Table of Contents

Acknowledgment

'Gratitude is a currency that we can mint for ourselves and spend without fear of bankruptcy'

-Fred De Witt Van Amburgh

As appropriately stated, the success of my Ph.D. work would be incomplete without expressing gratitude towards each individual who succoured in accomplishment of my goal. First and foremost, I wish to thank the great **Almighty** for blessing me with enough fortitude, purpose, enthusiasm and confidence to consistently attain my target and pray for the same in my future aims.

Mere words are inadequate to express my sincere and deepest feelings of appreciation originating from the innermost core of the heart for my Ph.D. research supervisor **Dr. Anil Jindal,** Assistant Professor, Department of Pharmacy, BITS-Pilani, Pilani campus for believing and encouraging me throughout my PhD tenure. I am obliged to him for his timely advice, co-operative approach, critical assessment, constructive criticism, untiring relentless efforts throughout the period of research. His scientific approach to rectify research problems is really commendable. His constructive suggestions, patience and belief were the genuine propulsion behind the successful completion of this thesis work. Mere words would not suffice to express gratitude for the parental love received from him during the period of my research.

It was indeed an honour for me to join as a research scholar in BITS-Pilani. This thesis would not have seen the light of the day without the infrastructural and administrative support from the institute for which I owe a special thanks to **Prof. Souvik Bhattacharyya,** Vice-Chancellor, BITS-Pilani, **Prof. Sudhirkumar Barai,** Director, BITS-Pilani, Pilani campus, **Prof. Sanjay Kumar Verma,** Dean, Administration, BITS-Pilani. I wish to extend special thanks to **Prof. Jitendra Panwar,** Associate Dean, Academic - Graduate Studies and Research (AGSR) Division for their administrative support.

I sincerely thank **Dr. Deepak Chitkara**, Convener, Departmental Research Committee, and DAC member for his timely suggestions, intellectual guidance and support during various stages throughout the thesis work. This thesis work would not have been the same without the scientific inputs, enthusiastic discussions and evaluation from another DAC member for my proposed topic of research, **Dr. Aniruddha Roy**, Asst. Professor, Department of Pharmacy, BITS-Pilani. I wish to acknowledge **Prof. Hemant Jadhav**, Head-Department of Pharmacy, BITS-Pilani and **Prof. Atish Paul**, ex-HOD, Department of Pharmacy, BITS-Pilani for providing timely support and access to the departmental facilities required for my research work. Special gratitude to **Prof. Anil Gaikwad**, Associate Dean-Practice school division, Department of Pharmacy, BITS-Pilani for analysing the potential in me and lending me opportunity to become a part of esteemed Department of Pharmacy, BITS, Pilani after my PhD interview. I wish to thank **Dr. Sushil Yadav,** Veterinarian in charge, Central Animal Facility for supporting me during the experimentation. I would like to extend my sincere gratitude to the faculty members **Prof. R. Mahesh, Prof. S. Murugesan, Prof. Rajeev Taliyan, Dr. Anupama Mittal, Dr. Sunil Kumar Dubey, Dr. Gautam Singhvi, Dr. Murli Manohar Pandey, Dr. Sandeep Sundriyal, Dr. Vaibhav A. Dixit and Dr. Richa Shrivastava** who have supported me during my research and teaching practice. I wish to thank whole non-teaching staff **Mr. Rampratap Suthar, Mr. Puran, Mr. Lakshman, Mr. Navin, Mr. Sajjan, Mr. Tarachand, Mr. Shyam Sundar, Mr. Shiv, Mr. Vishal, Mr. Mukesh** and **Mr. Mahender** for their direct and indirect help and support during my doctoral program.

I am extremely thankful to my juniors, **Mr. Atharva and Mr. Kedar** for their untimely helping hand, motivation, companionship and unequivocal support during my research studies.

My stay in BITS Pilani was made commendable and enjoyable in large part due to friends like **Ms. Nisha, Ms. Pracheta, Mr. Kishan, Mr. Krishna, Mr. Ginson, Ms. Moumita and Ms. Geetika** who lighten up my mood and remained my rock-solid support throughout the ups and downs that came in my personal as well as professional life. I am happy to acknowledge my junior colleagues, **Mr. Vamhsi krishna, Mr. Sudeep, Mr. Sarath, Mr. Samrat, Ms. Swetha, Ms. Swati, Ms. Violina, Ms. Paramita, Ms Kavya, Mr. Karan, Mr. Arihant, Mr. Amritansh, Mr. Deepak, Ms. Sakshi, Ms. Sonia, Mr. Mani, Mr. Ajinath, Mr. Prashant** for their bright smiles and positive attitudes with which they met me each day and inspired me to keep working.

I am extremely rapturous to acknowledge my senior colleagues, **Dr. Satish Reddy, Dr. Archana Khosa, Dr. Almesh Kadakol, Dr. Anuradha Pandey, Dr. Vajir Malek** and **Mr. SNC Sridhar** for their unconditional love and support.

I acknowledge BITS-PILANI for providing me the financial assistance for my doctoral research.

My family deserves special mention for their unwavering support and blessings. I would like to acknowledge my parents **Mr. Hemant Rajaram Surve** and **Mr. Surekha Surve** for their immense love, care, support, motivation, constant counselling and making me fortunate enough to write a thesis. My father was a constant source of inspiration for me. I am thankful to my brother **Mr. Shubham Surve** for his perpetual love, support and wisecrack character to lighten my mood which was essential in completing this journey. I also want to thank my friends **Mr. Pranit More, Mr. Mitesh, Mrs. Kavita, Ms. Bhakti and Mrs. Dhanashree Lokegaonkar** who remained a keen listener to all my worries and motivated me to keep going.

Last but not the least, I owe a great debt of gratitude to the **innocent rats** who sacrificed their lives to unearth the facts that are the necessary foundation of this research.

List of Abbreviations

AOT	1,4-bis-(2-ethylhexoxy)-1,4-dioxybutane-2-sulfonate
ACN	Acetonitrile
API	Active pharmaceutical ingredient
ARV	Antiretroviral
ATLAS	Antiretroviral therapy as long acting suppression
AUC	Area under curve
BCS	Biopharmaceutical Classification System
BDDS	Biopharmaceutical Drug Disposition Classification System
CAB LAP	Cabotegravir Long acting parenteral
cART	Combination antiretroviral therapy
CD	Circular Dichroism
C_{max}	Maximum concentration
DAPI	4',6-diamidino-2-phenylindole
DIR	1.1'-Dioctadecyl-3,3,3',3'-Tetramethylindotricarbocyanine iodide
DMEM	Dulbecco's modified eagle medium
DcNP	Combination drug nanoparticle
DS	Docusate sodium
DSC	Differential scanning calorimetry
% DL	% drug loading
%EE	% entrapment efficiency
Efa	Efavirenz
Enf	Enfuvirtide
FESEM	Field emission scanning electron microscopy
FTIR	Fourier transform infrared
GIT	Gastrointestinal tract
HIV	Human immunodeficiency virus
HIC	Hydrophobic ionic complex
HETP	Height equivalent to theoretical plate
HQC	High quality control
RP-HPLC	Reverse Phase High Performance Liquid Chromatography

ISMM	Isometamidium chloride
ISMM-DS LNP	Isometamidium chloride-docusate sodium complexed lipid nanoparticles
FACS	Fluorescence activated cell sorting
HAART	Highly active antiretroviral therapy
IM	Intramuscular
IV	Intravenous
LA	Long acting
LASER	Long acting slow effective release
LDN	Lipid drug nanoaprticle
LOD	Limit of detection
LLOQ	Lower limit of Quantification
LQC	Low quality control
MDM	Monocyte derived macrophage
MEC	Minimum effective concentration
MQC	Medium quality control
MTT	Thiazolyl blue tetrazolium bromide
PDI	Polydispersity index
PLN	Polymer-lipid hybrid nanoparticles
PSA	Polar surface area
MRT	Mean residence time
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NRTI	Nucleoside reverse transcriptase inhibitor
PLGA	Poly(lactic-co-glycolic acid)
OLS	Ordinary least square
RPMI	Rosewell Park Memorial Institute
RSD	Relative standard deviation
SC	Subcutaneous
MTT	Thiazolyl blue tetrazolium bromide
PMA	Phorbol-12-myristate-13-acetate
WLS	Weighted least square

Abstract

Perennial relapse of infectious diseases in humans and animals emanating from failure in disease treatment is a major cause of solicitude for grave socio-economic loss. Long-acting (LA) nanoformulations would overcome the pitfalls associated with existing anti-infective agents including inadequate plasma and tissue concentration, incompetence in treatment adherence due to high pill burden, frequent dosing interval, injection site reaction and ascendancy in treatment cost; further worsen the disease conditions. LA nanotechnology involves secondary depot genesis in immune cells infiltered at the injection site (primary depot) after subcutaneous/intramuscular (SC/IM) administration. Furthermore, the slow effective release of drugs from the immune cell depot transpires due to their traverse through the lymphatic vesicles, sequestration into lymph node, and slow drainage into systemic circulation preceding thoracic duct which upon reaching various organs differentiate into tissue macrophages forming secondary tissue depot. Therefore, current research work involved the development and evaluation of LA nanoformulations for targeted delivery of antiretroviral and anti-trypanosomal drugs. Although hydrophobic drugs with longer half-lives were found to be suitable for LA potential; yet LA nanoformulations are desirable for drugs with a short half-life (<10 h) with frequent dosing. Therefore, the aim of the present work involves the development and characterization of LA nanoformulations incorporating hydrophilic high molecular weight peptide (Enfuvirtide; Enf) with short half-life (3.8 h) co-loaded with hydrophobic Efavirenz (Efa) in polymer-lipid hybrid nanoparticles (PLN). It further involved the development and characterization of hydrophilic charged anti-trypanosomal drugs (Isometamidium chloride; ISMM) loaded into solid lipid nanoparticles (ISMM-DS LNP) eliciting LA effect.

The development of suitable analytical and bioanalytical methods is indispensable for the estimation of each drug from its nanoformulation, release media, and biological matrices.

Therefore, the present work involved RP-HPLC and spectrophotoflurimetric method development for the estimation of Efa and Enf from Efa-Enf PLN. While, RP-HPLC analytical and bioanalytical method development was done for estimation of ISMM from ISMM-DS LNP, release media, and different biological matrices. The bioanalytical method development involved a novel ion-pairing approach for estimation of ISMM using 0.1% v/v formic acid in sodium lauryl sulfate buffer (5 mM, pH 3, 55% v/v) and 45% v/v acetonitrile. The method could suitably elute ISMM from biological matrices due to decreased interaction of free ISMM with free silanol groups and ion-pairing with ISMM. The developed analytical and bioanalytical method were validated as per ICH Q2(R1) and USFDA guidance for industry, respectively.

LA Efa-Enf PLN were prepared by a double emulsion solvent evaporation method. Box-Behnken design was utilized to optimize three high-risk factors namely, Efa amount, sonication time for primary emulsion, and sonication time for aqueous nano-dispersion obtained from preliminary studies. The optimized Efa-Enf PLN was found to have with particle size, PDI % EE of Efa and Enf of 346.4 nm ±30.41 nm, 0.440±0.06, 27.1%±0.78% and 69.7%±3.79% respectively. Lyophilized Efa-Enf PLN using trehalose elicited spherical morphology, drug amorphization on incorporation depicted by DSC studies, and absence of drug-excipient interaction evaluated by FTIR spectroscopy. *In vitro* release studies revealed extended-release of both drugs from PLN with differential release profile of Efa and Enf upto 33 h and 120 h; respectively. Blood compatibility studies of Efa-Enf PLN revealed low hemolytic, platelet and leukocyte aggregation. While, Efa-Enf PLN exhibited low cytotoxicity in Jurkat E6.1 T cells and U937 macrophage cells. Circular dichroism spectra confirmed the presence of an α-helix form of Enfuvirtide after encapsulation in PLN. Coumarin-6 loaded PLN exhibited enhanced cellular uptake in Jurkat E6.1 T cells and U937 macrophage cells in comparison to free coumarin-6 as evidenced by fluorescent microscopy

and flow cytometry. *In-vivo* biodistribution studies after intravenous administration of near-infrared dye (DIR) loaded PLN (surrogate for Efavirenz-Enfuvirtide PLN) using bioimaging technique revealed non-uniform distribution within 2h in order of spleen ≥ liver > lymph node > thymus > lungs >female reproductive tract (FRT)> heart > kidneys > brain. While, subcutaneous administration caused non-uniform biodistribution after 3 days eliciting long-acting slow release from injection site depot until day 5 in infection spread site (lymph nodes and female reproductive tract), reservoir sites (liver and spleen), and difficult-to-access site (brain). Furthermore, it presents a vital illustration of available tissue-specific drug concentration prediction from simulated surrogate PLN.

The second aim of current research involved the development of LA nanoformulation for a charged hydrophilic drug with a short half-life (<10 h) to attain protracted plasma drug release as well as secondary tissue depot. ISMM was adopted as a representative molecule and incorporated suitably into solid lipid nanoparticles (LNP) to achieve the established goal. The ISMM LNP were developed by a novel *in situ* complexation and solvent evaporation method. Docusate sodium (DS) was selected as an anionic complexing agent upon pretesting the available complexing agents by n-octanol and water solubility studies. ISMM-DS elicited maximum complexation (358.3±108.41 µg/ml ISMM-DS LNP solubility in n-octanol) at 1:4 charge molar ratio of ISMM and DS respectively. ISMM-DS LNP were developed using Precirol® ATO 5 and tween-80 as solid lipid and surfactant respectively to yield particle size, PDI, zeta potential, and ISMM % EE of 173.17 nm ± 10.70 nm, 0.22 ± 0.02, -48.3 mV ± 2.02 mV, and 69.14% ± 1.27%, respectively. *In vitro* drug release studies of ISMM-DS LNP revealed 46.3%±8.9% drug release within 1 h. Thereafter, sustained drug release was observed leading to plateau until 24 h, mimicking the behaviour of ISMM-DS LNP upon exposure to systemic circulation. Blood compatibility studies revealed non-haemolytic potential (0-2.52% haemolysis) of ISMM-DS LNP. While, cytotoxicity studies of ISMM-DS LNP revealed

74.6±3.87% cell viability in Vero cells at the minimum effective concentration (MEC; 1-4 µg/ml) of ISMM. Furthermore, cellular uptake studies in THP-1 macrophage-like cells using fluorescence microscopy with apotome attachment revealed 2.3-folds higher uptake of ISMM-DS LNP compared with free ISMM. While, the presence of a significantly higher amount of ISMM in PBMC (68.4±19.0 ng/ml) at day 7 upon subcutaneous (SC) administration of ISMM-DS LNP confirmed the potential of ISMM-DS LNP for secondary depot genesis *in vivo*. Moreover, the ISMM-DS LNP engender protracted plasma drug release for 7 days with 3.0-, 4.5-, and 4.2- folds enhanced $AUC_{0-\infty}$, $MRT_{0-\infty}$, and $t_{1/2}$, compared with free ISMM. Further, the Cl/F was decreased by 4.2-folds upon SC administration of ISMM-DS LNP compared with free ISMM. Therefore, ISMM-DS LNP overcome the drawbacks associated with free ISMM including miniscule plasma retention and quick disposition leading to parasite relapse. Biodistribution studies revealed accumulation of ISMM in various reticuloendothelial (RES) organs and non-RES organs in the order of liver>spleen>kidneys>lymph node>lungs on day 7. Interestingly, the amount of ISMM was 2.9-, 4.2- and 2.0-folds higher in RES organs like liver (Kupffer cells), spleen (spleenotropic macrophages and 15% T-lymphocytes), and lymph nodes (75% T-lymphocytes), respectively in LA ISMM-DS LNP group compared with free ISMM which confirmed the mechanism of primary and secondary depot genesis of LA ISMM-DS LNP. To conclude, current research work was an invaluable contribution for long-term treatment and prophylaxis of drugs with varied physicochemical properties, short half-life (<10 h), desirable of frequent administration, and high adherence.

List of Figures

List of Tables