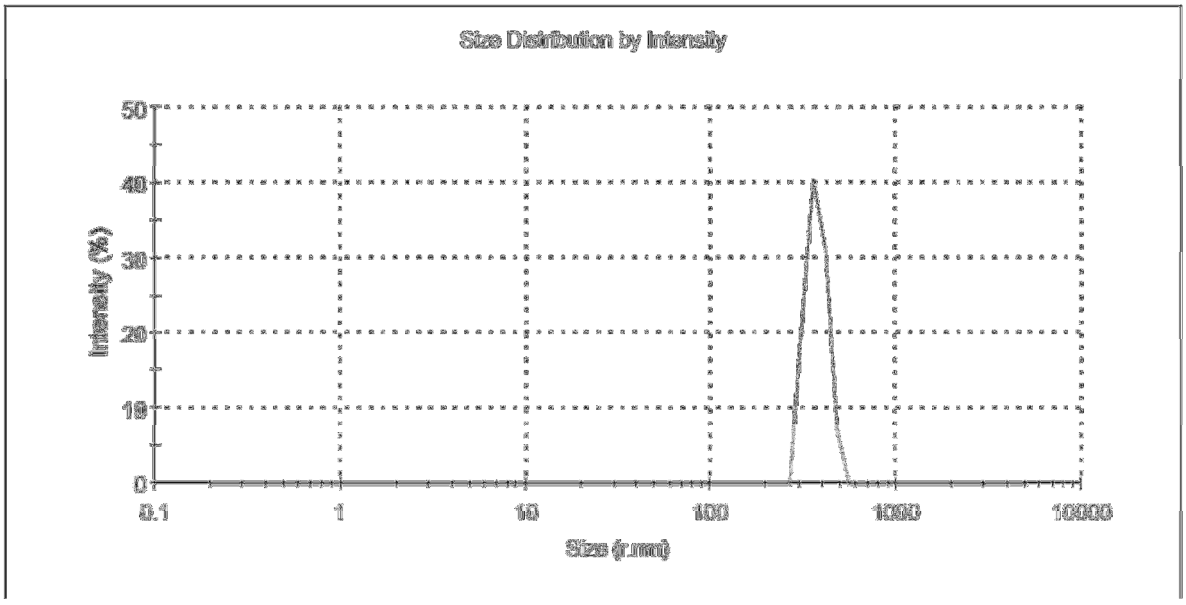

Figure 5.75: Particle size distribution of sildenafil citrate loaded emulsion for large porous lipospheres after 3 cycles of homogenization

5.7 Scanning Electron Microscopy

Scanning electron micrographs of conventional DPI formulation of sildenafil citrate CD3 **(Figure 5.76)**, (prepared with 70:30 LH 200 and P350M), Sildenafil citrate-sugar composites DSS7 **(Figure 5.77-5.78)**, sildenafil citrate-lipid composites DLS7 **(Figure 5.79-5.80)**, sildenafil citrate loaded liposomal dry powder for inhalation DPL7 **(Figure 5.82-5.84)** and sildenafil citrate loaded large porous lipospheres LPL16 **(Figure 5.85-5.87)** reveals different surface morphological characteristics of different dry powder formulations. Conventional DPI (CD3) showed crystalline structure of the carrier with drug crystals. Rest all other formulations were found to be spherical in nature. As evident from SEM photographs, the surface of sugar composites **(Figure 5.77-5.78)** and lipid composites of sildenafil citrate **(Figure 5.79-5.80)** prepared spray dried with sugar carriers like mannitol and trehalose respectively were smooth, spherical non–porous and having some depressions on the surface. The surface topography of Sildenafil citrate loaded liposomal dry powder prepared from HSPC and DPPC **(Figure 5.82-5.84)** revealed slightly porous surface having few depressions on surface with some minute pores on their surface.

Spray drying of submicron liposomal dispersion in suspension/emulsion form rather than spray drying drug with sugar or lipids in solution form, can impart porosity to the particle surface. However, this porosity can be increased to large extent by the inclusion of any blowing agent in emulsion. This technique can lead to the formation of highly porous, large sized lighter particles. The surface topography of large porous lipospheres prepared from emulsion feed stock containing a blowing agent is shown in **Figure 5.85-5.87**. These lipospheres were spherical and highly porous in nature. This highly porous nature in lipospheres might be attributed to a faster rate of evaporation of the solvent on surface and then solvent from the bulk diffuse out through the surface creating highly porous particles during evaporation (Ungaro F *et al*. 2006, Duddu SP *et al*. 2002). Surface topography of large porous lipooospheres revealed bigger, porous and hollow aerodynamically light particles that would be more resistant to be phagocytosed, which is also consistent with results of macrophage uptake study and its better lung deposition and longer stay in lungs observed during in-vivo studies.

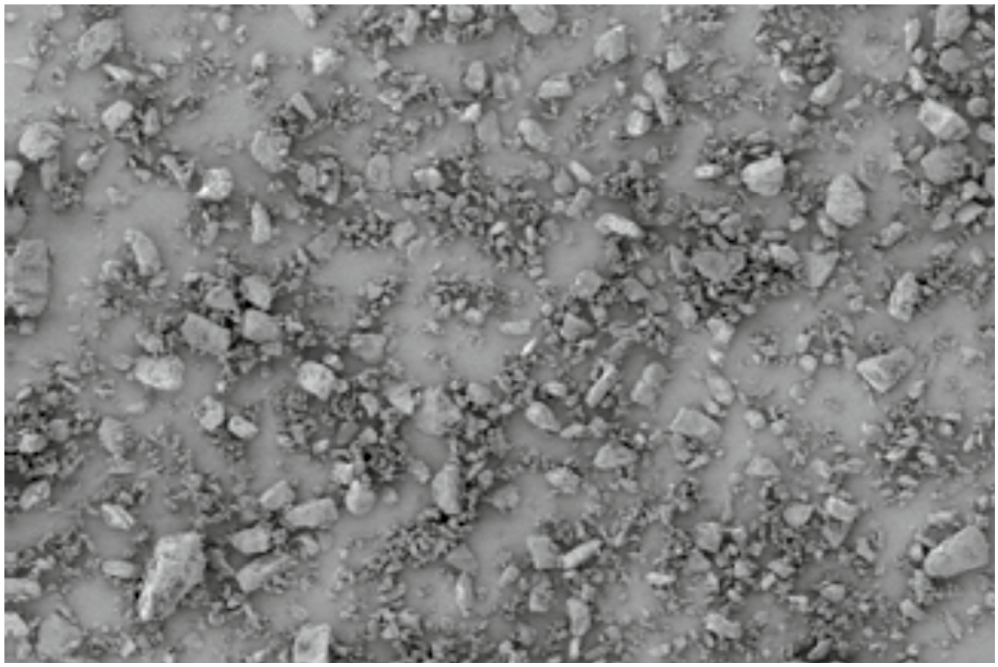

Figure 5.76: Scanning electron micrograph of Conventional DPI of sildenafil citrate

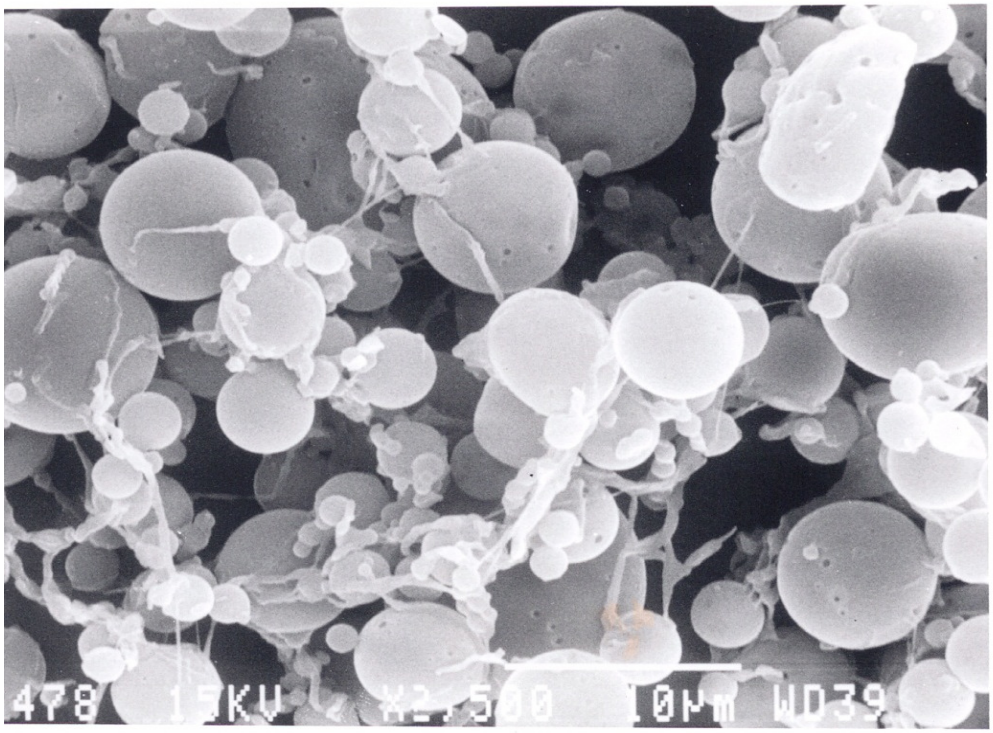

Figure 5.77: Scanning electron micrograph of sildenafil citrate loaded -sugar Composites

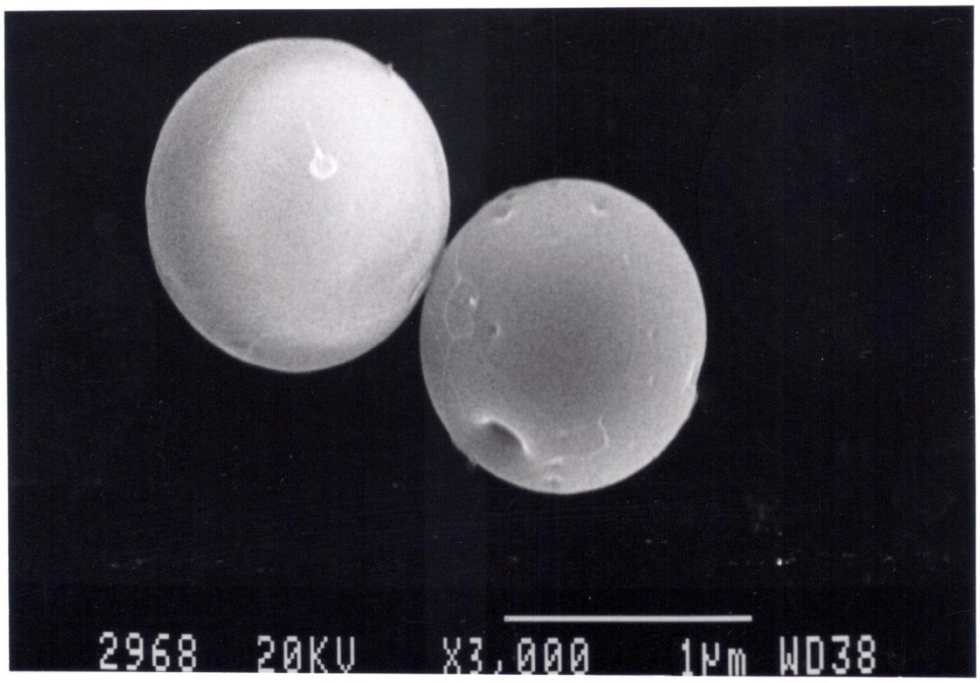

Figure 5.78: Scanning electron micrograph of sildenafil citrate loaded sugar Composites

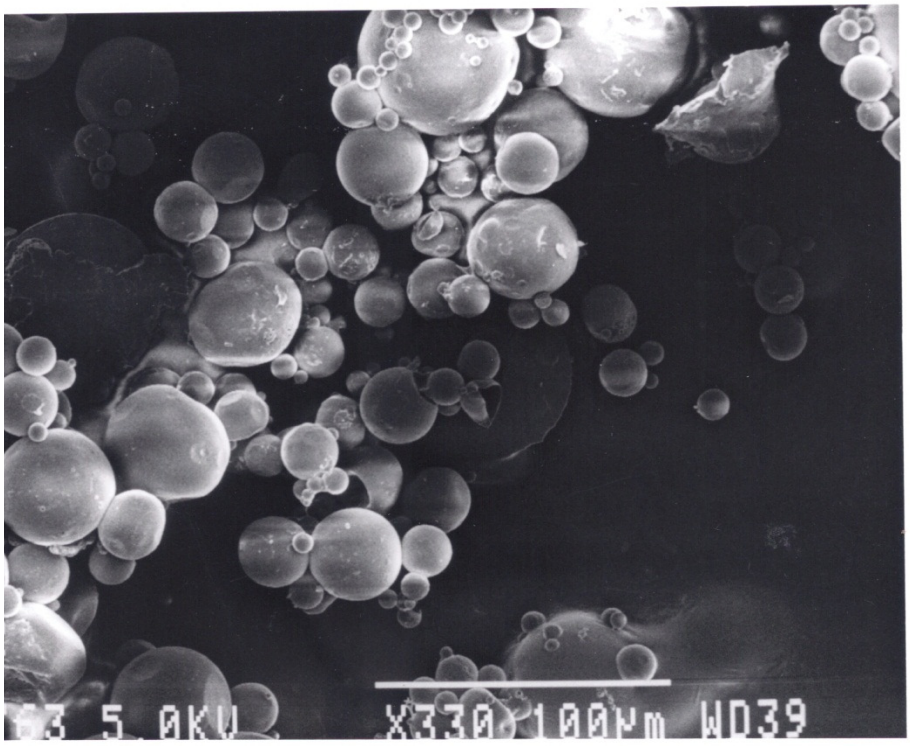

Figure 5.79: Scanning electron micrograph of sildenafil citrate loaded -lipid Composites

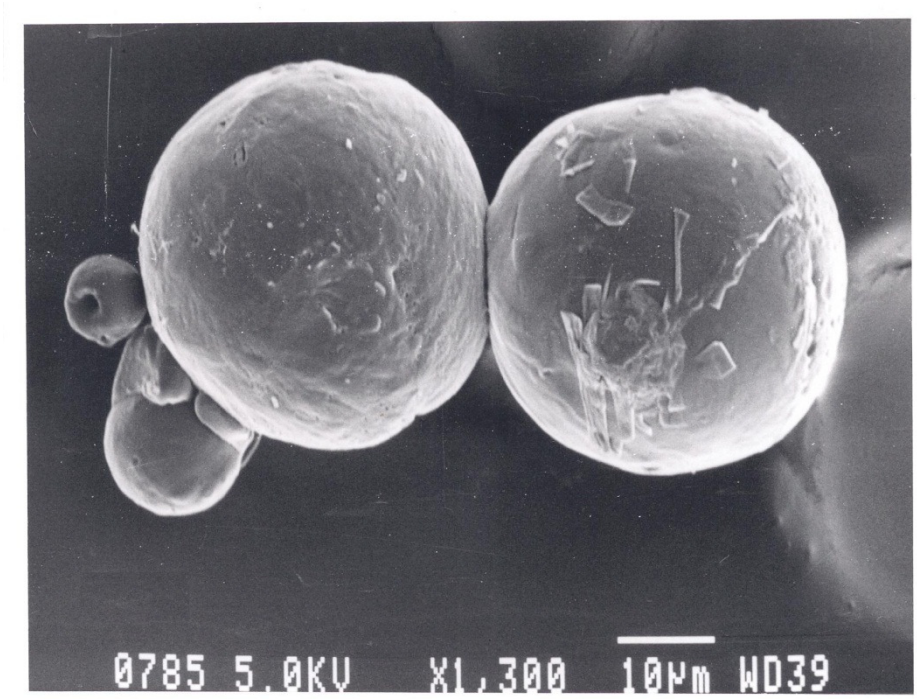

Figure 5.80: Scanning electron micrograph of sildenafil citrate loaded -Lipid Composites

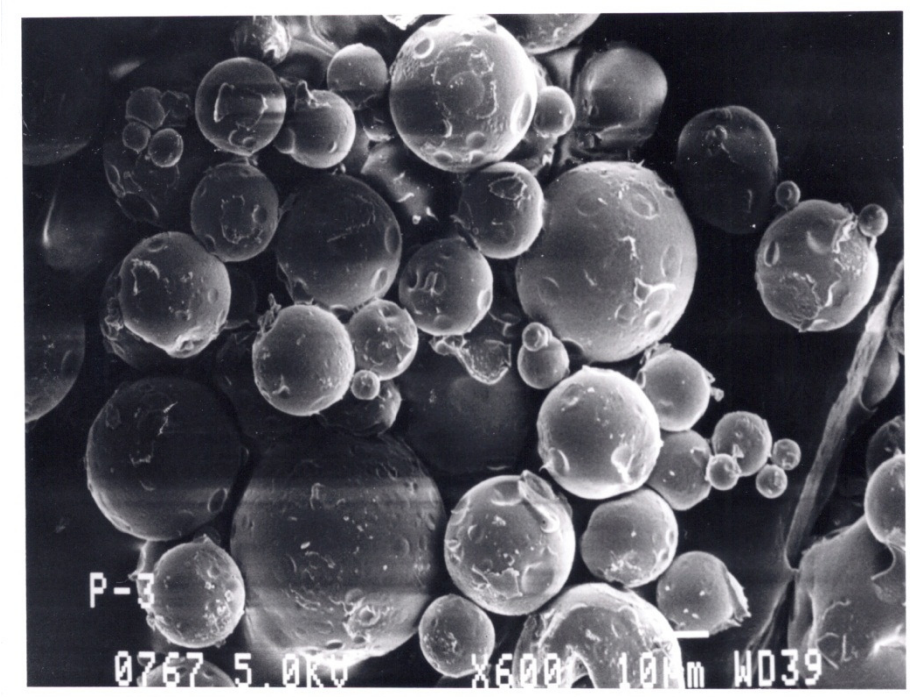

Figure 5.81: Scanning electron micrograph of sildenafil citrate loaded -Lipid Composites

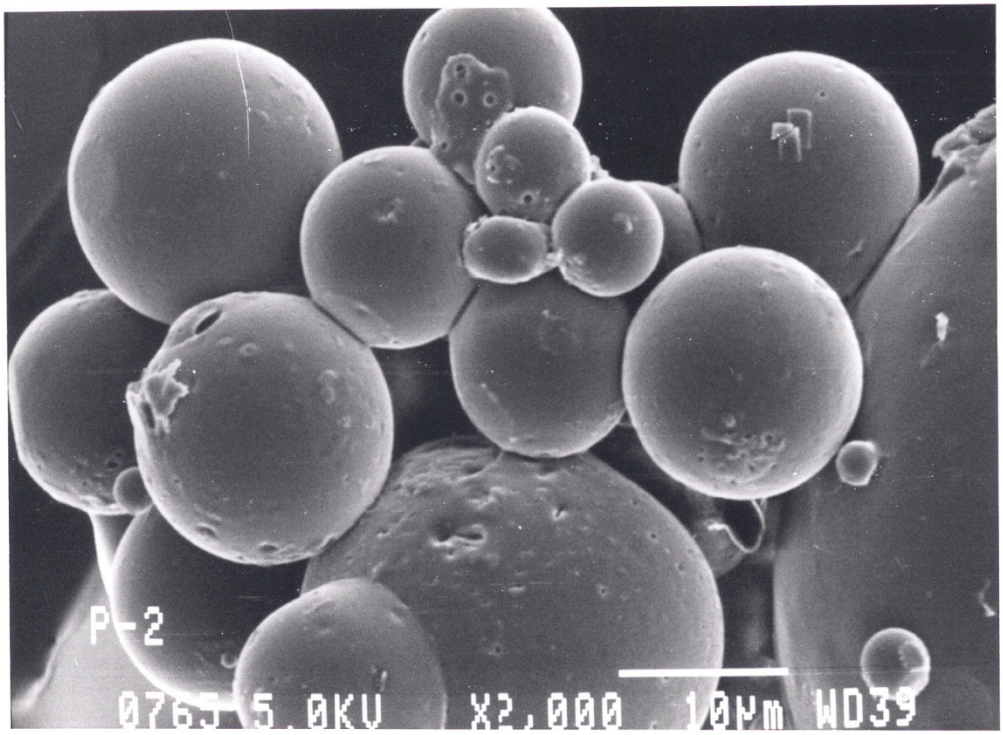

Figure 5.82: Scanning electron micrograph of sildenafil citrate loaded liposomal dry powder

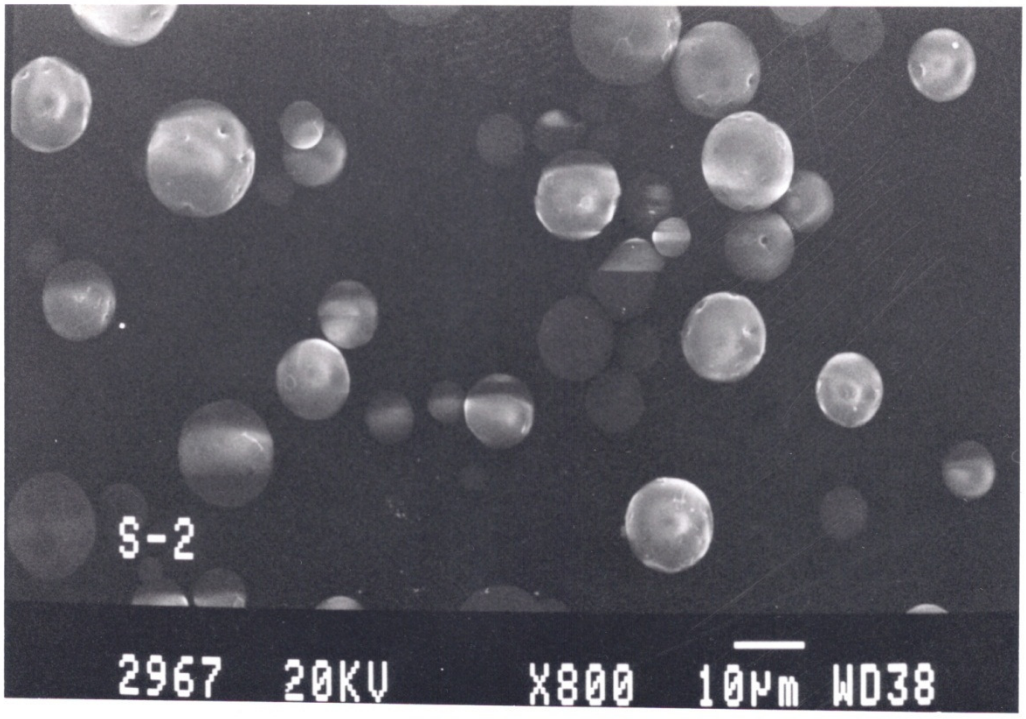

Figure 5.83: Scanning electron micrograph of sildenafil citrate loaded liposomal dry powder

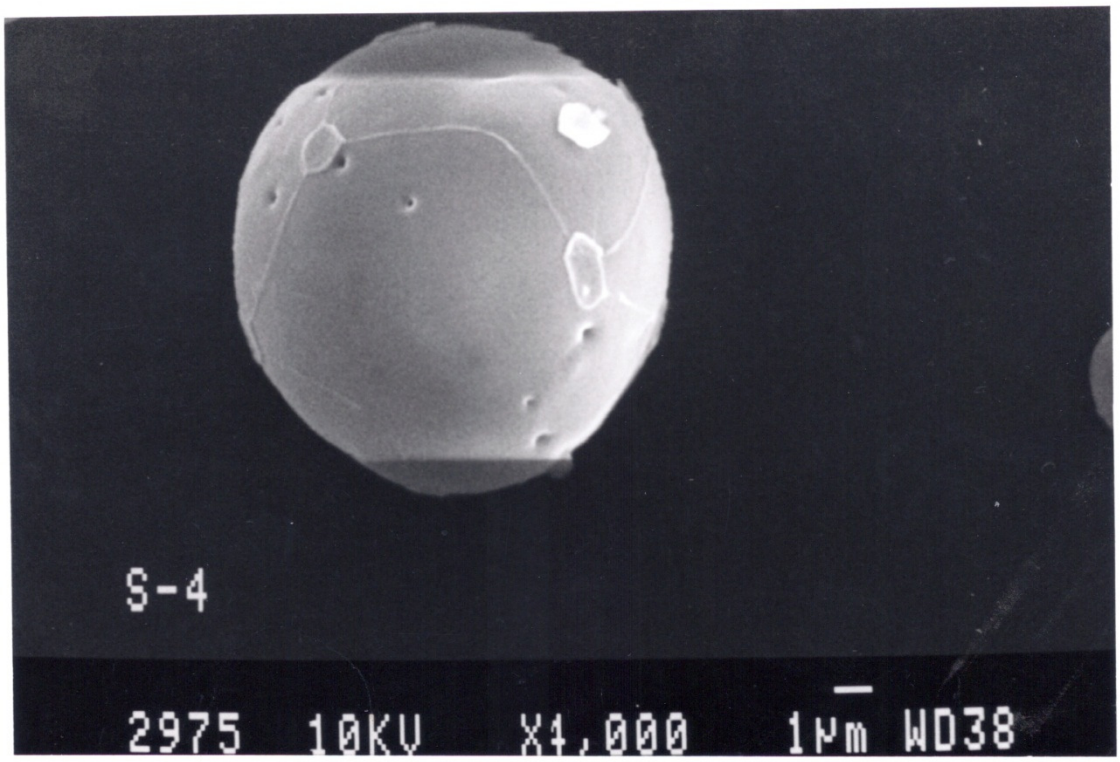

Figure 5.84: Scanning electron micrograph of sildenafil citrate loaded liposomal dry powder

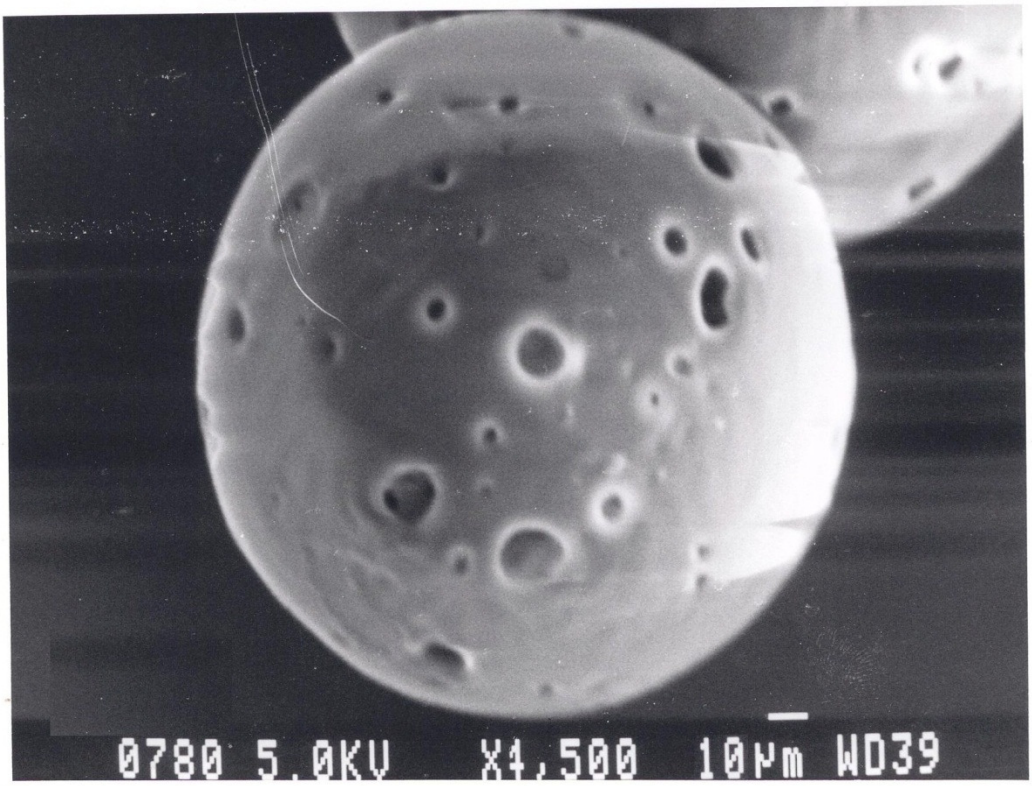

Figure 5.85: Scanning electron micrograph of sildenafil citrate loaded Large Porous Lipospheres

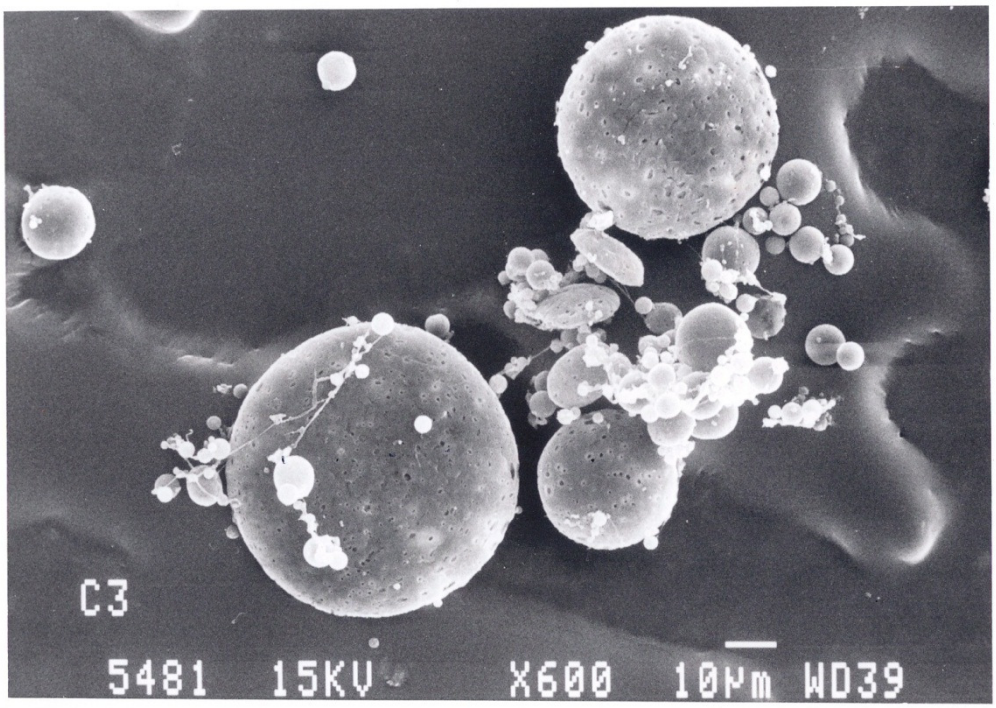

Figure 5.86: Scanning electron micrograph of sildenafil citrate loaded Large Porous Lipospheres

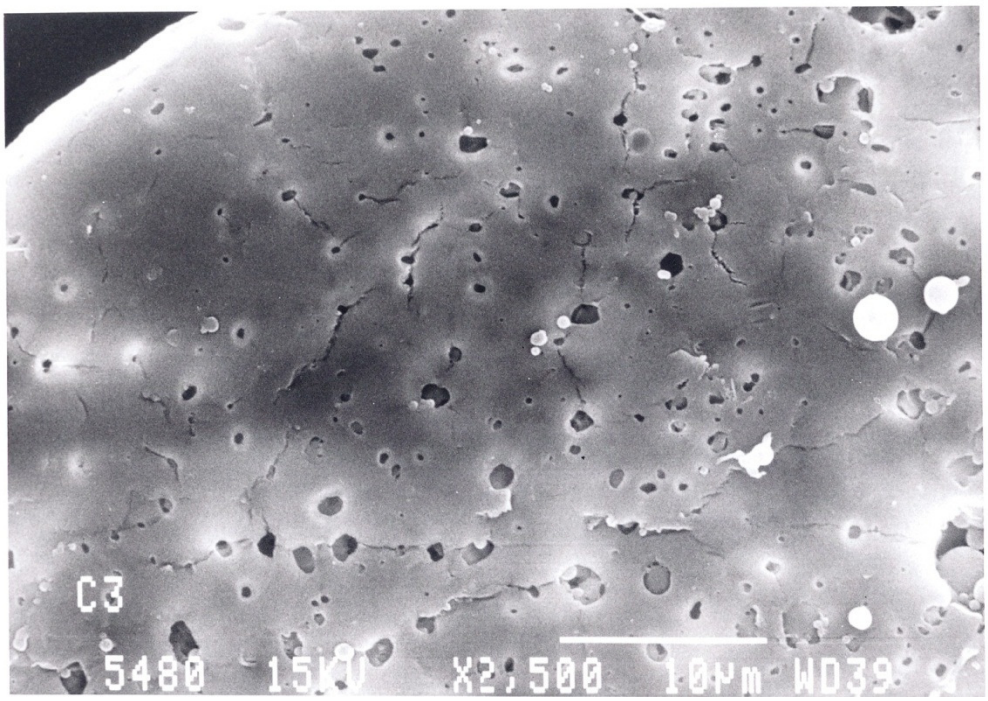

Figure 5.87: Scanning electron micrograph of sildenafil citrate loaded Large Porous Lipospheres

5.8 Differential Scanning Calorimetric studies

DSC thermograms of sildenafil citrate, mannitol (stabilizing sugar used as carrier for drug-sugar composites, liposomal dry powder for inhalation and large porous lipospheres), D (+) trehalose dihydrate (stabilizing sugar in drug-lipid composites), mixture of lipids used in the formulations are shown in the **Fig.s 5.88-5.94.** Different optimized formulations of sildenafil citrate and their respective placebos are overlapped for DSC peaks. Drug-lipid composites prepared using trehalose as the carrier for spray drying showed peak near the glass transition temperature (105°C) of D (+) trehalose dehydrate.

The thermograms of drug-loaded lipid composites did not show any endothermic peak of drug (197ºC) 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. The thermograms of drug-loaded formulations did not show any endothermic peak of drug (197ºC) indicative of the complete protection of drug with mannitol in drug-sugar composites and complete encapsulation of drug in liposomal dry powder and large porous lipospheres.

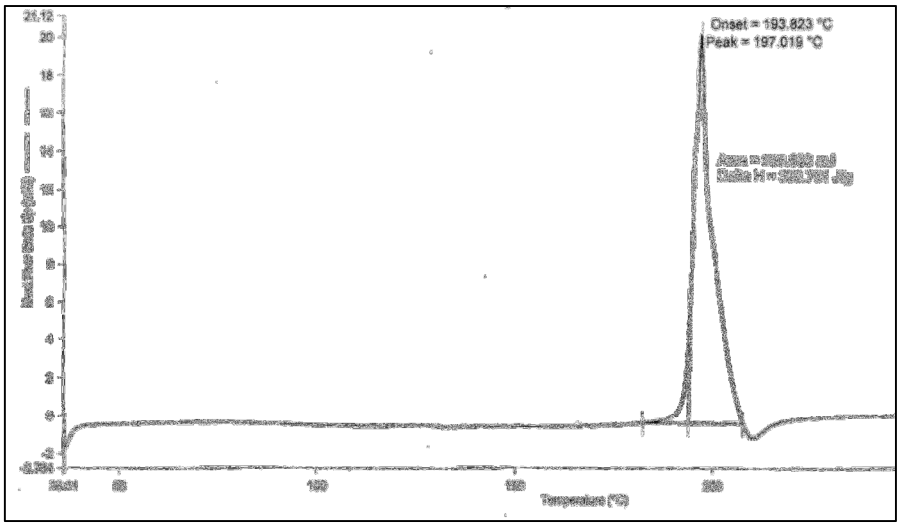

Fig. 5.88: DSC Thermogram of sildenafil citrate

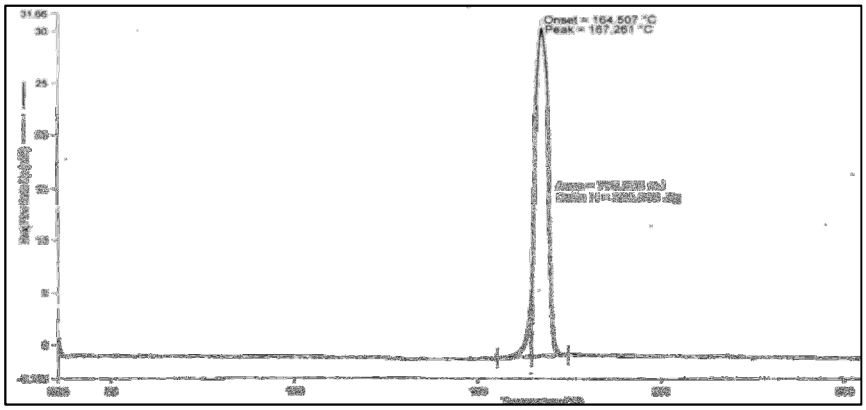

Fig. 5.89: DSC Thermogram of Mannitol

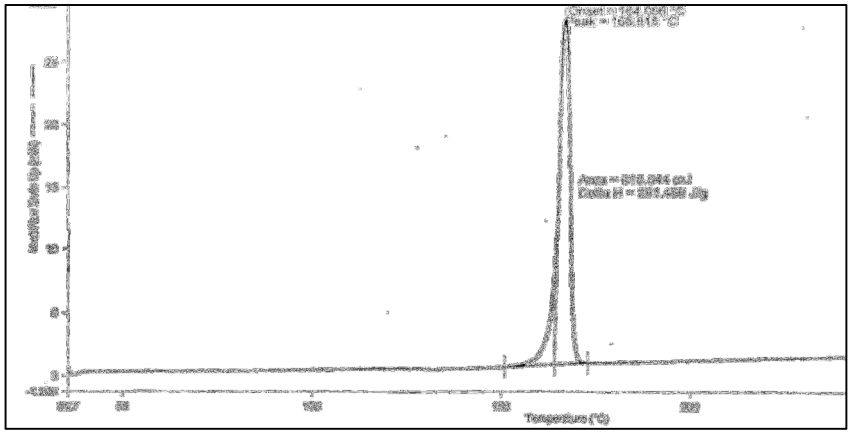

Fig. 5.90: DSC Thermogram of drug-sugar composites of sildenafil citrate

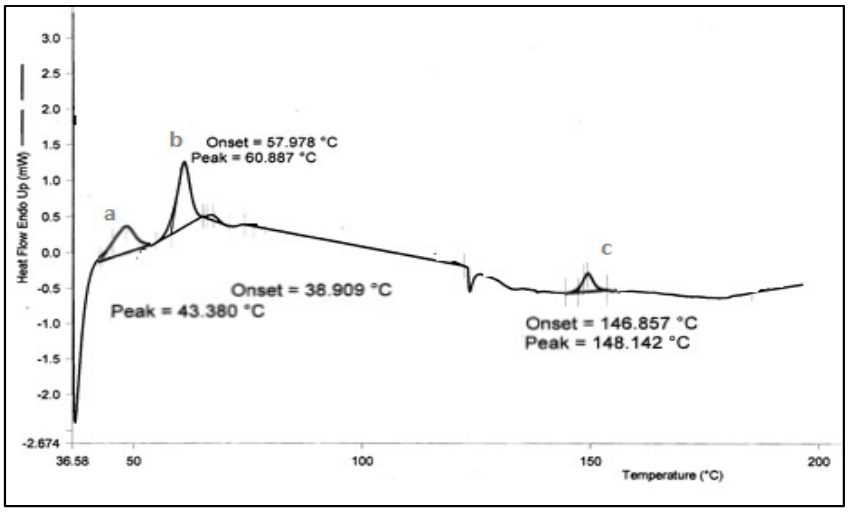

Fig. 5.91: DSC Thermogram showing peaks of a) DPPC, b) HSPC and c) Cholesterol

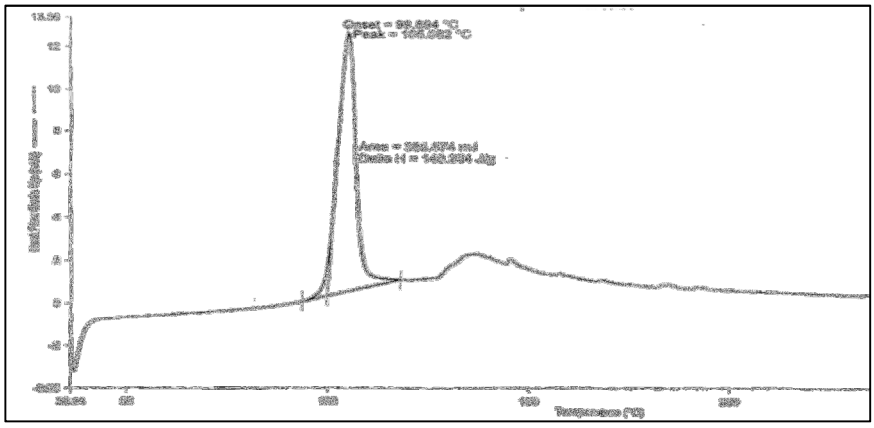

Fig. 5.92: DSC Thermogram of D (+) Trehalose dihydrate

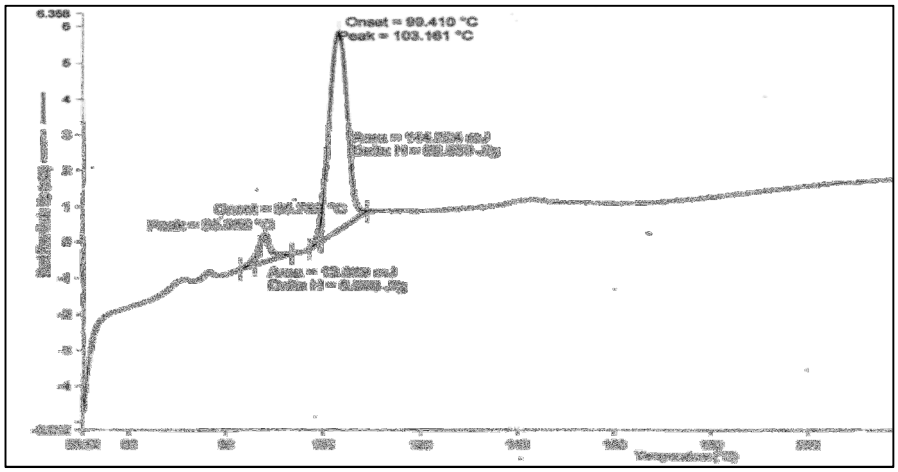

Fig. 5.93: DSC Thermogram of sildenafil citrate-lipid composites spray dried with Trehalose

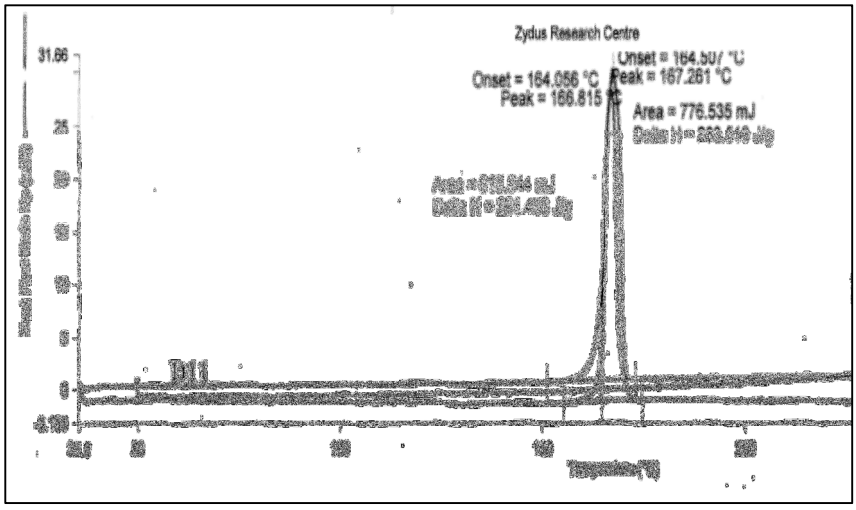

Fig. 5.94: Overlapped DSC Thermogram of placebo and sildenafil-citrate loaded liposomal dry powder for inhalation

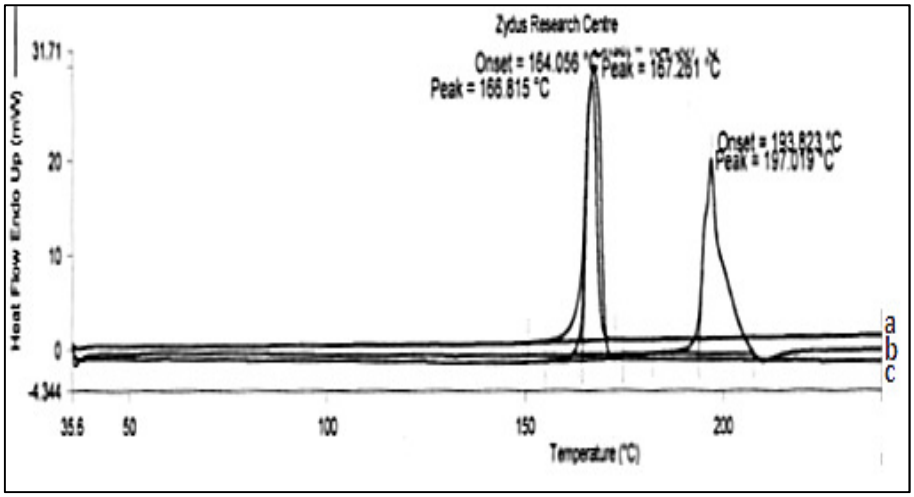

Fig. 5.95: Overlapped DSC Thermogram of a) placebo, b) sildenafil citrate and c) sildenafil-citrate loaded large porous lipospheres

5.9 X-ray Diffraction studies (XRD):

X-ray Diffraction (XRD) results of various sildenafil citrate formulations compared to API and respective placebos have been shown in the **Fig 5.96-5.100**. 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 at two theta degree values of 7.97, 10.17, 14.29, 19.7, 22.55 and 22.88 as well as those seen for mixture of LH 200 and P350M revealing the crystalline nature of the drug in mixture. Other formulations were prepared using spray drying technique. It is clear from the X-Ray diffractograms that there was drastic change in the crystallinity of drug after processing with excipients using spray drying. Crystalline peaks of sildenafil citrate at two theta degree of 7.97, 10.17, 14.29, 19.7, 22.55 and 22.88 were either not seen or were of reduced intensity in case of drug-sugar composites, drug-lipid composites and liposomal dry powders. However, in case of large porous lipospheres there was no crystalline peak representing sildenafil citrate was seen revealing highly amorphous nature of the product. Large porous lipospheres were prepared by the technique that yielded very light, porous and hollow particles with highly amorphous nature, which is a desirable characteristic for dry powder formulations for inhalation.

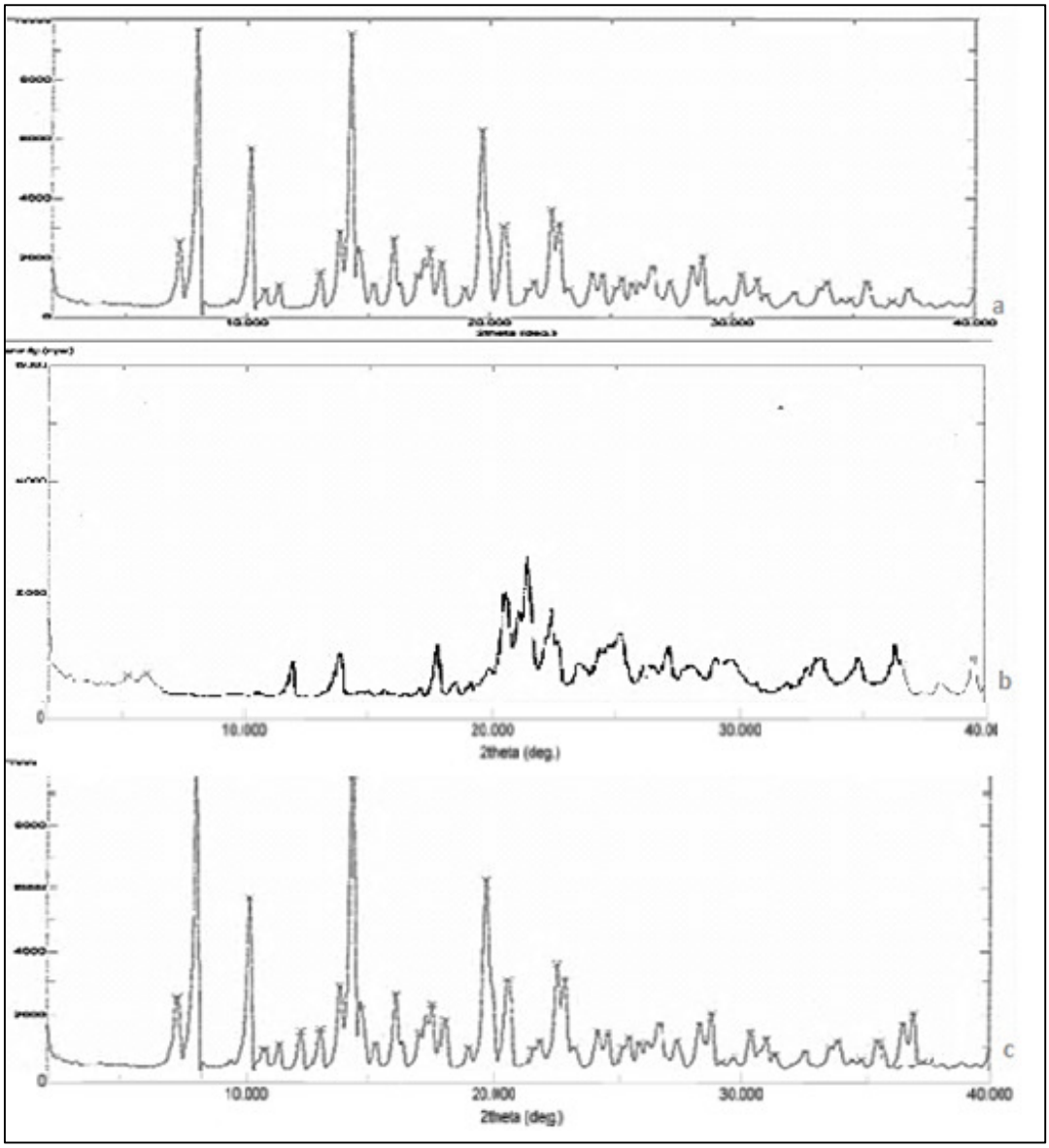

Fig. 5.96: X-Ray diffractogram of a) sildenafil citrate b) Mixture of LH 200 and P 350M (70:30) c) Sildenafil citrate-conventional dry powder

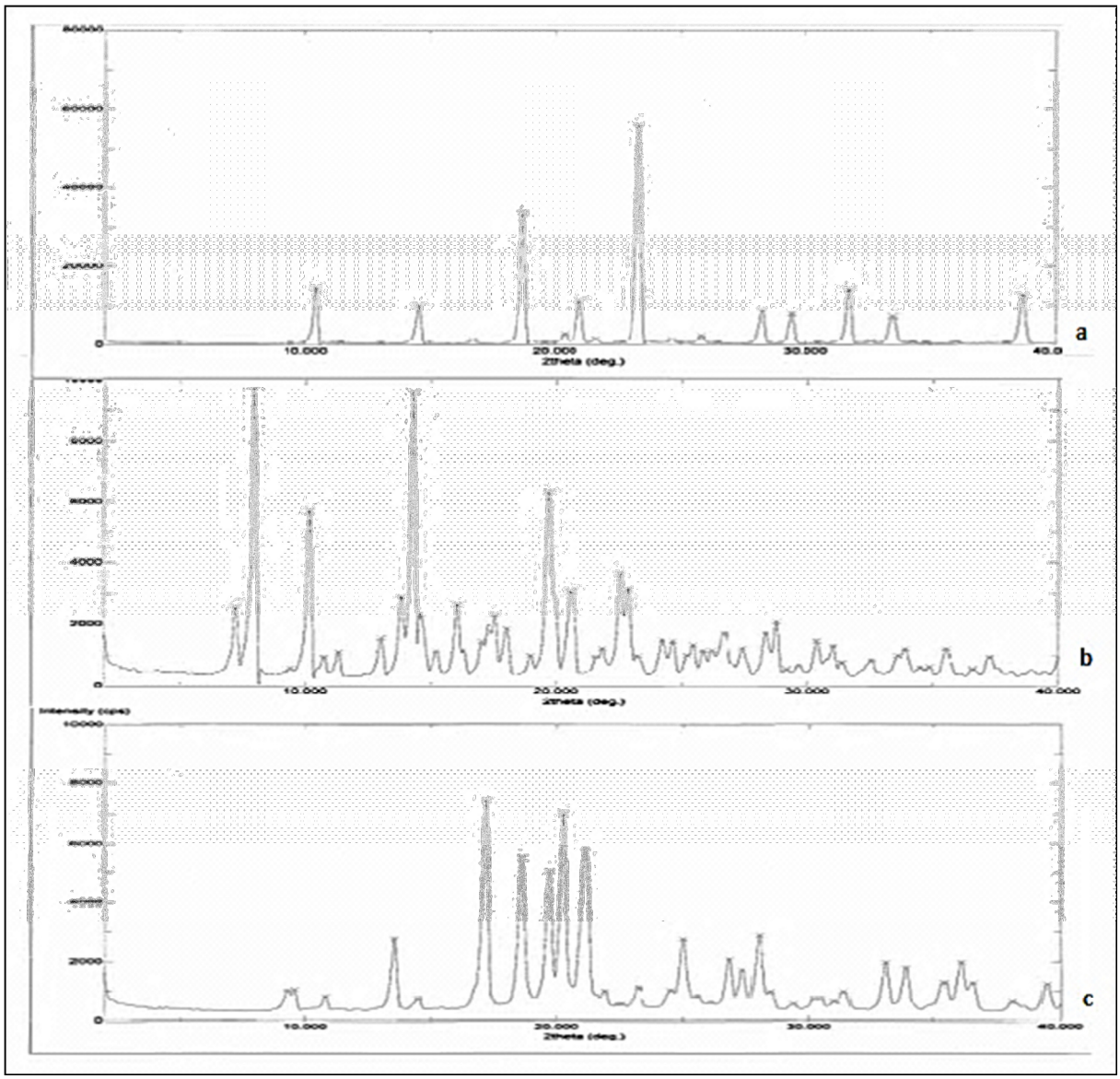

Fig. 5.97: X-Ray diffractogram of a) Placebo for drug-sugar composites (spray dried mannitol) b) Sildenafil citrate c) Sildenafil citrate-mannitol composites (spray dried sildenafil citrate with mannitol)

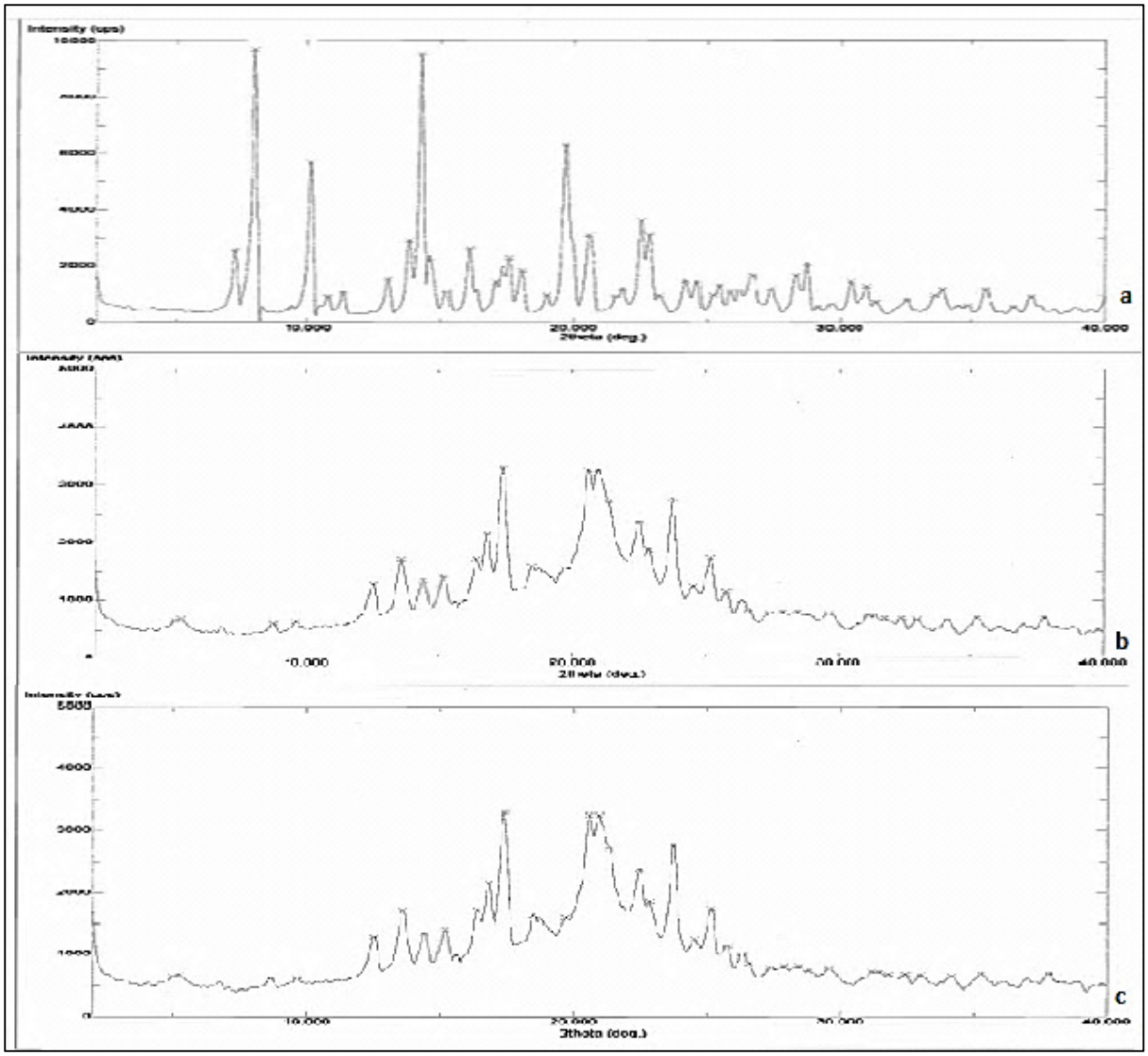

Fig. 5.98: X-Ray diffractogram of a) Sildenafil citrate b) Placebo for sildenafil citrate lipid composites c) sildenafil citrate lipid composites

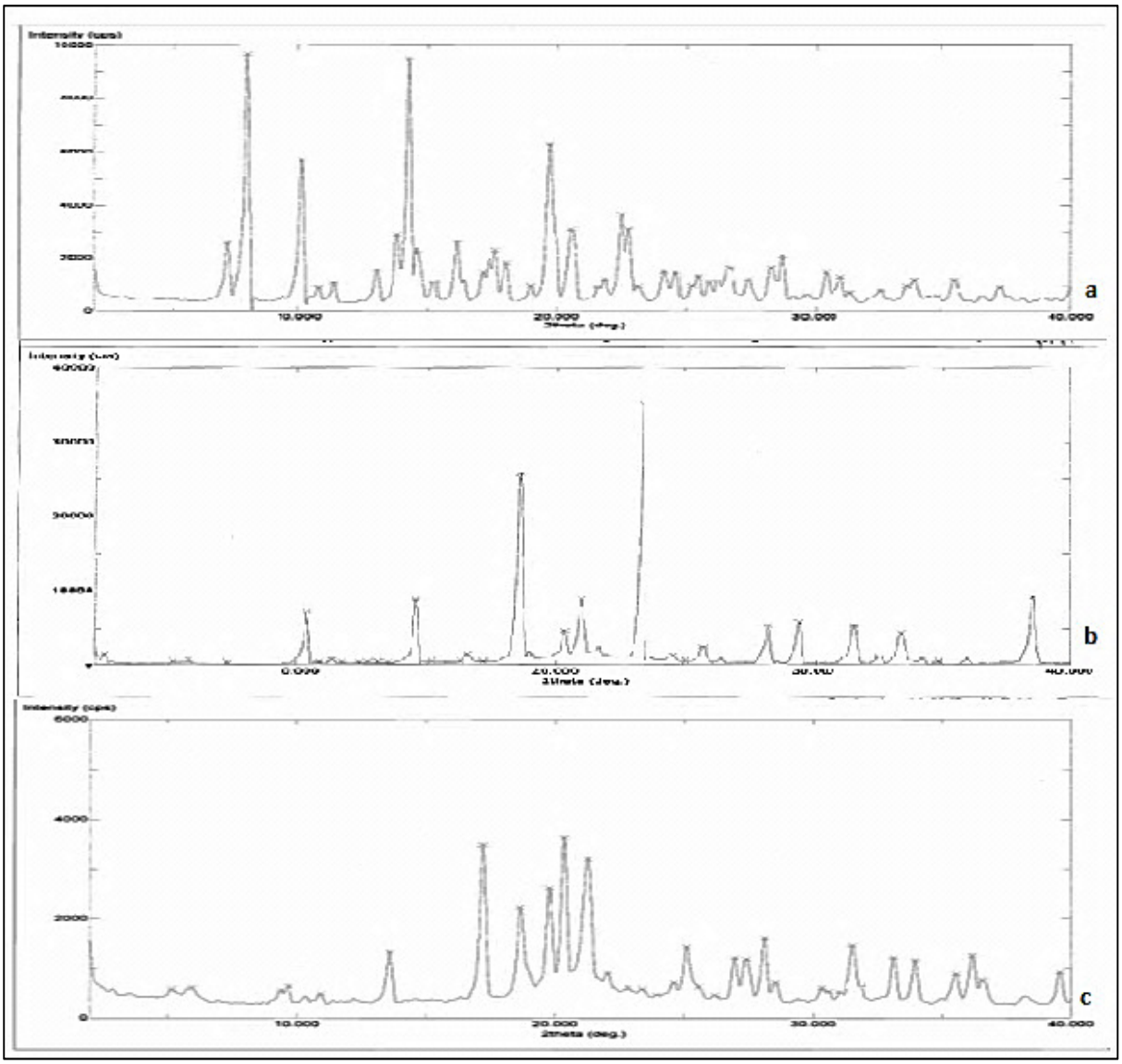

Fig. 5.99: X-Ray diffractogram of a) Sildenafil citrate b) Placebo for liposomal dry powder for inhalation c) Sildenafil citrate loaded liposomal dry powder for inhalation

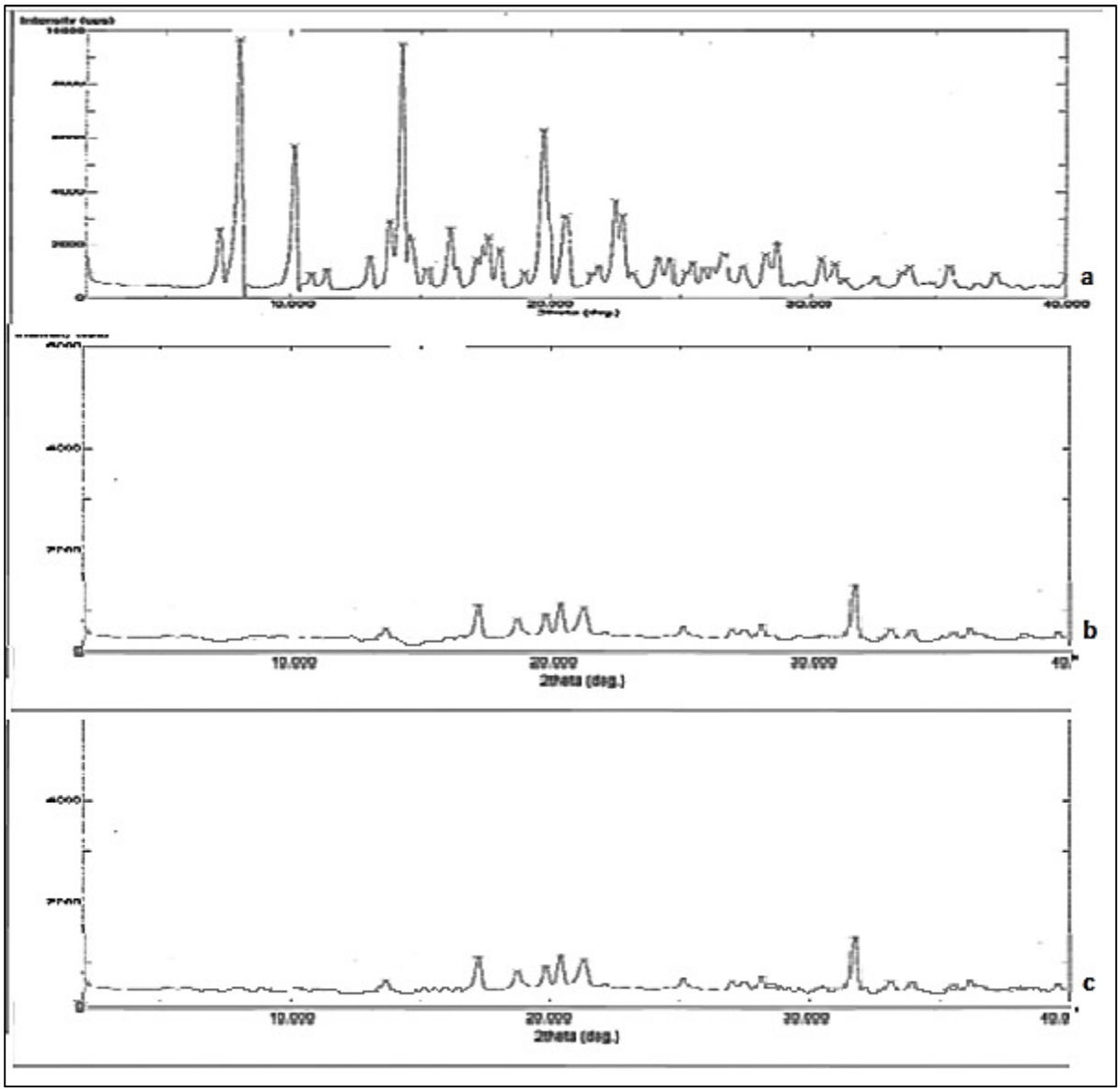

Fig. 5.100: X-Ray diffractogram of a) Sildenafil citrate b) Placebo for large porous lipospheres c) Sildenafil citrate loaded large porous lipospheres

5.10 Evaluation of other physical characteristics and geometric particle size:

Hausner's ratio and Carr's index are considered as appropriate methods of evaluation of the flow properties of solids. These were determined from tapped and bulk density values. Hausner's ratio is measure of flowability of powder and has a negative correlation with flowability and value < 2 means that the powder has a high flowability. Particles having bulk density less than 0.4g/cc and geometric particle size (volume mean diameter) greater than 10µ are considered to have better lung deposition and reduced macrophage uptake. Carr's index values of <25% are usually taken to indicate good flow characteristics; values beyond 40 indicate poor powder flowability. Carriers like LH 100, 200 and P 350, 450 had bulk density greater than 0.4g/cc and CI values around 48 for LH 200 and P 450M. Flow parameters were improved on combining these fine carriers with coarse carriers and it was particularly best for the formulation CD3 at ratio 70:30 of LH 200: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±1.05µm geometric diameter. Since the particles were of crystalline nature with irregular shape, the aerodynamic diameter calculated from the density value and volume mean diameter was (3.16± 2.23 µm) different from the actual MMAD value obtained from ACI experiment. It had a good flow with 27.05 ± 1.19° angle of repose, %CI of 18.2% and 1.22 Hausner's ratio. For all formulations, Hausner's ratio was in the range of 1.2–1.95 **(Table 5.6, 5.7)**, indicating good flowability. Carr's index values **(Table 5.7)** for almost all formulations prepared with combination of fine and lactose carriers were found to be <25% indicating good powder flow properties.

All the spray dried formulations were optimized to achieve better flow characteristics. The target was to achieve low bulk density and higher volume mean diameter for better lung deposition and least clearance by macrophages from the lungs.

In case of drug-sugar composites, the best flow characteristics were found with formulation DSS7 having 1:10 drug: mannitol ratio at 4% mannitol spray dried at parameters optimized for the formulation. It showed a bulk density of 0.211 ± 2.03g/cc and geometric diameter of 6.44± 0.66µm**.** Tapped density was found to be 0.246 ± 0.18g/cc. Angle of repose was <30° (27.03 ± 0.86°), %CI of 14.59% and 1.16

Hausner's ratio revealed good flow charcteristics of the formulation **(Table 5.10** and **5.11**).

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. Thus, liposomal dry powder found to have more desirable characteristics.

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 aerodymanic 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 (large enough to escape macrophage uptake) could be achieved by spray drying an emulsion containing chloroform as blowing agent to form very light and large porous particles. Calculated aerodynamic diameter was 4.22 µm which is small enough for good lung deposition. Tapped density was 0.118 ± 1.25g/cc, 26.34 ± 0.81° angle of repose, 14.40% carr's index and 1.17 Hausner's ratio **(Table 5.21, 5.22)**. Large porous liposphere showed excellent and desirable characteristics for dry powder inhalation formulations.

Table 5.25: Comparison of moisture content, volume mean diameter, density, calculated aerodynamic diameter and actual aerodynamic diameter from Andersen cascade impactor, of various optimized sildenafil citrate formulations:

S. No.	Batch No.	Formulation Code	Description	Moisture content (%w/w)	Bulk density (ρb in g/cc)	Volume mean diameter VMD in µm (span)	Aerodynamic Particle Size µm from ACI (calculated by VMD *√(ρb)
1	CD3	F1	Conventional dry powder for inhalation (CDPI) of sildenafil citrate with lactose carriers	5.11 ± 1.08	0.418 ± 0.25	6.76±1.05 (2.364)	6.12 ± 2.23 (3.16± 2.23)
2	DSS7	F2	Drug-sugar composites of sildenafil citrate with mannitol prepared by spray drying technique	3.07 ± 0.82	0.211± 2.03	6.44± 0.66 (1.803)	2.96 ± 1.83 (2.95±0.98)
3	DPL7	F3	Liposomal dry powder for inhalation (LDPI) prepared by thin film hydration and spray drying	1.16 ± 2.24	0.201± 2.05	8.99± 1.26 (2.137)	4.095± 0.52 (4.03±1.04)
4	DLS7	F4	Drug-lipid composites of sildenafil citrate prepared by spray drying technique	2.69 ± 0.09	0.207± 2.11	5.12± 0.91 (1.667)	2.125± 0.5 (2.33±1.22)
5	LPL16	F5	Large porous lipospheres prepared by emulsification and spray drying technique	0.97 ± 1.16	0.101± 1.43	13.28± 0.51 (1.918)	4.64± 0.71 (4.22±1.51)

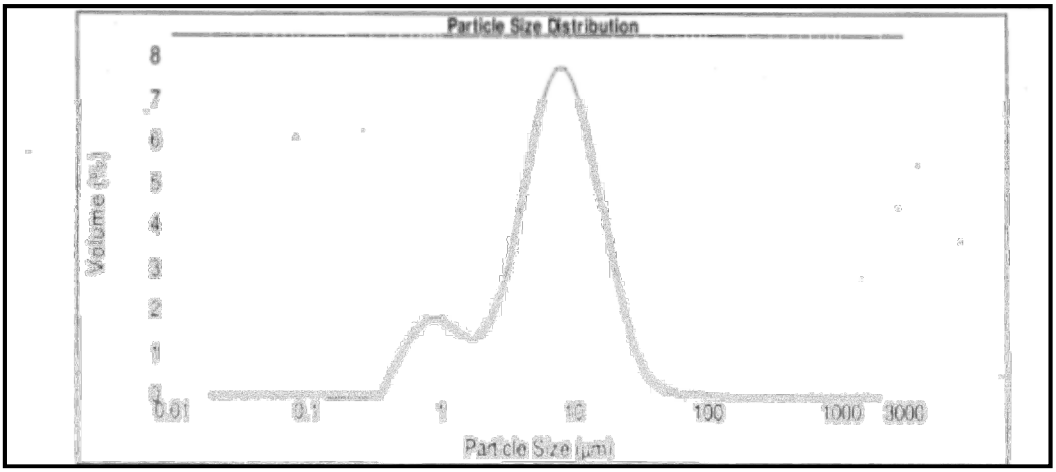

Fig. 5.101: Particle size distribution of conventional DPI formulation (F1)

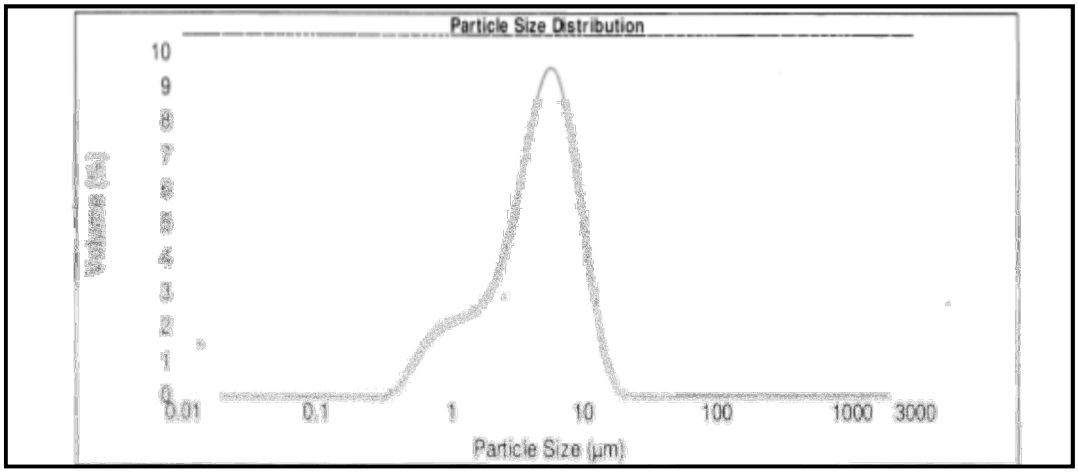

Fig. 5.102: Particle size distribution of Drug-sugar composites (F2)

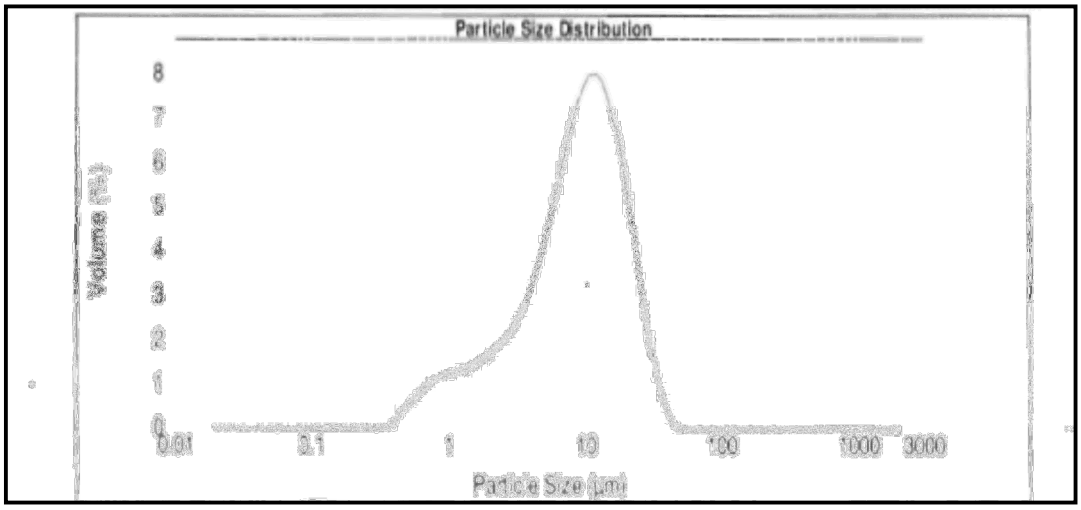

Fig. 5.103: Particle size distribution of liposomal dry powder for inhalation (F3)

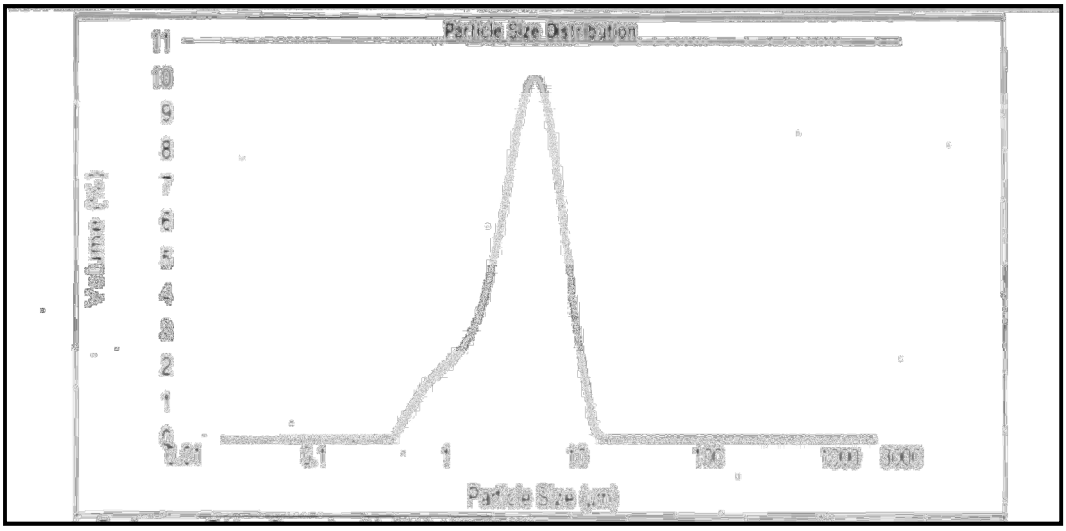

Fig. 5.104: Particle size distribution of Drug-lipid composites (F4)

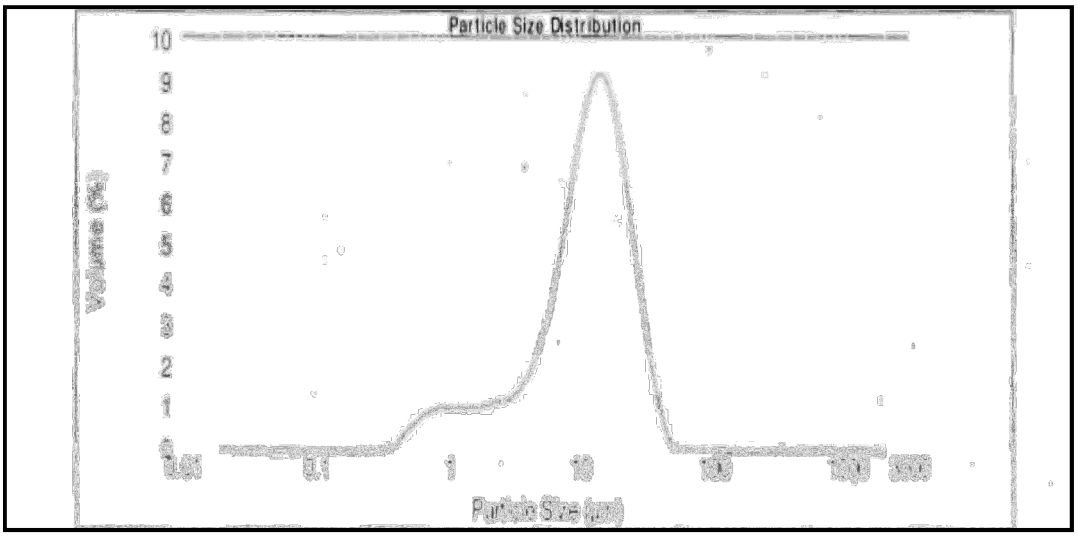

Fig. 5.105: Particle size distribution of large porous lipospheres (F5)

5.11 Moisture content and residual solvent:

Moisture content of dry powder formulations is one of the most important characteristics that determine the flow properties, dispersibility, particle size and hence respirable fraction. Moisture content of DPI formulations should be less than 4%. Maximum moisture content of 5.11±1.08% w/w was there in conventional DPI formulation (CD3). Effect of spray drying on moisture content was studied for all spray dried formulations and has been discussed in optimization of spray drying process for the respective formulation **(Table 5.10, 5.16, 5.19** and **5.22** respectively). 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 (0.97±1.16% w/w) was found in large porous lipospheres **(Table 5.22).** Residual solvent content was determined in liposomal dry powder, drug-lipid composites and large porous liposphere dry powder formulations using gas chromatography. Chloroform and methanol were used in formulation of liposomal dry powder and large porous lipospheres and methanol for drug-lipid composites. Residual solvent limit for chloroform is 60ppm and that for methanol is 3000ppm. In any of our formulations, residual solvent was not detected using gas chromatography and hence indicating safety for administration.

5.12 Aerosolization performance of the formulations:

Aerosolization behaviour of the developed formulations was determined based on delivered dose uniformity and Mean median aerodynamic particle size using Dosage unit sampling apparatus (DUSA) for DPI and Andersen cascade impactor (ACI) respectively of Copley Scientific.

5.12.1 Delivered Dose Uniformity:

"Delivered Dose Uniformity" test of spray dried optimized formulations was performed using Dosage Unit Sampling Apparatus (DUSA by Copley Scientific) for DPIs. Testing was done for ten doses of each formulation and one dose was considered to be equal to two capsules discharge from the inhaler. According to USP, the test is passed if not less than nine out of ten doses are between 75-125% of

average dose calculated and none is outside the range of 65-135% of average dose calculated as per USP. All the prepared formulation passed the test for delivered dose uniformity.

5.12.2 Aerodynamic particle size using Andersen Cascade Impactor (ACI):

Ten capsules were discharged into the apparatus one by one from rotahaler device at appropriate flow rate to give a pressure drop of 4kPa. Different aerosolization parameters like Recovered dose (RD), emitted dose (ED), Fine particle dose (FPD) were determined and were expressed as percent of the average assay amount of sildenafil citrate discharged from the inhaler. Fine particle dose was considered as the amount of sildenafil citrate found below effective cut-off diameter < 4.7µ. Fine particle fraction (FPF) was the ratio of FPD to RD, expressed as percentage, while dispersibility was expressed as the percentage of FPD to ED. Percent fine powder fraction is the actual respirable fraction. It was determined for all prepared conventional DPI formulations with two different devices Rotahaler® and Handihaler®. 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®.

Handihaler® exhibits higher air resistance, thus more drag force might be required to achieve turbulence of the air stream for deaggregation and detachment of drug particles from the surface of carrier for better %FPF. Therefore, the results obtained for %FPF of sildenafil citrate particles from formulations which were aerosolised using Rotahaler® were significantly better as compared to Handihaler®. The highest % FPF (36 ± 0.02%) as found with formulation CD3 (containing 70:30 of LH 200:P350M). Amongst spray dried products, %FPF was significantly improved which might be due to the better control on size, shape and structural morphology of the spray dried formulations. Drug-sugar composites showed significantly improved %FPF as compared to conventional DPI formulation CD3. Maximum % FPF (53.08 ± 0.28%) was recorded with DS3 formulation prepared at 1:10 ratio of sildenafil citrate and mannitol and 54.04 ± 0.33% when this composition was spray dried at optimized spray drying parameters (DSS7). However, in case of optimized drug-lipid composites formulation (DLS7), the %FPF was significantly lower (30.05 ± 0.39%) as compared to drug-sugar composites and even conventional DPI. This might be due

to the hygroscopicity of the formulation prepared using trehalose dihydrate as the carrier for spray drying.

However, liposomal dry powder formulation of sildenafil citrate (DPL7 containing HSPC, DPPC and cholesterol) revealed significantly improved %FPF (62.01 ± 0.09%) as compared to CD3, DSS7 and DLS7. There was significant effect of lipid composition on % FPF of liposomal formulations. Inclusion of DPPC in smaller proportion in liposomal formulation (DPL7) could significantly improve the %FPF from 51.73 ± 0.43% (DPL14 prepared with HSPC only) to (62.01 ± 0.09%). Liposomal dry powder formulation prepared with only DPPC (DPL15) showed significantly lower %FPF (47.88 ± 0.49) as compared to DPL7. Amongst all the prepared dry powder formulations, large porous lipospheres (LPL16) showed maximum %FPF of 82 ± 0.42%. Dispersibility is the percent of emitted dose that reaches the lungs and is determined as the percent ratio of FPD to ED. Conventional DPI formulation was found to be least dispersible (76.41±0.32%) among all prepared dry powder formulations.

However, spray dried formulations like drug sugar composites (85.95±0.54%) and drug lipid composites (84.77±0.48%) showed dispersibility above 80%. In case of liposomal dry powder formulation it was 90.55±0.31% and the best was found with large porous lipospheres (95.91±0.38%). Porosity in the particle help to improve the dispersiblity of the formulations due to better impact of inertial force at a given flow rate as compared to non-porous particles. SEM pictures revealed that LPL16 formulation consist of highly porous, hollow spheres showing improved dispersibility as compared to non-porous spray dried products. This characteristic is desirable for better aerosolization of the formulation to achieve maximum lung deposition which was further supported by the highest Cmax value and longest mean resident time in lungs of LPL16. The aerosolization characteristics of various fomulations are summarized in **Table 5.26.**

There was no significant difference in the aerodynamic diameter calculated from the density and geometric diameter and the actual MMAD value obtained from ACI in case of all dry powder formulations prepared by spray drying revealing the spherical nature of these particles due to which shape factor became equal to unity. However, there was significant difference in the calculated and actual MMAD value in case of conventional DPI which might be due to the crystalline nature of CDPI reflecting presence of particle with shape significantly different from spherical.

Table 5.26: Summarized aerosolization characteristics of various sildenafil citrate-sugar composite formulations (mean ± SD, n=3):

S. No.	Batch code	Drug: Sugar	Mannitol % w/w	Recovered Dose (%)	Emitted dose (%)	%FPF	MMAD (μm)	GSD	DDU (75-125% of Average as per USP)
1	DS1	1:5	2	99.48 ± 1.24	66.43 ± 1.75	19.8 ± 0.87	9.05 ± 1.11	1.95 ± 0.77	(Between 78.51-115.04%) Passed
2	DS2	1:10	2	97.2 ± 0.93	83.3 ± 0.86	23.04 ± 0.68	4.16 ±0.62	1.63 ± 0.33	(Between 77.59-122.94%) Passed
3	**DS3**	**1:10**	**4**	99.05 ± 1.78	85.13 ± 1.15	53.08 ± 0.28	2.96 ± 1.83	1.21 ± 0.95	(Between 81.08-120.75%) Passed
4	DS4	1:20	8	98.31 ± 0.38	84.78 ± 0.92	25.32 ± 0.47	3.14 ± 1.96	1.66 ± 1.01	(Between 79.44-121.82%) Passed

Table 5.27: Summarized aerosolization characteristics of optimized sildenafil citrate conventional DPI and other formulations prepared at optimized spray drying conditions (mean ± SD, n=3):

S.No.	Batch code	Lipids composition	% Recovered dose	% Emitted dose	%FPF	% Dispersibility	MMAD (μm)	GSD	DDU (75-125% of Average as per USP)
1	**CD3**	**Drug with LH200 and PH 350M**	99.30 ± 0.02	75.88 ± 0.43	36 ± 0.02	76.41±0.32	6.12 ± 2.23	2.26± 1.92	(Between 75.07-124.09%) Passed
2	**DSS7**		99.05 ± 1.78	85.13 ± 1.15	54.04 ± 0.33	85.95±0.54	2.96 ± 1.83	1.21 ± 0.95	(Between 81.08-120.75%) Passed
3	**DLS7**	**HSPC, DPPC and cholesterol**	98.65 ± 0.39	83.63 ± 0.46	30.05 ± 0.39	84.77±0.48	2.125± 0.5	1.23 ± 0.52	(Between 78.29-110.13%) Passed
4	**DPL7**	**HSPC, DPPC and cholesterol**	98.69 ± 0.31	89.36 ± 0.48	62.01 ± 0.09	90.55±0.31	4.095± 0.52	1.21± 0.76	(Between 89.87-106.66%) Passed
5	DPL14	HSPC and cholesterol	98.18 ± 0.46	82.25 ± 0.29	51.73 ± 0.43	83.77±0.26	3.265± 0.6	1.76± 2.61	(Between 79.43-117.52%) Passed
6	DPL15	DPPC and cholesterol	98.83 ± 0.69	80.11 ± 0.38	47.88 ± 0.49	81.06±0.15	4.81± 0.41	2.68± 1.57	(Between 75.99-120.68%) Passed
7	**LPL16**	**HSPC and DPPC**	98.76 ± 0.56	94.72 ± 0.36	82 ± 0.42	95.91±0.38	4.64 ± 0.17	1.01± 0.89	(Between 95.02-108.17%) Passed

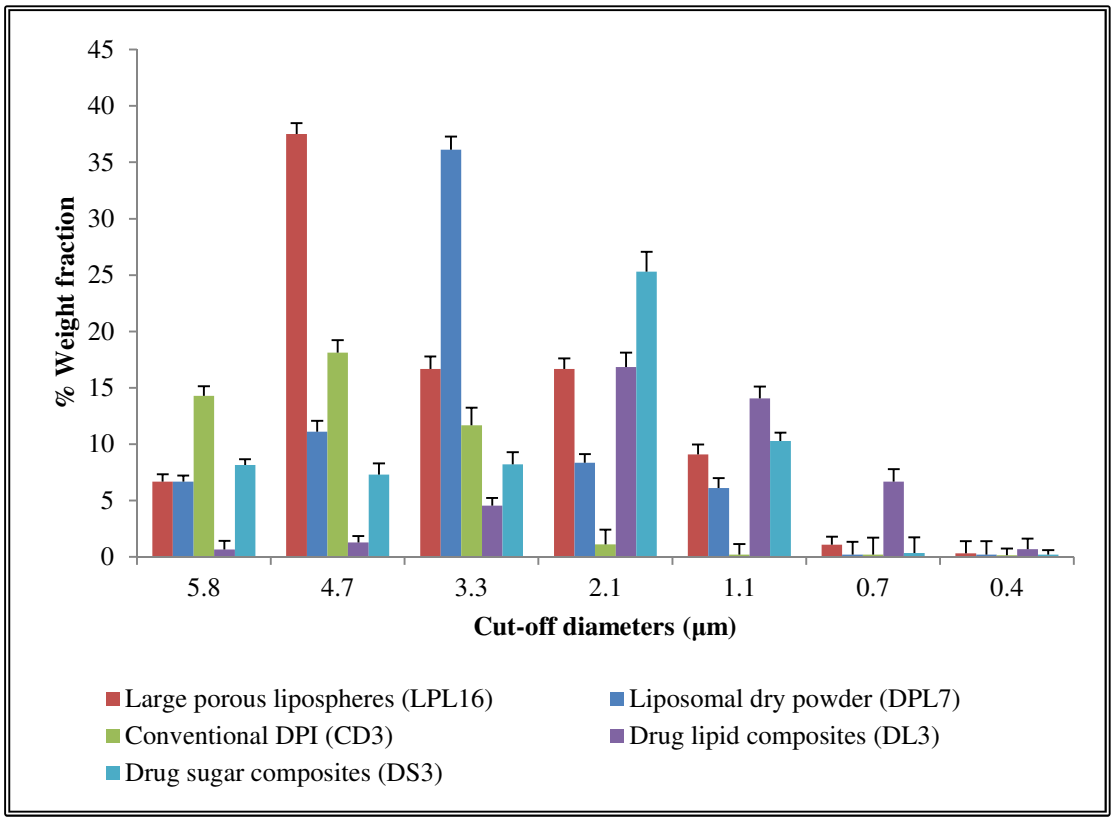

Fig. 5.106: Comparison of *in-vitro* powder deposition on various stages of Andersen Cascade Impactor among different dry powder formulations of sildenafil citrate.

5.13 *In-Vitro* Release study:

Formulations optimized with respect to percent yield, percent drug content, moisture content and percent respirable fraction i.e. %FPF were considered further for *in-vitro* and *in-vivo* studies. An *in-vitro* release study of the sildenafil citrate dry powder formulations was performed by using dialysis bag method in rotating bottle dissolution apparatus (Electrolab). The samples drawn at different time intervals for different optimized dry powder formulations were analysed to further calculate the percent release from various formulations. The concentrations of sildenafil citrate were determined by the UV spectrophotometer UV–Visible system (Shimadzu UV-1601, Japan) at 292 nm. Each test was conducted six times and data from all experiments are expressed, as mean ± SD (**Table 5.29** and **5.30**). The data were compared using ANOVA and Student's t-test and difference at $p<0.05$ were considered significant. According to the determined concentrations of released sildenafil citrate, cumulative percent release (Q) was determined at each time point

(t). Different graphs Q vs t for Zero order, log Q vs t for First order, Q vs √ t for Higuchi model, cube root of Q vs t for Hixson crowell and log Q vs log t for Korsmeyer-peppas were plotted to evaluate the best fit release kinetic model for various sildenafil citrate dry powder formulations.

Table 5.28: Dry powder formulations of sildenafil citrate selected for *in-vitro* release studies:

S. No.	Batch No.	Formulation Code	Description
1	CD3	F1	Conventional dry powder for inhalation (CDPI) of sildenafil citrate with lactose carriers
2	DSS7	F2	Drug-sugar composites of sildenafil citrate with mannitol prepared by spray drying technique
3	DPL7	F3	Liposomal dry powder for inhalation (LDPI) prepared by thin film hydration and spray drying
4	DLS7	F4	Drug-lipid composites of sildenafil citrate prepared by spray drying technique
5	LPL16	F5	Large porous lipospheres prepared by emulsification and spray drying technique

In case of conventional DPI (formulation F1), almost all the drug was released within 1h. Spray dried formulation, drug-sugar composites (F2) showed even faster release of 55.93±0.01% within 5min and whole amount of drug was released in less than 45min. This might be due to the increased surface area of spray dried sildenafil citrate due to its decreased particle size and spherical particle shape as compared to the crystalline nature of formulation F1. Formulations F1 and F2 showed best fitting with first order release kinetic model **(Table 5.29** and **Fig. 5.109**). However, all lipid based formulations were able to sustain the release for more than 24hours **(Table 5.30).** *In-vitro* release profile suggested a biphasic release pattern for liposomal dry powder formulation (F3). The phenomenon of burst release was seen because of the release of drug adsorbed on the surface of the particles and development of a high concentration gradient across the particle surface. Formulation F3 showed *in-vitro* release closer to Korsmeyer-peppas kinetic model and release could be sustained upto 34h. 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 **(Fig. 5.112)**. Summary of model fitting parameters for *in-vitro* release pattern of various sildenafil citrate dry powder formulations is shown in **Table 5.31.**

Table: 5.29 Release profile of conventional DPI and drug sugar composites of sildenafil citrate

S. No.	Time (h)	Mean cumulative percent drug release (Mean±S.D., n=6)	
		Conventional DPI (F1)	Drug sugar composites (F2)
1	0.083	29.167±1.01	55.938±0.01
2	0.167	39.219±0.95	63.719±0.83
3	0.25	48.422±0.29	78.333±0.35
4	0.334	56.984±2.44	87.161±0.62
5	0.5	68.380±0.35	96.286±1.63
6	0.75	85.328±1.42	101.599±1.48
7	1	99.578±0.94	

Table: 5.30. Release profile of lipid based dry powder formulations of sildenafil citrate

S. No.	Time (h)	Mean cumulative percent drug release (Mean±S.D., n=6)		
		Liposomal dry powder for inhalation (F3)	Drug lipid composites (F4)	Large porous lipospheres (F5)
1	0.083	0.019±1.65	10.992±2.20	4.869±1.11
2	0.167	0.551±2.98	22.314±1.87	9.582±3.00
3	0.25	1.553±1.80	31.224±0.11	19.263±0.42
4	0.334	2.231±1.39	42.593±3.45	26.754±2.10
5	0.5	2.952±1.74	48.017±0.17	33.772±0.25
6	0.75	3.389±1.86	56.667±0.95	36.205±1.58
7	1	3.853±3.85	64.778±1.42	39.908±1.44
8	1.5	9.476±1.73	70.02±0.53	46.339±0.82
9	2.5	20.754±2.04	75.078±0.68	53.376±0.31
10	4	32.762±2.65	80.841±1.89	59.652±1.26
11	6	45.314±0.39	86.620±2.34	65.524±2.6
12	8	53.702±0.17	92.103±6.12	70.930±0.28
13	10	60.522±0.69	95.861±2.08	77.517±1.92
14	12	67.745±0.49	98.348±0.17	83.994±0.37
15	16	73.680±0.88	98.876±1.76	89.245±0.74
16	18	78.014±3.42	99.529±0.66	92.818±1.87
17	20	79.955±0.76	99.981±1.83	94.232±0.49
18	24	85.020±1.23	100.725±1.68	96.546±0.75
19	30	94.620±0.86	-	98.690±1.54
20	36	99.063±3.31	-	99.839±0.86

Table 5.31: Summarized model fitting parameters for *in-vitro* release of sildenafil citrate dry powder formulations

Model	Parameter	Conventional DPI (F1)	Drug sugar composites (F2)	Drug lipid composites (F4)	Liposomal dry powder for inhalation (F3)	Large porous lipospheres (F5)
Zero Order	Equation	y = 12.785x - 4.1456	y = 14.605x + 10.586	y = 5.6067x + 11.016	y = 5.5121x - 22.596	**y = 4.9119x + 1.7289**
	R square	R2 = 0.9732	R2 = 0.8314	R2 = 0.9252	R2 = 0.9456	**R2 = 0.9651**
First Order	Equation	**y = 0.0866x + 1.3189**	**y = 0.0537x + 1.6544**	y = 0.0435x + 1.3353	y = 0.1302x - 0.4252	y = 0.0477x + 1.1118
	R square	**R2 = 0.9925**	**R2 = 0.9649**	R2 = 0.7574	R2 = 0.7692	R2 = 0.7704
Higuchi	Equation	y = 14.605x + 10.586	y = 14.605x + 10.586	y = 5.6067x + 11.016	y = 5.5121x - 22.596	**y = 4.9119x + 1.7289**
	R square	R2 = 0.9732	R2 = 0.8314	R2 = 0.9252	R2 = 0.9456	**R2 = 0.9651**
Hixson-Crowell	Equation	y = -0.518x + 5.5141	y = -0.656x + 4.6295	**y = -0.2457x+ 4.9361**	y = -0.1864x + 5.5411	y = -0.2015x + 5.1615
	R square	R2 = 0.7701	R2 = 0.8855	**R2 = 0.9817**	R2 = 0.8808	R2 = 0.9596
Korsmeyer-peppas	Equation	y = 0.0816x + 1.5949	y = 0.0495x + 1.7773	y = 0.0038x + 1.9819	**y = 0.0228x + 1.7861**	y = 0.0108x + 1.9075
	R square	R2 = 0.9895	R2 = 0.9452	R2 = 0.8783	**R2 = 0.9634**	R2 = 0.8313

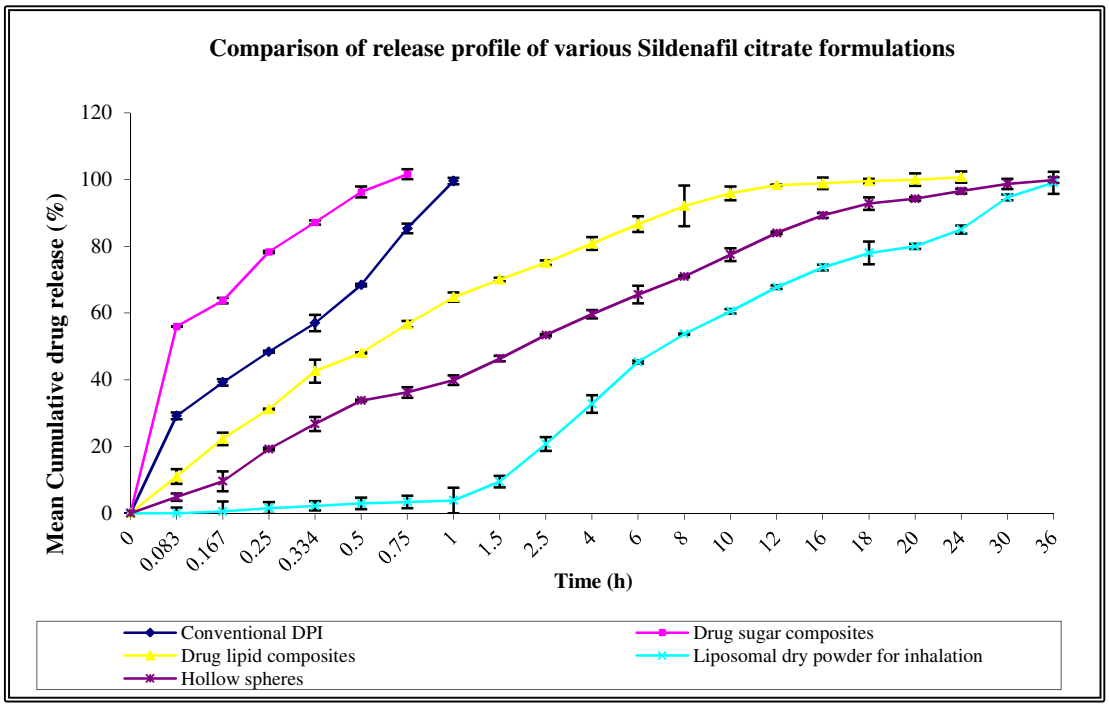

Fig. 5.107: Comparison of mean cumulative percent release profile of various Sildenafil citrate dry powder formulations.

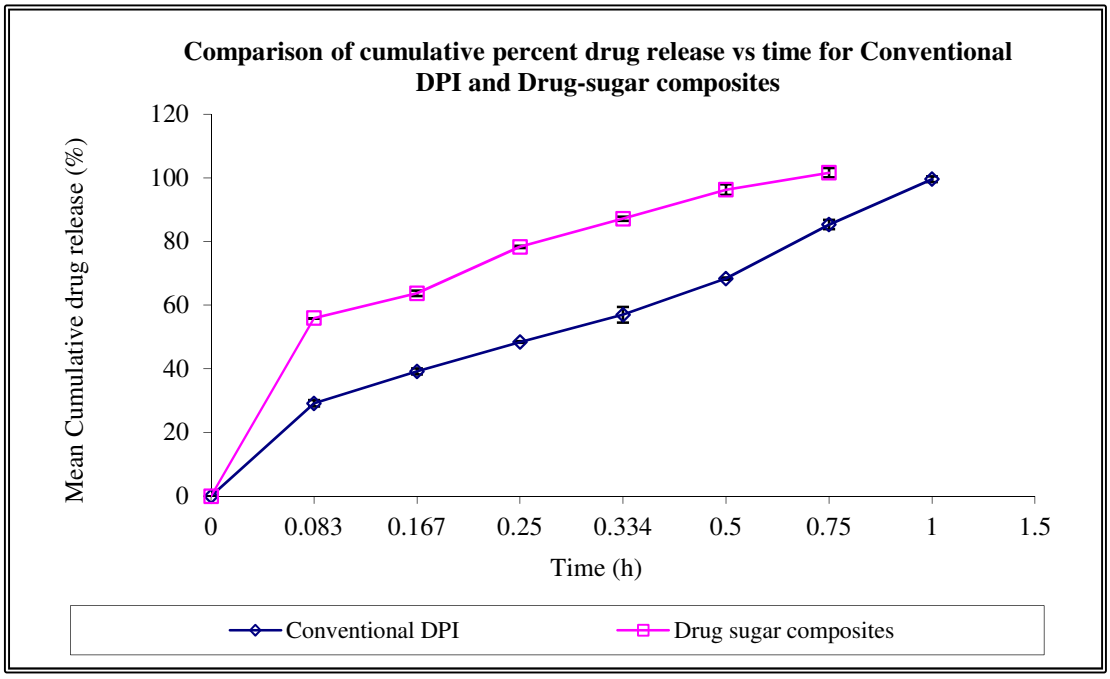

Fig. 5.108: Comparison of cumulative percent drug release vs time for Conventional DPI and Drug-sugar composites

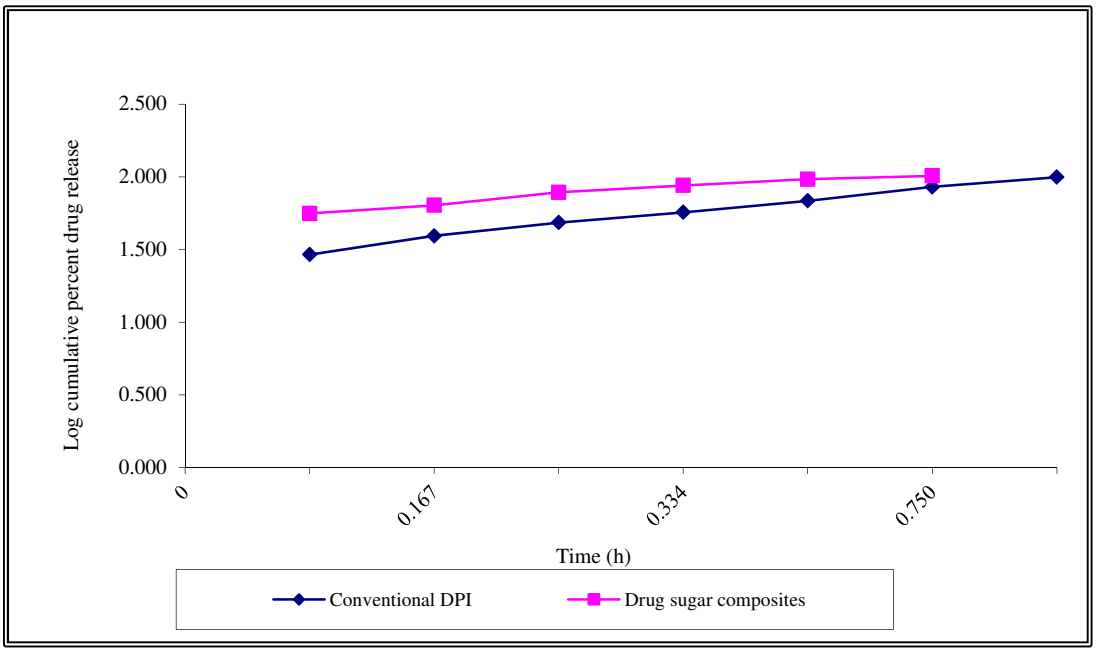

Fig. 5.109: Comparison of log cumulative percent drug release vs time (First order model fitting) for Conventional DPI and Drug-sugar composites

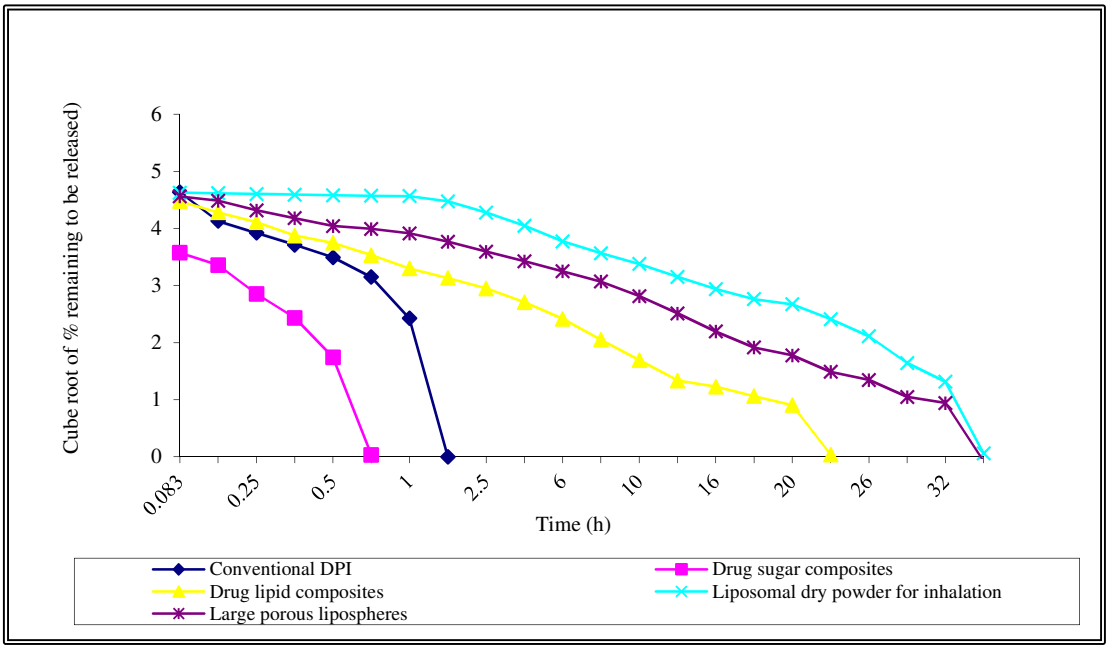

Fig. 5.110: Hixson's Crowell model fitting for various Sildenafil citrate dry powder formulations

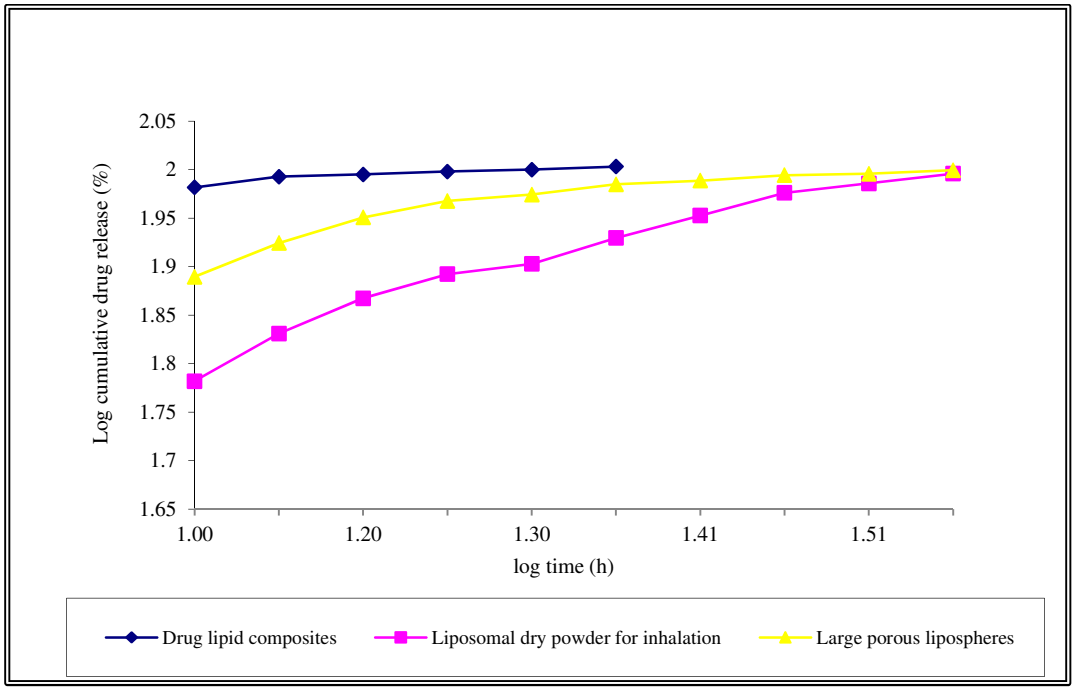

Fig. 5.111: Korsmeyer-peppas model fitting graph [Log cumulative drug release (%) vs log time (h)] for lipid based dry powder formulations of sildenafil citrate

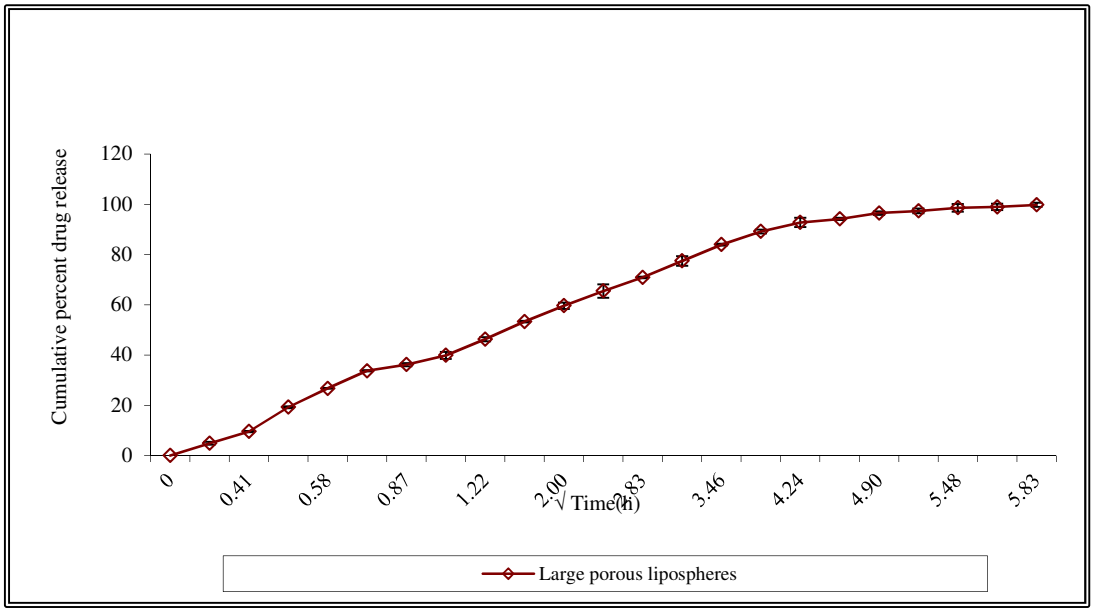

Fig. 5.112: Higuchi's model fitting graph (Cumulative percent drug release vs √ Time in h) for sildenafil citrate loaded large porous lipospheres

5.14 Stability studies of sildenafil citrate dry powder formulations:

Samples of all dry powder formulations F1 to F5 were taken out at specified times during the period of stability study and evaluated with respect to % assay, % drug retained, %w/w of moisture content, MMAD , % emitted dose and % FPF. HDPE bottles were allowed to reach room temperature and wiped dry with tissue paper, before opening, to avoid any moisture uptake by capsules during sampling. All the parameters were determined as per the methods described earlier in "Preparation and Characterization section".

In case of conventional DPI (Formulation F1), there was no significant loss in Percent drug assay in 6M (97.93 ± 0.79%) at 40°C/75%RH as compared to initial value (102.15 ± 0.30). Initial moisure content of the formulation was 5.11±1.08%w/w and there was approximately 2% increase observed during 6M storage (7.91 ± 0.99% w/w) at accelerated stability condition. %FPF was dropped from 36 ± 0.02% to 28 ± 0.48% after 12M long term storage stability condition. This might be due to the increase in moisture content during storage that led to decrease in %ED (from 75.88 ± 0.43% to 53.08 ± 0.31%) and %FPF dropped to 22 ± 0.49% at 6M accelerated condition too. Must be due to same reason, MMAD was also increased from 6.12 ± 2.23µm to 9.23 ± 2.23µm **(Table 5.32).**

Spray dried formulation of drug-sugar composites, Formulation F2, showed significantly ($p<0.05$) enhanced stability as compared to conventional DPI as there was no significant change in any of the parameters even after 6M of accelerated stability condition and 12M of long term stability. Spray dried formulation does not consist of any drug agglomerates as compared to conventional DPI and the surface of the spherical particles was smooth and stable due to the presence of carrier mannitol with an excellent free flowing property with negligible tendency of moisture uptake (Naini V. *et al*. 1998). This was the reason for maintained %FPF (54.04 ± 0.33) of co-spray dried drug-sugar composites even after 6M (52.04 ± 1.09%) of accelerated stability condition and 12M (53.11 ± 1.07%) of long term stability **(Table 5.33).**

Spray dried lipid based formulation need different storage stability conditions as compared to the only sugar based dry powder formulations. Since lipids have tendency to degrade and leach drug at higher temperatures, 30°C/65%RH was

considered as accelerated stability condition and samples were also analysed at 5±3°C (2-8°C) to decide the better storage conditions for the lipid based dry powder formulations.

Liposomal dry powder formulation (F3) showed better stability at 25°C/60%RH (Intermediate stability) and at 5±3°C (2-8°C). Although there was only 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%. There was no significant ($p<0.05$) change in % assay, % drug retained (97.23 ± 1.99% and 98.11 ± 0.96%), % moisture content (1.21 ± 1.11%w/w and 1.89 ± 2.00% w/w) and any of the aerosolization parameters (%FPF 60.62 ± 0.33% and 60.77 ± 0.27%) at 25°C/60% RH and at 5±3°C (2-8°C) respectively **(Table 5.34).** 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.

Sildenafil citrate-lipid composite 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 12M when compared to initial value (30.05 ± 0.39%). At other storage conditions 30°C/65%RH accelerated stability (11.89± 1.55% w/w) and 25°C/60%RH (13.22±1.39% w/w) formulation F4 showed significant moisture uptake ($p<0.05$) as compared to initial value (2.69 ± 0.09%w/w) which ultimately affected the aerosolization properties of the formulation. There was significant drop in %ED (67.61 ± 1.56%) at 12M 25°C/60%RH as compared to initial value of 83.63 ± 0.46% and % FPF was reduced to 18.69 ± 1.36% and 19.45 ± 1.02% at 6M 30°C/65%RH accelerated stability condition and 12M 25°C/60%RH respectively **(Table 5.35).** Presence of trehalose in the formulations can make the product hygroscopic during storage. It has been reported in literature that storing trehalose in ambient air having a relative humidity of 40-50%, a sticky residue is formed due to its supersaturation which changes its glass transition temperature from 97°C to 31°C (F. Franks *et al*. 1993; B.J. Aldous *et al*. 1995; L.M. Crowe *et al*. 1996). Thus trehalose can stabilize the product only if it is stored under less humid conditions at 5±3°C.

Table 5.32: Stability data of Conventional DPI Formulation (CD3 or Formulation F1)

S. No.	Parameters	**Conventional DPI Formulation (CD3 or F1) Mean** ± S.D. (n=3)							
		Initial	40°C/75%RH			25°C/60%RH (Long term stability)			
		0	1M	3M	6M	3M	6M	9M	12M
1	Appearance	WFP	WFP	WFP	WFP	WFP	WFP	WFP	WFP
2	Assay (%)	102.15 ± 0.30	100.02 ± 0.56	98. 75 ± 0.81	97.93 ± 0.79	102.23 ± 0.54	101.96 ± 0.30	99.62 ± 1.05	99.05 ± 0.46
3	Moisture content (%w/w)	5.11 ± 1.08	6.45 ± 0.51	7.12 ± 0.65	7.91 ± 0.99	4.99 ± 1.02	5.43 ± 1.18	5.98 ± 0.72	6.03 ± 0.58
5	Emitted dose (%)	75.88 ± 0.43	70.52 ± 0.62	62.15 ± 0.27	53.08 ± 0.31	73.02 ± 0.33	74.58 ± 0.28	72.03 ± 0.37	67.29 ± 0.5
6	%FPF	36 ± 0.02	31 ± 0.51	25 ± 0.96	22 ± 0.49	40 ± 0.36	33 ± 0.51	30± 0.43	28 ± 0.48
7	MMAD (µm)	6.12 ± 2.23	7.66 ± 2.23	7.91± 2.23	9.23 ± 2.23	6.08 ± 2.23	7.11 ± 2.23	7.25 ± 2.23	9.13 ± 2.23
8	GSD	2.26± 1.92	2.22 ± 0.89	2.76 ± 2.03	2.41 ± 1.55	2.11 ± 0.93	2.32 ± 0.85	2.21 ± 2.03	2.75 ± 1.24

WFP= White free flowing powder

Table 5.33: Stability data of Drug-sugar composites (DS3 or Formulation F2)

WFP= White free flowing powder

S. No.	Parameters	**Drug-sugar composites (DS3 or F2) Mean** ± S.D. (n=3)							
		Initial	40°C/75%RH			25°C/60%RH (Long term stability)			
		0	1M	3M	6M	3M	6M	9M	12M
1	Appearance	WFP	WFP	WFP	WFP	WFP	WFP	WFP	WFP
2	Assay (%)	99.61 ± 0.53	100.1 ± 0.88	99.81 ± 0.36	98.82 ± 0.91	99.75 ± 0.56	100.22 ± 0.91	100.05 ± 0.86	99.14 ± 0.33
3	Moisture content (%w/w)	3.07 ± 0.82	3.11 ± 0.42	3.24 ± 0.57	3.55 ± 0.43	3.04 ± 0.87	3.17 ± 0.39	3.22 ± 1.22	3.26 ± 0.94
5	Emitted dose (%)	85.13 ± 1.15	83.22 ± .075	81.42 ± 0.54	80.27 ± 0.61	84.99 ± .050	85.06 ± 0.79	82.58 ± 0.83	82.01 ± 1.22
6	%FPF	54.04 ± 0.33	54.04 ± 1.02	54.04 ± 0.87	52.04 ± 1.09	55.04 ± 0.39	62.17 ± 0.71	53.04 ± 1.11	53.11 ± 1.07
7	MMAD (µm)	2.96 ± 1.83	3.01 ± 1.11	3.15 ± 1.34	3.62 ± 0.97	2.33 ± 1.25	2.85 ± 0.95	2.92 ± 1.03	3.11 ± 1.06
8	GSD	1.21 ± 0.95	1.32 ± 0.87	1.22 ± 1.15	2.12 ± 1.12	1.24 ± 1.22	1.36 ± 0.86	1.66 ± 0.53	1.68 ± 1.09

Sildenafil citrate loaded large porous liposphere formulation (F5) was stable at 25°C/60%RH and at 5±3°C (2-8°C) storage conditions. At 6M 30°C/65%RH accelerated stability condition a drop in the initial assay (from 99.02 ± 0.93% to 94.17± 1.65%) and significant ($p<0.05$) fall in percent drug retained (from 97.86 ± 1.42% to 86.40± 2.14%) was observed. %FPF was also dropped from 82.00 ± 0.42% to 65.15 ± 2.01%. However, there was no significant ($p<0.05$) change in % assay, % drug 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 **(Table 5.36).** Thus sildenafil citrate loaded large porous liposphere formulation (F5) is recommended to be stored below 25°C/60%RH or at 5±3°C (2-8°C) to attain its excellent storage stability.

Table 5.34: Stability data of sildenafil citrate liposomal Dry powder for inhalation (DPL7 or F3)

S. No.	Parameters	Liposomal Dry powder for inhalation (DPL7 or F3) Mean ± S.D. (n=3)											
		Initial	30°C/65%RH (Accelerated stability)			25°C/60%RH (Intermediate stability)				2-8°C (Long term stability)			
		0	1M	3M	6M	3M	6M	9M	12M	3M	6M	9M	12M
1	Appearance	WFP	WFP	PYP	PYP	WFP	WFP	WFP	WFP	WFP	WFP	WFP	WFP
2	Assay (%)	100.11 ± 1.83	100.02 ± 1.14	97.11 ± 1.22	95.11 ± 0.96	101.05 ± 1.03	100.03 ± 1.11	99.99 ± 0.87	98.85 ± 1.55	100.26 ± 2.03	100.41 ± 1.16	99.87 ± 0.87	100.00 ± 1.38
3	Percent drug retained	98.79 ± 1.42	97.79 ± 1.12	95.79 ± 1.17	92.79 ± 1.01	98.21± 2.02	97.54 ± 0.69	98.28 ± 0.73	97.23 ± 1.99	99.01 ± 1.07	97.52 ± 1.38	97.46 ± 2.14	98.11 ± 0.96
4	Moisture content (%w/w)	1.06 ± 2.24	2.01 ± 1.12	2.16 ± 0.77	4.25 ± 1.35	0.97 ± 0.69	1.32 ± 1.12	1.87 ± 1.57	1.21 ± 1.11	1.23 ± 2.05	1.16 ± 1.93	1.91 ± 1.66	1.89 ± 2.00
5	Emitted dose (%)	89.36 ± 0.48	85.22 ± 0.71	82.07 ± 0.85	78.11 ± 1.07	88.58± 0.22	87.99 ± 0.75	88.01 ± 0.50	86.22 ± 0.46	86.27 ± 0.37	85.29 ± 0.28	86.22 ± 0.56	86.98 ± 1.05
6	%FPF	62.01 ± 0.09	58.82 ± 0.51	55.71 ± 0.97	48.18 ± 0.53	62.27 ± 0.50	61.38 ± 0.49	60.62 ± 0.33	60.96 ± 0.52	64.55 ± 0.37	60.83 ± 0.39	61.97 ± 0.18	60.77 ± 0.27
7	MMAD (µm)	4.09± 0.52	5.02± 1.14	6.03± 0.55	9.13± 0.62	4.96± 0.83	4.58± 0.75	5.11± 1.43	4.96± 2.12	5.66± 0.99	4.92± 1.33	4.76± 1.69	5.54± 0.83
8	GSD	1.21± 0.76	2.22± 1.02	2.24± 2.15	2.35± 2.26	1.02± 1.99	1.13± 0.92	1.33± 1.05	1.34± 1.72	1.66± 2.11	1.37± 0.91	1.39± 0.89	1.28± 1.04

WFP= White free flowing powder; PYP= Pale yellow powder

Table 5.35: Stability data of Drug-Lipid composites (DL3 or F4)

S. No.	Parameters	Drug-Lipid composites (DL3 or F4) Mean ± S.D. (n=3)											
		Initial	**30°C/65%RH (Accelerated stability)**			**25°C/60%RH (Intermediate stability)**				**2-8°C (Long term stability)**			
		0	**1M**	**3M**	**6M**	3M	6M	9M	12M	3M	6M	9M	12M
1	Appearance	WFP	WFP	PYP	PYP	WFP	WFP	WFP	WFP	WFP	WFP	WFP	WFP
2	Assay (%)	98.90 ± 0.06	96.22 ± 1.14	95.13 ± 1.28	92.18 ± 2.01	97.55 ± 1.09	95.76 ± 1.22	93.33 ± 1.64	90.19 ± 0.95	99.01 ± 0.88	98.61 ± 1.33	96.92 ± 1.90	95.15 ± 2.14
3	Percent drug retained	98.82 ± 1.33	94.02 ± 1.02	91.60 ± 2.54	84.52 ± 2.063	96.43 ± 1.04	94.11 ± 0.83	94.62 ± 1.65	90.15 ± 1.63	99.31 ± 2.01	99.08 ± 2.65	98.66 ± 1.18	98.10 ± 1.31
4	Moisture content (%w/w)	2.69 ± 0.09	3.57±1.11	6.44± 1.04	11.89± 1.55	3.12±1.17	4.88± 0.99	9.36± 1.62	13.22±1.39	3.04± 1.81	4.11± 1.05	5.52±2.01	6.53± 0.68
5	Emitted dose (%)	83.63 ± 0.46	78.22 ± 0.57	73.99± 0.95	67.61 ± 1.56	83.63 ± 0.84	83.63 ± 1.67	83.63 ± 0.66	83.63 ± 0.89	82.16 ± 0.27	82.59 ± 1.05	80.47 ± 1.26	75.55 ± 0.77
6	%FPF	30.05 ± 0.39	25.03 ± 0.48	21.22 ± 1.98	18.69 ± 1.36	32.64 ± 2.48	28.87 ± 0.29	23.56 ± 0.60	19.45 ± 1.02	31.19 ± 0.55	29.66 ± 0.25	30.85 ± 0.47	28.66 ± 0.44
7	MMAD (µm)	2.13± 0.5	5.66± 0.93	11.89± 1.66	13.25± 0.58	3.29± 0.87	4.55± 0.54	5. 26± 0.28	10.25± 0.79	2.86± 0.88	3.95± 0.32	4.45± 0.91	3.98± 0.75
8	GSD	1.23 ± 0.52	2.26 ± 1.12	2.52 ± 0.98	2.39 ± 2.11	1.67 ± 0.66	1.66 ± 0.93	1.73 ± 1.05	2.23 ± 0.83	1.39 ± 1.12	1.41 ± 0.69	1.36 ± 2.01	1.66 ± 1.86

WFP= White free flowing powder; PYP= Pale yellow powder

Table 5.36: Stability data of sildenafil citrate loaded Large porous lipospheres (LPL16 or Formulation F5)

S. No.	Parameters	Large porous lipospheres (LPL16 or F5) Mean ± S.D. (n=3)											
		Initial	30°C/65%RH (Accelerated stability)			25°C/60%RH (Intermediate stability)				2-8°C (Long term stability)			
		0	1M	3M	6M	3M	6M	9M	12M	3M	6M	9M	12M
1	Appearance	WFP	WFP	WFP	PYP	WFP	WFP	WFP	WFP	WFP	WFP	WFP	WFP
2	Assay (%) ± SD	99.02 ± 0.93	95.21±2.35	96.12± 2.01	94.17± 1.65	102.43± 1.32	100.12± 2.09	99.95± 2.54	97.62± 1.82	101.98± 2.34	100.29± 1.08	100.78± 1.47	101.0± 1.25
3	Percent drug retained ± SD	97.86 ± 1.42	90.22± 3.29	83.73± 2.47	86.40± 2.14	99.49± 0.59	98.27±2.08	98.36± 2.43	99.07± 1.79	99.34± 2.21	100.46± 2.83	98.06± 3.85	99.98± 2.36
4	Moisture content (%w/w) ± SD	0.97 ± 1.16	2.07±2.7	3.24± 3.45	4.39± 2.75	0.7± 2.02	1.11±1.15	1.23± 2.08	1.87± 1.98	1.21± 2.54	1.25± 2.78	1.81± 2.14	1.06± 2.86
5	Emitted dose (%)	94.72 ± 0.36	92.56 ± 1.04	90.11 ± 1.98	85.47 ± 0.63	94.00 ± 0.45	92.91±0.39	92.33 ± 0.27	94.15 ± 0.49	95.25 ± 0.55	93.66 ± 0.83	91.92 ± 0.08	93.97 ± 1.53
6	%FPF	82.00 ± 0.42	78.10 ± 0.48	70.23 ± 0.33	65.15 ± 2.01	84.11 ± 0.66	81.69±0.28	85.66 ± 0.09	80.17 ± 0.17	83.58 ± 0.95	80.96 ± 1.11	79.59 ± 0.38	80.99 ± 1.39
7	MMAD (μm)	4.64 ± 0.17	4.867± 1.08	5.12±0.9	9.07± 3.24	4.08± 0.8	4.33±0.60	4.03± 1.85	3.17± 2.37	3.10± 1.25	4.81± 0.8	4.43± 0.4	4.98± 2.45
8	GSD	1.01± 0.89	1.67± 0.91	1.66± 2.11	1.75± 1.34	1.36± 0.78	1.32± 0.56	1.29± 2.24	1.65± 0.21	1.67± 0.63	1.36± 0.93	2.01± 1.81	1.22± 0.59

WFP= White free flowing powder; PYP= Pale yellow powder

5.15 Macrophage uptake study:

5.15.1 Calibration curve of fluorescein and percent entrapment efficiency:

A calibration curve of fluorescein (free acid) in methanol and Dulbecco's Modified Eagle Medium (DMEM) showed good linearity (R^2=0.99 for both) in a concentration range from 0 to 200 nM **(Fig. 5.113, 5.114)**. Fluorescein RFU of the labeled plates was read on Fluorescence Microplate reader (FL_x 800, Biotec Instruments Inc. with Tungsten Halogen light source and a sensitivity of Fluorescein 5 pM typical, 1 fmol/well 96-well plate) using filters Ex: 485/20 and Em: 575/15. The same plates were covered well with aluminium foil and read again after 1day and there was no significant ($p<0.05$) change in %RFU value with time. The prepared formulations were coded from M1 to M4 for macrophage uptake study **(Table 5.37).** All the formulations were assayed for drug and fluorescein using UV spectroscopy and fluorescence plate reader respectively. Assayed amount of sildenafil citrate was 98.39 ± 1.02%, 99.76 ± 0.85%, 98.35 ± 0.49% and 101.25 ± 1.66% for formulations M1, M2, M3 and M4 respectively **(Table 5.37).** Fluorescein was added in 1:2.5 molar ratio with drug in all the formulations. Percent entrapment efficiency of fluorescein was 44.12 ± 1.64%, 56.23 ± 1.15%, 50.77 ± 0.83% and 58.47 ± 1.56% for formulations M1, M2, M3 and M4 respectively.

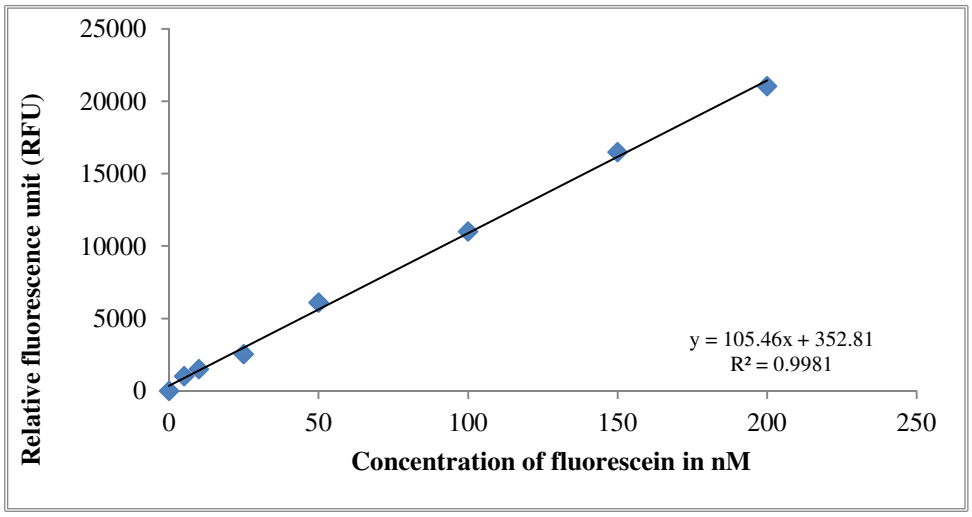

Fig. 5.113: Calibration curve of fluorescein in Methanol

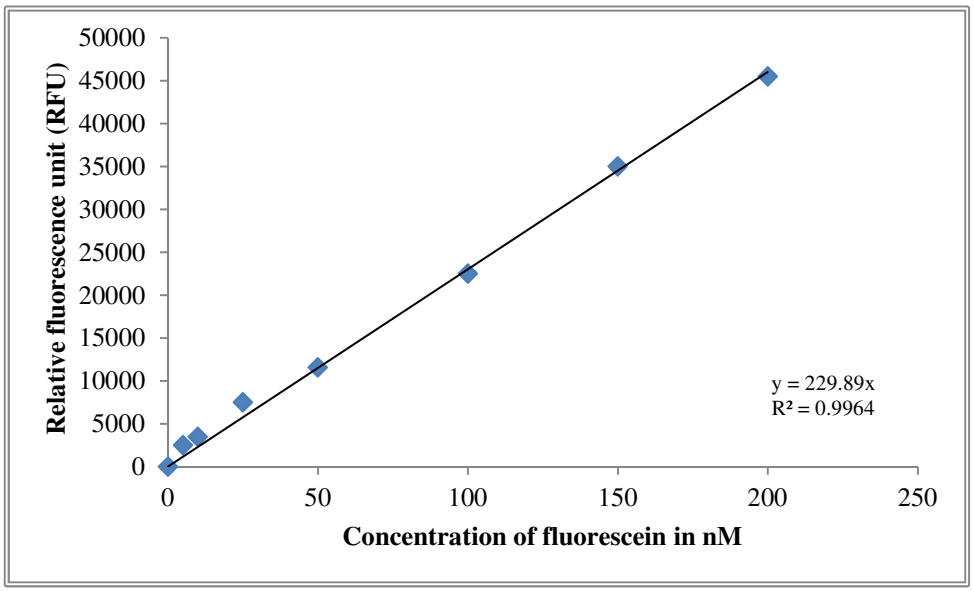

Fig. 5.114: Calibration curve of fluorescein in Dulbecco's Modified Eagle Medium (DMEM)

5.15.2 Percent macrophage uptake of different formulations:

During macrophage uptake study, initial RFU of the labeled plates was read on fluorescence microplate reader and compared with RFU in the samples after stopping the reaction at particular time points (30min, 1h, 2h, 4h, 12h and 24h). 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. Results of macrophage uptake are shown in **Table 5.37.**

The plates were also imaged using inverted olympus microscope with camera and DP controller software. Images of the alveolar macrophages, prepared formulations and standard beads diluted in DMEM were also taken while counting on hemacytometer **(Fig. 5.116-**

5.121). All lipid based formulations showed delayed and reduced macrophage uptake as compared to the standard fluorescent beads. Standard 2µm polystyrene fluorescent beads were taken upto 48.28±1.68% within 2h and all were phagcytozed by 4h. All views in its images showed standard beads too numerous to count **(Fig. 5.122, 5.123)**. However, uptake could be detected in drug- lipid composites (M3) after 1h and only 25.67±1.22% could be taken up by macrophage cells even after 24h **(Fig. 5.126).**

Liposomes composition also affects the macrophage uptake. Till 2h no macrophage uptake could be detected in case of liposomal dry powder formulation (M2) prepared with HSPC, cholesterol and inclusion of DPPC **(Fig. 5.125)**, however uptake could be observed within 2h with Liposomal dry powder formulation (M1) prepared with HSPC and cholesterol **(Fig. 5.124)**, but without DPPC. Even the extent of uptake of M2 was significantly ($p<0.05$) lower (3.28±0.99%) as compared to M1 (11.47±1.19%). It suggests that the addition of DPPC in the formulation may reduce the macrophage uptake by its presence on the surface of the particles and altering the cellular interactions occurring in the alveoli (Evora C, *et al*. 1998; Jones BG *et al*, 2002). No macrophage uptake was detected for even 12h in case of Large porous lipospheres (M4) and uptake was almost negligible (1.90±1.06%) even after 24h **(Fig. 5.127)** as compared to 100% uptake of Standard 2µm polystyrene fluorescent beads in 4h **(Fig. 5.115).**

Thus, it can be inferred from this study that though geometric particle size of 2µm-5µm is favorable for inhalation, but it shows enhanced uptake by alveolar macrophages at this size. Thus, light, large particles having aerodynamic particle size in range of 2µm-5µm, but geometric size >10µm are the best options for better lung deposition and longer stay in the lungs by avoiding phagocytosis by alveolar macrophages. Sildenafil itself has also been shown to have the tendency to reduce the influx of macrophages, eosinophils and neutrophils (Toward TJ *et al*. 2004; Haddad JJ *et al*. 2002). Thus, it seems encouraging to incorporate sildenafil citrate in lipid based formulations to have better control of the disease due to synergistic effects. Results are also consistent with *in-vivo* pulmonary kinetics of these formulations.

Table 5.37: Comparison of percent macrophage uptake of various sildenafil citrate dry powder formulations with 2µm polystyrene fluorescent standard beads

Time (h)	% Macrophage uptake (% of initial RFU)				
	M1	M2	M3	M4	Standard beads
	(Liposomal dry powder with HSPC and cholesterol)	(Liposomal dry powder with HSPC, DPPC and cholesterol)	(Drug-lipid composites)	(Large porous lipospheres)	(2µm polystyrene fluorescent beads)
0.50	0.00	0.00	0.00	0.00	7.24±1.05
1.00	0.00	0.00	0.00	0.00	25.65±0.98
2.00	0.32±0.98	0.00	0.43±1.11	0.00	48.28±1.68
4.00	3.35±1.24	0.29±1.16	4.59±1.02	0.00	99.76±1.21
12.00	6.98±1.55	3.17±1.51	7.70±1.45	0.37±1.33	-
24.00	11.47±1.19	3.28±0.99	25.67±1.22	1.90±1.06	-

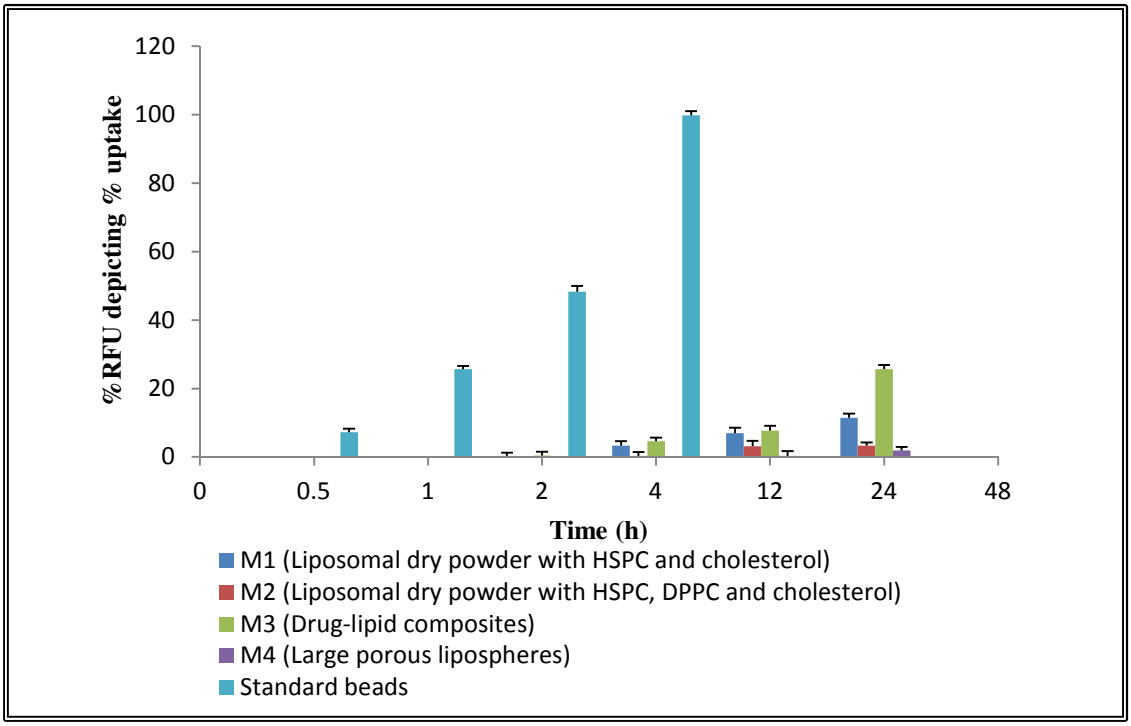

Fig. 5.115: Comparison of percent macrophage uptake (in terms of % RFU) of various sildenafil citrate dry powder formulations with 2µm polystyrene fluorescent standard beads

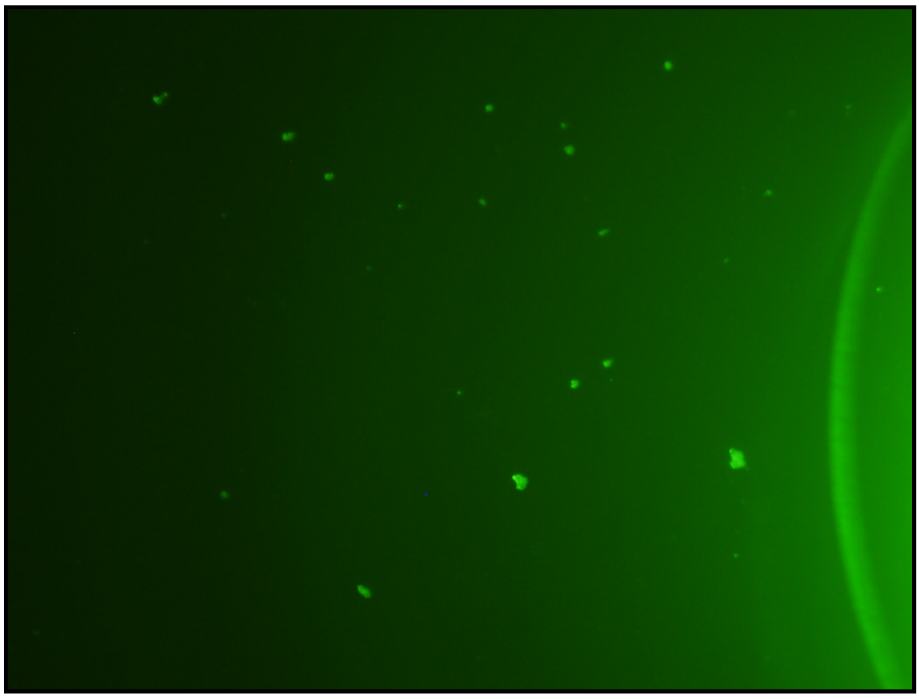

Fig. 5.116: Fluorescein and drug loaded Liposomal dry powder (M1) diluted in DMEM

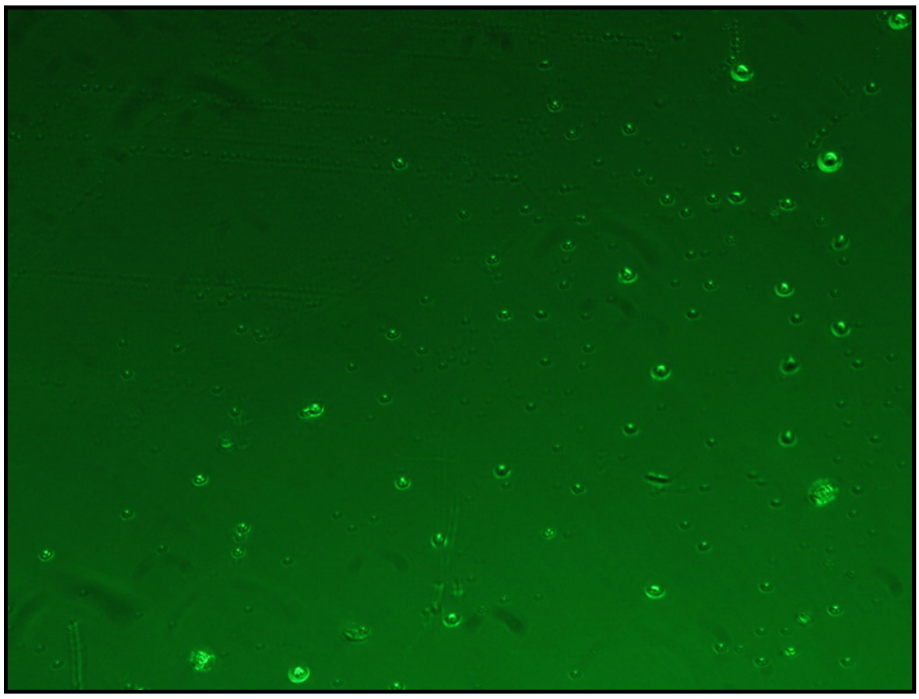

Fig. 5.117: Fluorescein and drug loaded Liposomal dry powder (M2) diluted in DMEM

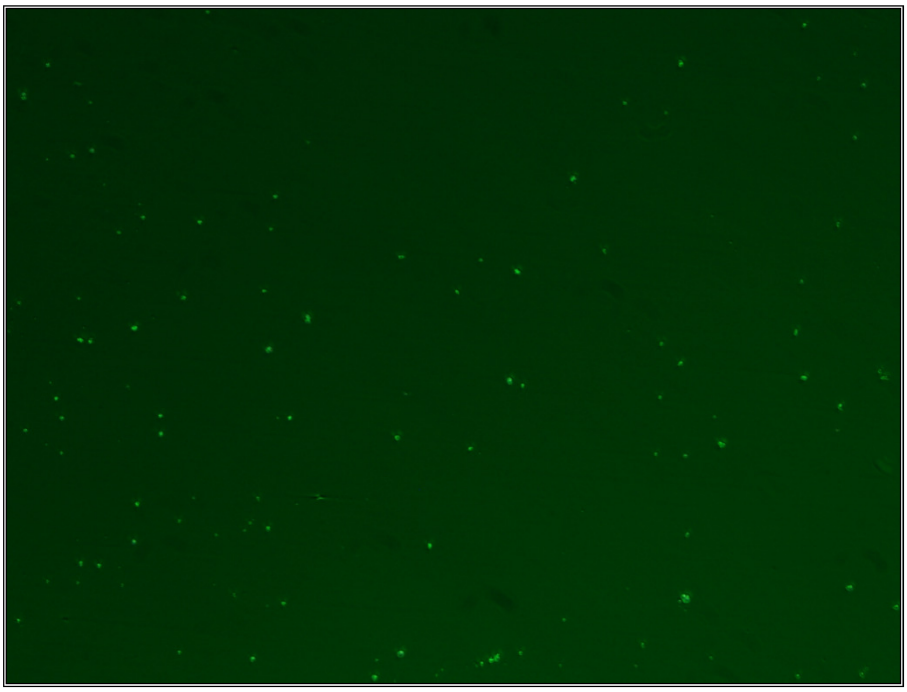

Fig. 5.118: Fluorescein and drug loaded lipid composites (M3) diluted in DMEM

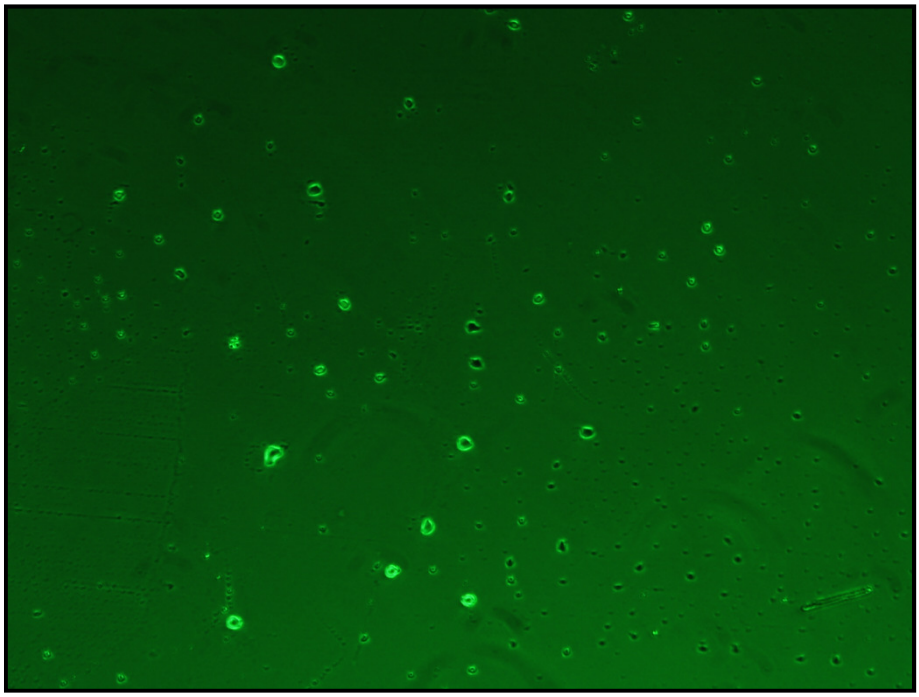

Fig. 5.119: Fluorescein and drug loaded large porous lipospheres (M4) diluted in DMEM

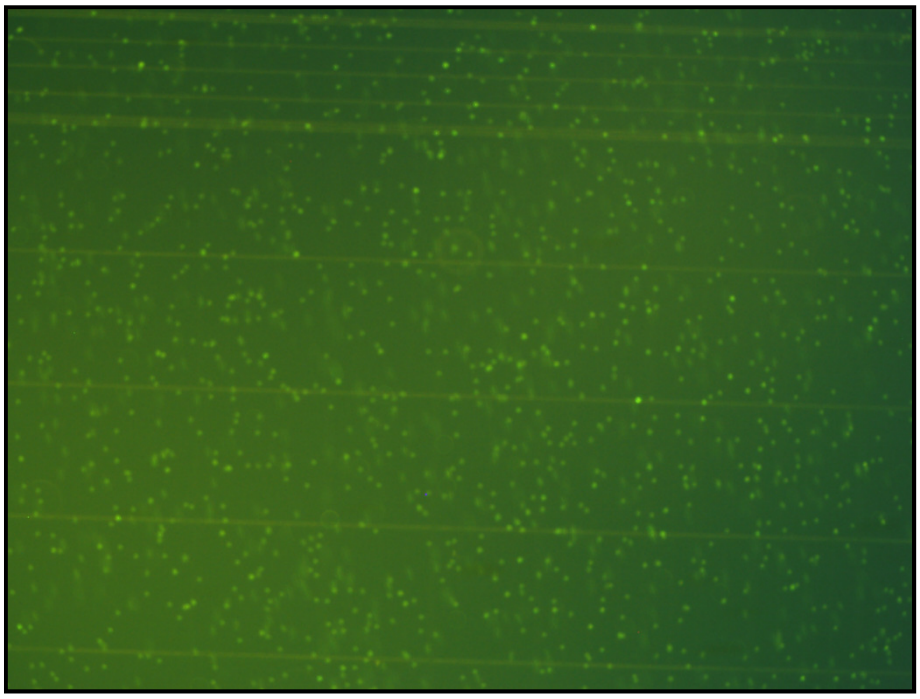

Fig. 5.120: Standard beads (2µm polystyrene fluorescent beads) diluted in DMEM

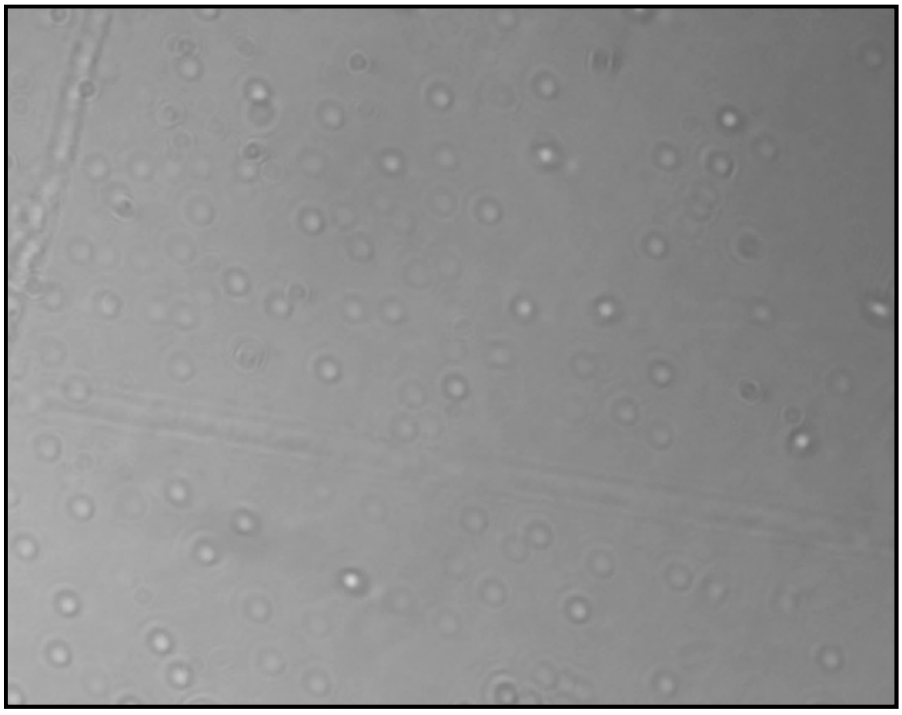

Fig. 5.121: Alveolar macrophages adhered to the walls

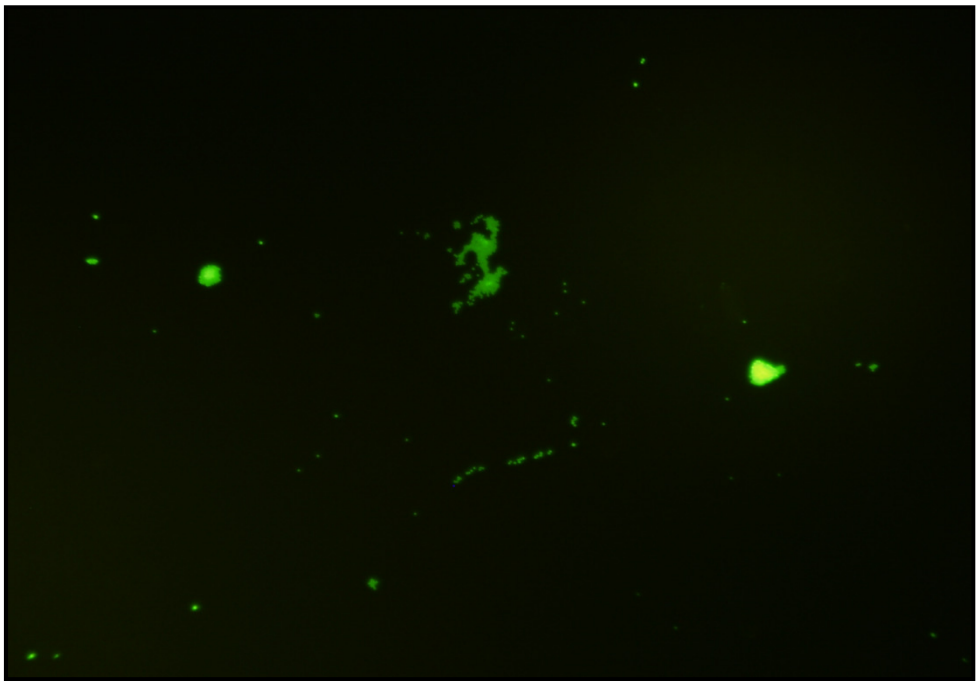

Fig. 5.122: A contrast view of standard beads (2µm polystyrene fluorescent beads) taken up by alveolar macrophage after 4h of study

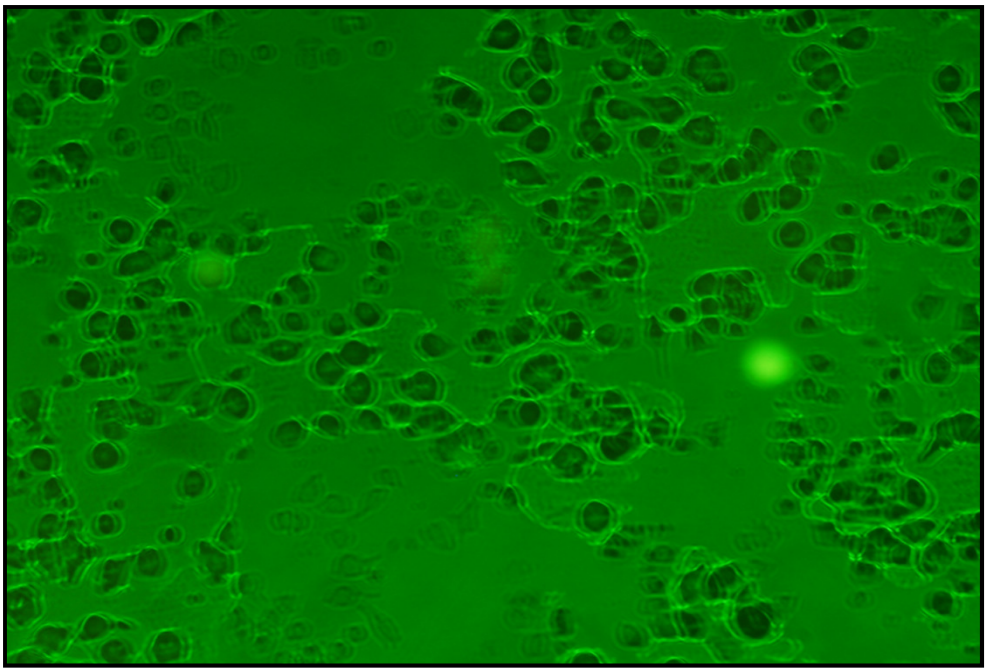

Fig. 5.123: A view of above figure revealing clear view of alveolar macrophage with blurred view of glowing standard beads (2µm polystyrene fluorescent beads) after 4h of study

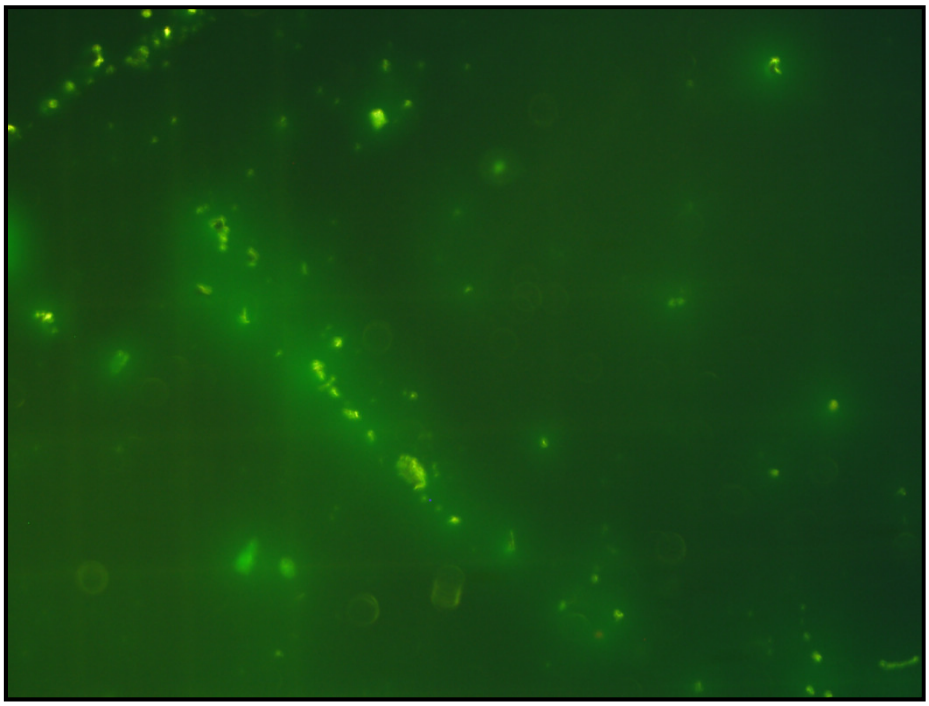

Fig. 5.124: A contrast view of formulation M1 taken up by alveolar macrophage after 24h of study

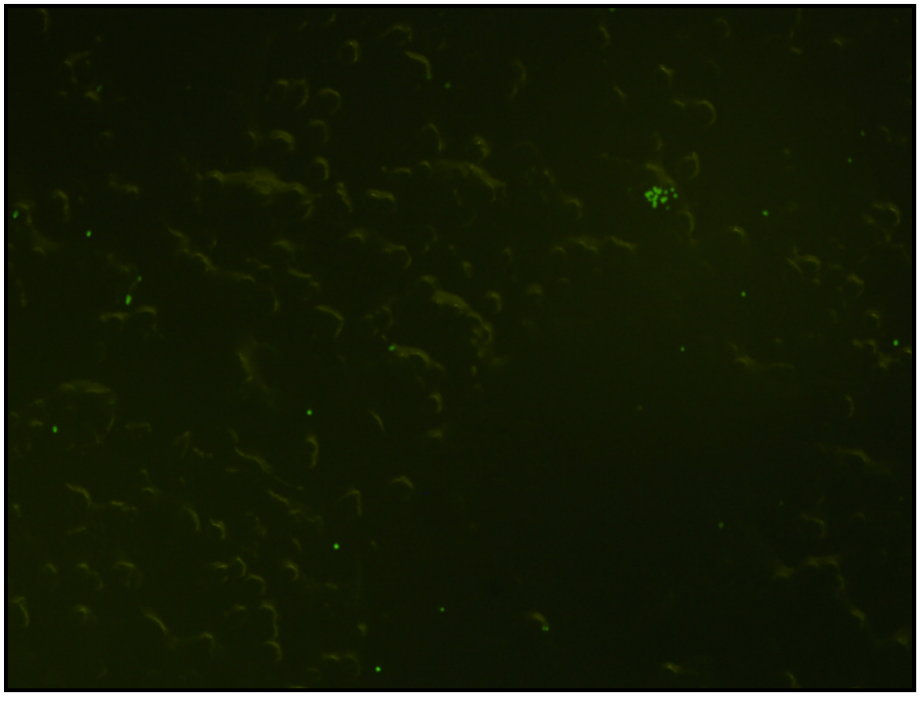

Fig. 5.125: A contrast view of formulation M2 taken up by alveolar macrophage after 24h of study

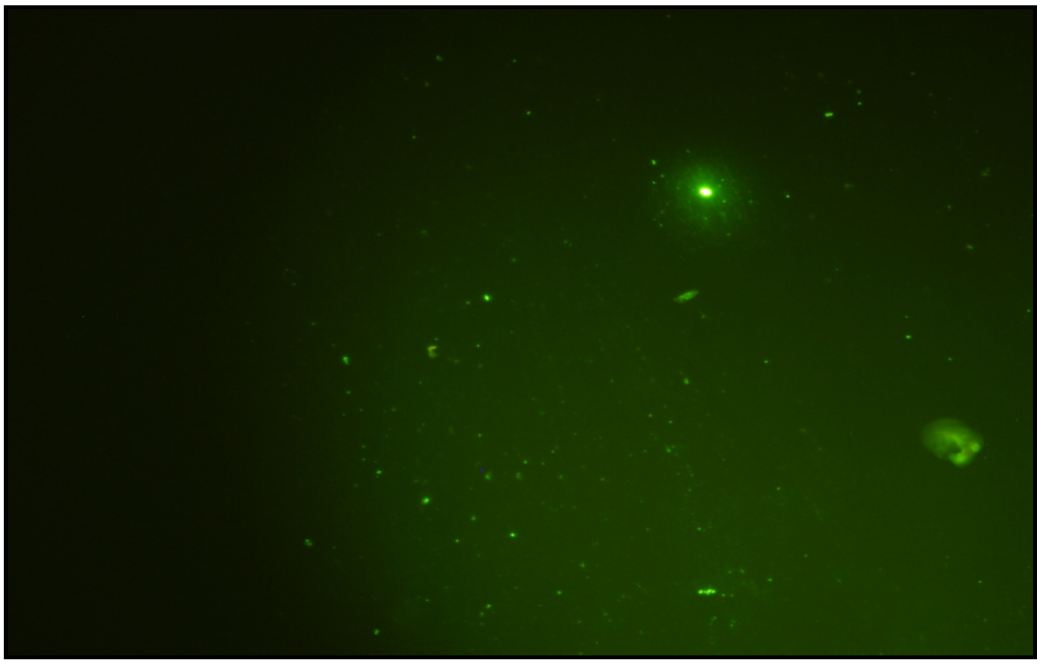

Fig. 5.126: A contrast view of formulation M3 taken up by alveolar macrophage after 24h of study

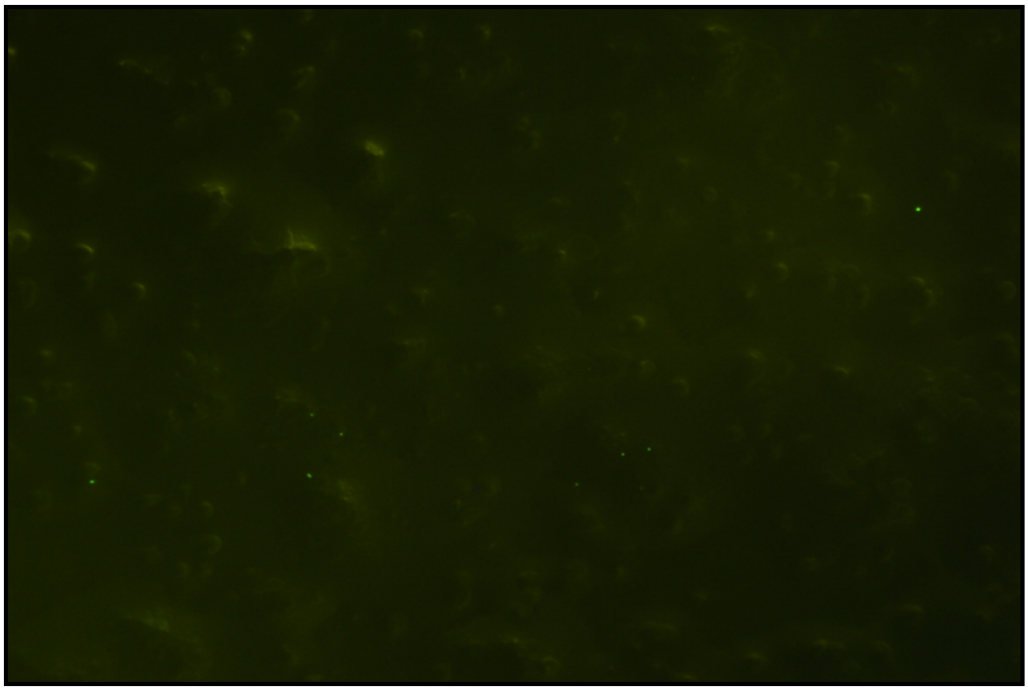

Fig. 5.127: A contrast view of formulation M4 taken up by alveolar macrophage after 24h of study

5.16. *In-vivo* pharmacological evaluation of sildenafil citrate dry powder formulations for inhalation

Sildenafil citrate dry powder formulations were administered to male Wistar rats using endotracheal intubation technique **(Fig. 5.128 A-D)**. Pulmonary delivery of different sildenafil citrate dry powder formulations **(Table 5.38)** demonstrated following observations associated with the prevention and treatment of PAH.

5.16.1 Preventive study:

Mean right ventricular systolic pressure (Mean RVSP) was significantly increased ($p<0.05$) in Only-MCT_{14} treated rats (40.00 ± 4.472 mm Hg) after 14 days of monocrotaline injection as compared to the saline treated control (11.83± 4.215 mm Hg) animals. All the groups administered with the inhaled sildenafil citrate dry powder formulations for 14 days after MCT injection, prevented the increase in mean RVSP **(Table 5.39).** The preventive effect was more significant ($p<0.05$) in case of lipid based sustained release formulations than that with conventional DPI and spray dried drug-sugar composites ($P<0.05$ when compared with Only-MCT_{14}).

Moreover, out of all the three lipid based formulations, animals treated with large porous liposphères, showed no significant increase of mean RVSP (13.50 ±3.082 mmHg) as compared to the control animals ($p>0.05$). Similar trend was seen for right ventricular hypertrophy (% RVH) that was significantly high in case of Only-MCT_{14} treated rats (46.948% ± 3.845) as compared to the control (28.569% ± 1.762) rats. No evident hypertrophy of right ventricles could be observed with liposomal dry powder for inhalation (27.359% ± 2.735) and large porous liposphères (28.389% ± 1.324) treated animals.

Lower %RVH was there with inhalation of drug-lipid composites (30.366% ± 0.776) which was better as compared to conventional DPI (38.178 ± 1.403) and drug-sugar composites (38.608 ± 3.299). Biochemical assay of cGMP levels in lung homogenates was performed as supportive data to assess the localization of the drug in the lungs. There was significant increase in the nucleotide levels in all the formulation groups when compared with control (471.229 ± 7.076) and Only-MCT_{14} treated (454.065 ± 7.436) rats ($p<0.05$) with highest cGMP levels detected (1014.661 ± 14.175) in animals treated with large porous

lipospheres. There was no mortality found till the 14 days of the study in any group. However, the percent weight gain was less in only-MCT_{14} treated rats as compared to the control and formulation treated groups. Thus inhalation of sildenafil citrate dry powder formulations could significantly prevent the development of PAH and better protection could be achieved with lipid based formulations **(Table 5.39, Fig. 5.129 A-C**).

Table 5.38: Description of the groups and formulations used in the study

Formulation code*	Group	Description
F1	A for preventive study B for therapeutic study	Conventional dry powder for inhalation (CDPI) of sildenafil citrate with lactose carriers
F2	A for preventive study B for therapeutic study	Drug-sugar composites of sildenafil citrate with mannitol prepared by spray drying technique
F3†	A for preventive study B for therapeutic study	Liposomal dry powder for inhalation (LDPI) prepared by thin film hydration and spray drying
F4†	A for preventive study B for therapeutic study	Drug-lipid composites of sildenafil citrate prepared by spray drying technique
F5†	A for preventive study B for therapeutic study	Large porous lipospheres prepared by emulsification and spray drying technique
Control	For preventive and therapeutic study	Animals received a single subcutaneous injection of normal saline
Only-MCT_{14}	For observations on 14^{th} day of study as final for preventive and initial for therapeutic study	Single subcutaneous injection (40mg/Kg) of monocrotaline
Only-MCT_{28}	For final observations on 28^{th} day of study	Single subcutaneous injection (40mg/Kg) of monocrotaline

*Respective placebos of formulations F1 to F5 were also prepared without drug to see any effect on the study parameters

† Lipid based formulations

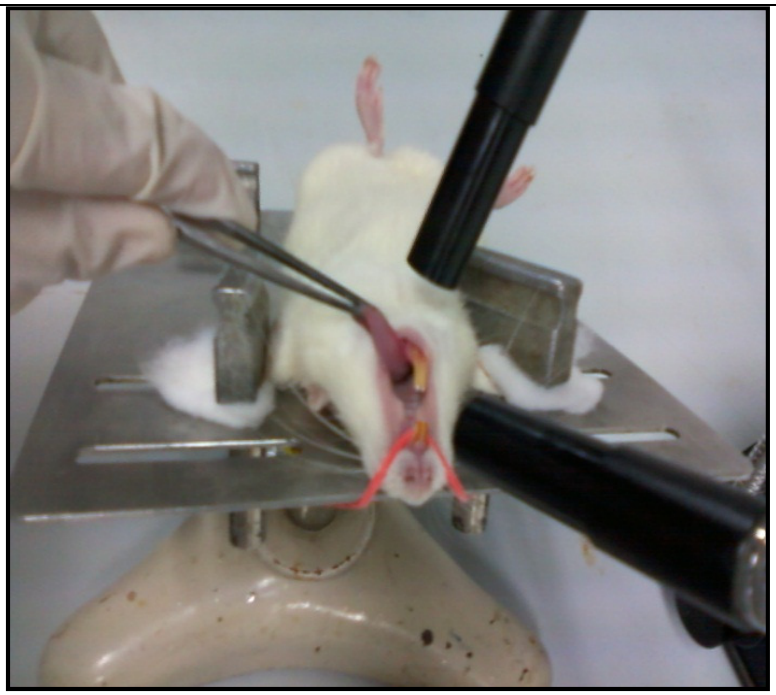

Fig. 5.128A: Pulling aside the tongue of the rat hung at 45° angle on a metal plate

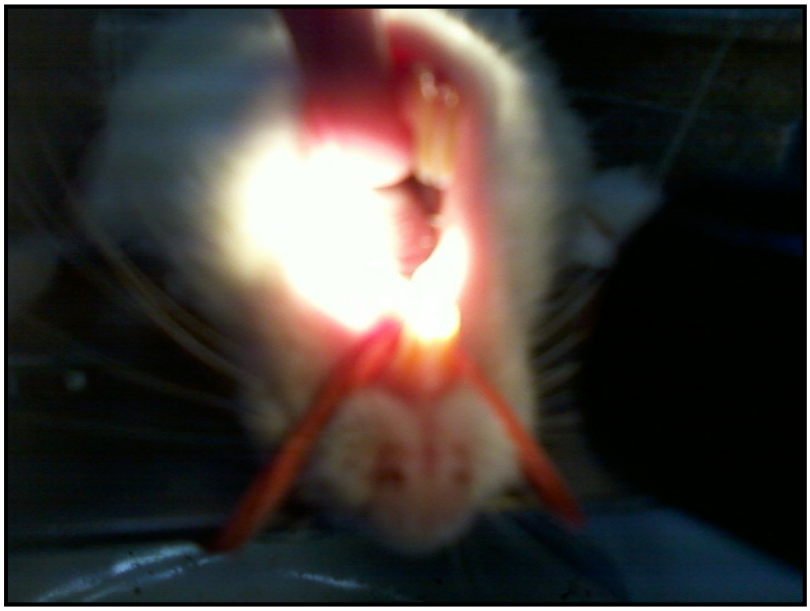

Fig. 5.128B: Illuminating the tracheal view using Fibre optic lamp with two flexible optical arms

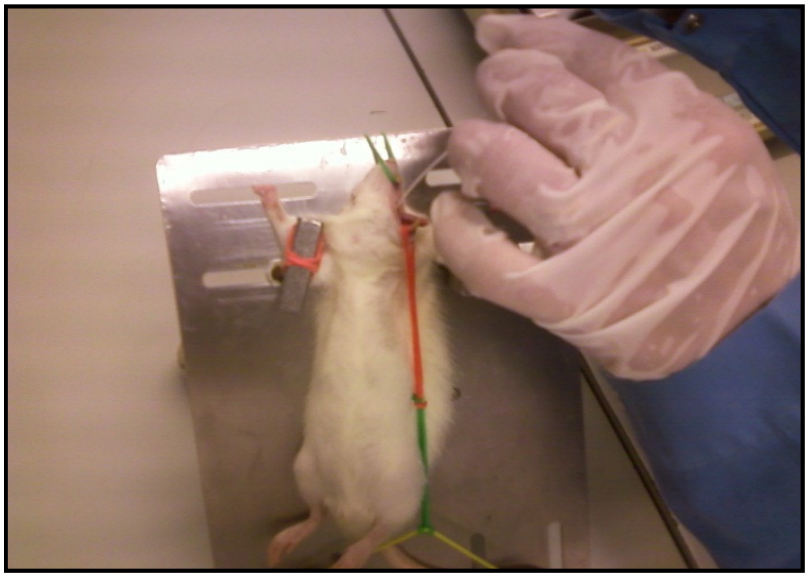

Fig. 5.128C: Careful insertion of the PE30m catheter tubing into the trachea

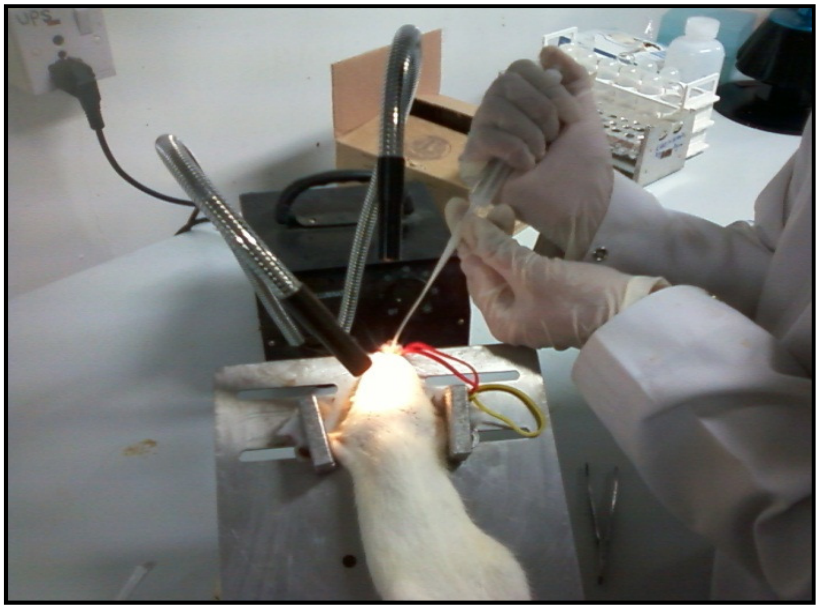

Fig. 5.128D: Inhalation of the drug contained in microtip attached to the catheter at one end and Finntip syringe barrel at another, by pushing the plunger

Fig. 5.128. A-D: Illustration of pulmonary drug administration in rats using endotracheal intubation technique

Table 5.39: Summarized data of hemodynamics, right ventricular hypertrophy and cGMP levels in the lungs of male wistar rats after preventive study with various sildenafil citrate inhalation formulations compared to control and only-MCT_{14} treated

Group	Mean RVSP (mm Hg)	Standard deviation (n=6)	Mean Right ventricular hypertrophy (%)	Standard deviation (n=6)	Mean cGMP pmol / mg of protein)	Standard deviation (n=6)
	After 14days of preventive study					
F1A	33.500	6.091	38.178	1.403	559.83	4.67
F2A	32.677	3.777	38.608	3.299	579.63	3.77
F3A	19.667	3.830	28.389	1.324	727.62	7.30
F4A	18.833	6.616	30.366	0.776	682.86	3.78
F5A	13.500	3.082	27.359	2.735	1014.66	14.17
Control	11.833	4.215	28.569	1.762	454.06	7.44
MCT	40.000	4.472	46.948	3.845	471.22	7.07

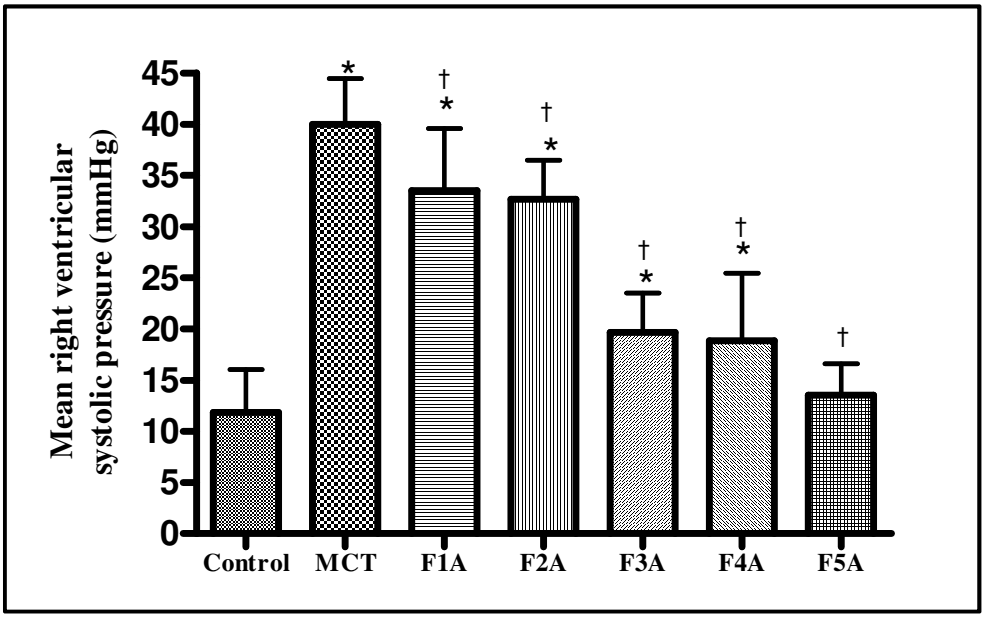

Fig. 5.129A: Effect on mean right ventricular systolic pressure (mean RVSP, mmHg)

(*p<0.05 as compared to control, †p<0.05 as compared to Only MCT_{14} treated animals)

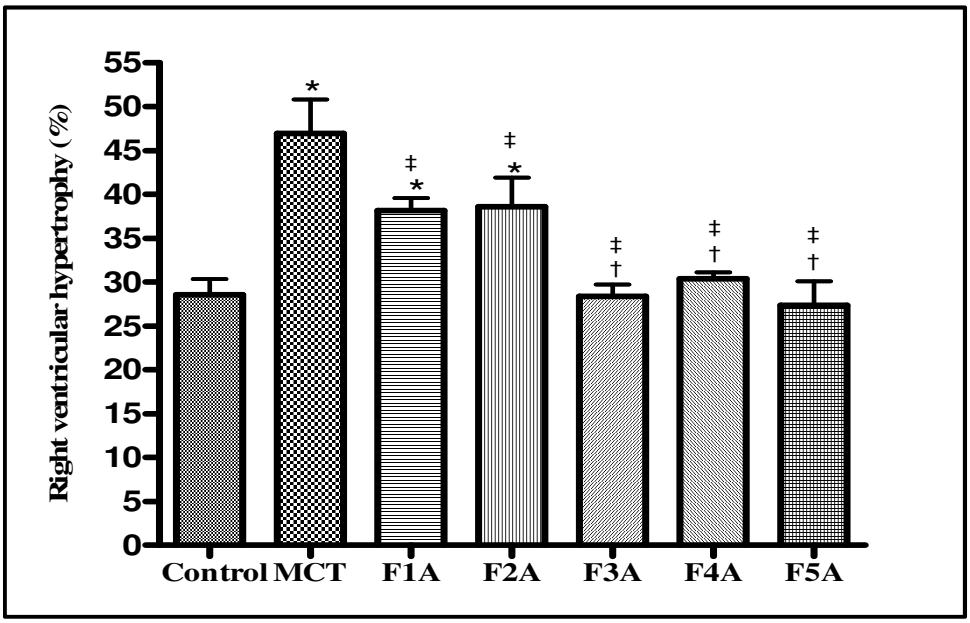

Fig. 5.129B: Effect on % right ventricular hypertrophy

(*p<0.05 as compared to control, †p<0.05 as compared to F1 and F2 treated animals, ‡p<0.05 as compared to Only MCT_{14} treated animals)

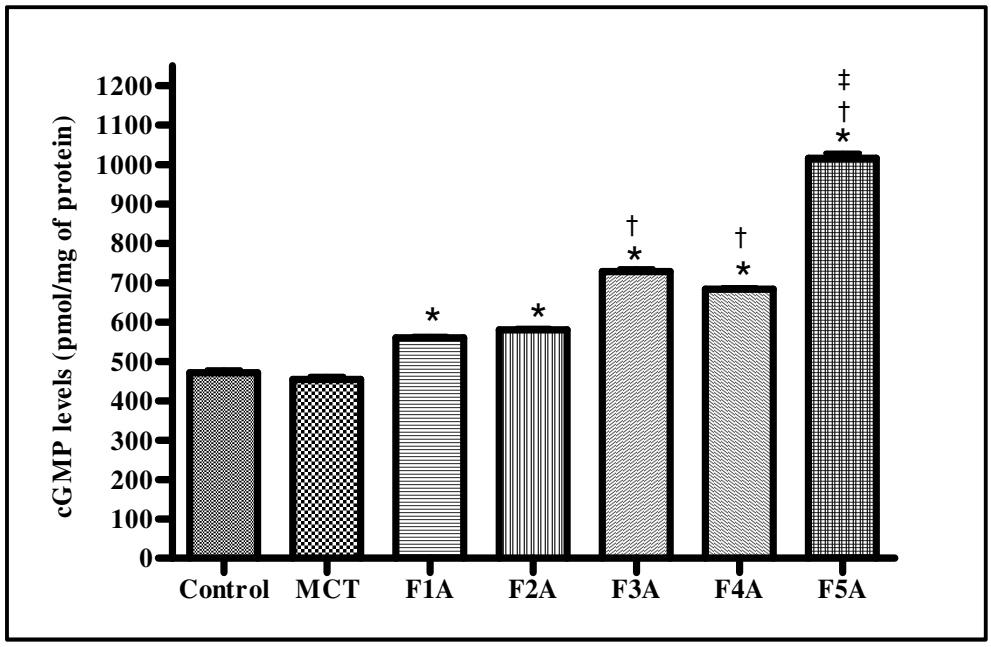

Fig. 5.129C: Effect on cGMP levels (pmol/mg of protein) in lung homogenates

(*p<0.05 as compared to control and Only MCT_{14} treated animals, †p<0.05 as compared to F1 and F2 treated animals, ‡p<0.05 as compared to F3 and F4 treated animals)

Fig. 5.129. A-C: Effect of different sildenafil citrate formulations compared with control and MCT treated rats on hemodynamic and biochemical parameters of male wistar rats after 14 days of preventive study

5.16.2 Therapeutic study

Development of disease was evident with the significant increase in mean RVSP and increased right ventricular hypertrophy after 14 days of MCT injection. We further evaluated the prolonged therapeutic potential of these formulations in the disease induced rats and the treatment was started on 14th day of the study. After first administration of different formulations in respective groups, various parameters were studied at different time points till 48h of the first administration **(Table 5.40).** Significant reduction in mean RVSP **(Fig. 5.131A)** was observed till 8h in case of conventional DPI treated animals and drug-sugar composites when compared with Only-MCT_{14} treated rats. Faster onset was seen with inhalation of drug-sugar composites (9.533mmHg ± 2.483) as compared with conventional dry powder inhalation (17.167mmHg ± 2.137) as there was significantly lower mean RVSP

values ($p<0.05$) within 2h, but in both these cases mean RSVP reduction could not be maintained after 8h. In case of animals treated with liposomal dry powder formulation and large porous lipospheres significant reduction ($p<0.05$) in mean RVSP could be maintained till 48h (15.50mmHg ± 4.764 and 16.167mmHg ± 3.125 respectively) and till 24h in case of drug-lipid composites (22.50mmHg ± 3.391) treated animals. Correspondingly, significant higher ($p< 0.05$) cGMP levels could be maintained till 48h in case of liposomal dry powder formulation (1072.31pmol/mg of protein ± 9.47) and large porous lipospheres (1008.64 pmol/mg of protein ± 7.49) and till 24h in case of drug-lipid composites (617.12pmol/mg of protein ± 11.34) as compared to Only-MCT_{14} treated rats (454.07pmol/mg of protein ± 7.44). However, in case of conventional DPI treated animals (565.77pmol/mg of protein ± 4.64) and drug-sugar composites (608.77pmol/mg of protein ± 8.74), significantly higher cGMP levels were observed only till 2h **(Table 5.40, Fig. 5.131B).**

Table 5.40: Mean Right ventricular systolic pressure and mean cGMP levels (± Standard deviation, n=6) at different time points after first administration of sildenafil citrate inhalation formulations in disease induced rats compared to control and Only-MCT_{14} treated rats

Group	Mean RVSP (mm Hg)	Mean cGMP pmol / mg of protein)	Mean RVSP (mm Hg)	Mean cGMP pmol / mg of protein)	Mean RVSP (mm Hg)	Mean cGMP pmol / mg of protein)	Mean RVSP (mm Hg)	Mean cGMP pmol / mg of protein)
	2h		8h		24h		48h	
F1B	17.16± 2.137	565.77±4.64	16.00± 2.898	456.28±5.00	36.16± 5.981	450.79±7.10	35.83± 7.414	455.57±11. 60
F2B	9.83± 2.483	608.77±8.74	16.16± 3.488	455.52±10.49	35.66± 6.563	458.36±13.20	35.66± 4.227	456.27±9.5 7
F3B	8.66± 2.160	514.04±9.42	15.00± 4.000	1097.52±7.52	17.50± 4.848	1101.65±6.96	15.50± 4.764	1072.31±9. 47
F4B	15.00± 3.899	622.33±8.68	11.50± 2.258	805.23±9.93	22.50± 3.391	617.12±11.34	33.50 ±4.680	452.00±12. 16
F5B	13.66± 1.506	802.51±10.72	18.83± 2.483	1112.08±4.67	12.33± 3.011	1095.4±11.52	16.16± 3.125	1008.64±7. 49
Control	11.83± 4.215	471.23± 7.08	Measured on 14th day of the study					
MCT	40.00± 4.472	454.07± 7.44						

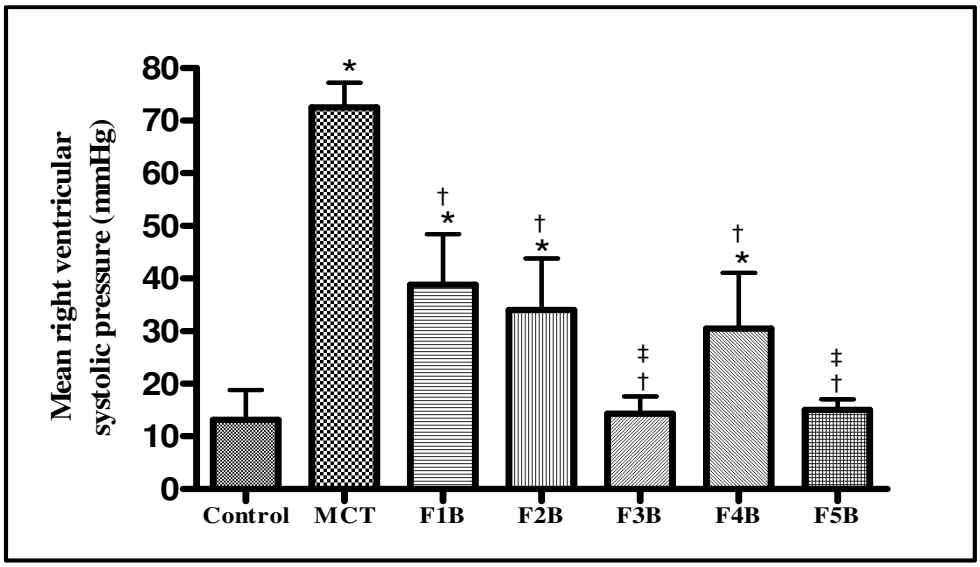

Fig. 5.130A: Effect on mean right ventricular systolic pressure (mean RVSP, mmHg)

(*p<0.05 as compared to control, †p<0.05 as compared to Only MCT_{14} treated animals, ‡p<0.05 as compared to F4 treated animals)

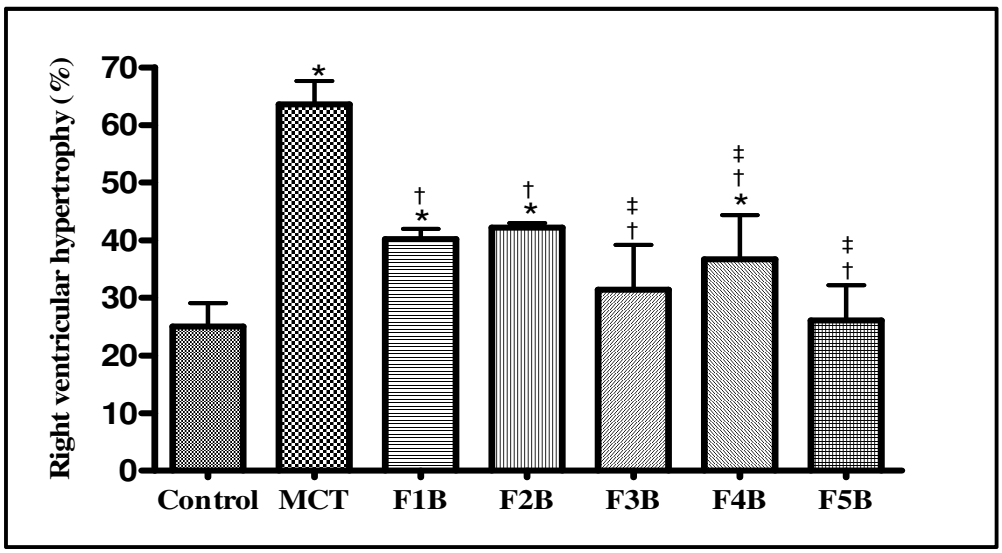

Fig. 5.130B: Effect on % right ventricular hypertrophy

(*p<0.05 as compared to control, †p<0.05 as compared to Only MCT_{14} treated animals, ‡p<0.05 as compared to F1 and F2 treated animals)

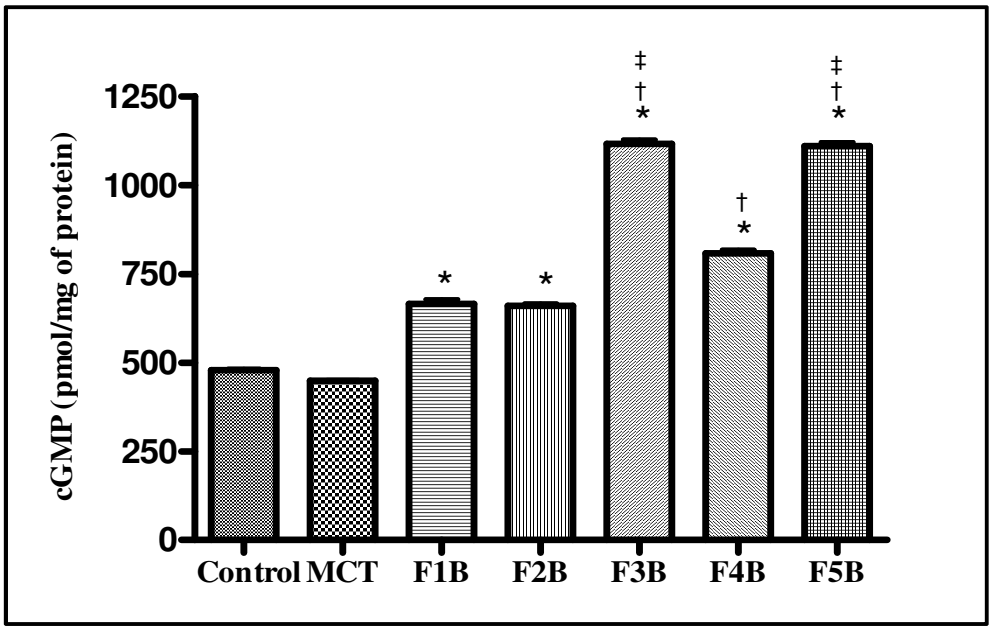

Fig. 5.130C: Effect on cGMP levels (pmol/mg of protein) in lung homogenates

(*p<0.05 as compared to control and Only MCT_{14} treated animals, †p<0.05 as compared to F1 and F2 treated animals, ‡p<0.05 as compared to F4 treated animals)

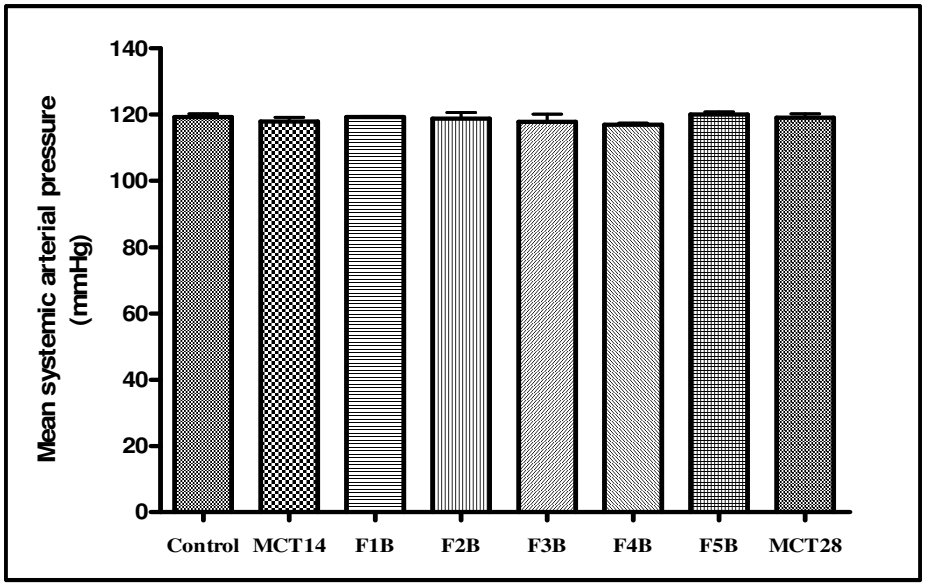

Fig. 5.130D: Effect on mean systemic arterial pressure (mean SAP, mmHg)

Fig. 5.130 A-D: Effect of different sildenafil citrate formulations compared with control and MCT treated rats on hemodynamic and biochemical parameters of male wistar rats after 28 days of therapeutic study

Efficacy of the formulations was also evaluated after chronic treatment for further two weeks in disease induced rats by once daily administration of the formulations in the respective groups **(Table 5.41).** On 28th day of the MCT injection, Only-MCT_{28} 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-MCT_{14} treated animals **(Fig. 5.130 A,B)**. 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-MCT_{14}, Only-MCT_{28} and all formulation treated groups **(Fig. 5.130 D).** Almost 30% of the animals died in Only-MCT_{28} group by this time which might be due to right heart failure caused due to extensive vasoconstriction and hypertrophy in untreated animals. Treatment for two weeks with sildenafil citrate dry powder formulations through pulmonary delivery showed significant reduction in mean RVSP and mean %RVH in all the formulation treated groups ($p<0.05$). After daily administration for two weeks, animals treated with large porous lipospheres showed no significant difference in mean RVSP and %RVH when compared with control animals **(Fig. 5.130A, B)**. The increase in cGMP nucleotide levels induces relaxation and anti-proliferative effects on vascular smooth muscle. Thus the above seen trend in hemodynamics and RVH was further supported by the cGMP levels found in the lung homogenates of the rats in different groups.

All the formulations were able to increase cGMP levels in rat lungs **(Fig. 5.130C)** as compared to the control and only-MCT_{28} treated animals. Extent of increase in cGMP levels was same with no significant difference in case of conventional dry powder and drug-sugar composites treated animals. Drug lipid composites showed higher cGMP level hike (1.74 times than control and 1.2 times than conventional DPI and drug-sugar composites) but it was even higher for liposomal dry powder formulation and large porous lipospheres treated animals (2.4 and 2.39 times respectively compared to control and 1.4 times compared to drug-lipid composites).

Table 5.41: Summarized data of hemodynamics, right ventricular hypertrophy and cGMP levels in the lungs of Wistar rat after treatment with various sildenafil citrate inhalation formulations compared to control and Only-MCT_{28} treated

Group	Mean RVSP (mm Hg)	Standard deviation (n=6)	Mean Right ventricular hypertrophy (%)	Standard deviation (n=6)	Mean cGMP pmol / mg of protein)	Standard deviation (n=6)
	After 28 days of therapeutic study					
F1B	38.833	9.600	40.247	1.701	664.684	13.038
Placebo F1B	70.211	3.145	65.243	1.011	458.999	8.014
F2B	34.000	9.818	42.196	0.773	659.678	5.984
Placebo F2B	74.552	4.601	62.829	2.304	461.521	6.665
F3B	14.333	3.204	31.392	7.863	1116.34	11.329
Placebo F3B	68.124	2.124	57.991	2.224	452.363	5.201
F4B	30.500	10.616	36.708	7.633	807.615	9.745
Placebo F4B	56.225	8.445	59.431	6.385	455.988	4.982
F5B	15.000	2.000	26.092	6.146	1109.480	11.092
Placebo F5B	71.225	3.285	60.785	3.229	451.268	9.568
Control	13.167	5.636	25.031	4.032	462.846	4.108
MCT	72.500	4.764	63.607	4.033	455.833	8.360

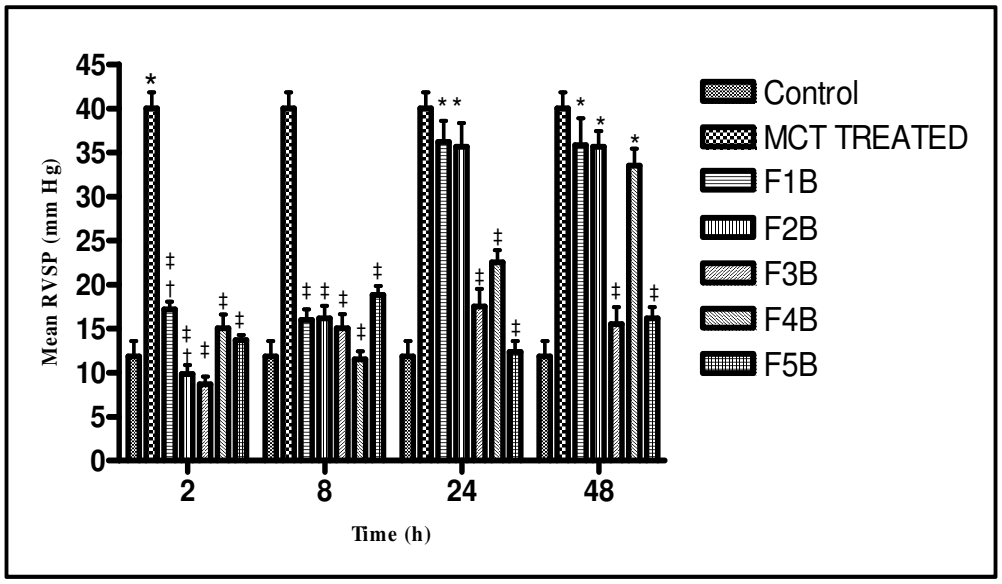

Fig. 5.131A: Effect on mean right ventricular systolic pressure (mean RVSP, mmHg)

(*p<0.05 as compared to control, †p<0.05 as compared to lipid based formulations (F3, F4 and F5) ‡p<0.05 as compared to Only MCT_{14} treated animals)

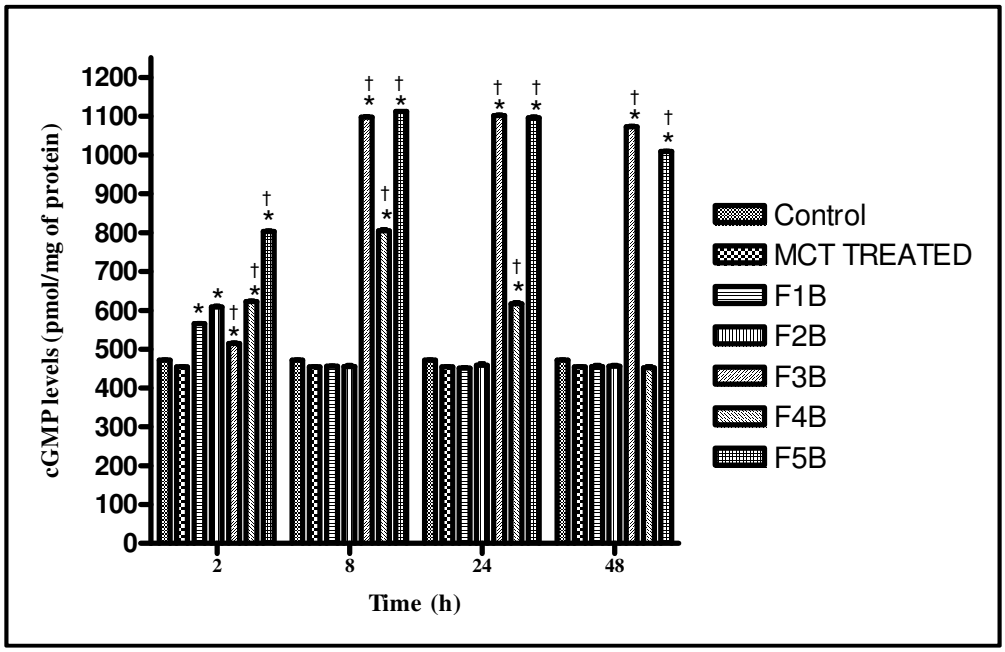

Fig. 5.131B: Effect on cGMP levels (pmol/mg of protein) in lung homogenates(*p<0.05 as compared to control and Only MCT_{14} treated animals, †p<0.05 as compared to F1 and F2 treated animals)

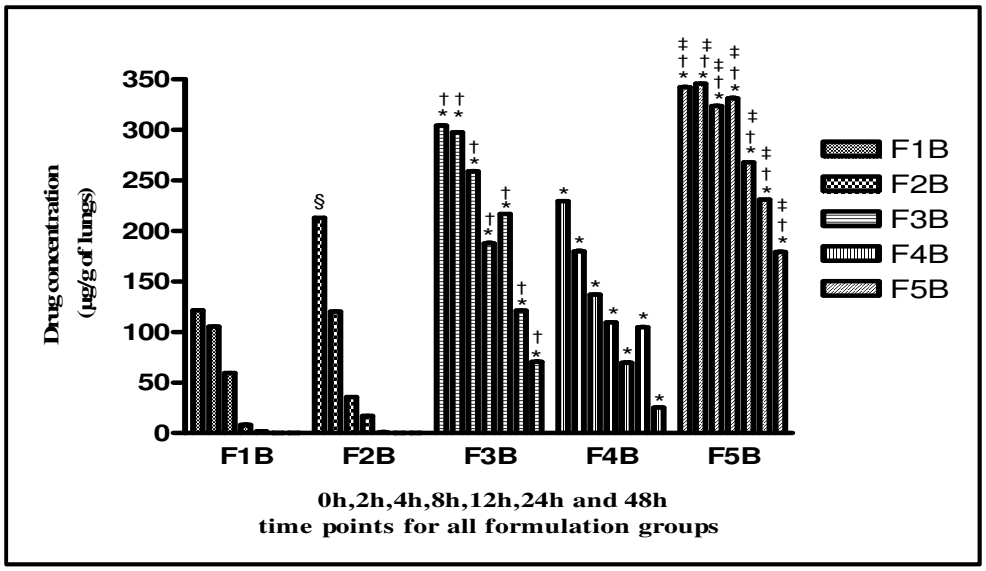

Fig. 5.131C: Effect on sildenafil citrate levels (µg/g of lungs) in lung homogenates

(*p<0.05 as compared to F1 and F2 treated animals, †p<0.05 as compared to F4 treated animals
‡p<0.05 as compared to F3 and F4 treated animals)

Fig. 5.131A-C: Therapeutic study to evaluate sustained potential of sildenafil citrate dry powder formulation through hemodynamic and biochemical parameters of male wistar rats till 48hours after single administration

5.16.3 Histopathological findings:

Arterial medial thickening in the small pulmonary arteries and inflammatory changes were observed after 14 days of MCT injection **(Fig. 5.132B)** as compared to control animals **(Fig. 5.132A)** and these were further aggravated alongwith the muscularization of intra-acinar arteries till 28th day **(Fig. 5.133 A, B).** Arterial medial thickening in the pulmonary microvasculature and inflammatory reaction were curbed by the inhalation of all sildenafil citrate dry powder formulations during preventive study **(Fig. 5.132 C-G).** Chronic treatment for two weeks with pulmonary administration of sildenafil citrate dry powder formulations in male Wistar rats revealed suppression of the arterial medial thickening, reduced muscularization of pulmonary arteries and moderated inflammatory reaction (De Visser YP *et al*. 2008) with conventional dry powder, drug-sugar composites and drug-lipid composites treated groups (**Fig. 5.133 C, D, F** respectively). 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 hyperreactivity (Toward TJ *et al*. 2004; Haddad JJ *et al*. 2002). Moreover, in case of liposomes dry powder **(Fig. 5.133E)** and large porous lipospheres treated groups **(Fig. 5.133G)**, significantly negligible inflammatory response, medial thickening and muscularization could be observed which might be due to their prolonged stay in the lungs that maintained the required pulmonary drug levels for longer time to prevent vasoconstriction and vascular remodelling.

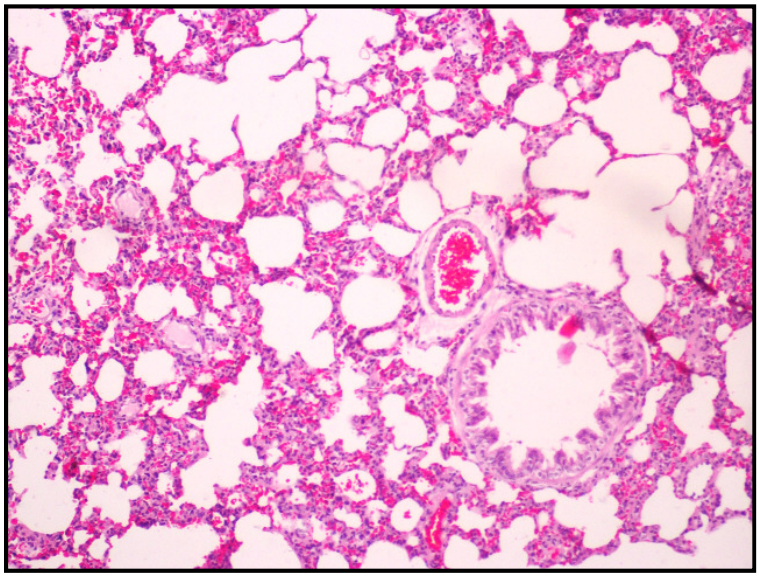

Fig. 5.132A: Control animals

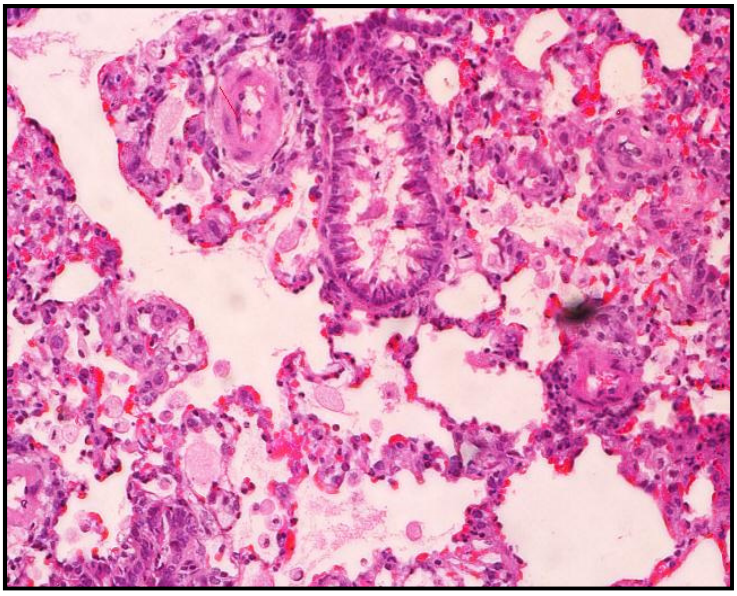

Fig. 5.132B: Only-MCT$_{14}$

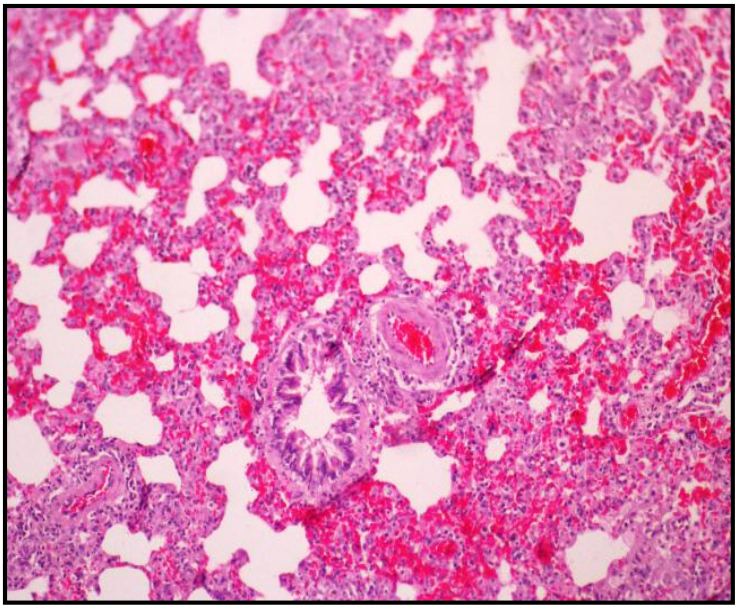

Fig. 5.132C: F1A

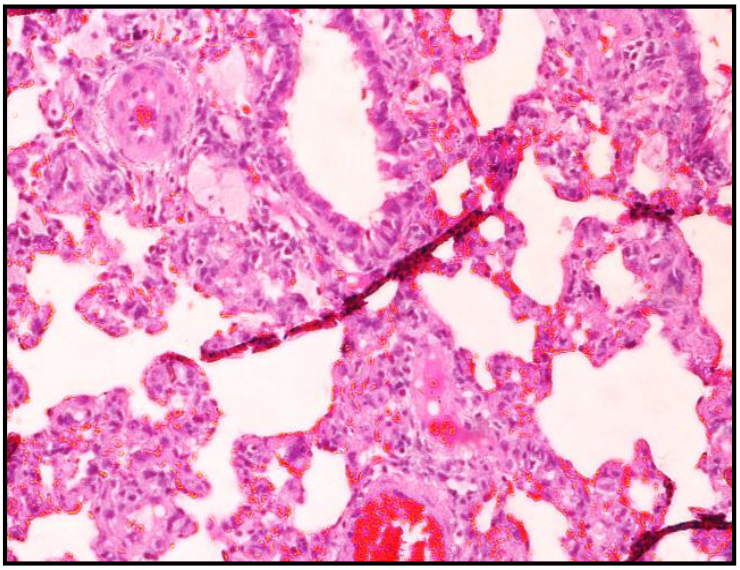

Fig. 5.132D: F2A

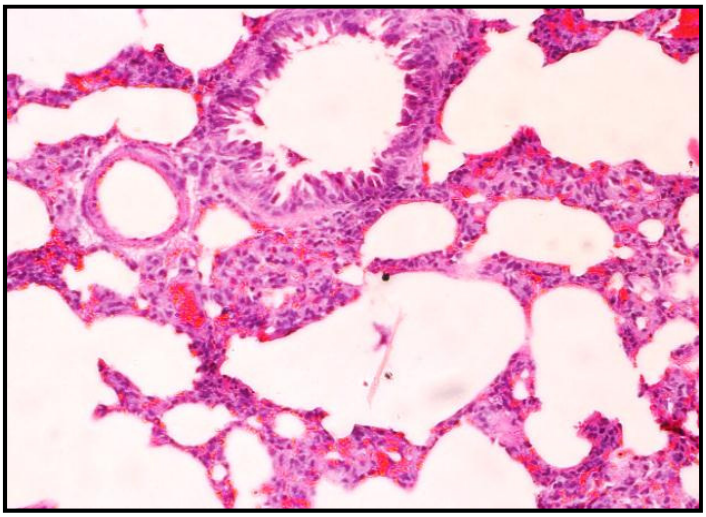

Fig. 5.132E: F3A

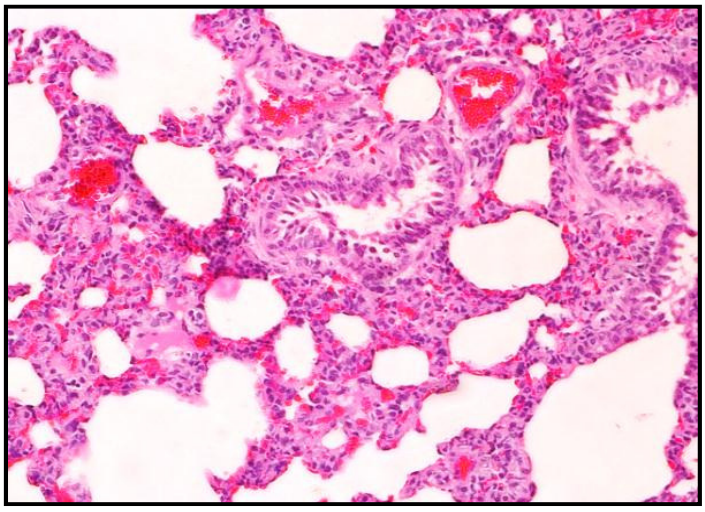

Fig. 5.132F: F4A

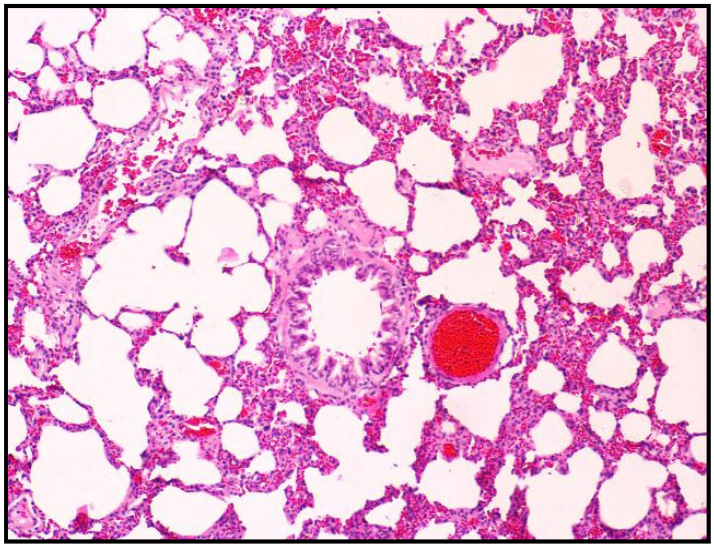

Fig. 5.132G: F5A

Fig. 5.132 A-G: Histopathological findings on 14th day of preventive study

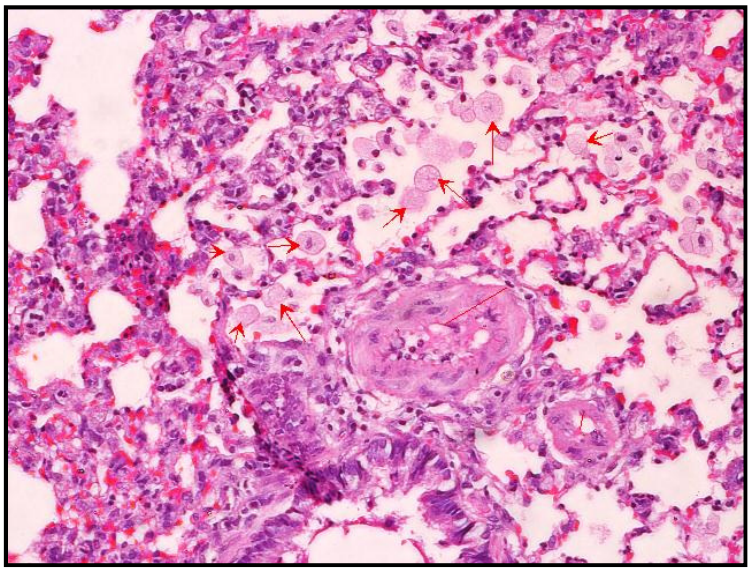

Fig. 5.133A: Only-MCT_{28}

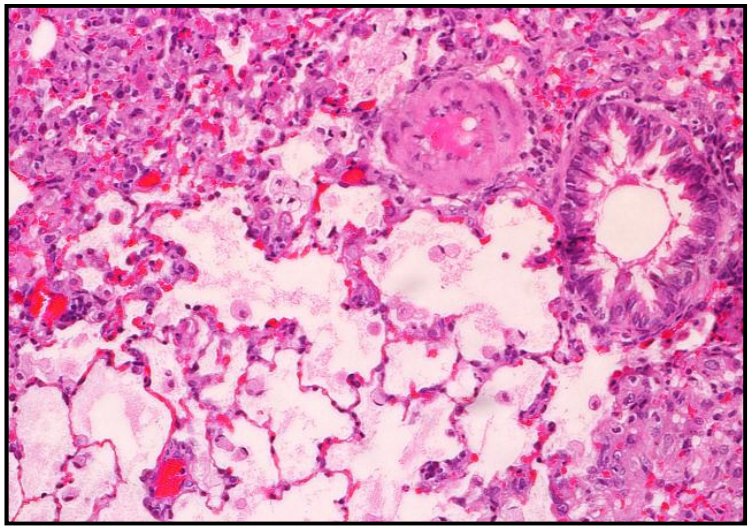

Fig. 5.133B: Only-MCT$_{28}$

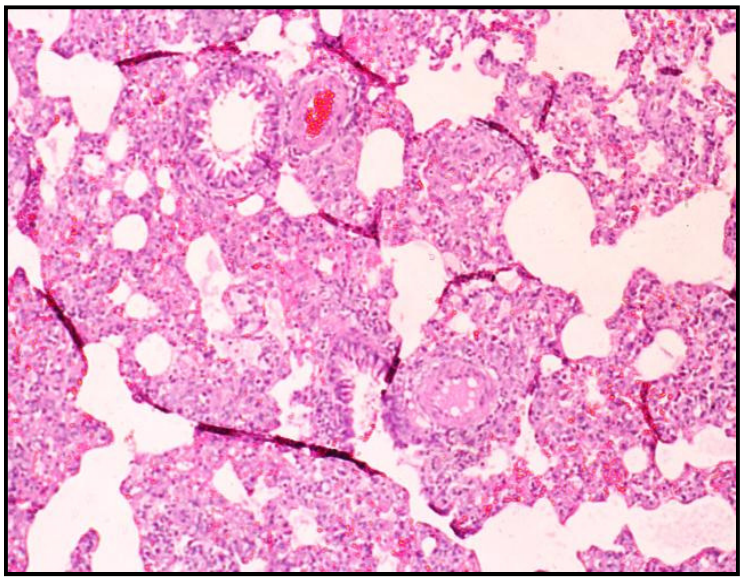

Fig. 5.133C: F1B

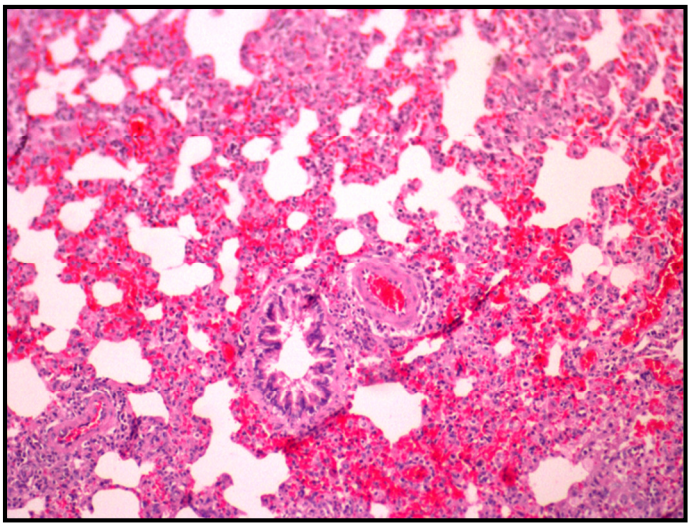

Fig. 5.133D: F2B

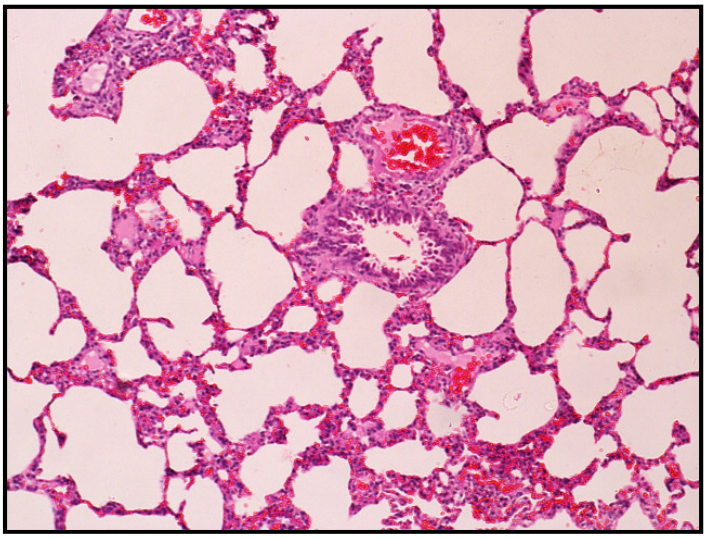

Fig. 5.133E: F3B

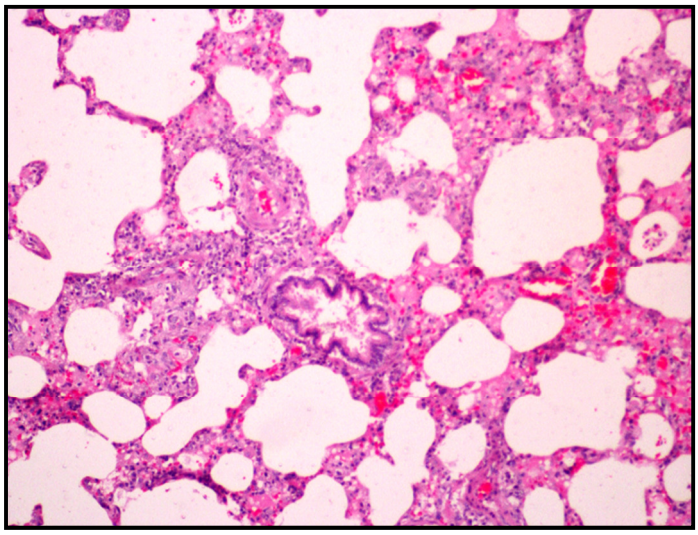

Fig. 5.133F: F4B

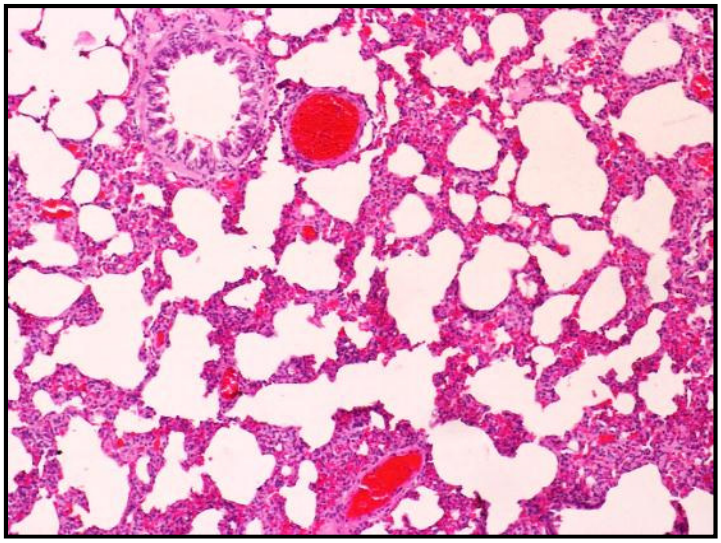

Fig. 5.133G: F5B

Fig. 5.133 A-G: Histopathological findings on 28th day of therapeutic study

5.16.4 Pulmonary pharmacokinetics:

The concentration of drug found in the lung homogenates was significantly different ($P<0.05$) at all the time points for sustained release sildenafil citrate dry powder formulations **(Table 5.42, Fig. 5.131C)** when compared with conventional DPI and spray dried drug sugar composites. HPLC chromatograms of these formulations revealing drug peaks in lung homogenates at different time points are shown in **Fig. 5.134 A-E**. 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 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. Moreover the Cmax reached was also 1.75 times more than that with conventional dry powder which might be due to the better lung deposition due to improved aerodynamic behavior of the spray dried drug-sugar composite formulation. All Lipid based formulations have shown significantly higher ($P<0.05$) Cmax (2.5, 1.88 and 2.82 times higher than conventional dry powder and spray dried drug sugar composites and results signify the best lung deposition with large porous lipospheres treated animals, followed by liposomal dry powder and then drug-lipid composites.

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. Investigation of drug levels in the lung homogenates of the rats revealed a striking increase in $t_{1/2}$ i.e. 11.35 times with drug lipid composites, 14.03 times with dry powder liposomal inhalation and 32.73 times with large porous lipospheres as compared to conventional DPI. Mean pulmonary residence time was prolonged 6.5 to 7.5 times in case of liposomal dry powder and drug lipid composites as compared to conventional DPI and drug sugar composites. The most prolonged pulmonary mean residence time **(Table 5.43)** of 21.24 hours was seen with large porous lipospheres.

It has been well documented that the large porous particles due to its better aerosolization performance, show better lung deposition than the non-porous particles and that might be the

reason for maximum Cmax achieved with it. Moreover, being larger in size, it is less likely to be phagocytosed and relatively less aqueous affinity make these inhalation aerosols more proficient to stay integrated longer with alveolar membrane and hence release the drug into the lungs for prolonged period of time (D.L. French *et al*. 1996; R. Vanbever, J.D. *et al*. 1999).

Table 5.42: Drug levels (µg/g of the lungs ± Standard deviation, n=6) at different time points in lung homogenates of disease induced rats after first administration of sildenafil citrate inhalation formulations

Group	Concentration (µg/g)						
	0h	2h	4h	8h	12h	24h	48h
F1B	121.52±1.07	105.16±2.04	59.54±0.958	8.04±2.16	1.66±1.907	ND	ND
F2B	212.98±1.11	120.21±1.47	35.52±1.132	16.84±1.418	0.36±3.049	ND	ND
F3B	304.24±2.35	297.48±1.84	258.88±2.44	187.66±3.04	216.62±1.26	121.04±1.87	70.6±2.166
F4B	229.50±1.57	179.91±3.16	136.88±1.88	109.56±1.25	69.52±3.103	104.76±2.15	25.28±1.935
F5B	342.36±2.31	345.56±1.79	323.68±2.09	331.00±3.00	267.66±1.96	230.84±1.58	179.04±2.52

Table 5.43: Pulmonary pharmacokinetic parameters of different sildenafil citrate dry powder inhalation formulations

Group	AUC (µg*h/g)	Cmax (µg/g)	$T_{1/2}$ (h)	MRT (h)
F1B	505.74	121.52	1.62	2.75
F2B	583.73	212.98	1.19	2.44
F3B	7065.99	304.24	22.73	18.16
F4B	3938.23	229.50	18.39	18.10
F5B	11736.88	345.56	53.03	21.24

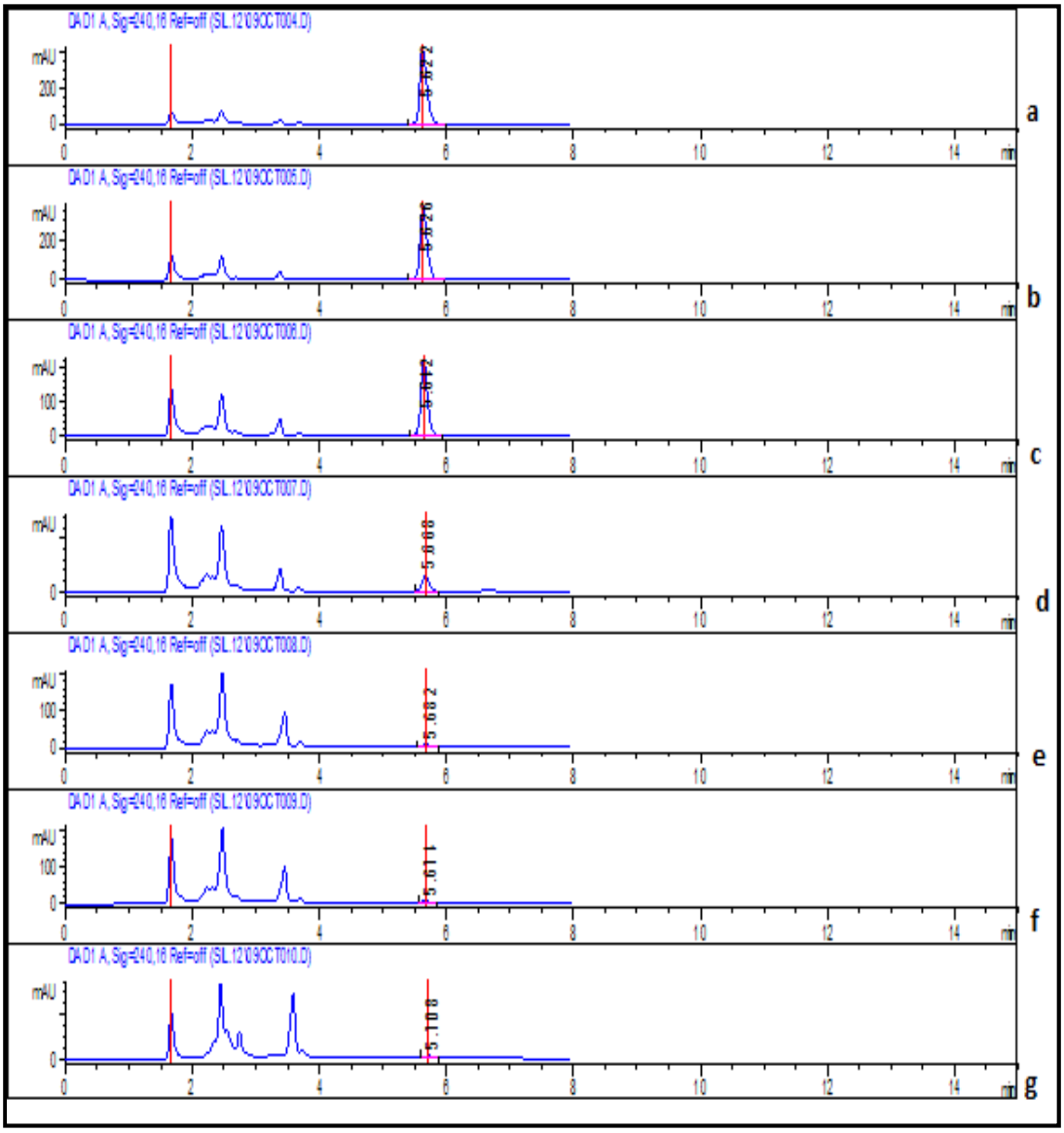

Fig. 5.134A: HPLC chromatogram for formulation F1B showing peaks for sildenafil citrate in rat lung homogenates at a) 0h b) 2h c) 4h d) 8h e) 12h f) 24h g) 48h

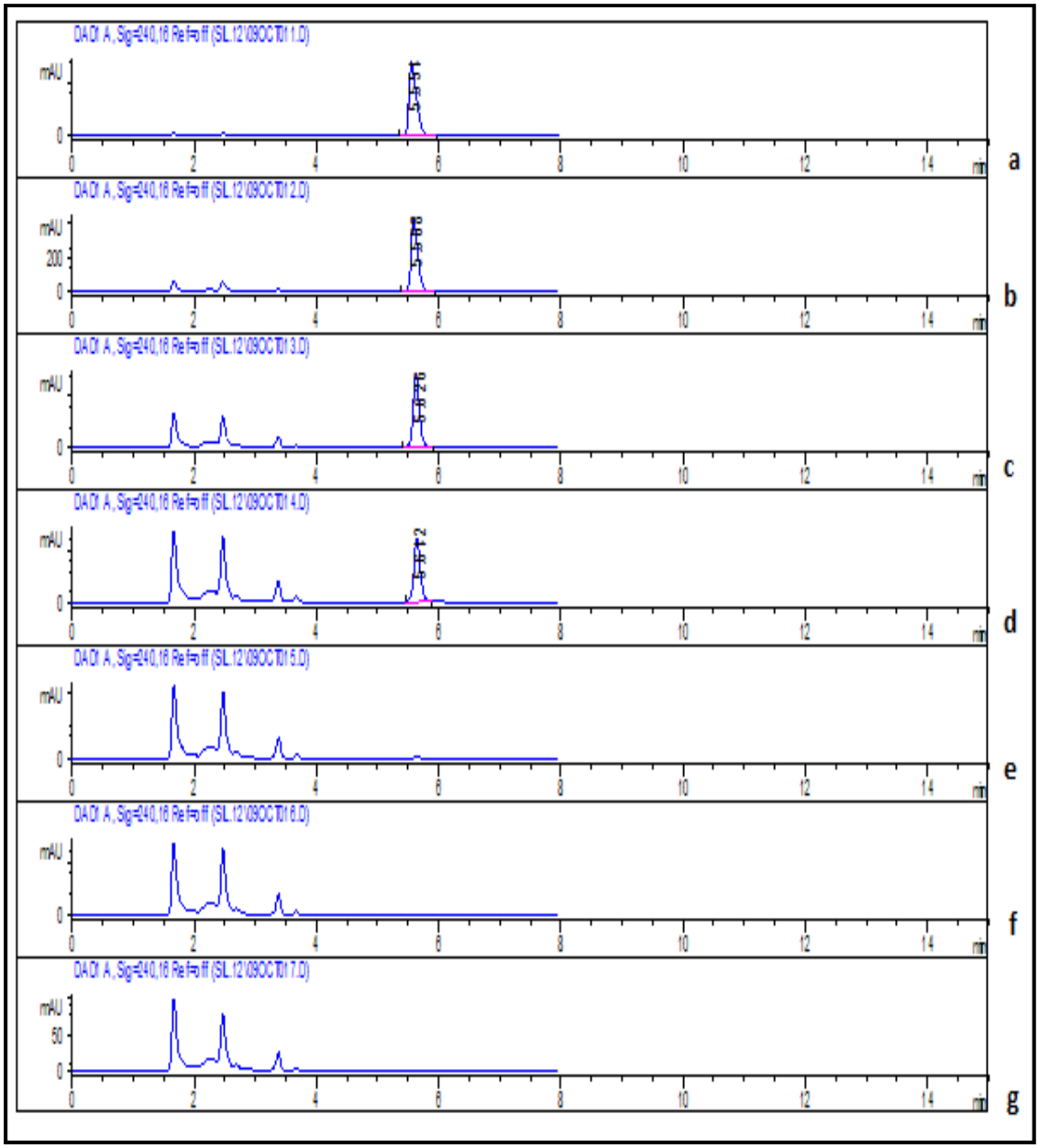

Fig. 5.134B: HPLC chromatogram for formulation F2B showing peaks for sildenafil citrate in rat lung homogenates at a) 0h b) 2h c) 4h d) 8h e) 12h f) 24h g) 48h

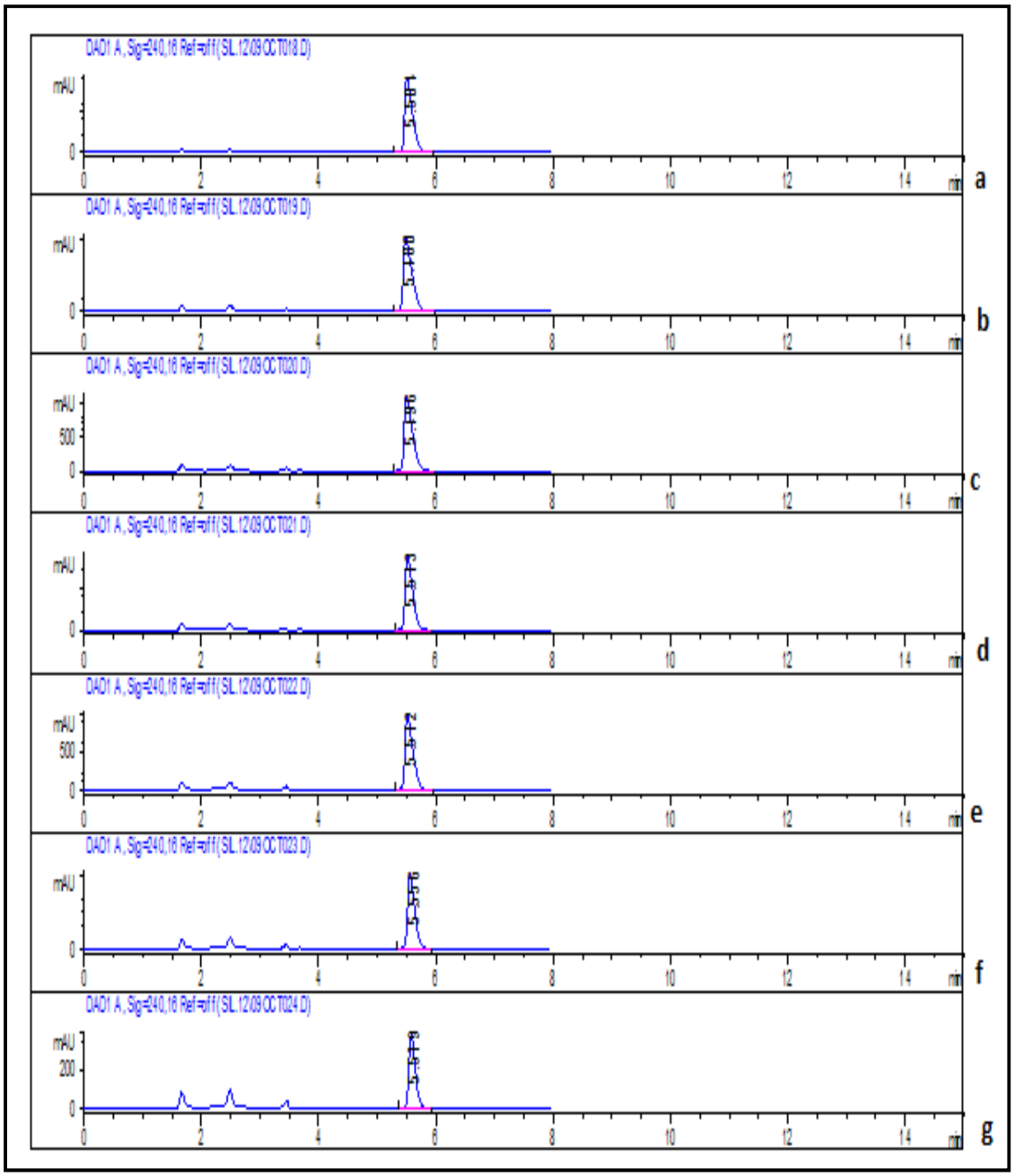

Fig. 5.134C: HPLC chromatogram for formulation F3B showing peaks for sildenafil citrate in rat lung homogenates at a) 0h b) 2h c) 4h d) 8h e) 12h f) 24h g) 48h

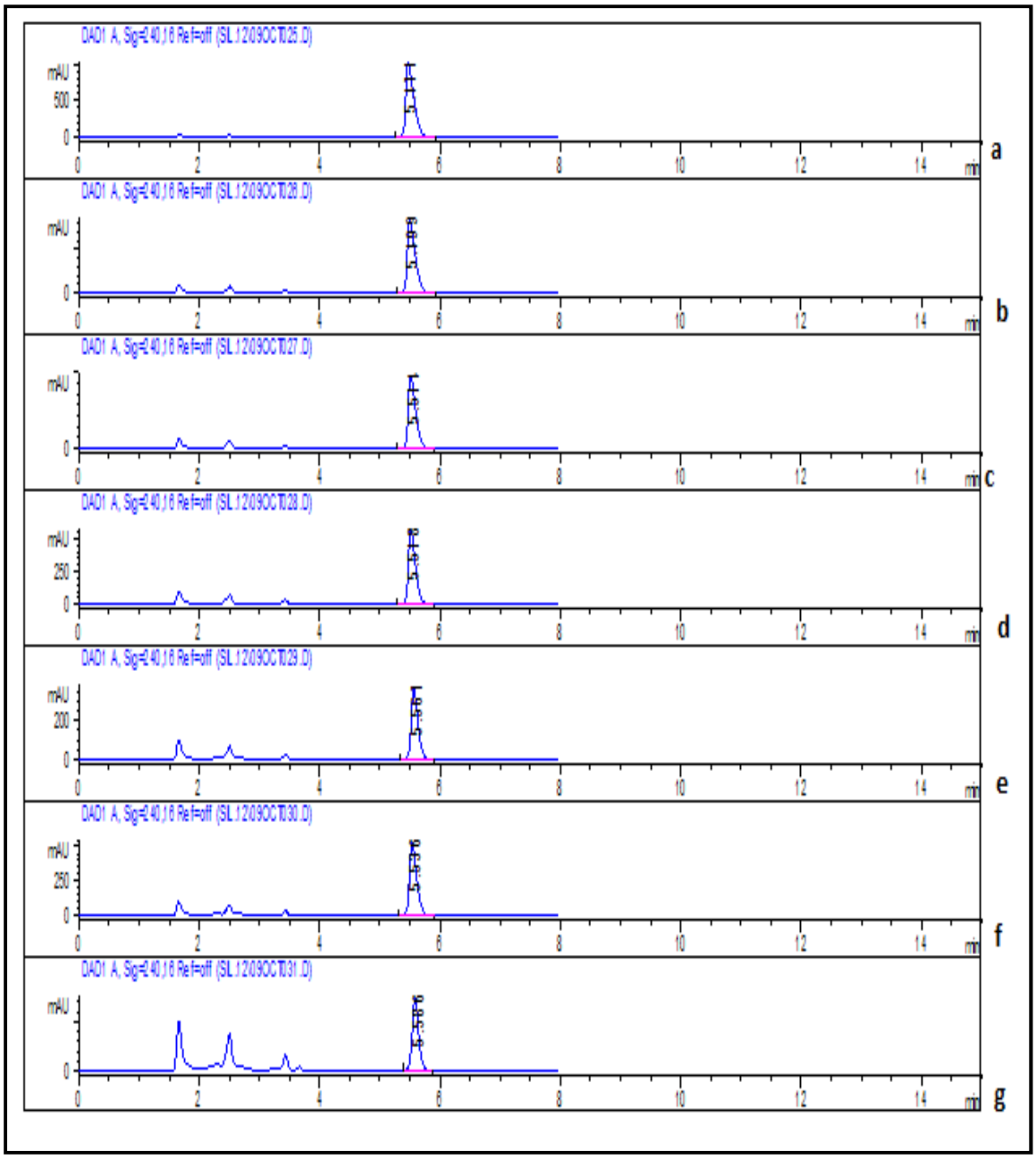

Fig. 5.134D: HPLC chromatogram for formulation F4B showing peaks for sildenafil citrate in rat lung homogenates at a) 0h b) 2h c) 4h d) 8h e) 12h f) 24h g) 48h

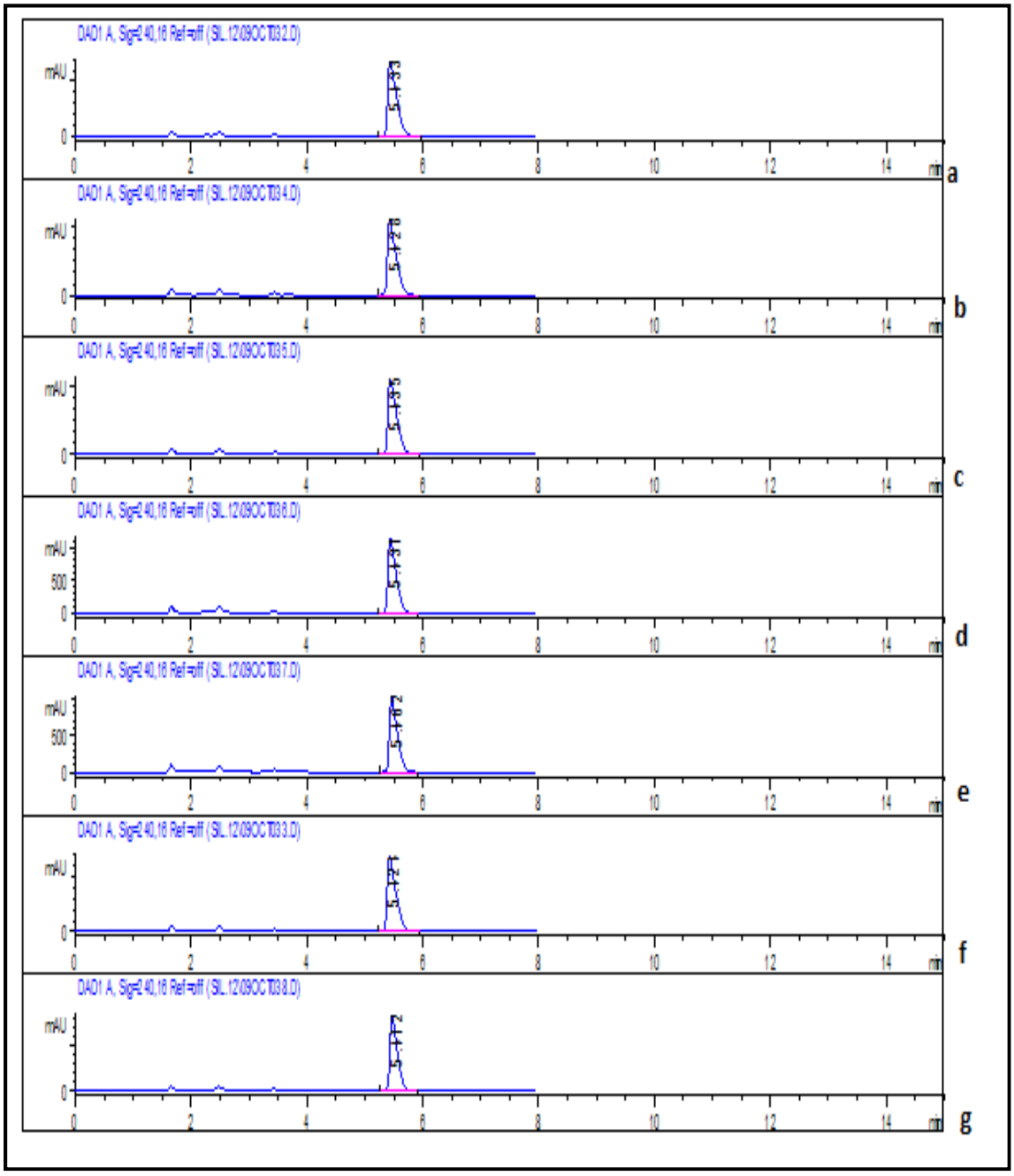

Fig. 5.134E: HPLC chromatogram for formulation F5B showing peaks for sildenafil citrate in rat lung homogenates at a) 0h b) 2h c) 4h d) 8h e) 12h f) 24h g) 48h

On the basis of *in-vivo* results, it could be inferred that the pulmonary administration of sildenafil citrate dry powder formulations could significantly prevent and reverse PAH without affecting the 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 the lipid based formulations as compared to Conventional DPI and spray dried drug-sugar composites linking the improved hemodynamic and biochemical parameters for better prevention and treatment of PAH in MCT model (Jaspart S, Bertholet P, *et al*, 2007).

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 reconnoitred to reduce the patient inconvenience of frequent administration of currently available therapies while exerting the minimum systemic side effects.

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 therapeutic agent directly 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 inhlation) 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 24mcg/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 (197ºC) 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 (0.97±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, % drug 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-MCT_{28} 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-MCT_{14} 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-MCT_{14},

Only-MCT_{28} 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.

Chapter: 7

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List of Publications

1. **S**. **Abrol (Trehan)**, A. Trehan, and O. P. Katare, "Comparative study of different silymarin formulations: formulation, characterisation and in vitro/in vivo evaluation," Current **Drug Delivery**, vol. 2, no. 1, pp. 9–21, 2004.

2. **Sonia Abrol (Trehan)**, Aman Trehan and O. P. Katare. Formulation, Characterization, and In Vitro Evaluation of Silymarin-Loaded Lipid Microspheres 2004, Vol. 11, No. 3, Pages 185-191.

3. Misra Ambikanandan, **Trehan Sonia**. Pulmonary delivery of sustained release pharmaceutical Compositions of PDE5 inhibitors and combinations thereof for pulmonary arterial hypertension. **Indian Patent** Application No. 1237/MUM/2011.

4. **Trehan S**, Sharma G, Misra A. siRNA: Sojourn from discovery to delivery challenges and clinics. Syst Rev Pharm 2010;1:1-16

5. Khatri NI, Rathi MN, Kolte AA, Kore GG, Lalan MS, **Trehan S**, Misra AR Patents review in siRNA delivery for pulmonary disorders. Recent Pat Drug Deliv Formul. 2012 Apr 1; 6(1):45-65.

6. NI Khatri, MN Rathi, DP Baradia, S **Trehan**, AR Misra. *In Vivo* Delivery Aspects of miRNA, shRNA and siRNA. Critical Reviews™ in Therapeutic Drug Carrier Systems. 29(6), 487-527 (2012).

7. Dipesh Baradia, Nirav Khatri, **Sonia Trehan,** Ambikanandan Misra, Inhalation therapy to treat pulmonary arterial hypertension. Pharmaceutical Patent Analyst, November 2012, Vol. 1, No. 5, Pages 577-588.

8. Gitanjali Kher, **Sonia Trehan**, Ambikanandan Misra. Chapter 7:Antisense Oligonucleotides and RNA interference. In Challenges in Delivery of Therapeutic Genomics and Proteomics. Ed: Ambikanandan Misra. First Edition. Elsevier. USA, 2011, 325-386.

9. **Sonia Trehan**, Ambikanandan Misra. Chapter 1:Polymers in Drug Delivery Systems. In Applications of Polymers in Drug Delivery. Eds: Ambikanandan Misra and Aliasgar Shahiwala. Smithers Rapra. England (To be published in 2013).

10. **Sonia Trehan**, Mohan Rathi, Ambikanandan R Misra. Pulmonary delivery of sustained release dry powder formulations to treat pulmonary hypertension. (Communicated Research Work).

Summary

Pulmonary Arterial Hypertension (PAH) is WHO Group I class of pulmonary hypertension (PH) defined by the blood pressure higher than 25 mmHg at rest or 30 mmHg during physical activity, in the pulmonary arteries. Pulmonary hypertension is a severe pathophysiological condition in which the right heart needs to work harder to force the blood to the lungs through constricted small pulmonary arteries eventually leading to right heart failure. New treatment guidelines and increasing awareness of PAH is now attracting R&D investment to enter this compact but lucrative market due to the high unmet needs and high treatment values per patient. Commercially available treatments like Flolan® (Epoprostenol sodium) and Remodulin® (Treprostinil) as a continuous intravenous infusion; Tracleer® (Bosentan, twice a day), Revatio® (Sildenfil, thrice a day) as tablet dosage form are associated with limitations of frequent dosing and patient incompliance. Further, once a day available treatments like Thelin® (Sitaxsentan), Letairis® (Ambrisentan) and Adcirca® (Tadalafil) are associated with non-specific vasodilation and other toxic effects in whole vasculature instead of reducing pulmonary vascular resistance.

For respiratory conditions, non-invasive pulmonary route of delivery is preferred to deliver the drug for instant and enhanced local (pulmonary arteries) action while reducing the exposure of drug to the systemic circulation and hence potentially minimizing adverse effects. Ventavis® (Iloprost sterile solution) and Tyvaso® (Treprostinil sterile solution) are the only commercially available inhalations, but with a drawback of high inconvenience to patient due to very frequent dosing of 6-9 times and four times (3 inhalations per treatment) per day respectively.

Several lipid and biodegradable polymer based sustained-release systems have been explored as potential carriers for pulmonary delivery. Components of the delivery system must be non-toxic, non-immunogenic, biodegradable and without inflammatory and alloreactive reactions. Using lipid based dry powder inhaler formulations like liposomes and lipid particles, these limitations can be largely circumvented due to its ability to act as pulmonary sustained release reservoir. Furthermore, the key challenge with the pulmonary delivery of dry powders is the high dispersibility of the powder for reproducible and higher deposition at the required site. Large porous particle technology has been shown to improve the dispersibility of the powders and hence reproducible delivery of the drugs to the patients via lungs. Use of spray

drying technique can further perk up the lung deposition by enhanced dispersibility of the particles with required aerodynamic diameter and narrow particle size distribution.

Further, in pulmonary circulation, cGMP plays a major role on pulmonary vascular resistance. PDE5, the enzyme that specifically hydrolyzes cGMP, is abundantly expressed in the whole lung and predominates in pulmonary artery smooth muscle cells and both the activity and the expression of PDE5 are increased in pulmonary arteries with PAH. Recently, sildenafil citrate, a potent and selective PDE5 inhibitor successfully used for the treatment of erectile dysfunction has now been approved to treat PAH. It is available as tablet and injection dosage forms and is associated with non-specific vasodilation and other toxic effects.

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:

1. Formulation of various dry powder formulations of sildenafil citrate for pulmonary delivery to achieve practically feasible, scalable and stable formulations with desired formulation characteristics for better lung deposition and sustained release effect.
2. Characterization and comparison of these formulations with respect to desired parameters of dry powder formulations for inhalation like geometric and aerodynamic particle size, solid state characteristics, moisture content, aerosolization behaviour and storage stability.
3. In-vitro evaluation of the optimized formulations with respect to release pattern or kinetics to compare sustained release potential of the prepared formulations. Comparison of macrophage uptake of lipid based formulations with standard 2µ fluorescent latex beads to intimate the existence time of formulation in lungs.
4. Pulmonary delivery of sustained release dry powder formulations of sildenafil citrate to evaluate its prolonged local efficacy in monocrotaline-induced pulmonary hypertensive rats.

Aforementioned extensive experimentation disclosed following outcomes and inferences:

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

- Formulations prepared by spray drying technique presented better particle size distribution and aerosolization behaviour as compared to conventional DPI formulations. Spray drying parameters like feed rate, air pressure, inlet temperature and vacuum have individual as well as combined impact on formulation characteristics.
- 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.
- 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.

CERTIFICATE

This is to certify that the following enlisted publications (publications 1-3 are attached herewith) belong to my PhD. student Ms. Sonia Trehan who wishes to submit thesis entitled **"Treatment of Pulmonary Arterial Hypertension by Pulmonary Drug Delivery"** herewith to The Maharaja Sayajirao University of Baroda, Vadodara for the award of Ph.D. in Pharmacy.

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