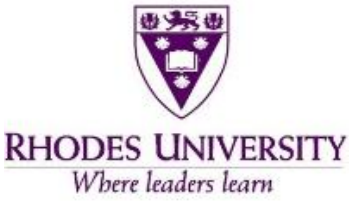
FORMULATION AND EVALUATION OF LIPOSOMAL FILMS FOR BUCCAL DELIVERY OF ANTIRETROVIRAL DRUG.

A Dissertation

Submitted in Fulfilment of the

Requirements for the Degree of

Master of Science (Chemistry)



By Nnamdi Ikemefuna Okafor

Supervised by: Prof. Rui Werner Krause

November 2018

DEDICATION

To almighty God and to the entire family of Ezinne Helen Akpunku Okafor.

ACKNOWLEDGMENTS

I would like to thank my supervisor Prof. Rui Werner Krause for accepting me as his student and his immense contributions for the success of the research project. I greatly wish to thank my mother Ezinne Helen Okafor for her prayers and words of encouragement. Truly mama, your prayers and motivation has been my source of strength and I owe everything I am and whatever I have to you. To my brother Mr Christian Ndidi, thank you for your amazing financial support throughout the course of this work.

My gratitude goes to National Research Foundation (NRF) for the block grant given to me which went a long way in minimizing my financial burdens.

I sincerely wish to appreciate Prof. R.B. Walker, Department of Pharmacy, Prof. A.N Hodgson, Department of Zoology and Entomology and Prof. Isadore Kanfer, Department of Pharmacy for the opportunity given to me to make use of their equipment in their various laboratories.

I would like to acknowledge my colleagues in F22 Laboratory Chemistry department, especially Mr Christian Nkanga Isalamboto for his tremendous contribution and guidance throughout my research, Dr. Mpho Ngoepe for his opinion and suggestions, Dr. Xavier Noundou for his outstanding input and help, Mr Nnaemeka Nnaji for his wonderful advice and contributions, and Rev. Idowu Akinloye law department for his prayers and invaluable input throughout my work.

Last but not the least, I wish to extend my acknowledgement to Seepra Rath, Department of Pharmacy for her generosity in the course of my research, to Mrs Stella I. Aghahiwe for her encouragement and assistance and to the entire members of St' Michael and St' George students ministry for being my family away from home.

I express my deep gratitude and apologize to people whose their inputs could not be mentioned here.

ABSTRACT

The human immune deficiency virus (HIV) infection has been ranked as one of the most devastating microbial infections in the world. This status is a result of the HIV rapid genetic variation, which limits discovery of a vaccine. Use application of antiretroviral therapy (ARVT) in treatment of the disease caused by the HIV infection (known as acquired immunodeficiency syndrome, HIV-AIDS) is frequently compromised by several factors such as the low bioavailability and severe adverse effects associated with the existing antiretroviral drugs (ARVDs). This underlines the need for controlling the pharmacokinetics profiles of ARVD using effective vehicles that can modify drug biodistribution. The same is true for many other conditions, where delivery systems can determine the success or failure of treatment by controlling pharmacokinetic and dynamic properties. The mucosal linings of the oral cavities in addition offer adorable route of administration for systematic drug delivery, improving drug therapeutic performance and often preferred by clinicians and patients. Liposomes are tiny spherical sacs of phospholipid molecules enclosing water droplets, formed (artificially) to carry drugs or other substances into the tissues by crossing and targeting to specific organelles. This work therefore focused on preparation of liposomes and liposomal buccal films (BFs) for potential buccal delivery of efavirenz, an ARVD model endowed with poor solubility and several side effects.

The liposomes were prepared by thin film hydration method using crude soybean lecithin (CL) and cholesterol. Efavirenz loaded liposomes were evaluated for particle size, Zeta potential (ZP), morphology, encapsulation efficiency (EE%) and release kinetics studies. The physiochemical properties of the liposomes were also evaluated using Differential Scanning Calorimetry (DSC), X-ray diffraction (XRD), energy dispersity spectroscopy (EDS), and Fourier transform infrared (FTIR), while the formulation with the best encapsulation efficiency was used as the solvent medium for the buccal film formation. The buccal films were prepared using solvent casting method, where the liposomal suspension was used as the dispersing medium. The films were optimized for physical properties (thickness, weight variation and folding endurance) using digital Vernier calliper and digital weighing balance. The physiochemical properties of the selected BFs films made of Carbopol (CP) and its combination with Pluronic F127 (PF127) were further characterized using XRD, DSC, FTIR, Transmission Electron Microscopy (TEM), EDS and Scanning Electron Microscopy (SEM). The

permeation study of the selected BFs was investigated using Franz diffusion cell. The BFs composed of CP alone or its combination with PF127 demonstrated much better bio-adhesive properties than the films made of other polymers (like Hydroxyl propyl methyl cellulose, HPMC) alone or in combination with PF127.

The developed liposome formulation showed high encapsulation 98.8 ± 0.01 % in CL to cholesterol mass ratio of 1:1 and total lipid to drug mass ratio of 2:1. The average particle size 104.82 ± 2.29 nm and Zeta potential -50.33 ± 0.95 mV of these liposomes were found to be attractive for targeted delivery to the HIV infected cells. The CP based BFs (without and with PF127) exhibited good film thickness 0.88 ± 0.10 and 0.76 ± 0.14 mm, with weight uniformity 68.22 ± 1.04 and 86.28 ± 2. 16 mg, satisfactory flexibility values 258 and 321, and slightly acidic pH 6.43 ± 0.76 and 6.32 ± 0.01. The swelling percentage was found to be 50 % for CP film alone and 78 % for CP film with PF127. The cumulative amount of drug that permeated through the buccal epithelium over 24 hours was about 66 % from CP film alone and 75 **%** from CP film with PF127.

Since no evidence of the liposomal encapsulation of EFV have been reported to our knowledge, we find the insights from the present study valuable as a set of preliminary data to encourage further investigations of the encapsulation and delivery of EFV like antiretrovirals for enhanced solubility, site targeting and prolonged release using crude soybean lecithin and mucoadhesive polymers, which holds some added economical values as naturally occurring lipid and polymeric mixtures as a promising delivery systems for buccal delivery of ARVDs.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1:

LITERATURE REVIEW

1.1. Acquired Immunodeficiency Syndrome (AIDS)

1.1.1. Definition and History

The acquired immunodeficiency syndrome (AIDS) is ranked as one of the most devastating and major life-threatening diseases to humans. AIDS is caused by the human immunodeficiency virus (HIV), a lentivirus that infects the human white blood cells and leads to gradual and persistent decline and failure of the immune system. This results in heightened risk of contracting various infections and cancers (UNAIDS, 2016). HIV is known to have been transmitted to humans by non-human primates in Africa as a consequence of zoonotic transfers of viruses infecting primates (Gao *et al.*, 1999; Sharp and Hahn, 2015).

AIDS was first recorded on the 5th June 1981, when the U.S. Centre for Disease Control (CDC) reported a cluster of *pneumocystis carinii* pneumonia (currently named as PCP, but known to be caused by *pneumocystis jirovecii*) in five homosexual men in Los Angeles (Greene *et al.*, 2007; MMWR, 2011).

The earliest known positive identification of the HIV-1 virus emanated from Congo in 1959 and 1960, this is one of the most accepted theories that the virus probably was transmitted to the human population from chimpanzees (Hooper *et al.*, 2000; Sharp and Hahn, 2015).

In 1983, HIV was first identified as a reverse transcriptase containing virus (retrovirus), after being isolated from the lymph node of a man with persistent lymphadenopathy syndrome (LAS) by Barre-sinoussi at the Pasteur institute (Barre-SINOUSSI *et al.*, 1983; Levy, 1993). This led to the hypothesis that LAS was hugely associated with the HIV infection (AIDS), but no conclusive evidence was provided (Barre-SINOUSSI *et al.*, 1983).

In 1984, from a report by Gallo supported the investigation from the Pasteur institute that the virus isolated from lymph was a member of a well-known retrovirus group. However, many other physicians suggested the LAS emerged from the Epstein-Barr virus (EBV) or the cytomegatovines (CMV), since the enlarged lymph nodes were found in numerous viral infections (Levy *et al.*, 1993).

Later on, the HIV-1, previously known as retrovirus, was identified as the causative agent of AIDS, which then became one of the high risk diseases to have surfaced in recent history (Barre-SINOUSSI *et al.*, 1983; Popovic *et al.*, 1984). Since the discovery of HIV-1 many decades

ago, the pandemic has infected more than 60 million individuals and has caused over 25 million deaths (Merson *et al.*, 2008). A high rate of morbidity, mortality and prevalence of HIV-1 has been witnessed in the sub-Saharan Africa and other developing countries, especially among adults (UNAIDS, 2016).

During the late 1980s, azidothymidine (AZT) or Retrovir was the first drug for HIV treatment. Subsequently, in the mid-1990s, antiretroviral therapy (ARVT) was based on the combination of azidothymidine, didanosine and zalcitabine for management of AIDS. This combination therapy slows down the viral replication and prevents the evolution of AIDS to advanced stages (UNAIDS, 2016).

1.1.2. Epidemiology

In 2017, the global HIV incidence was an estimated 22.0 million, associated with about 510,000 deaths in sub-Saharan Africa. Sub-Saharan Africa remains the most affected region in the world, with South Africa accounting for the most HIV populated nation in the world. About 5.9 million people were diagnosed in 2014 (UNAIDS, 2018).The overall epidemiology showed a decrease in HIV incidence and mortality in sub-Saharan Africa from 2000 to 2012. The decrease in HIV incidence among adults was by more than half, which corresponds to an estimate of 1 million fewer new infections in 2012 compared to 2000. The number of AIDS related deaths was found to have fallen from approximately 1.4 million in 2000 to 1.2 million in 2012 (Fettig *et al.*, 2014; UNAIDS, 2015).

There has been also a slight drop in in the HIV incidence in Asia. AIDS related deaths among the adults and children in Asia have slowly decreased from 330,000 to approximately 280,000 in the 2015-2017 period. Both China and India were reported to have the highest HIV burden due to their large populations (UNAIDS, 2018).

In 2008, there were more than 1.2 million HIV-positive people in the United State, of which 30 % were not aware of the HIV infection. This led to 17,500 deaths per annum from 1999 to 2008, as reported by the Centre for Disease Control and Prevention (CDC) in 2011 (Lazzarini *et al.*, 2009). 516 deaths were reported in Australia from approximately 86,500 HIV cases by Health and Protection agency in 2010 and 23 deaths were earlier reported in 2009 from about 21,171 cases of HIV cases (UNAIDS, 2016). In Canada, the 2009 HIV and AIDS surveillance report recorded about 65,000 cases of HIV and 53 deaths in 2008. According to the WHO,

over 36.7 million people are living with HIV globally, with about 1.8 million cases in children (UNAIDS, 2016). In 2016, there was 1 million HIV related deaths in sub-Saharan Africa. However, there was a remarkable drop in the number of new HIV cases in 2016, from 2.1 million new infections to 1.8 million, which was a successful result of increased HIV awareness and other preventive measures (UNAIDS, 2016). **Figure 1.1** shows the HIV prevalence in the age range of 15–49, with Africa exhibiting the highest number of HIV cases.

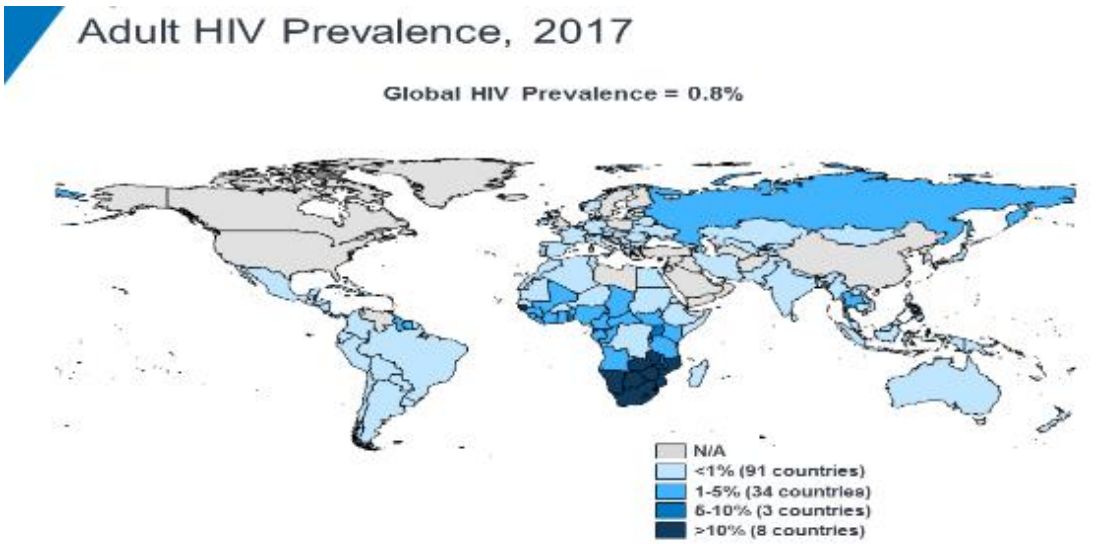


Figure 1.1. Global HIV prevalence among adults aged 15 to 49, 2017 (UNAIDS, 2018)

1.1.3. Pathogen

HIV, the causative agent of AIDS, is a lentivirus that forms a separate genus of the retroviridae family, which includes various viruses that affect a diverse group of animal species (Coffin *et al*., 1992; Levy *et al*., 1993; Kirchhoff, 2010; Brown *et al*., 2012).

Lentiviruses are considered as an equine infection anaemia virus, since it induces episodic autoimmune haemolytic anaemia in horses and has proved to have devastating effects on the equine population in many parts of the world, especially in Japan (Sellon *et al.,*1994). Although the lentivirus agent initially isolated exhibited some retrovirus characteristics previously, it was identified as an RNA virus containing a reverse transcriptase and a member of the lentivirus genus (Charman *et al.*, 1976).

The International Committee on Taxonomy of Viruses endorsed the name human immunodeficiency virus (HIV), in order to resolve the host and to refer to a major biological

property of the virus, i.e. the detrimental effect it has on the human immune system (Anderson *et al.*, 1986; Coffin *et al.*, 1986).

HIV enters cells through the cluster of differentiation molecule ($CD4^+$) chemokine co-receptors (CXCR4 and CCR5) as shown in **Figure 1.2** (Gupta and Jain, 2010). The effect of HIV on the immune system was extensively investigated due to the development of cytometry in the 1970s, which helped clinical and research laboratories to discover the number of the $CD4^+$ and $CD8^+$ cells using monoclonal selection antibodies (Gomez and Hope, 2005).

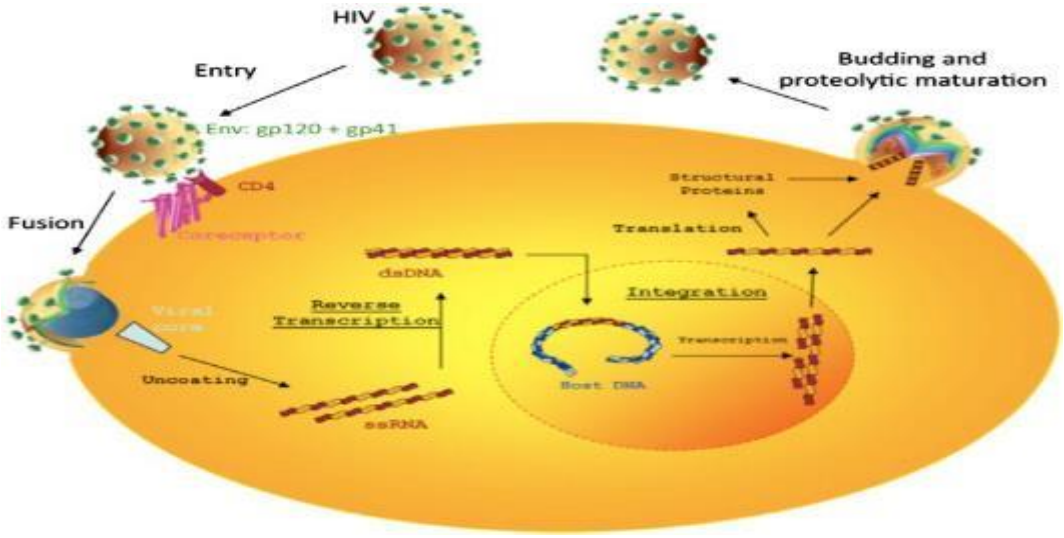


Figure 1.2. HIV replication adapted from (Teixeira *et al*., 2011)

The $CD4^+$ lymphocyte was later discovered to be the main target for HIV replication and the receptor on $CD4^+$ cells were identified as the major attachment site for the virion (**Figure 1.2**). The presence of the HIV RNA in the blood at detectable levels allows treatment monitoring and better control of the viral load drop and replication (Levy *et al.,* 2006*;* Texeira *et al.,* 2011).

The viral dynamics were formerly used to evaluate the mono-therapy and later a combined therapy, currently referred as a highly active antiretroviral therapy (HAART), which generally corresponds to a three drug-combination (Fauci *et al.*, 2003; Levy *et al.*, 2006). However, many novel therapies (including dolutegravir and cabotegravir) that are under consideration that promote RNA interference, target specific viral genes and use drugs that block virus interaction with the chemokine co-receptors or fusion at entry into cells (Cammack *et al.*, 2001; Levy *et al.*, 2006; Escape *et al.*, 2018).

1.1.4. Pathogenesis, symptoms and treatment

The virus typically gains entry into the host cells by binding to the cell surface of the CD4 receptor and a chemokine co-receptor (CCR5 and CXCR4) (Lawn *et al.*, 2004). The CD4 receptor molecules, the main target of HIV-1, undergo conformational change that allows the glycoprotein 41 (gp41) to mediate fusion of the viral envelope with the host cell membrane (Lawn *et al.*, 2004). HIV weakens the immune system by infecting and destroying $CD4^+$ cells, which in turn leads to immunodeficiency at a later stage of the disease (Chun and Fauci, 2012).

The interaction between the chemokine co-receptors and the virus triggers irreversible conformational changes and induces complete fusion between the virus and the receptor as well as pore formation, whereby the viral core is released into the cytoplasm (Eckert and Kim, 2001; Simon, Ho and Karim, 2006).

A dramatic depletion of the $CD4^+$ cells in the peripheral blood brings the HIV infection at the acute phase. The $CD4^+$ T-cells depletion may lead to a massive depletion of the $CCR5^+$ memory cells (Moir *et al.*, 2010).

Infected individuals present virus-like illness within 1-3 weeks of infection. The associated symptoms include muscle ache, headache, retro-orbital pain, sore throat, swollen lymph nodes, low grade or high-grade fever, non-pruritic macular erythematous rash involving the trunk and later the extremities (Cooper *et al.*, 1985; Connolly *et al.*, 1989). There are also signs of oral candidiasis and ulceration in the oesophagus, with disorder in the central nervous system as shown in **Figure 1.3** (Carne *et al.*, 1982; Rabeneck *et al.*, 1990). Cases of diarrhoea, pneumonia and some other gastro intestinal complaints have been reported in acutely infected individuals (Carr *et al.*, 1991). All these symptoms exist for 1 to 3 weeks, except lymphadenopathy, lethargy and malaise, which can persist for many months and the treatment requires long course of combined drugs.

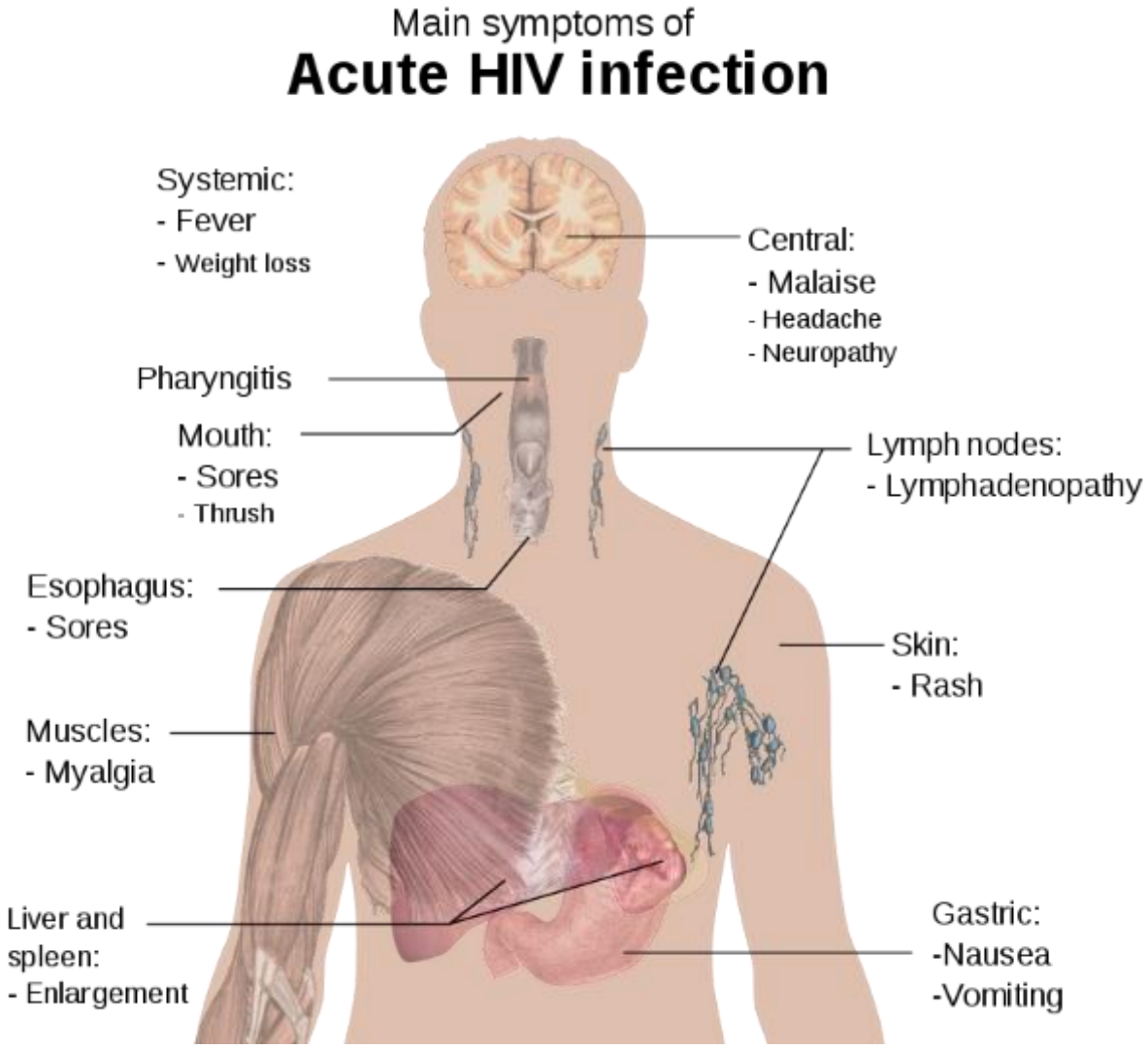


Figure 1.3. Major symptoms of HIV with numerous variant at different stages, from (Häggström *et al*., 2014)

The primary HIV-1 infection is accompanied by an asymptomatic period of several months to years, but an estimate of up to 40 % of the infected individuals show a history of virus-like illness (Tindall *et al.*, 1988; Carr *et al.*, 1991). The infected individuals develop an AIDS status when the plasma HIV load is high and the $CD4^+$ T count is less than 200 mm^3. Nevertheless, only the availability and accessibility of the HAART will determine whether or not an HIV infected person will develop AIDS. Hence the need for advanced drug delivery strategies to improve the biodistribution of the drug and enhance the therapeutic response (Naif *et al.*, 2013).

Antiretroviral therapy (ART) has been successfully used in the management of the viral infection as well as controlling the disease progression, thereby improving the quality of life of the patient (Gupta and Jain, 2010; Ramana *et al.*, 2012). ART treatment remains the best

obtainable means for long lasting viral suppression and, subsequently, for reduction of morbidity and mortality (Simon *et al.*, 2006). ART consists of a clinical combination of 3 or 4 drugs that target different components of the virus, hence this therapy is referred as HAART and currently prescribed for the HIV treatment (Gupta and Jain, 2010).

These drugs (**Figure 1.4**) commonly target the viral reverse transcriptase or protease. The main mechanism of action encompasses inhibition of viral replication, by eight nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), three non-nucleoside reverse transcriptase inhibitors (NNRTIs) and eight protease inhibitors (PIs), which prevents virion maturation and leads to the development of non-infectious particles (Simon *et al.*, 2006). Some of these ARVDs includes; zidovudine, zalcitabine (NRTI), nevirapine, efavirenz (NNRTIs), fosamprenavir, ritonavir (PIs), enfuvirtide (FIs), dolutegravir, and raltegravir (IIs).

Zidovudine was approved in 1987 as the first NRTI. Due to its structural similarity with the building block of the nucleic acids (RNA, DNA), Zidovudine causes anaemia, rash, myopathy, nausea that have been observed as the major side effects. The use of the zalcitabine as one of the NRTIs was contraindicated because of its weak antiviral activity and unfavourable toxicity and pharmacokinetics profiles (Mitsuya and Broder, 1987; Warnke *et al.,*2007; Hawkins *et al.*, 2010).

Nevirapine, delavirdine and efavirenz are the three approved NNRTIs to date. Nevirapine is the first NNRTIs and typically the primary choice for efficient viral suppression. Its use is known to cause liver toxicity, CNS toxicity, and epidermal necrolysis. Its use has been restricted except in cases where the benefit to the patient exceeds the risk. Delavirdine is only rarely used in clinical practice due to the large pill burden and its low potency. The use of efavirenz remains problematic due to induction of the hepatic cytochrome CYP3A4 and associated central nervous system side effects, such as dizziness, insomnia, somnolence, aggressive behaviour, abnormal dreams, impaired concentration, and severe depression. (Witvrouw *et al.*, 2004, Warnke, Barreto and Temesgen, 2007, Ramana *et al.*, 2010, Teixeira *et al.*, 2011).

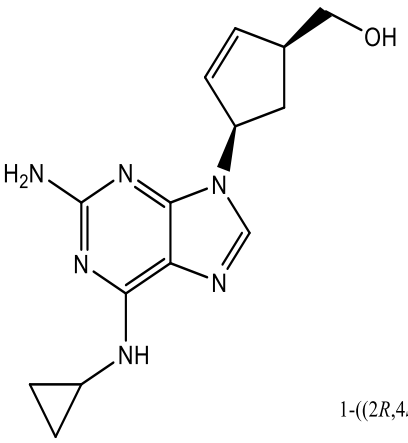
The improved pharmacokinetic profile of PIs fosamprennavir compared to its active metabolite has led to fosamprenavir being produced alone, and it remains the only amprenavir available. The full dose ritonavir is no longer used due to its low antiviral effects,

associated remarkable drug–drug interactions with other protease inhibitors currently prescribed (Pillay and Richman, 1995).

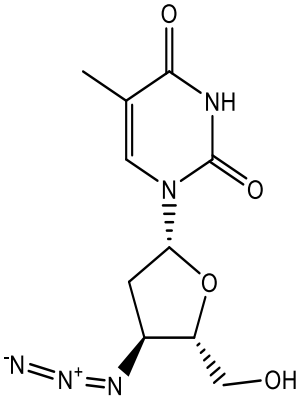
Enfuvirtide, also known as T-20, is the first and the only approved fusion inhibitor (FI). T-20 is indicated in the treatment of HIV-1 infection , but not active against HIV-2 (Warnke *et al.*, 2007). Enfuvirtide is only administered by subcutaneous injection and its bioavailability is about 84 % (Eckert and Kim, 2001, Cooley and Lewin, 2003) .

While the HAART represents the only hope for the management of HIV, there therapeutic limitations worsen the devastating status of HIV infection. These problems include poor pharmacokinetics and severe side effects of most of the ARVDs, which in turn reduce patient compliance (adherence) and can lead to drug resistance as well as unsatisfactory clinical outcome (Shah and Amiji, 2006; Ramana *et al.*, 2012). In fact, most of the ARVDs exhibit low bioavailability because of their first pass metabolism, degradation in the gastrointestinal tract and their low half-life, resulting in frequent administration of high doses for clinical success (Li and Chan, 1999; Ramana *et al.*, 2012). In general, the most common side effects of HAART include skin rashes, vomiting, diarrhoea and liver disorder (Ramana *et al*., 2014). Cross side effects ranging from dizziness to hallucinations, insomnia, nightmares, worsened psychiatric illness and hepatotoxicity. These reactions occur in most patients in the first few days to several weeks of drug administration (Carr and Cooper, 2000). Highlighted below are structures of some commercially approved ARVDs used in treatment of HIV. A fixed-dose combination of stavudine, lamivudine, and nevirapine is extensively used as an antiretroviral regimen in developing countries because of its affordability. The virological failure with this regimen has become more common, attributed to patients having lamivudine and nonnucleoside reverse-transcriptase inhibitor resistance (Sungkanuparph *et al.*, 2007) just as in the case of efavirenz. This therefore necessitates options for a second-line regimen in this regard and scintillated the present study as it concerns improved drug delivery technique to address this major concern.

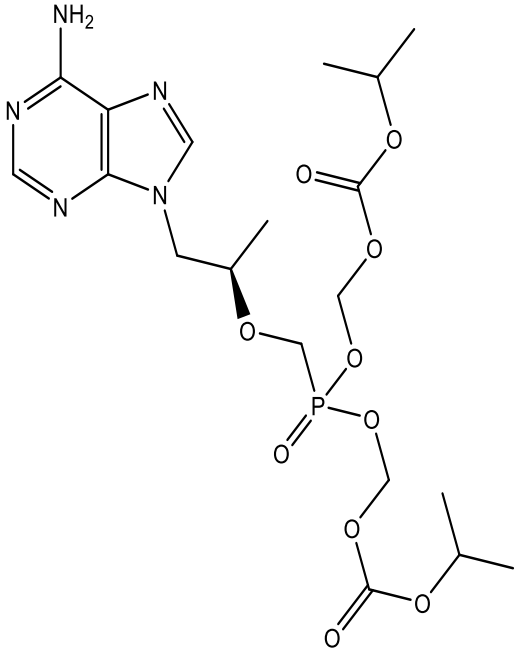
Chemical structures of some NRTIs



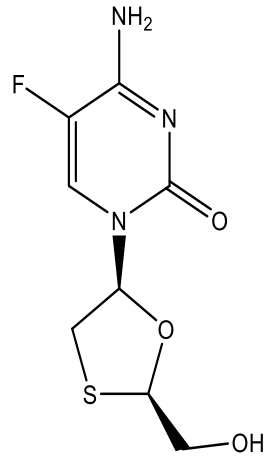
((1*S*,4*R*)-4-(2-amino-6-(cyclopropylamino)-9*H*-purin-9-yl)cyclopent-2-en-1-yl)methanol
(**Abacavir**)



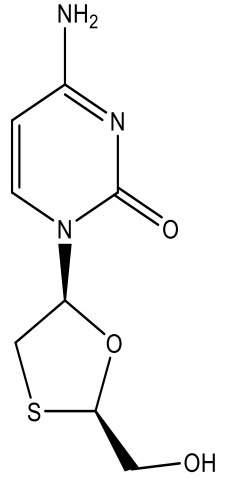
1-((2*R*,4*S*,5*S*)-4-azido-5-(hydroxymethyl)tetrahydrofuran-2-yl)-5-methylpyrimidine-2,4(1*H*,3*H*)-dione
(**Zidovudine**)



(*R*)-(((((1-(6-amino-9*H*-purin-9-yl)propan-2-yl)oxy)methyl)phosphoryl)bis(oxy))bis(methylene) diisopropyl bis(carbonate)
(**Tenofovir disoproxil**)

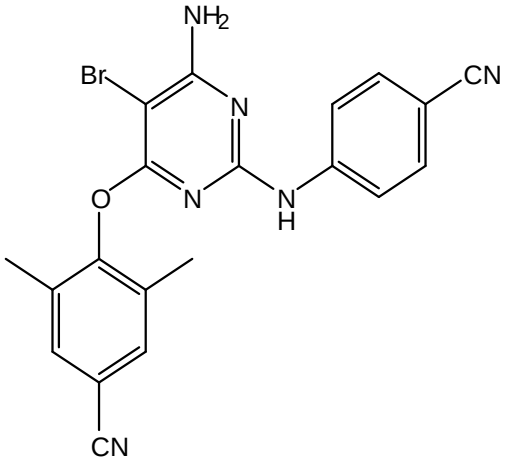


4-amino-5-fluoro-1-((2*R*,5*S*)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl)pyrimidin-2(1*H*)-one
(**Emtricitabine**)

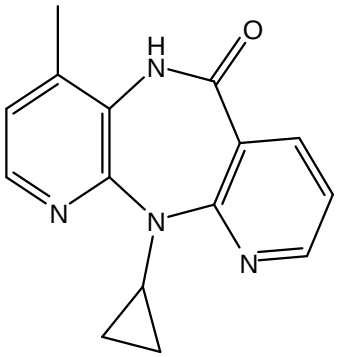


4-amino-1-((2*R*,5*S*)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl)pyrimidin-2(1*H*)-one
(**Lamivudine**)

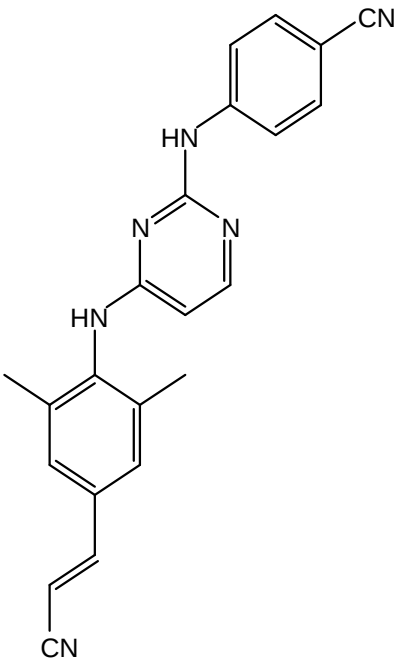
Chemical structures of some NNRTIs



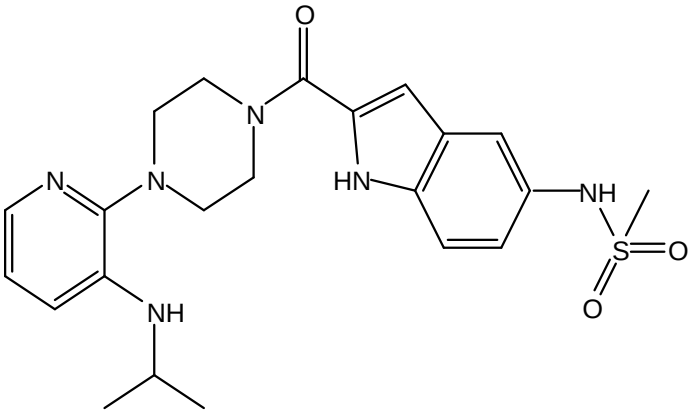
4-((6-amino-5-bromo-2-((4-cyanophenyl)amino)pyrimidin-4-yl)oxy)-3,5-dimethylbenzonitrile
(**Etravirine**)



11-cyclopropyl-4-methyl-5,11-dihydro-6*H*-dipyrido[3,2-*b*:2',3'-*e*][1,4]diazepin-6-one
(Nevirapine)

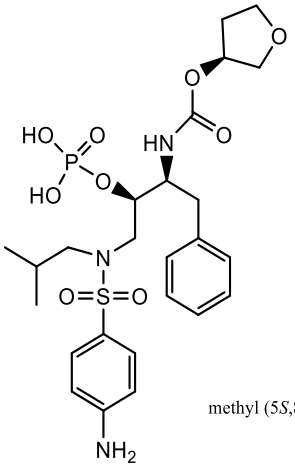


(*E*)-4-((4-((4-(2-cyanovinyl)-2,6-dimethylphenyl)amino)pyrimidin-2-yl)amino)benzonitrile
(**Rilpivirine**)

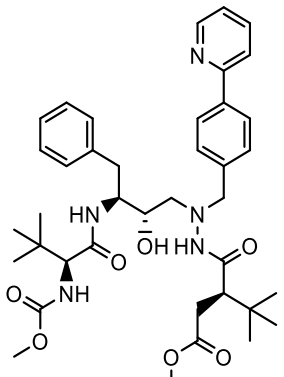


N-(2-(4-(3-(isopropylamino)pyridin-2-yl)piperazine-1-carbonyl)-1*H*-indol-5-yl)methanesulfonamide
(***Rescriptor***)

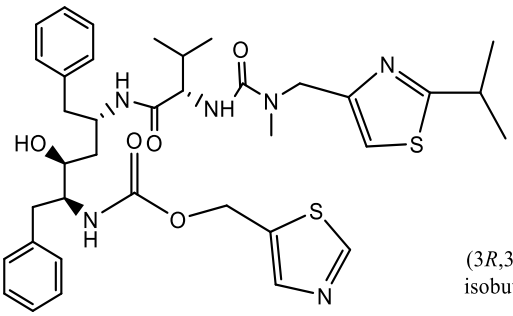
Chemical structures of some PIs



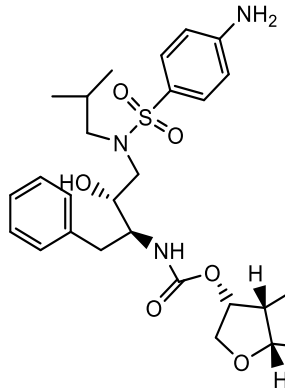
(*S*)-tetrahydrofuran-3-yl ((2*S*,3*R*)-4-((4-amino-*N*-isobutylphenyl)sulfonamido)-1-phenyl-3-(phosphonooxy)butan-2-yl)carbamate
(**Fosamprenavir**)



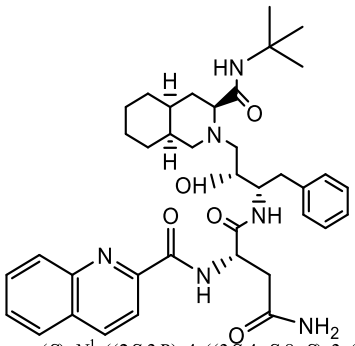
methyl (5*S*,8*S*,9*S*,14*R*)-8-benzyl-5,14-di-*tert*-butyl-9-hydroxy-3,6,13-trioxo-11-(4-(pyridin-2-yl)benzyl)-2-oxa-4,7,11,12-tetraazahexadecan-16-oate
(**Atazanavir**)



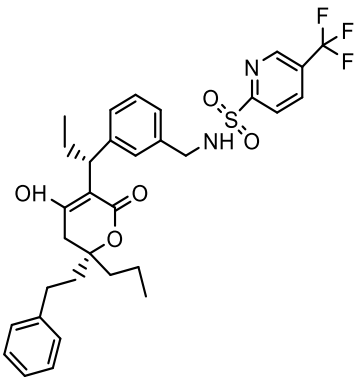
thiazol-5-ylmethyl ((2*S*,3*S*,5*S*)-3-hydroxy-5-((*S*)-2-(3-((2-isopropylthiazol-4-yl)methyl)-3-methylureido)-3-methylbutanamido)-1,6-diphenylhexan-2-yl)carbamate
(**Ritonavir**)



(3*R*,3a*S*,6a*R*)-hexahydrofuro[2,3-*b*]furan-3-yl ((2*S*,3*R*)-4-((4-amino-*N*-isobutylphenyl)sulfonamido)-3-hydroxy-1-phenylbutan-2-yl)carbamate
(**Darunavir**)

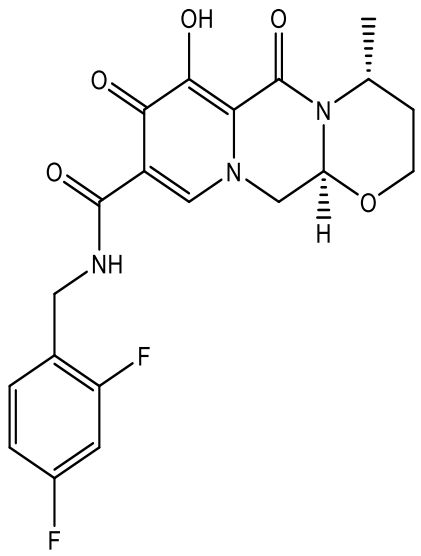


(*S*)-*N*^{1}-((2*S*,3*R*)-4-((3*S*,4a*S*,8a*S*)-3-(*tert*-butylcarbamoyl)octahydroisoquinolin-2(1*H*)-yl)-3-hydroxy-1-phenylbutan-2-yl)-2-(quinoline-2-carboxamido)succinamide
(**Saquinavir**)

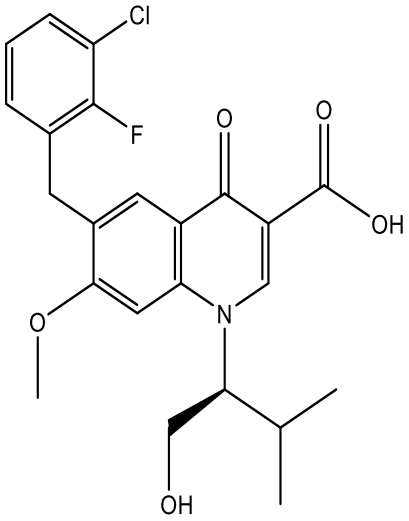


N-(3-((*R*)-1-((*R*)-4-hydroxy-2-oxo-6-phenethyl-6-propyl-5,6-dihydro-2*H*-pyran-3-yl)propyl)benzyl)-5-(trifluoromethyl)pyridine-2-sulfonamide
(***Tipranavir***)

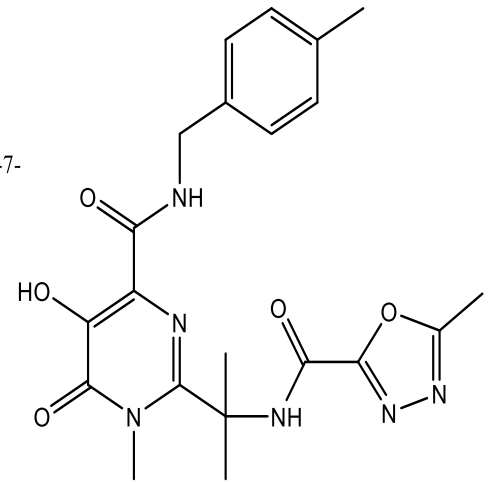
Chemical structures of some IIs



(4*R*,12a*S*)-*N*-(2,4-difluorobenzyl)-7-hydroxy-4-methyl-6,8-dioxo-3,4,6,8,12,12a-hexahydro-2*H*-pyrido[1',2':4,5]pyrazino[2,1-*b*][1,3]oxazine-9-carboxamide
(**Dolutegravir**)

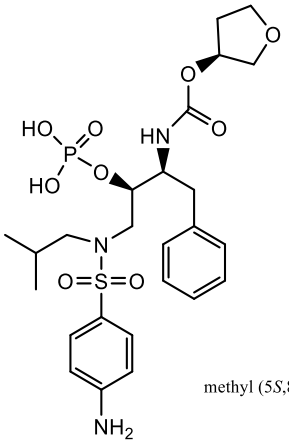


(*S*)-6-(3-chloro-2-fluorobenzyl)-1-(1-hydroxy-3-methylbutan-2-yl)-7-methoxy-4-oxo-1,4-dihydroquinoline-3-carboxylic acid
(**Elvitegravir**)

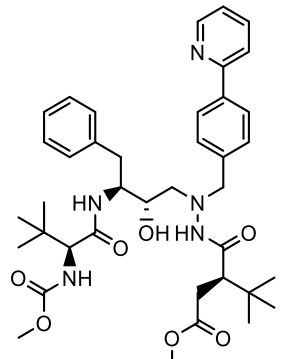


N-(2-(5-hydroxy-1-methyl-4-((4-methylbenzyl)carbamoyl)-6-oxo-1,6-dihydropyrimidin-2-yl)propan-2-yl)-5-methyl-1,3,4-oxadiazole-2-carboxamide
(***Raltegravir)***

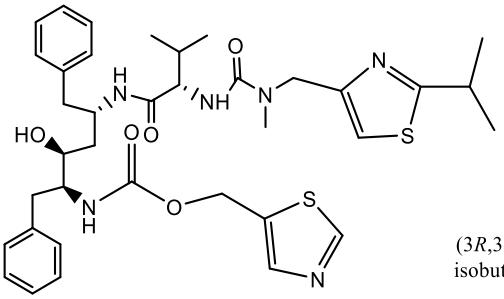
Chemical structures of some PIs



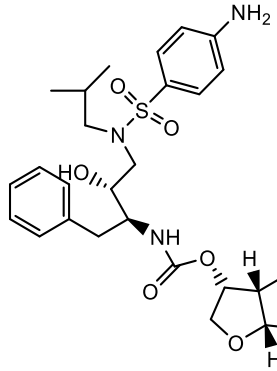
(*S*)-tetrahydrofuran-3-yl ((2*S*,3*R*)-4-((4-amino-*N*-isobutylphenyl)sulfonamido)-1-phenyl-3-(phosphonooxy)butan-2-yl)carbamate
(**Fosamprenavir**)



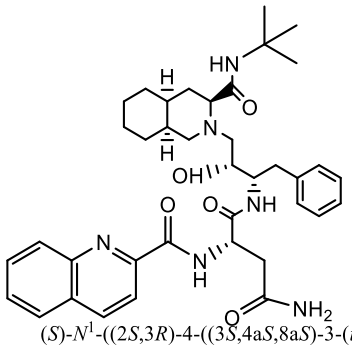
methyl (5*S*,8*S*,9*S*,14*R*)-8-benzyl-5,14-di-*tert*-butyl-9-hydroxy-3,6,13-trioxo-11-(4-(pyridin-2-yl)benzyl)-2-oxa-4,7,11,12-tetraazahexadecan-16-oate
(**Atazanavir**)



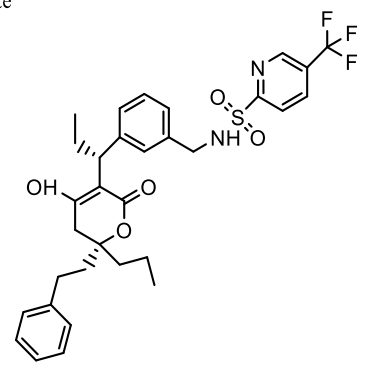
thiazol-5-ylmethyl ((2*S*,3*S*,5*S*)-3-hydroxy-5-((*S*)-2-(3-((2-isopropylthiazol-4-yl)methyl)-3-methylureido)-3-methylbutanamido)-1,6-diphenylhexan-2-yl)carbamate
(**Ritonavir**)



(3*R*,3a*S*,6a*R*)-hexahydrofuro[2,3-*b*]furan-3-yl ((2*S*,3*R*)-4-((4-amino-*N*-isobutylphenyl)sulfonamido)-3-hydroxy-1-phenylbutan-2-yl)carbamate
(**Darunavir**)



(*S*)-N^1-((2*S*,3*R*)-4-((3*S*,4a*S*,8a*S*)-3-(*tert*-butylcarbamoyl)octahydroisoquinolin-2(1*H*)-yl)-3-hydroxy-1-phenylbutan-2-yl)-2-(quinoline-2-carboxamido)succinamide
(**Saquinavir**)



N-(3-((*R*)-1-((*R*)-4-hydroxy-2-oxo-6-phenethyl-6-propyl-5,6-dihydro-2*H*-pyran-3-yl)propyl)benzyl)-5-(trifluoromethyl)pyridine-2-sulfonamide
(***Tipranavir***)

Figure 1.4. Chemical structures of some commercially available ARVDs

1.2. Efavirenz Profile (EFV)

1.2.1. General Review

Efavirenz (**Figure 1.5**) is an NNRTI characterized by a prolonged half-life of 56–76 hours after single doses and 40–55 hours after multiple doses. Efavirenz (EFV) is dosed once daily in combination with other ARV drugs (ARVDs), thus representing an important component of HAART. Due to its relatively low potential against the HIV mutants, EFV is subject to a high level of phenotypic resistance. Over 20–40 % of patients receiving efavirenz have experienced several side effects in the central nervous system (CNS), including dizziness, hallucination, insomnia and nightmares (Adkins and Noble, 1998; Marzolini *et al.*, 2001).

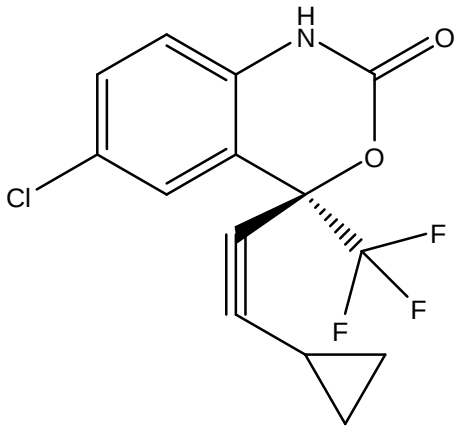
EFV was chosen in this research based on its therapeutic importance in the treatment and management of HIV infection, severe side effects compared to other antiretrovirals and poor solubility in aqueous media.

1.2.2. Physicochemical properties

Molecular formula: $C_{14}H_9ClF_3NO_2$

Molecular weight: 315.68 g/mole

Chemical structure:



(*S*)-6-chloro-4-(cyclopropylethynyl)-4-(trifluoromethyl)-1,4-dihydro-2*H*-benzo[*d*][1,3]oxazin-2-one

Figure 1.5. Chemical structure of Efavirenz

Organoleptic features: EFV is a white to off white crystalline powder, that is insoluble in water, and soluble in lower alcohol.

Melting point: 139 – 141 °C

1.2.3. Pharmacological properties

Drug category: Antiretroviral agent

Dosage forms: Capsules (50 mg, 200 mg), tablets (600 mg)

Mechanism of action: EFV binds non-competitively to the allosteric site on the HIV reverse transcriptase enzyme, preventing the conversion of RNA and DNA. EFV does not require activation through phosphorylation but is a substrate and inhibitor of CYP3A4.

Metabolism: EFV is highly metabolised by the polymorphic cytochrome P450 enzyme called CYP2B6 (Wang *et al.*, 2006). Studies in humans (di Lulio *et al.*, 2009) have shown that the drug is metabolized into 8-hydroxy-EFV, 7-hydroxy-EFV and N-glucuronide-EFV (**Figure 1.6**).

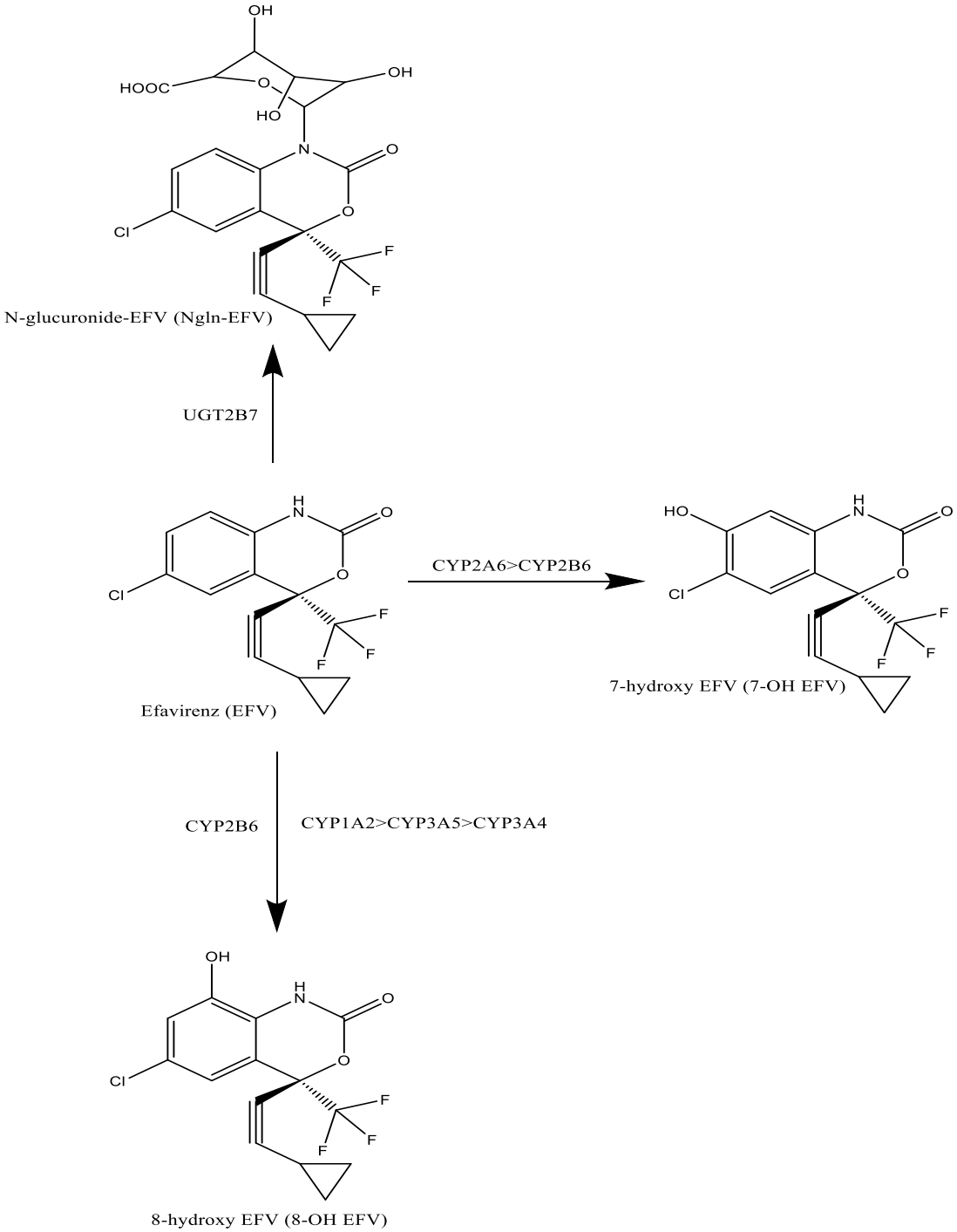


Figure 1.6. Pathways of Efavirenz metabolites adapted from (di Lulio *et al.,* 2009)

The CYP3A and CYP2B6 are the main isoenzymes responsible for EFV metabolism. EFV exhibits CYP enzyme inducing properties, which causes its own metabolism (Ogburn *et al.*, 2010).

Elimination: EFV has a relatively long terminal half-life of 52–76 hours after a single dose and 40–50 hours after multiple doses. Approximately 14–34 % of the radiolabelled dose of efavirenz was recovered in the urine and <1 % of the dose was excreted in urine as unchanged

EFV.

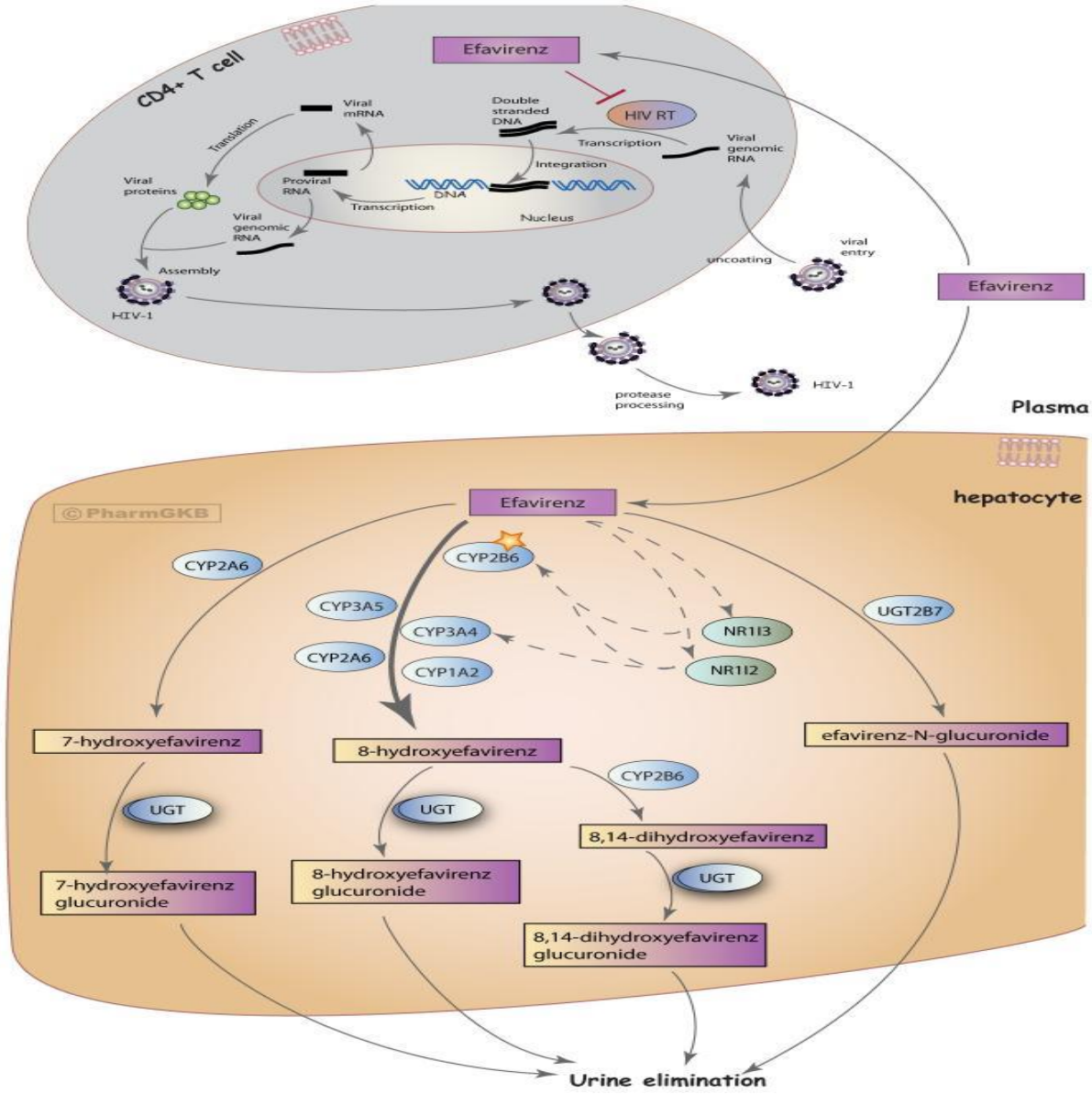


Figure 1.7. Efavirenz metabolism from (Mcdonagh *et al.*, 2015)

Indication: EFV is used in combination with NRTIs or PIs in the treatment of HIV-1 infection in adults and in babies at least 3 months old, weighing 3.5 kg.

Contraindication: EFV is contraindicated in patients with previously demonstrated hypersensitivity such as Steven Johnson syndrome, erythemia multiforme or skin eruptions.

Adverse effects: Cross side effects range from dizziness to hallucinations, insomnia, nausea, fatigue, vomiting, abnormal dreams and worsening of psychiatric illnesses. These reactions occur in most patients in the first few days to weeks (Carr and Cooper, 2000).

1.3. Delivery Routes

1.3.1. Oral Delivery Route

1.3.1.1. Anatomical description of the gastrointestinal tract

The mouth is the entry to the alimentary canal and the site at which the digestive process begins (**Figure 1.8**)**.** Most medicines are not chewed but swallowed whole as good number of the drugs have a coating of some sort. In addition to mouth carrying enzymes that initiate the breakdown of carbohydrates and fats, saliva also lubricates the ingested material to aid in swallowing (deglutition). The pharynx and oesophagus are the hollow muscular structures that serve as tandem conduits for the transfer of the ingested material from the mouth to the stomach. (DeSesso and Jacobson, 2001; Lotan and Zysman, 2006).

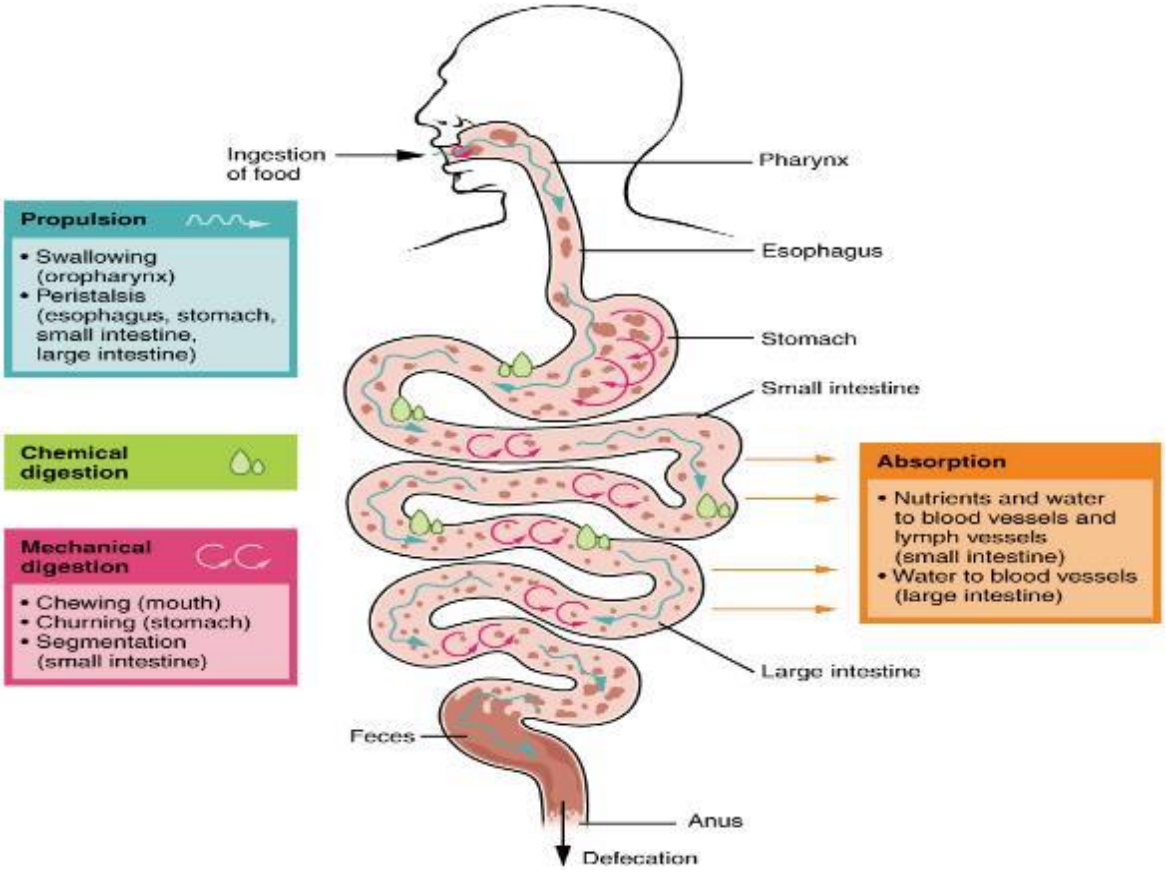


Figure 1.8. Anatomical demonstration of the human digestive system from (Lotan and Zysman)

1.3.1.2. Oral drug delivery- challenges and potential solutions

Oral delivery route is the most widely accepted route for drug administration among other routes such as parental, transdermal, submucosal routes etc., because of its low therapy cost, good comfort of the administration and self-medication (Goyal *et al.*, 2012; Hanif *et al.*, 2015).

Over 70 % of the marketed drugs today are in the recommended dosage form due to the avoidance of pain and patient adaptability to pills. However, despite the wide acceptance, about 50 % of the population, generally paediatric and elderly patients avoid taking solid oral preparations (such as tablets and capsules) because of the potential choking hazard or difficulty in swallowing. This can lead to low patient compliance (Patwa *et al.*, 2011; Singh *et al.*, 2013; Hanif *et al.*, 2015). This and other significant limitations in oral administration, especially those due to the drug's peculiar physiochemical characteristics, demand other options. In addition, even for drugs that are successfully delivered by this route, physiological barriers such as the pre-systemic metabolism and gastrointestinal instability remain a problem (Gupta *et al.*, 2009; Singh *et al.*, 2013).

Challenges in swallowing Pills: There is enormous array of solid oral dosage forms in use, or undergoing research and development, although swallowing remains a true problem for the older patients, who represent the largest group of tablet takers. More than 50 % of adults over the age of 60 years are affected by dysphagia, (a condition involving difficulty in eating as a result of disruption in swallowing) (Hansen *et al.*, 2008; Cichero *et al.*, 2013; Lau *et al.*, 2018). Many paediatric patients also have difficulty in swallowing or chewing solid dosage forms. Both groups of patients are often reluctant to take these solid preparations due to the fear of choking. Swallowing difficulties also occur in the case of long term medication, and when treating paediatric patients with high sensitivity to bitter tasting substances (Amin *et al.,* 2015). Because solid dosage forms almost always show lower bioavailability, long onset time and dysphagia, buccal delivery such as the buccal delivery of oral films have gained considerable attention over tablets (Bhattarai and Gupta, 2015).

Gastrointestinal destruction of labile molecules: The small intestine is an absorptive organ in the uptake of the orally administered drugs and metabolises drugs in various pathways (Lin *et al.*, 1999). The amount of drug administered through the oral route that reaches the systemic circulation can be significantly reduced by both intestinal and hepatic metabolism, with the liver being the major site of the drug metabolism because of its high content of enzymes (Ilett *et al.*, 1990, Lin *et al.*, 1999). Studies have suggested that the intestinal metabolism of the drugs is relatively greater than that of the hepatic metabolism in the overall first pass effect (Wu *et al.*, 1995). The poor responses elicited to orally delivered drugs mostly result from enzymatic degradation (Lavelle *et al.*, 1995). One idea to reduce the

gastrointestinal metabolism and to ensure that the labile molecules are shielded until they can reach the target is to incorporate the drug molecules into biodegradable nano-particles such as liposomes. These can in addition be coated using polymers and applied to buccal delivery (Solaro *et al.*, 2010).

Uncontrolled drug release and poor absorption: Controlled delivery in oral route is one of the major constraints due to random absorption of drug molecules throughout the gastrointestinal tract (GIT) (Chawla *et al.*, 2003). Many drugs administered orally are preferably absorbed in a particular segment of the GIT at a specific absorption window and only the drug released in the segment and in close vicinity to the absorption window is available for absorption. Beyond the absorption window, the released drug goes into the waste with negligible or no absorption, thereby minimizing drastically the rate of drug absorption and bioavailability. This suggests the need for an alternative delivery route such as the buccal delivery that leads to direct entry of the drugs into the systemic circulation, avoiding the gastrointestinal degradation and hepatic metabolism thereby promoting drug bioavailability (Nayak *et al.,* 2010; Jones *et al.*, 2014)

Severe side effects: Orally administered drugs with poor bioavailability fail to reach the minimum concentration required to achieve the best pharmacological action. In response, oral doses of drugs showing poor solubility or bioavailability are usually high. This results in various side effects such as diarrhoea, nausea, abdominal discomfort, dyspepsia, flatulence, vomiting, etc. (Kumar *et al.*, 2013). An example of such a drug is Saquinavir, a highly potent HIV protease inhibitor, whose minimum effective concentration is 100 mg/ml daily, but the oral dose required is 1200 mg/day. This suggests the need for solubility improvement and controlled delivery using drug vehicles like liposomes or polymeric materials (Eron *et al.*, 2006; Kumar *et al.*, 2013). Again, the buccal delivery route may be beneficial in overcoming this problem. This is achievable since the entrapping the drug in a delivery system would solve the solubility challenge of the drug, and further aided by the incorporation of polymeric materials for site targeting and sustained release through drug permeation into the systemic circulations.

1.3.2. Buccal Route Delivery- Anatomy of the Buccal Mucosa

The buccal mucosa comprises three main layers (**Figure 1.9**): (1) the epithelium, (2) the basement membrane, and (3) the connective tissue. The epithelium is found within the oral

cavity, and below the epithelium lies the supporting basement membrane which is supported by the connective tissue (Kaul *et al.*, 2011). The epithelium consists of: (A) the non-keratinized surface in the mucosal lining of the soft palate, which is the tongue ventral surface, the floor of the mouth, alveolar mucosa, vestibule, lips, and cheeks, (B) keratinized epithelium found in the hard palate and non-flexible regions of the oral cavity.

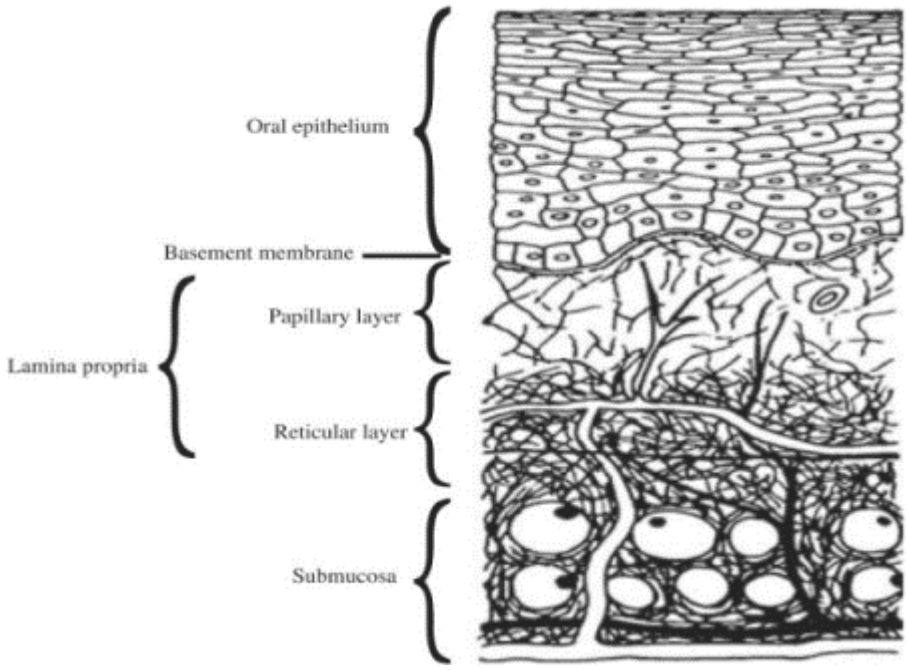


Figure 1.9. Anatomy of the buccal mucosa from (Kaul *et al.,* 2011)

The buccal epithelium is a non-keratinized tissue having 500 – 600 µm thickness and surface area of 50.2 cm^2 that is penetrated by conical connective tissue (Kaul *et al.*, 2011; Bobade *et al.*, 2013). The connective tissues consist of the collagen fibres, a supporting layer of the connective tissues, blood vessels, and smooth muscles. The buccal artery and other parts of the facial artery; the posterior alveolar artery, and the infra-orbital artery are the primary sources of blood supply for the lining of the cheek in the buccal cavity. By buccal administration, drugs held or applied in the buccal area diffuse through the oral mucosa and enter directly into the bloodstream. The basement membrane, which forms a distinctive layer between the connective tissues and the epithelium, serves as the mechanical support for the

epithelium, and the underlying connective tissues that offers mechanical properties to the oral mucosa (Bobade *et al.*, 2013).

1.3.3. Buccal Delivery

Buccal delivery is the administration of the drug through the lining mucosal membranes of the cheeks (buccal mucosa) (Patel *et al.*, 2013). Unlike the oral delivery technique that involves drugs through the first pass-metabolism, buccal delivery technique does not involve this stage (Puratchikody *et al.*, 2011) . Over the years, drug administration through the buccal route has gained huge consideration and demonstrated numerous advantages over other routes. Amongst these advantages (explored below) is improved drug bioavailability. By avoiding degradation in the GIT and hepatic first pass-metabolism, a 10 % improvement was observed (Şenel and Hincal, 2001). The preferred site for retentive transmucosal delivery systems with sustained and controlled release features is the buccal mucosa. This is mainly because of the differences in permeability between the regions of the buccal and sublingual mucosa, and the buccal mucosa's expanse of smooth and relatively immobile mucosa, which allows direct access to the systemic circulation through the internal jugular vein (Patel *et al.*, 2011).

The advantages of buccal delivery over other delivery strategies includes (Kaul *et al.*, 2011; Lekshmi*et al.*, 2016).

1. Buccal delivery offers direct entry of the drug into the systemic circulation, thereby bypassing the first-pass metabolism that is characteristic of the orally administered drug. The rate of drug absorption is not influenced by food or gastric emptying rate, and the drug is protected from degradation in the GIT.
2. Buccal delivery is known to improve patient compliance because of the elimination of pains associated with other administration routes, such as oral administration and injection.
3. The presence of the saliva ensures relatively large amount of water for drug dissolution, unlike other routes such as the rectal route.
4. Significant reduction in doses can be achieved, reducing thereby the dosing frequency and dose dependent side effects.

5. Unlike the oral delivery, an onset of action can be easily achieved with buccal delivery and the formulation can be removed in case the therapy has to be discontinued or terminated.
6. There is rapid cellular recovery at the smooth surface of the buccal mucosa.
7. The large surface contact of the oral cavity contributes to rapid and extensive drug absorption.
8. Possible localization of the drug for a prolonged time and facile administration of the drug, even to unconscious or traumatised patients.

The use of muco-adhesive polymers with bio-adhesive properties (polymers that can effectively bind and interact to the mucin layer of the biological membrane), is now implemented in the formulation of buccal film dosage form to achieve systemic delivery of drugs through the mucosa. This allows the minimization of drawbacks associated with the buccal delivery of hydrophilic drugs (Patel *et al.*, 2011). Hydrophobic drugs, however, being poorly water soluble, cannot be successfully delivered via the buccal delivery, unless they are modified first (Q.Xu *et al.*, 2007). Hence, the first step of this work will be to encapsulate the drug in a hydrophilic lipid-based system (like liposomes), within which the drug will be dispersed at molecular level and further incorporate the drug loaded liposomes into the polymeric matrix using bio-adhesive polymers (Chen *et al.*, 2014)..This is possible since liposomes surface is hydrophilic, hence there is possibility of dispersing the drugs within the polymeric network. Like this, the drug though hydrophobic, could easily get absorbed since dispersed at molecular level as it is dissolved in lipid system (Chen *et al.,* 2014). Earlier work (Abd El Azim *et al*., 2015) studied delivery of water - soluble vitamins incorporated in liposomal buccal mucoadhesive films. This technique differs from the study of ARVD incorporated in didanosine loaded solid lipid nanoparticles (Jones *et al.,* 2014). Both works aforementioned highlight study trends in improved drug delivery. However, present study seeks to highlight improved delivery for Efavirenz embedded in liposomes incorporated in polymeric materials

1.4. Liposomes

Liposomes are small artificial vesicles of a spherical shape that can be formed from natural or synthetic phospholipids (Akbarzadeh *et al.*, 2013). Liposomes were first discovered and examined by A. Bangham and his colleague R.W. Thorne in 1964 at the Babraham Institute,

University of Cambridge. The dispersion of phospholipids in water yielded liposome structure that was visualized under electron microscope (Bozzuto and Molinari, 2015). Liposomes offer several advantages including biocompatibility, biodegradability, non-toxicity, ability to incorporate hydrophilic and hydrophobic drugs. They also gather a variety of physicochemical and biophysical features that can be modified to control their biological behaviour (Sahoo and Labhasetwar, 2003; Sercombe *et al.*, 2015). Compared to other nanoparticulate systems, the substantial clinical and manufacturing features of liposomes, i.e. the batch to batch variability, easy of synthesis, scalability, and biocompatibility have led to approval of several liposomes-based formulations by food and drug administration FDA, and many other regulatory bodies as presented in **Table 1.1** (Noble *et al.*, 2014). Liposomes face the challenges of being cleared from the bloodstream by the mononuclear phagocytic system (MPS), also known as the reticuloendothelial system (RES) (Longmire *et al.*, 2008).. The opsonins and serum proteins that adhere to the surface of the nanoparticles prime the particles for detection by MPS and target them for phagocytosis (Longmire *et al.*, 2008; Moghimi and Farhangrazi, 2013). However, formulation of stealth or PEGylated liposomes using poly(ethylene) glycol (PEG) has been found to be an effective strategy to prevent liposomes clearance by the MPS, which results in increased *in vivo* circulation time. This approach possesses the advantage of facile chemical synthesis, inhibition of nanoparticle aggregation and non-specific interactions. Through attachment of PEG units, liposomes surface turns into a physical barrier to opsonins and other serum proteins, which prevent their adsorption onto the nanoparticle surface and subsequent clearance (Charrois and Allen, 2003; Li and Huang, 2011).

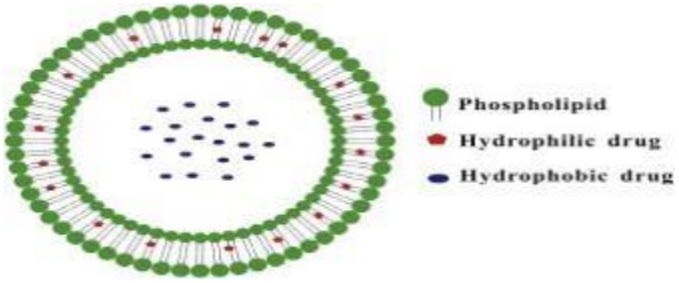


Figure 1.10. Figure 1.10: Structure of liposome showing encapsulated drugs from (Li *et al.*, 2014)

Table 1.1. List of some commercially approved Liposomal drugs and vaccines

Liposomal drug name	Trade name	Therapeutic Indications
Amphotericin B	Abelcet	Fungal infections
Amphotericin B	Ambisome	Fungal and protozoal infection
Cytarabine	Depocyt	Lymphomatous meningitis
Daunorubicin	DaunoXome	HIV related kaposi's sarcoma
Doxorubin	Myocet	Metastatic breast cancer
Vaccine	Epaxal	Hepatitis A
Vaccine	Inflexal V	Influenza
Morphine	DepoDur	Postsurgical analgesia
Verteporfin	Visudyne	Photodynamic therapy
PEG Doxorubicin	Doxil	Kaposi's sarcoma and solid tumors
Micellular	Estrasorb	Menopausal therapy

1.4.1. Components of Liposomes

Liposome membranes are mainly composed of natural or synthetic phospholipids. Usually liposomes are composed of phospholipids bilayers that are stabilized by cholesterol or other stabilizers depending on the intended application (Torchilin *et al.*, 2005; Karami *et al.,* 2018).

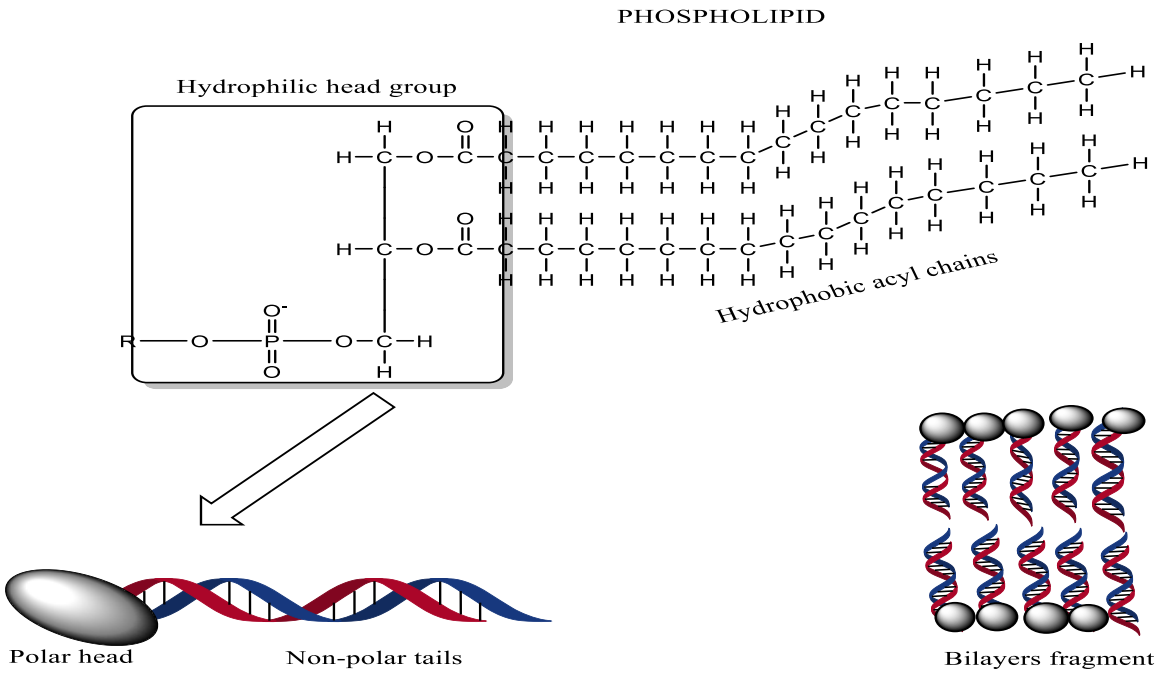


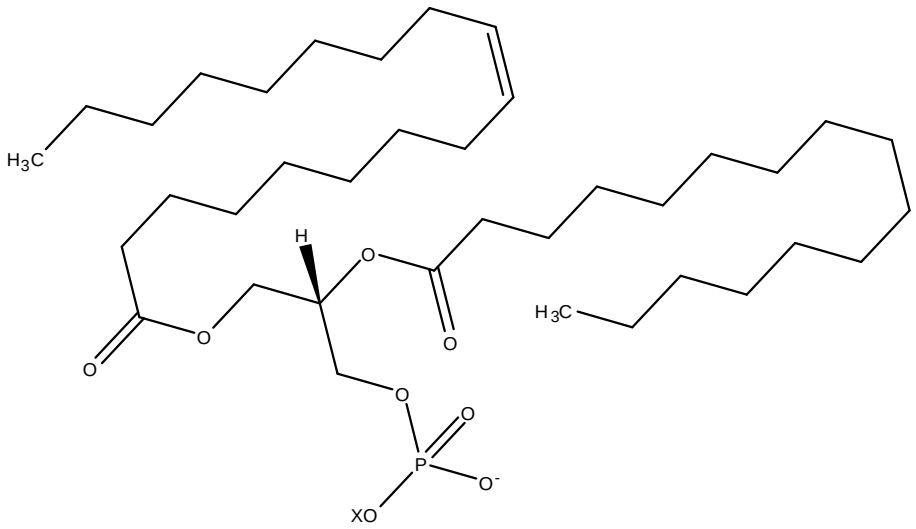
Figure 1.11. Graphical illustration of phospholipid molecule and lipid bilayers adapted from (Laouini *et al*., 2012)

1.4.1.1. Phospholipid

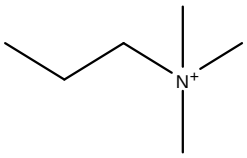
Phospholipids are a class of lipid molecules which are a key component of cell membranes. Phospholipids have amphiphilic properties that allow them to form lipid bilayers. They consist of two hydrophobic fatty acid '' tails'' and a hydrophilic phosphate ''head'' joined together by a glycerol molecule (Mansoori *et al.*, 2012), **Figure 1.11**. The hydrophilic head is attracted to water while the hydrophobic tails consisting of two long fatty acid chains, which are repelled by water, have the propensity to aggregate. The most commonly used phospholipid (**Figure 1.12**) for formation of liposomes are the phosphatidylcholine (PC), also referred as lecithin, phosphatidylethanolamines (PE), phosphatidylserines (PS) and phosphatidylglycerols (PG) (Li *et al.*, 2014; Pattni *et al.*, 2015).

Phospholipid name -X moiety

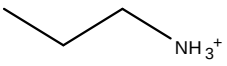
'Glycerol Phospholipid tail'



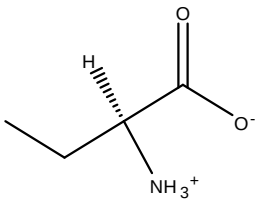
PC



PE



PS



PG

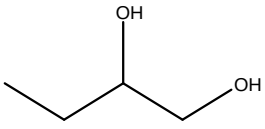


Figure 1.12. Illustration of -X moieties of various glycerol- phospholipid molecule (adapted from Li *et al.,* 2014)

Among all the phospholipids, phosphatidylcholine represents the most popular and commonly used lipid for liposomes preparation. Therefore, a naturally occurring lipid mixture that contains phosphatidylcholine as the major component was used in this research because of its relatively low cost and high availability and accessibility, which would facilitate wider development of liposomes in developing countries, which are the most affected by HIV/AIDS. The phospholipid membrane stability depends upon the transition temperature (Tc) of the phospholipid, which is a temperature at which the lipid undergoes change in physical state, from gel to liquid crystalline state. The Tc is proportional to acyl chain length: the longer is the chain, the higher is the Tc. The liposome membrane shows good rigidity and stability when lipids with high Tc are incorporated, hence the use of cholesterol as permeability controller (Sharma and Sharma, 1997). Addition of cholesterol helps to stabilise the liposomes by increasing the Tc of the membrane, decreasing the permeability of the bilayer and protecting the cargo form premature release and potential *in vivo* degradation and toxicity. Due to the hydrophobic feature of cholesterol, it is thought to reside in the interior segment of the lipid bilayers and serves to fill the gap created because of the imperfect packaging of phospholipid molecules, thus preventing flip flop of membrane components and movement across the membrane (Kirby *et al*, 1980; Pandey *et al.*, 2016).

1.4.1.2. Lecithin

Lecithin is the name given to a naturally occurring lipid mixture composed of glycolipids, triglycerides, and phospholipids (i.e. phosphatidyl choline (PC), phosphatidyl ethanolamine (PE), and phosphatidyl inositol (PI). In some instances, the term lecithin refers to pure phosphatidyl choline that represents the main component of the phosphate fraction of the naturally occurring lipid mixture (Adriana *et al.*, 2014). Lecithin derives from egg yolk and various oil seeds like flax corn germ, sunflower, cotton seed, rape seed and soybeans. Soybean lecithin was used in this work because of its relatively high availability and low cost, which may lead to new openings towards cost-effective liposome developments (Nkanga *et al.*, 2017).

Production of crude soybean lecithin: Soybean lecithin is mainly produced (**Figure 1.13**) through hydration and separation from the crude (Adriana *et al.*, 2014).

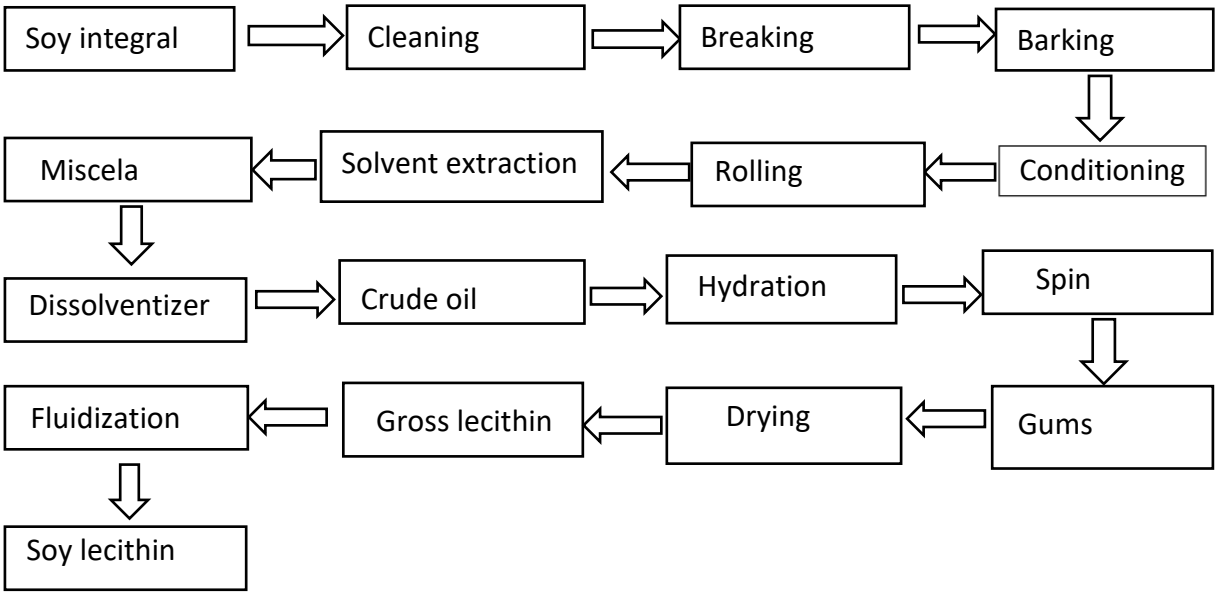


Figure 1.13. Flowchart illustrating the production of crude lecithin from soybean adapted from (Adriana *et al.,* 2014)

Uses of lecithin: Lecithin is commonly used as an emulsifier and lubricant in various formulation activities such as food or pharmaceutical processing. It is considered to be a non-toxic surfactant and well tolerated by organisms, hence part of the reason for choosing this component (Adriana *et al.*, 2014).

1.4.1.3. Cholesterol

Cholesterol (Chol) (**Figure 1.14**) is a lipid molecule comprised majorly of carbons and hydrogens, a four ring molecule that contributes to the biological functions of the cell membrane. There is a hydrocarbon tail linked to one end of the steroid and a hydroxyl group linked to the other end. The hydroxyl group is able to form hydrogen bonds with nearby carbonyl oxygen of phospholipid. It is a common component of liposome that controls membrane permeability and rigidity, strengthens the bilayer structure and plasma stability. Cholesterol is primarily found in meat, poultry, eggs, dairy and sea food (Haeri *et al.*, 2014; Albuquerque *et al.*, 2016).

Chemical formula: $C_{27}H_{46}O$

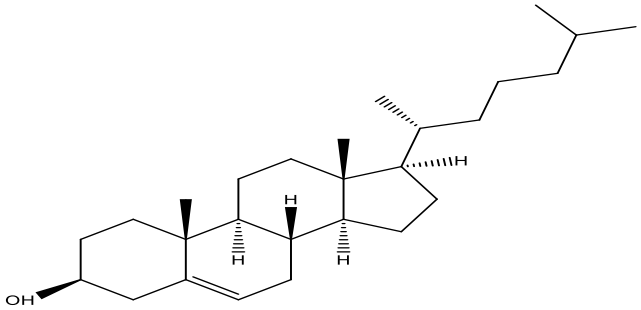


Figure 1.14. Chemical structure of cholesterol

Molecular mass: 386.65 g/mol

Melting point: 148.5 –150 °C

Boiling point: 360 °C

Density: 1.052 g/cm^3

Odour: Almost odourless

Appearance: White or faintly yellow pearly granules or crystals

Solubility: Insoluble in water, moderately soluble in acetone, benzene, chloroform, ethanol, ether, hexane, isopropyl myristate, and methanol

Source and physiological functions: Cholesterol became available to the enterocytes from dietary sources, bile and intestinal mucosa epithelium turnover. Cholesterol enters into the intestine in the free form by the action of the pancreatic cholesterol esterase and becomes packed into more hydrophilic micelles containing conjugated bile acids, monoglyceride and lysolecithin to be absorbed by cells (Dinh *et al.*, 2011).

Cholesterol is highly important for construction and maintenance of the cell membrane integrity. It contributes to the cell membrane's fluidity, viscosity and stability over a wide temperature interval. More than 30 % of cholesterol contributed to biogenesis of some physiological compounds such as steroidal hormones and vitamin D. Cholesterol also acts as an antioxidant (Salisbury *et al.*, 1995; Dinh *et al.*, 2011).

1.4.2. Formation of Liposomes

Liposomal systems are formed by dispersing phospholipids in aqueous media. The polar end of phospholipid molecule is exposed to water surface (hydrophilic), while the hydrophobic tails of one lipid layer face the tails of the other layer, resulting to formation of an anhydrous zone. Within the self-assembled bilayered structure, phospholipid molecules are oriented in

a manner that the polar ends remain in contact with the polar region of other molecules as represented in **Figure 1.15**, and at the same time shielding the non-polar chains (Mansoori *et al.*, 2012). The common macroscopic structure often formed are lamellar, hexagonal or cubic phases dispersed as colloidal nano-constructs or artificial membranes, referred to as liposomes, hexasomes or cubosomes, respectively (Uhumwangho and Okor, 2005, Tarkunde *et al.*, 2014).

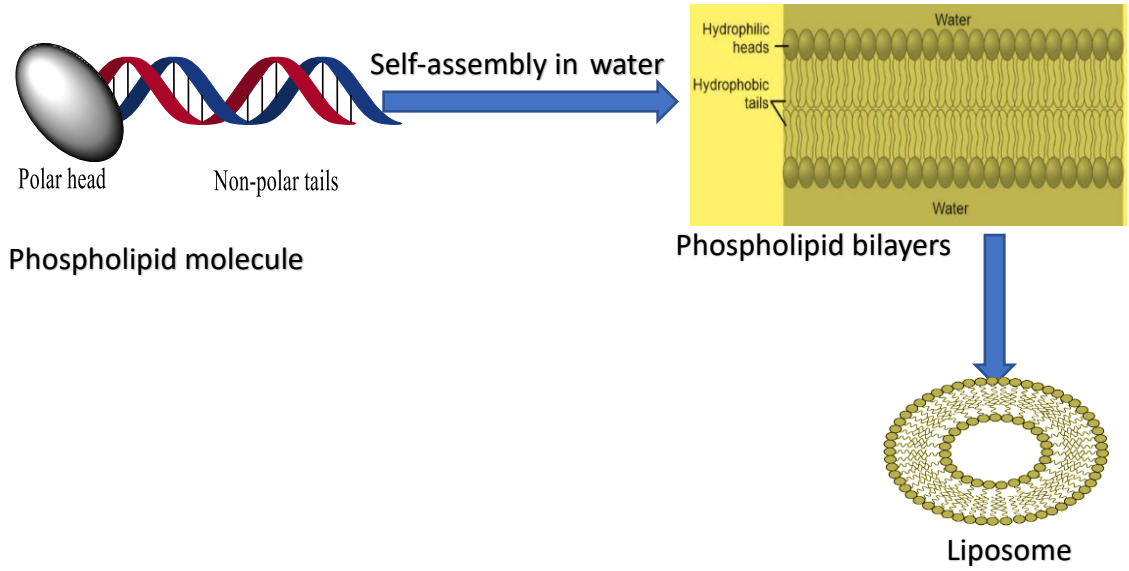


Figure 1.15. Illustrative demonstration of liposome formation from phospholipid adapted from (Dwivedi and Verma, 2013)

1.4.3. Classification of Liposomes

The classification of liposomes is based on their structural and functional characteristics, which arise typically from the method of preparation, the chemical composition as well as the designed functionalities (Pradhan *et al.*, 2015; Andhale *et al.*, 2016).

Liposomes classification based on liposome structure (size and lamellarity), (Rani *et al.,* 2013; Pradhan *et al.*, 2015).

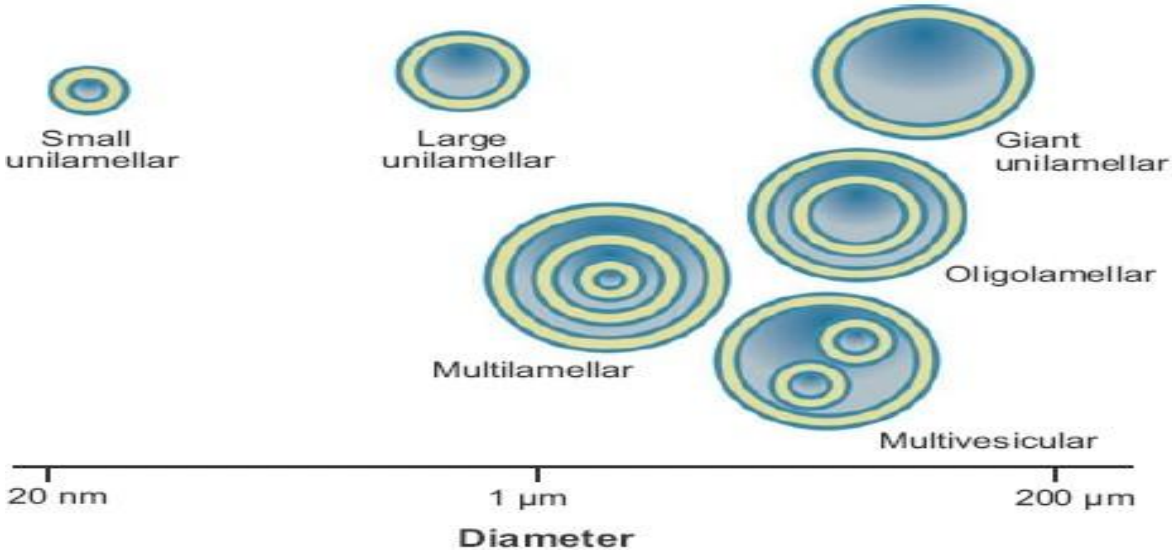


Figure 1.16. Liposome classification based on structure and size from (Rani *et al.,* 2013)

Based on the composition and functionality, liposomes can be classified as; (Storm and Crommelin, 1998; Pradhan *et al.*, 2015; Andhale *et al.*, 2016)**.**

I. Conventional liposomes (liposomes primarily composed of only phospholipid either neutral or negatively charged and cholesterol).
II. Fusogenic liposomes (liposomes composed of ultra-violet inactivated sendai virus on the surface).
III. Cationic liposomes (positively charged lipid made of by use of cationic lipids).
IV. Long circulating liposome (sterically modified liposomes modified through coating or grafting of the hydrophilic polymer, mainly the PEGylated, known to increase).
V. pH sensitive liposome (liposomes containing materials sensitive to change in pH, which allows control release of the cargo).
VI. Immuno-liposome (Liposomes with attached monoclonal antibody through surface modification).

1.4.4. Method of Liposomes Preparation

There are various methods of liposome preparation, which also influence the properties of liposomes such as the size, lamellarity, and the encapsulation efficiency (Pattni *et al.,* 2015).

1.4.4.1. Thin film hydration method

The thin film or Bangham method is one of the most widely used hydration methods. This method involves dissolving the lipid in an organic solvent followed by evaporation of the organic solvent and dispersion of the obtained lipid film in an aqueous media. Encapsulated drugs can be dissolved in aqueous media beforehand (for hydrophilic drugs) or in in the organic solution of the lipid (for lipophilic drugs). This method can lead to very high encapsulation efficiency of hydrophobic compounds. However, the drugs soluble in water are poorly encapsulated since they are widely dispersed in the aqueous hydrating medium, and only 10–15 % of the total volume gets entrapped (Bozzuto and Molinari, 2015; Andhale *et al.*, 2016).

1.4.4.2. Reverse phase evaporation method

This method promotes direct hydration of the lipid from solution in organic solvents to achieve an aqueous suspension of MLVs and ULVs. Firstly, the organic solution of lipids is purged with nitrogen and lipids are re-dissolved in an organic phase that is going to be mixed with an aqueous medium to form an emulsion which result in liposomes (called reverse phase evaporation vesicles) upon removal of the organic solvent under reduced pressure. High encapsulation efficiency of hydrophilic drugs (above 65 %) can be achieved through this method. However the exposure of the material to be encapsulated to organic solvent may affect the stability and activity of the formulation, hence the need for alternative method to escape from this major setback (Pradhan *et al.*, 2015).

1.4.4.3. Solvent injection method

The solvent injection method involves dissolving the lipids in an organic solvent (diethyl ether or ether methanol) and inject the mixture into an aqueous phase containing the compound to be entrapped. The lipids form a monolayer by aligning themselves at the interface of the organic and aqueous phase. The consequent removal of the organic solvent under reduced vacuum pressure leads to the formation of liposomes. The major disadvantage of this method is the exposure of materials to the organic solvent, high temperature and generation of a heterogeneous population of the liposomes (Akbarzadeh *et al.*, 2013; Andhale *et al.*, 2016).

1.4.4.4. Detergent removal method

The detergent removal method involves the lipid film hydration with detergent solution leading to formation of MLVs. The detergents can be removed using dialysis. The major advantage of this method is the excellent reproducibility and formation of liposomes with uniform size. The drawback of the method is the long preparation time required and poor trapping efficiency, hence this method is rarely used for liposome preparation (Torchilin *et al.*, 2003; Bozzuto and Molinari, 2015).

1.4.4.5. Heating method

Heating method is one the recent methods discovered for the formation of liposomes without use of any hazardous chemical or process. The method involves hydration of lipid components in an aqueous medium followed by the heating of the lipid components in the presence of 3 % v/v glycerol. Application of heat abolishes the need for any sterilization procedure, thus reducing time and cost for liposome production. This method was termed 'the heating method', since heating is the main step in the methodology and the resultant liposomes are called heating method (HM) liposomes (Torchilin *et al.*, 2003; Mozafari *et al.*, 2005).

1.4.4.6. Microfluidic method

Microfluidic method requires liquid flow inside the channel having a cross sectional dimension of 5 – 500 µm and above. In this method, lipids are dissolved in an accurate organic solvent (ethanol or isopropanol) and the resultant solution propelled perpendicularly or in the opposite direction to the aqueous medium within the micro-channels. The continuous axial mixing of the organic and aqueous solutions leads to formation of liposomes due to the local diffusion of phospholipids in aqueous phase, which encourages self-assembly. (Van Swaay and Demello, 2013; Patil and Jadhav, 2014).

1.4.4.7. Supercritical fluids method

The supercritical fluids (SCF) method possesses the desirable feature of using liquids and gas. This method has been reported to be a better alternative to for liposome formulation due to lack of organic solvent, easy and efficient separation and purification steps, overcoming the issue of poor stability and reduced activity as mentioned in organic solvent base methods. The most widely used SCF is carbon dioxide (CO_2), which is used for dissolution of the lipid

components at maintained supercritical temperature and pressure (Patil and Jadhav, 2014; Pattni *et al.*, 2015).

1.4.4.8. Drug loading

Loading of a drug in liposomes can be achieved either during the formation of liposomes (passive loading) or after liposome formation (active loading). Passive loading involves the combination of the hydrophobic drugs in the liposomes during the vesicle formation, and the dispersion of the hydrophilic drugs in the aqueous medium used for lipid hydration. The use of passive loading method results in good entrapment efficiency for hydrophobic drugs. This is due to the embedment of the drugs in the lipid bilayers, while low entrapment efficiency for hydrophilic drugs occurs because of large volume of the dispersing aqueous medium. However, the degree of entrapment efficiency depends on the method of liposome preparation, type of phospholipid, composition of the liposome (phospholipid to cholesterol ratio and lipid to drug ratio). Active loading involves initial preparation of the liposome in a particular pH buffer, followed by dialysis in excess of another buffer to form a transmembrane pH gradient. This method yields high entrapment efficiency, when compared to passive loading due to the diffusion effects during the establishment of gradient across lipid bilayers (Akbarzadeh *et al.*, 2013; Pattni *et al.*, 2015).

1.4.4.9. Freeze-drying

Freeze drying, also known as lyophilization, is the removal of water in the frozen state. This method helps in ensuring long term stability of some liposomal systems. Use of a cryoprotecting agent like sugar such as lactose, sucrose and trehalose have been reported to ensure liposome stability during freeze-drying, by overcoming some of the liposome stability issues such as leakage and loss of hydrophilic cargo (Vemuri and Rhodes, 1994; Akbarzadeh *et al.*, 2013).

1.4.4.10. Freeze thawing

The freeze thawing method includes freezing of liposome dispersion and thawing by keeping at higher temperature over a given time interval, followed by sonication for a limited duration. In this process, liposomes fuse with each other and enlarge in size. This method is quite easy and leads to formation of LUV. However, this method was reported to be

unsuccessful in case of neutral liposomes which exhibit poor thawing behaviour (Andhale *et al.*, 2016).

1.4.5 Characterization of Liposomes

Liposome properties including the size, shape, surface charge, lamellarity etc. influence the biological behaviour and fate of liposome. Therefore, these properties need to be extensively characterized prior to use of liposomes to ensure potential *in vitro* or *in vivo* performance (Patil and Jadhav, 2014; Bozzuto and Molinari, 2015).

1.4.5.1. Size and polydispersity

Evaluation of size and polydispersity has proved to be very critical in drug delivery and allows estimation of the batch quality and reproducibility (Pattni *et al.*, 2015). There are several analytical techniques used for measuring liposome size. These include dynamic light scattering (DLS), size exclusion chromatography (SEC), nuclear magnetic resonance (NMR), transmission electron microscopy (TEM), cryogenic TEM (cryo- TEM), and atomic microscopy (AFM). Amongst, DLS is the most widely used technique for the determination of size and polydispersity. This technique evaluates incident light scattering due to the Brownian motion of the dispersed particles, which result in time dependent fluctuation in the intensity of the scattered light. This allows the determination of the liposome distribution diffusion coefficient, which is converted into a size distribution using established theories. However, use of DLS is associated with some bottlenecks such as system inability to differentiate individual liposomes from aggregates and false readings may arise from measuring aggregates as single particles (Bozzuto and Molinari, 2015; Pattni *et al.,* 2015).

Use of HPLC-SEC in size determination involves passing of the samples through the columns with perfect or porous packing under pressure from HPLC pumps. This has the ability of separating particle size from low to moderate sizes. This method is reliable and reproducible. However, as deformable systems, liposomes can squeeze through the pores and lead to false readings (Pattni *et al.*, 2015). Electron microscopy including cryo-TEM and TEM are essential for size determination, as they provide much more precise liposome size reading by allowing direct particle visualization. The use of TEM can involve negative staining with uranylacetate or phosphotungstic acid, and liposomes are placed on a small copper grid and the aqueous medium is allowed to dry. Liposomes appear like bright dots on a dark background or blank

dots on a white background, depending on whether the staining agent was used or not. Although TEM has shown tremendous consideration for structural characterization, its application may be affected by formation of artefacts in the generated image, as the technique requires removal of the liposomes from their native environment. Cryo-TEM, in turn, allows liposomes to be visualized in their native environment, using flash freezing of the sample in liquid nitrogen, thereby preventing liposome damage, shrinkage or distortion. However, the use of these techniques is limited as they require expensive infrastructures (Bozzuto and Molinari, 2015; Pattni *et al.*, 2015). AFM uses a high-resolution method for measuring liposome size. This method is reliable, quick and requires no sample pre-treatments. Nanoparticle tracking analysis (NTA) is a newly developed technique that detects nanoparticles by evaluating the scattering of the illuminated laser lights. This technique works when the liposomes are injected into the laser beam view and the illuminated cell containing the liposomes is captured by the digital camera in the medium. This method quickly analyses liposomes in the range of 10 to 2000 nm size using the nanosight instrument software (Pattni *et al.,* 2015).

1.4.5.2. Zeta potential

The zeta potential involves measuring and calculating liposomal surface charge in a dispersion, as the individual particles can carry either negative, positive or neutral charges depending on the system composition as well as the attached ligand. The evaluation of liposome charge helps to predict and control the stability of the liposome under storage conditions, as highly charged liposomes show no aggregation due to repulsive forces in the medium, while low charged liposomes aggregate over time. DLS is widely used for surface charge determination. This measures the fluctuations in the scattered light as liposome moves due to the applied electric field. (Pattni *et al.*, 2015).

1.4.5.3. Encapsulation efficiency (EE)

EE is typically defined as the total amount of encapsulant found in liposome solution versus the initial amount used for encapsulation. This requires the separation of drug loaded liposomes from the free drug through centrifugation, dialysis or column chromatography, accompanied by quantification of the encapsulated drug and replacing the aqueous media with an organic solvent (e.g. acetonitrile, ethanol, methanol, Triton X-100). Several

techniques are used for estimation of the drug concentration. These encompass ultraviolet (UV) spectrometry, fluorescence spectroscopy, gel electrophoresis, enzyme or protein-based assays, HPLC, UPLC or LC-MS depending on the physicochemical properties of the drug (Škalko *et al.*, 1998; Pattni *et al.*, 2015).

1.4.5.4. Lamellarity

Lamellarity of a liposome is the number of bilayers forming the liposomal vesicles. This characteristic holds tremendous consideration since it can impact significantly the liposomes functionalities and fate *in vivo*. Several chemically labelled reagents or radiolabelled ions are used to estimate the number of lipids on the surface, based on the distribution of the reagents on the vesicle outer layer (Pattni *et al.*, 2015). Cryo-TEM is the most recommended method for study of lamellarity. Other techniques such as ^{31}P NMR determines the degree of lamellarity from signal ratio before and after addition of Mn^{2+}. X-ray scattering (SAXS) is also employed in the study of lamellarity (Škalko *et al.*, 1998; Fröhlich *et al.*, 2012; Pattni *et al.*, 2015).

1.4.5.5. *In vitro* drug release

The determination of the drug release profile is mostly performed using the dialysis. The liposomes dispersion is placed into a dialysis bag, with specific molecular weight cut offs, that is soaked in the release media (mostly a buffer), which is then maintained under specific temperature (37 °C) and continuous stirring in an enclosed condition in order to mimic the *in vivo* environment. The release medium is withdrawn at defined time intervals and replaced with a fresh portion of the buffer. The drug is analysed using any of the techniques like the UV spectrometry, fluorescence spectroscopy, HPLC, UPLC, LC-MS, etc. The plots of cumulative release percentage against time give the estimate of the release kinetics of the liposomes (Pattni *et al.*, 2015).

1.4.5.6. Phase behaviour

There are several techniques used in investigating liposome phase behaviour. These include differential scanning calorimetry (DSC), X-ray diffraction (XRD), and thermogravimetric analysis (TGA). These techniques provide thermal stability and crystalline profile of the various components of the formulation (Omwoyo *et al.*, 2014).

DSC is a primary technique for direct investigation of heat energy uptake which occurs in a sample upon regulated increase or decrease in temperature. DSC involves simultaneous introduction of energy into the sample cell (containing the formulation, sample, or kept empty, reference), which is a metal pan composed of aluminium, zinc or tin (Koyama *et al.*, 1999; Gill *et al.*, 2010). DSC differs from the conventional thermal analyser (DTA) in one fundamental aspect. DTA measures and records the temperature differences between the sample and reference channel, while the DSC measures the differential energy required to keep both sample and reference channel at the same temperature throughout the analysis (Watson *et al.*, 1964; Bond *et al.*, 2002). However, DSC is widely used because most transitions are accompanied by change in heat, whether chemical or physical transitions (like melting point or absorption). The resulting plot is heat flow against temperature, which gives information about both first order transition such as melting or crystallisation and second order transitions including the glass transition temperature in the samples (Bond *et al.*, 2002).

XRD is used primarily for characterisation of crystalline materials. This gives information on crystal structure phase, preferred crystal orientation (texture) and some other structural parameters. The XRD instrument produces characteristic peaks of the sample by constructive interference of monochromatic beam of X-rays diffracted at specific angles from each set of the lattice planes in a sample. The peak intensities are determined by the distribution of atoms within the lattice. The scattering of X-rays from atoms produce a diffraction pattern that contains information about the atomic arrangement. XRD is commonly used in pharmaceutical industries for the characterization of polymorph, monitoring of the stability, development and validation of methods for quantification and identification of drug products. In nanotechnology, XRD is used for crystallinity assessment and particle size measurement (Giron *et al.*, 2002; Chauhan A and Chauhan P, 2014).

TGA is a thermal method used for characterization of element, compound or mixture by measuring the changes in weights at high temperatures. The basic components of TGA instruments include mainly a precision balance and a furnace. The instrument is programmed for a linear rise of temperature over time. TGA experimental outcome corresponds to a weight loss curve that indicates the profile of mass changes against temperature. This is accompanied by a derivative curve (DTG) that is drawn to clarify the recorded decomposition step of the sample. This allows the removal of gaseous products from the vicinity of the

sample in order to maintain a constant composition of the atmosphere throughout the experiment, preventing the reaction taking place between the sample and air (Giron *et al.*, 2002; MacArio *et al.*, 2013).

1.4.6. Application of Liposome

Liposomes have now evolved from mere experimental tools of research to industrially established products for clinical and veterinary use. They have shown the ability to increase the therapeutic efficacy, whereby the negatively charged liposomes are predominantly taken up by cells in the cytosis process (Sharma and Sharma, 1997). Liposome encapsulation can alter the spatial and temporal distribution of the encapsulated drug molecules in the body, which may significantly reduce side effects and enhanced therapeutic response (Daraee *et al.*, 2016). Liposomes have been used for topical delivery and oral delivery in the treatment of various diseases, including cancer and HIV infection; but also in other applications including food and cosmetics industries (Uhumwangho and Okor, 2005).

1.4.6.1. Application in drug delivery

The use of liposomes in drug delivery has been widely studied. Liposomes known to be used as solubilizing, site targeting agents for poorly soluble and cytotoxic drugs, which can distribute non-specifically all over the body, thereby causing the death of normal and malignant cells (Lian and Ho, 2001) .The encapsulation of these drugs into liposomes has shown to target the infected cells, increase the solubility and stability of the drugs and improve the pharmacokinetic profile of drugs such in the encapsulation of anti-HIV, anti-cancer drugs ((Banerjee *et al.*, 2001)**.**

The use of antiretroviral agents has so far shown some success in the treatment of HIV-1 infection. These antiretroviral agents are effective in combating HIV replication through reverse transcriptase inhibition. However, the antiretroviral agents are known for dose related toxicity, severe side effects and poor solubility. Therefore, the use of liposomes (concentric lipid bilayers) for antiretroviral drug has been introduced as a potential strategy to minimize the aforementioned the dose related toxicity and provide controlled and sustained drug release over a longer period of time. The greater efficacy of the liposomal drug arises from the preferential penetration of the liposomes into the HIV host cells(Uhumwangho and Okor, 2005, Karami *et al.,* 2018) .

Liposomes have been used to deliver anticancer agents in order to increase solubility, circulation time as well as enhance efficacy and reduce side effects. Small and stealth liposomes (50-150 nm) can passively target several kinds of tumours and circulate for prolonged time due enhanced biological stability. Typical example could be the case of doxorubicin encapsulated in polyethylene glycol coated liposomes for refractory ovarian and breast cancer as an alternative standard therapy in kaposis's sarcoma (commonly known as malignancy in HIV-infected patients). (Uziely *et al.*, 1995; Lasic *et al.*, 1998; Banerjee *et al.*, 2001)

1.4.6.2. Cosmetic application

Liposome characteristics have shown useful properties for the delivery of cosmetic ingredients. The application is based on the similarity between the liposome lipid vesicle bilayer structure and the biological layers that compose the skin. Lipids can be hydrated and contribute to the reduction of skin dryness. While playing the role of attractive vehicles for cosmetic agents, liposomes provide a great source of skin ingredients including both lipids and essential fatty acids (such as linolenic acid). This has shown great potential for maintaining the skin and hair in good physiological standing, preventing the rise of some common topical diseases. The use of skin care formulations with empty or hydrating agents loaded liposomes helps to reduce the transdermal water loss, which is suitable for the treatment of dry skin. An example of liposomal cosmetic is the ''capture'' launched by Christian Dior in 1986 as an anti-ageing cream (Patravale and Mandawgade, 2008; Pradhan *et al.*, 2015).

1.4.6.3. Application in food

The application of liposomes in food industry has shown huge potential due to improved nutrients stability, since the encapsulated materials are protected from moisture, heat or other extreme conditions. This involves incorporation of food ingredients, enzymes, cells or other materials in small capsules, thus enhancing their stability and prolonged validity. Apart from stabilising the food ingredients during storage, liposomal encapsulation in food industry also aims at masking odours or tastes and performing controlled and targeted delivery to a particular tissue. Examples of food ingredients that have been subjected to liposomal encapsulation includes flavouring agents such as oils, spices, seasoning, sweeteners, acids,

alkalies, preservatives, lipids; redox agents, colourants, etc. These ingredients are encapsulated in order to extend the shelf-life from a few months to several years, but also for easier handling, improved stability and solubility. Examples like menthol, peppermint and other flavours in their encapsulated form has gained popularity due to their enhanced stability at high temperature (Gibbs *et al.*, 1999; Marques De Assis *et al.*, 2014).

1.5. Reports on Antiretroviral Delivery and Liposome

Ramana *et al.*, (2010) formulated liposomal nano-delivery for nevirapine from egg phospholipid using film hydration method. The liposomes were characterized using SEM, DSC, laser diffractometer (LD), UV and modified Eagle's medium for study of the influence of proteins on the release of nevirapine from the liposomes. They achieved higher and best encapsulation efficiency of 78.14 % with a prolonged release of nevirapine up to 1320 minutes at physiological pH. They discovered that the presence of proteins in the medium and external stimuli like low frequency ultra-sound enhanced the rate of release, with high magnitude of drug release due to use of ultra sound and presence of cholesterol stabilizing the vesicles against fluidizing action of proteins. The discoveries points to a potential novel approach towards antiretroviral therapy using liposomal formulation to improve target delivery of nevirapine drug and alleviating systemic toxic side effects.

Garg *et al.*, (2008) have evaluated the radiolabelling, and antiretroviral efficacy of stavudine loaded ^{99M}TC labelled galactosylated liposomes. They studied the radiolabelling to determine the activity of antiretroviral and the biodistribution of ^{99M} TC labelled galactosylated liposomes loaded with stavudine. Reverse phase evaporation method with extrusion through 200 nm polycarbonate membrane was employed in the preparation. The galactosylated liposomes were assessed for *in vitro* ligand activity. The free stavudine, stavudine loaded plain liposomes and galactosylated liposomes were all radiolabelled with $^{99\,M}$ Tc by direct labelling method using stannous chloride as a reducing agent. The activity of the antiretroviral was determined using HIV-1 infected MT2 cell lines. They discovered a maximum labelling efficiency of 95 %, a dose dependent inhibition of p24 production was detected upon treatment of HIV-1 infected MT2 cell with stavudine loaded liposomes and galactosylated liposomes. The imaging and quantitative biodistribution of the labelled drug and liposomes showed that liposomal formulations were taken up by the liver and spleen. Free drug solution was cleared from the blood with higher retention liver and spleen over a period of 24 hours

in the case of galactosylated liposomes as compared to free drug and plain liposomes. This revealed that the reduced uptake of the galactosylated liposomes in bone and prolonged accumulation in the mononuclear phagocyte system (MPs) rich organs indicates the excellent potential in the treatment of HIV infection.

Ogunwuyi *et al.*, (2016) have successfully achieved the formulation of antiretroviral drugs loaded nanoparticles of zidovudine, lamivudine, nevirapine and raltegravir fabricated by dispersion polymerisation for HIV/AIDS treatment. They synthesised two different types of nanoparticles using stealth methacrylate and stealth ε. Caprolactone based nanoparticles, and polyethylene glycol PEG as both co-monomer and stearic stabilizer to form the corona of the nanoparticles. They revealed through the result that the nanoparticles efficiently inhibited HIV-1 infection in cell, media T cells and peripheral blood mononuclear cells and released *in-vitro*. The ARV-loaded nanoparticles with PEG on the corona also facilitated tethering ligand for targeting specific receptors on the cells of the HIV reservoirs. The study showed that nanoparticles hold high promise in minimizing the virus replication in the cells and toxicity of the ARVDs.

Clayton *et al.*, (2009)developed sterically stabilized PEGylated liposomes coated with a targeting ligand derived from the fab' fragment of HIV gp120 monoclonal antibody F105 for targeted delivery of HIV-1 protease inhibitor. The liposomes were made of hydrogenated soy phosphatidylcholine and methoxy polyethylene glycol di-stearoyl phosphatidylethanolamine. They revealed that the formulated immunoliposomes were selectively taken up by HIV-1 infected cells, enabling the establishment of a cytoplasmic reservoir of protease inhibitor intracellularly. The study further revealed that the antiviral drug delivered by the immunoliposomes showed greater and longer antiviral activity than the concentrations of the free drug and drug encapsulated in non-targeted liposomes. This proved that by combining a targeting moiety with drug loaded liposomes, efficient and specific uptake by non-phagocytic HIV cells was facilitated and therefore encouraging for targeted delivery of antiretrovirals loaded immunoliposomes to the infected cells.

Sudhakar *et al.,* (2016) formulated and evaluated ritonavir loaded liposomes for parental delivery. They achieved formulation of stealth liposomes using ethanol injection method. The selected formulations were PEGylated and characterized for various physicochemical parameters such as the drug content, size, surface charge, entrapment efficiency and *in vitro*

release using SEM, DSC, FTIR etc. The stealth liposomes demonstrated better and *in vivo* resident time and longer circulation half-life compared to the conventional liposomes and pure drug solution due to the effect of the PEG-10000. This study suggests that stealth liposomes are promising tool in antiretroviral therapy.

Garg *et al.*, (2006) had prepared stavudine loaded mannosylated liposomes, for *in-vitro* HIV-1 activity, tissue biodistribution and pharmacokinetics. The mannosyalted liposomes were prepared by reverse phase evaporation method and evaluated for cell viability, *in-vitro* ligand aggulation assay, cellular drug uptake, anti-HIV assay and haematological assay. The study revealed that stavudine encapsulation in mannosylated liposomes improved cell uptake without increasing cytotoxicity and delivered their content to mononuclear phagocytes system tissues which are the reservoirs of HIV-1. The haematological study of the mannose coated and uncoated liposomes showed that the encapsulation of stavudine in mannosylated liposomes increased the elimination half-life and mean residence time of the drug and generated better pharmacological activity. They reported that mannosylated liposomes can be used for biostable, site-specific and mediated delivery systems for desired therapeutics, overcome the toxicity of antiretroviral encapsulated in mannosylated liposomes and reduce the dose of the antiretrovirals.

Roopa and Kusum, (2009) formulated lamivudine liposomes for transdermal delivery. Liposomes of lamivudine was prepared using thin film hydration and evaluated for potential transdermal *in vitro* delivery. The formulated liposomes were characterized for some parameters like the size distribution, entrapment efficiency, storage stability, drug retention in skin and *in vitro* permeation. The study showed that the formulated lamivudine liposomes were stable, showed appreciably controlled skin permeation as well as minimal retention of the drug molecules in the skin with ideal particle size for transdermal delivery. The study advocates for use of suitable liposomes for transdermal delivery of antiretrovirals.

Ramana *et al.*, (2012) had investigated the stability of saquinavir loaded liposomes, implication on stealth, release characteristics and cytotoxicity. Liposomes was prepared using thin film hydration method and the surface was modified using poly (ethylene glycol). The study reported high encapsulation efficiency for saquinavir loaded liposomes and sustained release. However, the PEGylated liposomes revealed more stability in the protein supplemented media, better colloidal stability due to cavitation threshold and more sustained

release of the drug. Moreover, the study showed that the viability studies using Jurkat T-cells further revealed that PEGylated liposomes loaded with saquinavir were less cytotoxic compared to the non-PEGylated liposomes or free drug. This study provided a safe and efficient strategy for sustained drug delivery and for targeting cells using PEGylated liposomes and can offer the benefit of drug stability, reduce dose frequency and drug toxicity.

Chudasama *et al.*, (2015)have formulated self-emulsifying drug delivery system using lipid base excipients to improve solubility and enhance oral absorption of the poorly water soluble nevirapine drug. They examined the influence of oil, surfactant and co-surfactant in the lipid-based formulations. The formulations were evaluated for morphology, droplet size and surface charge, cloud point and *in vitro* diffusion using rat model. The diffusion rate and oral bioavailability result of the lipid-based formulations were compared with marketed suspension. The result revealed increased absorption of nevirapine from the formulated self-emulsifying lipid-based delivery system and great increase in relative bioavailability compared with marketed suspension. Nevirapine also showed higher *ex vivo* stomach and intestinal permeability and *in vivo* absorption than a marketed suspension. This suggests that self-emulsifying lipid-based drug delivery systems can be a useful delivery system for targeting of antiretrovirals to the lymphoid organs.

Dubey *et al.,*(2010) have developed and characterized indinavir bearing ethanolic liposomes for potential transdermal delivery. The formulated liposomes were evaluated for vesicular shape, surface morphology, stability, entrapment efficiency and *in vitro* skin permeation etc. The result demonstrated unilamellar nano-ranged vesicles, smooth surfaced nano carriers and higher entrapment efficiency which play a vital role in providing enhanced flux. However, the permeation studies of indinavir across human cadaver skin resulted in enhanced transdermal flux from ethanolic liposomes that was significantly greater than that with a plain drug solution. Ethanolic liposomes also demonstrated the shortest lag time for indinavir, thus presenting a suitable approach for transdermal delivery of the antiretrovirals.

1.6. Buccal Films (BFs)

Buccal films are polymeric materials that meet several requirements for being used as a drug delivery platform. BFs remain excellent candidates for targeting the infected sites where oral tablets or liquid formulations exhibit poor bioavailability (Morales and McConville, 2011; Karki *et al.*, 2016). BFs (**Table 1.2**) are mainly used for patients having swallowing difficulties or

emetic conditions. BFs provide improved patient compliance due to their small size, thickness when compared to tablets (Chaturvedi *et al.*, 2011). In addition, they circumvent the relatively short residence time of the oral gels on the mucosa, which are easily washed away by saliva. In the case of the local delivery for oral diseases, the BFs help to protect the wound surface, thus minimising the pain (Boddupalli *et al.*, 2010). Most of the films having mucoadhesive properties are hydrophilic in nature and swell upon interaction with the mucin, which explains their good flexibility and elasticity while still being strong enough to withstand breakage due to stress from mouth movement (Boddupalli *et al*., 2010).They are primarily composed of adhesive polymers, plasticizers, sweeteners, colourants and flavours for masking of taste or improved palatability (Silva *et al.*, 2015). Polymers represent the major part of film formulations. There are numerous types of polymers can be used alone or in combination for films preparation. However, BF formulations are preferably made of hydrophilic polymers, regardless of the drug being hydrophobic or hydrophilic (Dixit and Puthli, 2009; Borges *et al.*, 2015). There are several advantages offered by BFs. These include the onset of action by direct absorption through buccal mucosa and entrance into the systemic circulation, ease of transportation, handling and storage, improved oral bioavailability through avoidance of first pass metabolism, accurate dosing in a safe efficacious format that is convenient and portable without requiring use of water or spoon. Because their multiple benefits; pharmaceutical industries and consumers have embraced BFs as an alternative to the traditional dosage forms (Andrews *et al.*, 2009; Singh *et al.*, 2013; Landova *et al.*, 2014; Silva *et al.*, 2015). This underlines the potential of BFs in drug product development and explains the existence of several commercially available BFs formulations that have shown some clinical success.

Table 1.2 . List of some marketed buccal film products (Montenegro-Nicolini and Morales)

Product name	**Therapeutic use**	**Absorption route**
Breakyl	Narcotic pain relief	Systemic
Onsolis	Narcotic pain relief	Systemic
Niquitin	Smoking cessation	Systemic
Seto film	Antiemetic	Systemic
Chloraseptic	Sore throat relief	Local

Donepezil Hexal SF	Alzheimer's treatment	Systemic
Gas X-	Anti-flatulence	Systemic
Risperidone Hexal SF	Neuroleptic	Systemic
Triaminic or Theraflu night-time	Cough and cold	Systemic
Triaminic Theraflu	Allergy	Systemic
Suboxone	Opioid addiction treatment	Systemic

1.6.1. Components of buccal films

Buccal films are composed of mainly the drug, polymers and plasticizers. The incorporation of sweeteners, colourants or flavours is sometimes relevant when dealing with drug substance having unpleasant taste or odour. All the excipients used in the preparation of BFs are regarded as safe and approved in pharmaceutical dosage forms (Patil and Shrivastava, 2014; Silva *et al.*, 2015).

1.6.1. Drug

Several class of drugs can be formulated in buccal films, either hydrophilic or hydrophobic. A typical film composition contains 1 to 25 % w/w of the drug (Patil and Shrivastava, 2014).

1.6.1.2. Polymers

Polymers are the most important ingredient in the preparation of BFs either alone or in combination, since they represent 40 – 50 % of the formulation. The polymers commonly used for BFs formulation are mainly hydrophilic, which allows system disintegration in the buccal cavity upon contact with saliva. The strength of the film depends on the type and amount of polymer in the formulation. The polymers employed in the film formulation should be non-toxic, non-irritating, devoid of leaching impurities, good wetting and readily available. Polymers commonly used for the formation of the films are water soluble grades of cellulose ethers, polyvinyl alcohol, polysaccharides, polyethylene glycols, carboxyl methyl cellulose, propyl methyl cellulose, hydroxyl propyl cellulose. (Bhyan *et al.*, 2011; Patil and Shrivastava, 2014). The polymers used in this study were selected because of their good bio-adhsive

properties (swelling ability, controlled release), and availability. They are briefly presented in the following paragraphs.

Hydroxyl propyl methyl cellulose (HPMC)

HPMC (**Figure 1.12**) is a non-toxic cellulose ether and one of the most important hydrophilic carrier materials used for the preparation of oral drug-controlled delivery systems. Its most important feature is its ability to swell, which has a significant effect on the release kinetics of an incorporated drug. HPMC is inexpensive and various available grades allows for desired release profiles (Liquid *et al.*, 1986; Brannon-Peppas, 1990; Siepmann and Peppas, 2012).

Chemical name: Cellulose hydroxy methyl ether

Molecular formula: $C_{56}H_{10}O_{30}$

Molecular weight: 1261.45 g/mol

Synonyms: Hydroxyl propyl methyl cellulose, HPMC, Hydroxyl propylene Glycol Ether, Metocel K4M; Hpromellose; Ultra Tears.

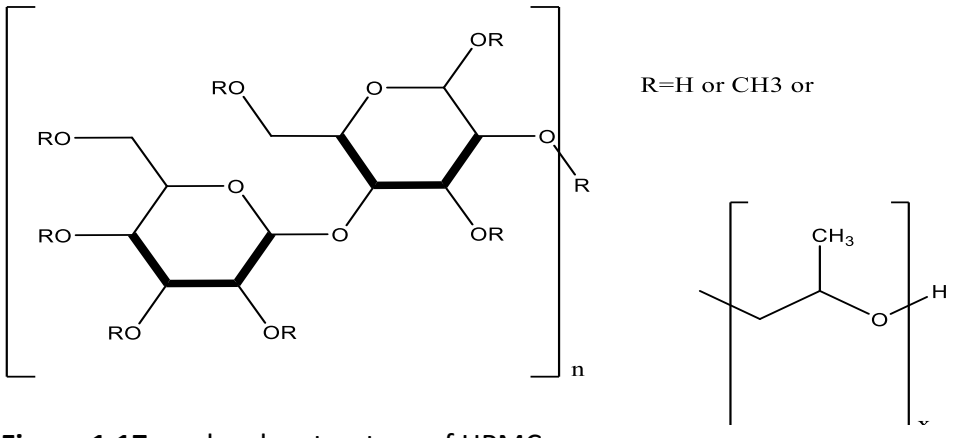


Figure 1.17. molecular structure of HPMC

Description:

HPMC is a white to off white fibrous or granular with no taste or odour.

Viscosity: HPMC is in different viscosity grades but are measured in 2% aqueous solution.

pH: 5.5 – 8.0

Melting point: Chars at 225 °C – 230 °C

Specific gravity: 1.26

Bulk density: 0.341 g/m^3

Solubility: Soluble in cold water, forming a viscous colloidal solution. Practically insoluble in chloroform, ethanol and ether. But soluble in mixture of ethanol and dichloromethane, mixture of methanol and dichloromethane, and mixture of water and alcohol.

Stability and Storage Conditions: HPMC powder is a stable material, although it is hygroscopic after drying and should be stored in a well-closed container, in a cool dry place.

Functional category: Coating agent, film-former, rate controlling polymer for sustained release, stabilizing agent, suspending agent, tablet binder, viscosity increasing agent.

Carbopol

Carbopol (**Figure 1.18**) is a synthetic polymer of acrylic acid cross-linked with poly-alkenyl ethers or divinyl glycol with a high molecular weight estimated from 740,000 to 4–5 million. They are produced from primary polymer particles of about 0.2 to 6.0 micron average diameter. The particles are viewed as a network structure of polymer chains interconnected via cross-linking (Hosmani *et al.,* 2013).

Chemical name: Carboxy poly-methylene

Molecular formula: $(C_3H_4O_2)n$

Synonyms: Acritamer; acrylic acid polymer; Carbopol; carboxy polymethylene, polyacrylic acid; carboxyvinyl polymer; Pemulen; Ultrez.

Chemical structure

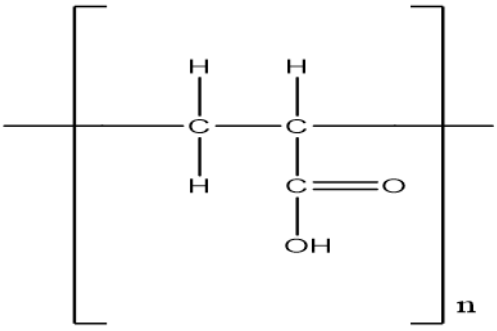


Figure 1.18. Chemical structure of Carbopol

Description: Carbopol is a white fluffy, hygroscopic, and acidic powder with a characteristic odour

pH: 2.7 – 3.5

Specific gravity: 1.41

Melting point: 260 °C

Bulk density: 1.76 – 2.08 g/cm^3

Tapped density: 1.4 g/cm^3

Solubility: Soluble in water and, after neutralization, in ethanol (95 %) and glycerine.

Functional Category: Bio-adhesive; emulsifying agent; release-modifying agent; suspending agent; tablet binder; viscosity-increasing agent.

Stability and storage: Carbopol is a stable hygroscopic material which can be heated below 104 °C temperature for close to 2 hours without the thickening efficiency affected. But excessive exposure to temperature may result in the discoloration and decreased stability. However, should be stored in an airtight, corrosion resistant, cool dry place.

Pluronic

Pluronic is a triblock copolymer consisting of a central hydrophobic block of propylene glycol flanked by two hydrophilic blocks of polyethylene glycol as shown in **Figure 1.19** (Bohorquez *et al.*, 1999). Pluronic F127 is synthetic non-ionic surfactant approved by the FDA and has been used in several biological applications such as drug delivery systems (Escobar-Chavez *et al.*, 2006). It has inverse thermoreversible ability. In other words, when temperature increases to body temperature, Pluronic forms a gel due to the low solubility of polypropylene glycol at high temperature (Escobar-Chavez *et al.*, 2006).

Chemical name: Pluronic

Molecular formula: $C_5H_{10}O_2$

Synonyms: Poloxomar 407, polyethylene -polypropylene glycol; polyoxyethylene-polyoxypropylene block copolymer; (Ethylene oxide-co-polypropylene oxide), Block; Block copolymer of ethylene oxide and propylene oxide.

Chemical structure:

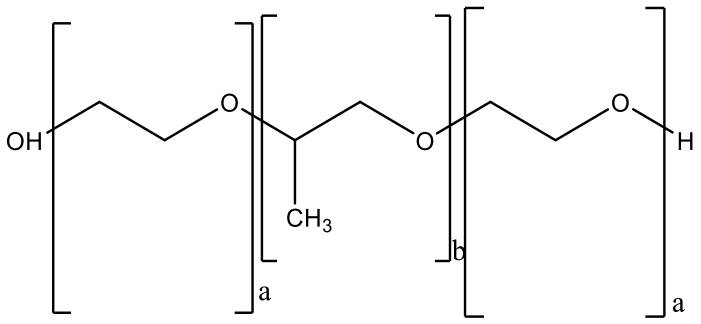


Figure 1.19. Chemical structure of Pluronic

Description: Pluronic is a white coarse, waxy powder with a faint specific odour.

pH value: 6 – 7

Melting point: 5357 °C

Molecular weight: 9840 – 14600 g/mol

Specific gravity: approx. 0.5 g/cm^3

Solubility: Soluble in cold water.

Stability and storage: Pluronic is a stable, non-toxic surfactant copolymer and should be kept in a cool dry environment.

Application of Pluronic: The unique thermoreversible and promising drug release characteristics of PF127 render it as a pharmaceutical vehicle for drugs via different routes of administration (Escobar-Chavez *et al.*, 2006).

1.6.1.3. Plasticizer

The use of plasticizers in the formulation of the films helps to enhance the mechanical properties, such as the tensile strength and elongation, by reducing the glass transition temperature of the polymer. Plasticizer also reduce the brittleness of the film, which improves its flexibility. Some of the plasticizers commonly used in BFs formulation include low molecular polyethylene glycols, castor oil, citrate derivates (such as triethyl, acetyl citrate), glycerol and phthalate derivates like dimethyl, diethyl and dibutyl phthalate. (Patil and Shrivastava, 2014; Silva *et al.*, 2015). The plasticizer used in this work is presented in below.

Propylene glycol (PG)

Propylene glycol (**Figure 1.20**) is a viscous, odourless and colourless liquid mostly used as drug solubilizer in topical, oral, buccal and injectable medications. It is hygroscopic, with an isotonic concentration of 2 % in water, and superior humectant capable of retaining moisture over a wide range of humidity. It serves an ideal vehicular agent, a superior solubilizer, spreader and emollient for pharmaceutical preparations (Catanzaro and Smith, 1991).

Chemical name: Propylene glycol

Synonyms and trade name: 1, 2, Dihydroxy propane, 1, 2 – propane diol, 1, 2 – propylene glycol, 2, 3 – propane diol, hydroxy propanol, alpha propylene glycol, methyl glycol, methyl ethyl glycol, mono propylene glycol, trimethyl glycol.

Chemical formula: $C_3H_8O_2$

Chemical structure:

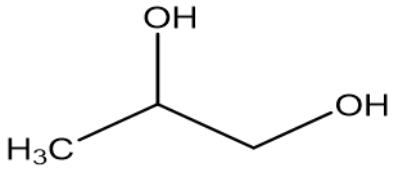


Figure 1.20. Chemical structure of propylene glycol

Appearance: Colourless liquid

Molecular weight: 76.095 g/mol

pH: 6 – 8

Melting point: -60 °C

Boiling point: >187 °C

Solubility: soluble in water, alcohol, ether, benzene, acetone, chloroform

1.6.2. Methods of preparation

There are several methods used for manufacturing the films. These include;

1.6.2.1. Solvent casting

The solvent casting method involves dissolution of the polymer and plasticizer in distilled water. The drug along with the other excipients are dissolved in a suitable solvent. The two solutions are mixed under stirring for one to two hours to form a homogeneous and viscous solution. The solution is then removed and kept aside in a desiccator to remove all the entrapped air bubbles. The preparation is further casted into petri dish and dried to form a film (Bhyan *et al.*, 2011; Patil and Shrivastava, 2014).

1.6.2.2. Semisolid casting

The semisolid casting method for film preparation from acid insoluble polymer dissolved in ammonium or sodium hydroxide. The obtained basic solution is added to water soluble film forming polymer. This mixture forms a gel by adding adequate amount of plasticizer. The product is then casted into the films or ribbons using heat-controlled drums (Chaturvedi *et al.*, 2011; Singh *et al.*, 2013).

1.6.2.3. Rolling method

In the rolling method, the solution or suspension of the drug and the film forming polymer is prepared in water or mixture of water and alcohol. The solution is suspended and dried on the rollers. The resultant dried product is cut into the desired shapes and sizes (Chaturvedi *et al.*, 2011; Patil and Shrivastava, 2014).

1.6.2.4. Hot melt extrusion

In the hot melt extrusion method, the drug is mixed with the carriers in solid form, and the extruder endowed with heaters melts the mixture. The melted product is shaped into films using the dyes. This method is also commonly used to prepare granules, sustained release tablets, transdermal and transmucosal drug delivery systems (Singh *et al.*, 2013).

1.6.2.5. Direct milling

The direct milling method affords film formation with no use of solvent. The drug and the excipients are mixed mechanically by direct milling or by kneading without any liquid. The resultant solid material is rolled on a release liner until the desired thickness is achieved. The

backing product is then laminated to the desired shapes and sizes (Raghavendra Rao *et al.*, 2012).

1.6.3. Evaluation of the characteristics of buccal films

There are numerous parameters considered in the preparation of the films. These embrace film thickness, weight variation, folding endurance, water absorption, surface pH, surface morphology, moisture content, etc. These parameters need to be evaluated using standard protocols. The techniques employed for BFs characterization are varied. This is based on the expected BFs properties, ranging from the physical through bucco-adhesive properties, *in vitro* permeation to *in vivo* performance. The mechanical parameter such as the tensile strength, elongation at break and the young's modulus vary significantly depending on the type of polymer and method of fabrication. In addition, the prepared films are also characterized using scanning electron microscopy SEM, XRD, FTIR, DSC and TGA (Nair *et al.*, 2013).

1.6.3.1. Thickness and weight variation

The measurement of the film thickness allows to ascertain the uniformity of the film, which is directly related to the dose accuracy. An optimum thickness of the film is essential as it can provide adequate bio-adhesion. An ideal film should show thickness between 50 and 1000 µm. Thickness of a film is measured using well calibrated electronic digital micrometre, screw gauge, Vernier calliper or SEM. The weight variation of the films is measured using digital weighing balance and the average weight is calculated by subtracting the average weight of the film from the individual weight of the films. A remarkable weight variation indicates inefficiency of the method used and may lead to non-uniform distribution of the drug (Cilurzo *et al.*, 2008; Nair *et al.*, 2013).

1.6.3.2. Folding endurance

This is an important physical parameter that determines the flexibility of the films, which allows easy application of the film at the site of administration. The flexibility of the films is determined by repeatedly folding the film at a 180 °C angle of the plane, and the same plane is folded up to 300 times (or at least up to film breakage). The number of times the film is folded without breaking describes the folding endurance (Nafee *et al.*, 2003; Sreeja *et al.*, 2014).

1.6.3.3. Water absorption

The water absorption or swelling ability of the BFs facilitates the release of the drug from the film, which occurs either by diffusion or erosion. The water absorption is essential in film formulation because wetted films provide better bio-adhesion onto the buccal mucosa, which is also dependent on the polymer matrix. Other parameters affecting the swelling behaviour of a film include the structure and composition of the polymer (Şenel *et al.*, 2000).

1.6.3.4. Moisture content

The amount of moisture present in the film is determined using fisher titration method or weighing method. The amount of moisture in a film is essential as it affects the brittleness and friability of the film. The polymer content of the product regulates the degree of moisture in a particular film (Nair *et al.*, 2013). The moisture content is calculated using the following equation:

$$\%\ Moisture\ content = Initial\ weight - Final\ weight\ X \frac{100}{Initial\ weight} \ldots.$$ Equation 1.1

1.6.3.5. Surface pH

Evaluation of the surface of the BFs is essential because both the acidity and alkalinity can affect the area of application, causing local damages or irritation to the buccal mucosa membrane, which leads to discomfort of the patients. This parameter is investigated by allowing the film to swell and keeping it in contact with distilled water over a time interval at room temperature. The pH of the resultant aqueous medium is measured using pH meter (Patel *et al.*, 2007).

1.6.3.6. Surface morphology

The surface morphology reveals the surface properties of the film such as the surface texture (rough or smooth), drug distribution, thickness etc. The morphology is basically evaluated using SEM or scanning tunnelling microscopy (STM). SEM is the most reputed because it reveals the size, shape and number of pores in the BFs as well as possible influence of the plasticizer (Boateng *et al.*, 2010; Nair *et al.*, 2013).

1.6.3.7. *In vitro* dissolution study

The drug dissolution study determines the cumulative drug release from the film over a given period. There is no specific *in vitro* method developed for BFs drug release studies. The most widely used method has been the USP 23 method. This method involves potassium dihydrogen phosphate (12 mM), sodium chloride (40 mM) and calcium chloride (1.5 mM) with an adjusted pH 6.2 (simulated saliva) as a dissolution medium. The BFs are placed on a glass plate and immersed in the dissolution medium and the paddle rotated at fixed speed. The dissolution studies have proven to be difficult many times due to the tendency of the film's strips to float on the dissolution medium (Nair *et al.*, 2013; Singh *et al.*, 2013).

1.6.3.8. *Ex- vivo* permeation studies

The *ex-vivo* permeation study gives information regarding possible drug transport mechanisms and the pathways across the buccal epithelium. The study further reveals a better insight into the drug absorption kinetics during pre-formulation study. Various permeability chambers such as the Franz diffusion cell (vertical or horizontal) or side by side diffusion cell have been used to study the permeability of the drug across the oral mucosa. The permeability diffusion cell comprises of the donor and the receptor compartments. Freshly processed buccal mucosa of animal of origin is used in the permeation studies. The oral epithelial tissue is mounted on the chambers of the cell membrane, and properly held to prevent leakage. The formulated film is placed under occlusion on the surface of the buccal mucosa in the donor compartment, the rate at which the drug permeates across the barrier membrane is determined from the receiver chamber containing the simulated saliva. The simulated saliva is stirred and continuously replaced to maintain sink condition at 37 ± 0.5 °C temperature (Nair *et al.*, 2013).

1.6.3.9. Tensile strength

A mechanical parameter that measures the weight applied at rupture or the maximum stress applied when film specimen breaks. The tensile strength measures the ability of a film to withstand rupture and can be calculated as:

$$Tensile\ strength = \frac{force\ at\ break\ (kg)}{Initial\ cross\ sectional\ area\ of\ the\ sample\ (mm^2)}$$ …. Equation 1.2

Several methods are used for measuring the tensile strength. These include the texture analyser (ATAXT2 analyser) with a 5 kg load cell, microprocessor based advanced force gauze with a motorized test stand, stationary and movable plates tensiometer etc. (Chaturvedi *et al.*, 2011; Nair *et al.*, 2013)

1.6.3.10. Young's modulus

The mechanical parameter young's modulus or elastic modulus is the measure of the film strip stiffness, hardness or rigidity. It is defined as the ratio of the strains (Chaturvedi *et al*., 2011, Sing *et al*., 2011). Young's modulus is calculated as:

$$young\ modulus = \frac{force\ at\ corresponding\ strain\ (kg)}{Cross\ sectional\ area\ of\ the\ film\ (mm^2)} X \frac{1}{Corresponding\ strain}$$ .... Equation 1.3

1.6.3.11. Percentage elongation at break

Elongation is defined as the increase in length relative to original length or form of deformation. The elongation of the BFs is measured using a texture analyser. The elongation is dimensionless and can be expressed as a percentage. A film can be analysed for either elastic elongation or ultimate elongation. The elastic elongation is essential and is referred as percentage elongation, which one can stretch the sample without deformation. Ultimate elongation is used when measuring the extent at which a sample can be stretched before it breaks (Chaturvedi *et al*., 2011, Nair *et al*., 2013). The percent elongation is calculated as:

$$\%\ elongation = \frac{Increase\ in\ lenth\ (mm)\ X\ Original}{Length\ (mm)}$$ .... Equation 1.4

1.7. Reports on Antiretrovirals and Mucoadhesive Films

Jones *et al.*, (2014) have designed and evaluated nano-enabled polymeric films for buccal delivery of didanosine antiretroviral drug for treatment of HIV and reducing related drug side effects. They evaluated optimal parameters for didanosine loaded solid lipid nanoparticle (SLN) incorporated in monolayered multi polymeric film (MMF) using hydroxyl propyl methyl cellulose HPMC and Eudragit RS100. The *in vitro* buccal permeability and *in vitro* drug release of the drug from SLN in the polymeric film were discussed, which showed the potential of drug permeability from SLN to the mucosa without adversely affecting the flux. The obtained results serve as a potential platform in using nano-enabled buccal polymeric films for antiretroviral drug delivery.

Machado *et al.*, (2016) have formulated tenofovir loaded nanoparticles in polymeric film for vaginal microbicide delivery using hydroxyl propyl cellulose and polyvinyl alcohol polymers for treatment of HIV disease and possible elimination of other related HIV infections. They used solvent casting method in formulation of the film and compared the drug loaded nanoparticle film with commercially available film. However, they reported favourable and sustained *in vitro* drug release for tenofovir loaded nanoparticles in the vagina than the commercially available film with no abnormal changes to the normal vaginal histology of mice used, which may arise from the polymer used.

Marimutho *et al.*, (2016) prepared zidovudine mucoadhesive films using the combination of hydroxyl propyl methyl cellulose, gelatin and sodium alginate to achieve a control release in the therapeutic range for an extended period as well as reduce dosing frequency in the treatment of HIV. They reported better uniform film thickness, uniform weight, good folding ability, salivary range pH, good moisture uptake and swelling ability for the formulated films using solvent casting method. The *in vitro* release studies also showed sustained drug release from use of the polymer alone and in combination, thereby showing potential in antiretroviral drug delivery.

Ramaya Teja *et al.*, (2015) have formulated oral thin films for paediatric medication. The oral was prepared using the combination of hydroxyl propyl methyl cellulose, sodium alginate, xanthan gum, guar gum. They evaluated several parameters of the formulated oral films like the film thickness, folding endurance, moisture uptake, tensile strength, percentage elongation measurement, drug uniformity and *in vitro* dissolution studies which revealed the combination of the polymers with lamivudine and were found to be the best formulation with prolonged drug release than when the polymers were used alone. The study is vital for administration of antiretroviral drugs orally in form of films which has shown lesser occurrence of drug side effects and improved bioavailability.

Chatterjee *et al.,* (2010) developed vaginal film of antiretroviral zidovudine using combination of film forming polymers of hydroxyl propyl methyl cellulose, ethyl cellulose and acry coat co-polymer. The film was prepared using the solvent casting method. The evaluated vaginal zidovudine films demonstrated good flexibility, high drug content with prolonged and diffused drug release due to combined effect of drug and polymer, good mechanical

properties and sufficient vaginal bio-adhesive strength in goat vagina, therefore paving way for use of muco-adhesive films in vaginal drug delivery of antiretroviral drugs.

Patel *et al.*, (2016) formulated fast mouth dissolving film of tenofovir fumarate using hydroxyl propyl methyl cellulose and polyvinyl pyrrolidone so as to overcome the fear of choking and difficult swallowing related to solid dosage form in paediatric patients. The data revealed good film thickness, high drug content, fast *in vitro* disintegration of the film which is desirable in dose delivery of tenofovir.

Ghosal *et al.*, (2016) formulated anti-HIV bio-adhesive vaginal film containing abacavir for treatment of HIV and minimizing of sexual infections. They used the combination of sodium alginate, hydroxyl propyl methyl cellulose film forming polymers using solvent casting method. The physicochemical evaluation of the vaginal films showed good film thickness, effective swelling ability of the film, good flexibility strength, high drug content bio-adhesive strength and sustained drug release suitable in delivery of antiretroviral through the vagina for effective treatment of HIV and related sexual infections.

1.8. Research Justification and Objectives

1.8.1. Justification of the Research

Though the use of the ARVD therapy has immensely improved the management of HIV/AIDS, its clinical use has suffered from several setbacks and inconveniences such as the extensive first pass metabolism and gastrointestinal degradation leading to low and erratic bioavailability, short half-life of the drugs resulting in frequent dosing, severe side effects arising from subsequent large doses and poor solubility due to inadequate drug concentrations at the site of action and poor bioavailability of the ARVDs (Oussoren *et al.*, 1999; Ojewole *et al.*, 2008).

The entrapment of the drugs into a particulate carrier delivery system such as liposomes represents an ideal and potential approach to overcoming the limitations of the ARVDs (Oussoren *et al.*, 1999; Torchilin *et al.*, 2005). Since some of the cells serve as the viral reservoirs, liposomes could potentially deliver ARVDs into the infected cells thereby improving the efficacy and reducing side effects offering targeted delivery (Ramana *et al.*, 2010).

However, the use of liposomal drug delivery system has suffered some major deficiencies like the high cost of liposome formulation, resulting from the use of expensive synthetic or purified natural phospholipids, lack of control over release rate and a lack of means to override biological barriers (Barenholz, 2001; Li *et al.*, 2014).

Consequently, use of less expensive and readily available bio-adhesive polymers have been employed in formulation of liposomal buccal films as one of the alternatives in overcoming the limitations of the liposomes in oral delivery. If these polymers are incorporated into the buccal formulations, the absorption of the drug by buccal mucosa layers would be enhanced, and drug released for extended period of time (Akhter *et al.*, 2012).

HIV also is one of the numerous viruses in the world affecting human which requires combination of several ARVDS in the treatment. The selection of EFV (a hydrophobic drug with associated limitations aforementioned) for study and incorporation in the liposomal buccal film formulation and is an ideal step that would bring to light new information (or results) on increased efficacy as it concerns target at the point of site. Hopefully, this would necessitate research of other numerous drugs in the market used for treatment of other viruses and diseases in this regard.

1.8.2. Aim

The aim of this research was to develop a liposome delivery system using crude lecithin and then embed the liposomes in bio-adhesive polymeric matrix to form buccal films for the delivery of EFV drug.

1.8.3. Objectives

The specific objectives of this research were;

Development of liposomes for entrapment of efavirenz using crude soybean lecithin.

Validation of the HPLC method for EFV quantification.

Preparation and characterization of EFV-loaded liposomes.

Formulation of liposomal buccal films using bio-adhesive polymers.

Ex-vivo permeation study of the EFV drug from the buccal films using chicken pouch.

Evaluation of the histological effects of the films on the chicken pouch.

Characterization of the liposomal buccal films.

CHAPTER 2:

FORMULATION OF EFAVIRENZ LOADED LIPOSOMES

This work has been presented as E-poster at the ''17th Edition of international conference and exhibition on pharmaceutics and novel drug delivery systems'' in Moscow Russia 2018, and orally during the 2017 Rhodes university chemistry postgraduate conference/ presentation with manuscript of the work on review.

2.1. Introduction.

HIV patients are mostly subjected to highly active antiretroviral therapy (HAART) which involves administration of a minimum of three ARV drugs (ARVDs). Unfortunately, the use of this therapy for effective management of the virus which can only limit the replication of the virus within the cells, thereby improving the quality of life of the patients (Gupta and Jain, 2010). This is significant as the virus resides mainly in the human anatomical and cellular reservoirs which are highly inaccessible to drugs, however most of the ARVDs and their combination are ineffective in controlling the viral proliferation (Ramana *et al.,* 2010). Antiretroviral drugs have been reported to show low oral bioavailability because of its metabolism in the gastrointestinal tract and its low half-life resulting in a steady administration of high doses, which in turn leads to low patient compliance (Li and Chan, 1999; Oussoren *et al.*, 1999; Ojewole *et al.*, 2008). Therefore, developing and optimising a drug carrier like liposomes remain an important treatment strategy for effective and efficient targeting and delivering of antiretroviral drugs, so as to increase its bioavailability and half-life and consequently reduce its side effects (Mallipeddi and Rohan, 2010; Ochubiojo *et al.*, 2012).

Liposomes or lipid vesicles are artificial vesicles of a small spherical shape which originate from natural phospholipids and non-toxic cholesterol and has been frequently studied and investigated since the 1970s as a delivery system for therapeutic improvement of a drug to the specific therapeutic sites (Dwivedi and Verma, 2013). Use of liposomes as a carrier for drug delivery has shown to improve the therapeutic index of an established or new drug by modification of drug adsorption, reducing metabolism, improving and prolonging biological half-life and minimizing toxicity of numerous antiretrovirals (Sathigari *et al.*, 2009; Chowdary and Naresh, 2011; Madhavi *et al.*, 2011; Chowdary and Enturi, 2013). Use of liposomes for drug delivery has had huge success, thereby influencing the potential of this treatment in clinics and hospitals. However there has not been any report on use of liposomes for EFV delivery. Therefore, the purpose of this work was to formulate EFV loaded liposomes with high entrapment efficiency and desired properties, using locally available crude soybean lecithin which has been approved by the FDA for the treatment of HIV/AIDS.

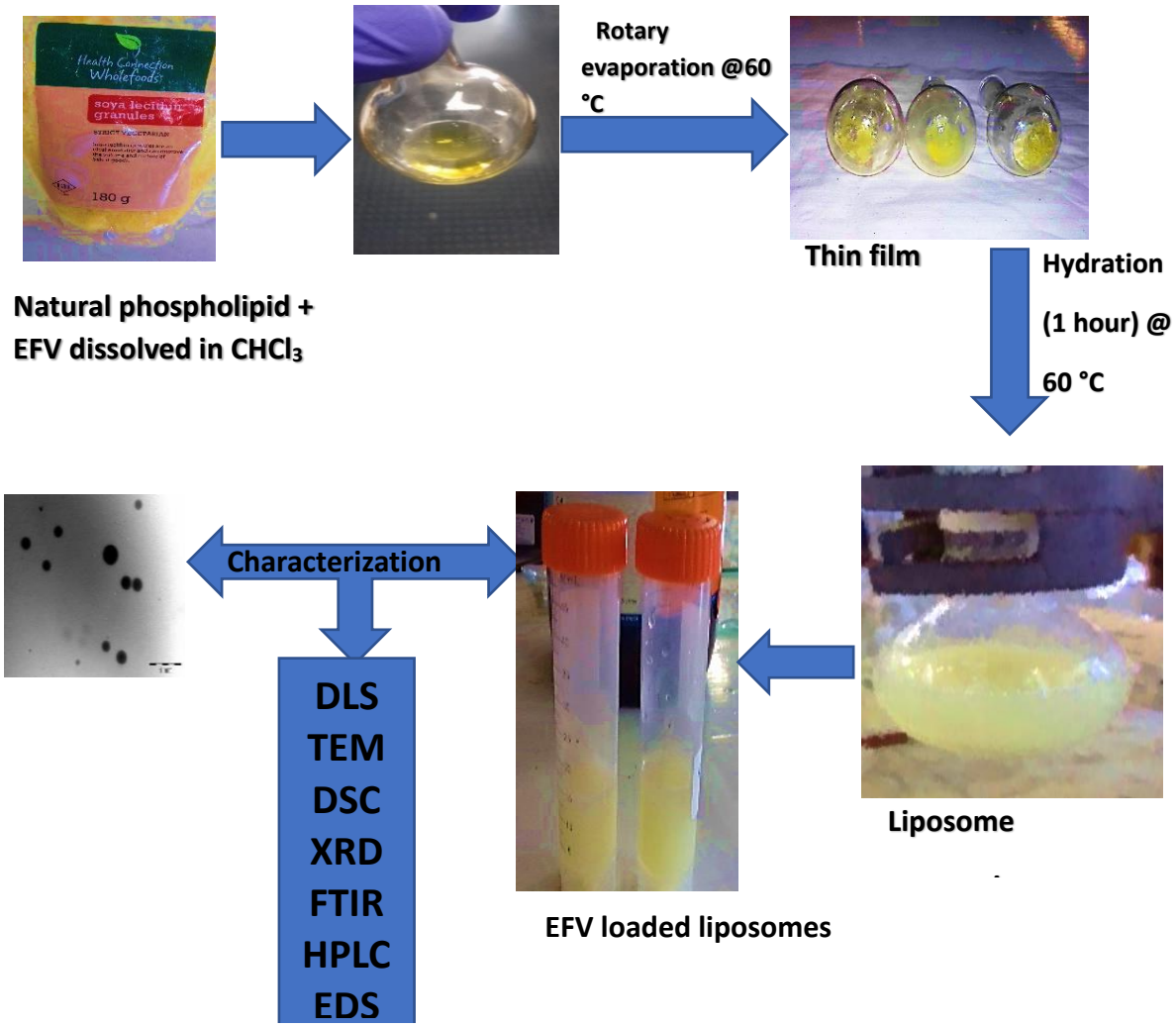


Figure 2.1. Schematic flowchart illustrating the formulation of EFV loaded liposomes

2.2 Experimentals- Materials and Equipments

The soybean lecithin phospholipids granules (100 g) used in this work was purchased from the Health connection wholefoods (USA), which contains (23 g) phosphatidylcholine, (14 g) phosphatidylinositol's, (35 g) polyunsaturated fats, (13 g) saturated fats, (8 g) glycaemic carbohydrates, (0.11 g) sodium and energy up to 2,940 KJ. Cholesterol was procured from carlotiba /Divisione chemical (Italy), Efavirenz, mono and dibasic sodium phosphate, Chloroform, Methanol, Acetonitrile (Chromatography grade), orthophosphoric acid were purchased from Merck South Africa, and were used as such without further purification.

The equipments used includes; Buchi rotary evaporator R-205 Switzerland, digital spellbound, 909 soniccator, Dynamic light scattering (Zetasizer nanoZEN Malvern instrument), Centrifuge (MSE – Mistral 1000), HP1100 Agilent LC-MSD high performance liquid chromatography (HPLC), Transmission electron microscopy (TEM), Differential scanning calorimeter (DSC-6000, PerkinElmer) X-ray diffractometer (XRD), Energy dispersive spectroscopy (VEGA TESCAN), Fourier transform infrared spectroscopy (FTIR), Freeze dryer (Apolo scientific Lyo Lab-300).

2.3. Methods

2.3.1. Preliminary Evaluation

2.3.1.1. Compatibility Study

The drug-excipient compatibility study was carried out to investigate any possible physical or chemical interactions between the drug and the excipients used. The preliminary screening is done to ascertain the stability of the drug in the dosage form and to limit any incompatibilities that could be detrimental to the drug's bioavailability and its efficacy (El-Maghraby and Abdelzaher, 2015).

The study was done using differential scanning calorimeter (DSC), X-ray diffractometer (XRD) and Fourier transform infrared spectroscopy (FTIR) for solid state analysis of the pharmaceutical ingredient and subsequent evaluation of the physicochemical of EFV drug. EFV was physically mixed with the crude soybean lecithin and cholesterol to make up a total mass of 20 – 30 mg (Helal *et al.*, 2012).

DSC. The thermal behaviour of the physical mixtures was assessed and compared with efavirenz, using a DSC-6000 PerkinElmer instrument to reveal endothermic peaks. 4 mg of the sample was heated from 50 to 170 °C, using an aluminium pan and an empty aluminium pan was used as the reference at 1 °C/min in a nitrogen environment. The data was collected and analysed using DSC manager series software.

XRD. The physical mixture of the drug and excipients were analysed for crystallinity using Bruker D8 equipment with an Eye Lynx detector and Cu-Kα radiation, set at 1.5404 angstrom along with nickel filter. The samples were evaluated within the range of 2θ from 10 to 80 °C at 1 °/min step size and 6.0 mm, and the samples were placed in a sample holder and the diffraction pattern recorded.

FTIR. The physical mixtures were evaluated using PerkinElmer Spectrum 100 FTIR Spectrometer to obtain the characteristic features of the drug with the excipients and assessing the presence of the functional groups of the drug and any possible interaction in the physical mixtures. The spectra were recorded in the frequency of 650 to 4000 cm^{-1} with 8 scans. The data was analysed using FTIR Spectrum software.

2.3.1.2. Preparation and Evaluation of Empty Liposomes

2.3.1.2.1. Preparation of Empty Liposomes

The preparation of empty liposomes was performed so as to examine and select some processes and parameters for formulation and development of EFV loaded liposomes. This involved evaluation of sonication, hydration and vortex time suitable for encapsulation of the drug. The empty liposomes were prepared and examined on the basis of their particle size distribution, polydispersity index (PDI) and zeta potential. The parameters that could affect the output variables were selected at different levels, as shown in the **Table 2.1**.

Table 2.1. Selected parameters for preparation of empty liposomes

Batches	**Parameters**	**Levels**		
		1	2	3
B1	Lecithin-cholesterol mass ratio	3:1	3:1	3:1
	Lipid-aqueous phase mass ratio	1:30	1:30	1:30
	Sonication time	15	30	45
	Hydration time	20	40	60
	Vortex time	5	10	20

The software for general statistic Minitab (version.12) was used to generate the full factorial design of experiment for 27 possible combinations, corresponding each to liposomal formulation. The evaluation and examination of the empty liposomes enabled us to define and ascertain other parameters which worked best for the preparation and encapsulation of efavirenz loaded liposomes.

2.3.1.2.2 Evaluation of empty liposomes

The formulated empty liposomes were examined and analysed using the dynamic light scattering to determine the particle size, polydispersity index and the zeta potential. The transmission electron microscopy (TEM) was used to determine the morphology of the liposomes, which revealed the aggregate or non-aggregate spherical shapes of the liposomes in the lipid suspension.

Particle size PDI and zeta potential. The formulated liposomal suspension was analysed using Zetasizer nano Malvern instrument (nano Zen-3600 MAL1043132) at a scattering angle of 173 °. The analysis was done in triplicate at 25 °C, using the ordinary capillary for particle size and folded capillary for zeta potential determination, respectively. The suspension was lightly diluted using deionized water to minimize opalescence.

Transmission Electron Microscopy. A drop of the empty liposomal suspension was spotted on the copper grid and the excess lipid suspension was absorbed using the filter paper. The samples were left to dry for 24 hours and then examined using Zeiss Libra-120 KV TEM instrument.

2.3.1.3. Validation of efavirenz quantification

The validation study was carried out to generate and ensure a suitable HPLC method for efavirenz quantification under appropriate conditions. The validation parameters mostly applied were; linearity, accuracy and precision (interday and intraday precision).

The chromatographic system comprised of HP1100 Agilent LC-MSD with a quaternary pump. In Line degasser, DAD detector, MSD1100, ChemStation for collection and analysis of data. A ZORBAX® Eclipse + C18 4.6 i.d.x 150 mm x 5 µm column was adopted for reverse phase HPLC analysis.

The chromatographic conditions that were used to validate efavirenz quantification were as follows: An isocratic system with mobile phase composed of acetonitrile, water and orthophosphoric acid (70:30:0.1), flow rate of 1 ml/min, oven temp of 30 °C, injection volume of 20 µL and a UV wavelength of 252 nm. Efavirenz stock solution was prepared in a mobile phase of ACN/H_20/H_3P04 (70:30:0.1), at a concentration of 2000 µg/ml. Dilutions were performed in order to obtain solutions with mobile phase concentrations between 8 and 40

µg/ml with an average 20 µg/ml defined as the 100 % and filtered using a 0.45 µm Millipore filter. The experiments were carried out over a 4-consecutive day period for comparison, with 3 injections daily with the exception of the 20 µg/ml which was injected 6 times daily and the solution was prepared fresh on a daily basis. The duration of the concentration elution was 7 minutes and the retention time was found to be at 5:254 minutes. The peak obtained was plotted against the concentration to generate a calibration curve for linearity range regression equation and correlation coefficient. The percentage recovery and relative standard deviation (RSD) was calculated so as to evaluate the precision and accuracy within the experimental duration.

2.3.2. Preparation of Efavirenz Loaded Liposomes

The Minitab software (version. 12) was used in generating the full factorial design of the experiment for 9 levels of mass ratio, crude lecithin (CL) to cholesterol (Chol), lipid to drug mass ratio and lipid to water mass ratio for 27 possible formulations for EFV loaded liposomes as shown in **Table 2.2** and **2.3**.

Table 2.2. Different mass ratio of crude lecithin (CL) to Chol, lipid to EFV and lipid to water mass ratio

Levels	Crude lecithin-Chol mass ratio	Lipid-EFV mass ratio	Lipid-H_20 mass ratio
1	1:0	4:1	1:20
2	3:1	2:1	1:40
3	1:1	1:1	1:80

Table 2.3. Composition Description of liposomal formulation

Formulation Code	Type of lecithin	CL-Chol mass ratio	Lipid-EFV mass ratio	Lipid-H_20 mass ratio
F1	Crude	1:0	4:1	1:20
F2	Crude	1:0	4:1	1:40
F3	Crude	1:0	4:1	1:80
F4	Crude	1:0	2:1	1:20
F5	Crude	1:0	2:1	1:40
F6	Crude	1:0	2:1	1:80
F7	Crude	1:0	1:1	1:20
F 8	Crude	1:0	1:1	1:40
F9	Crude	1:0	1:1	1:80
F10	Crude	3:1	4:1	1:20
F11	Crude	3:1	4:1	1:40
F12	Crude	3:1	4:1	1:80
F13	Crude	3:1	2:1	1:20
F14	Crude	3:1	2:1	1:40
F15	Crude	3:1	2:1	1:80
F16	Crude	3:1	1:1	1:20
F17	Crude	3:1	1:1	1:40
F18	Crude	3:1	1:1	1:80
F19	Crude	1:1	4:1	1:20
F20	Crude	1:1	4:1	1:40
F21	Crude	1:1	4:1	1:80
F22	Crude	1:1	2:1	1:20
F23	Crude	1:1	2:1	1:40
F24	Crude	1:1	2:1	1:80
F25	Crude	1:1	1:1	1:20
F26	Crude	1:1	1:1	1:40
F27	Crude	1:1	1:1	1:80

2.3.2.1. Encapsulation of Efavirenz

In preparing the empty liposomes, the parameters during the preparation of the empty liposomes (**Table 2.1**) were selected. Briefly, CL (75 mg) Chol (25 mg) was dissolved in chloroform (1 ml) in a clean round bottom flask. The organic solution generated was removed using rotary evaporator at 60 °C at 200 rpm for 5 minutes. The obtained thin film at the round bottom flask was detached from the rotary evaporator and stored in a desiccator overnight at room temperature. Millipore water (3 ml), was added to the thin film lipid and was heated at 60 °C for at different time intervals under stirring at 400 rpm to obtain a yellow liposomal suspension. The lipid suspension was homogenised using an oil bath sonicator at 60 °C to produce a homogeneous liposomal suspension. The control solution containing efavirenz with no lipid was also treated in the same manner for comparison purposes.

Passive loading method of drug loading, involving thin film hydration technique was used in the encapsulation of efavirenz, using crude soybean lecithin and following the selected parameters from the empty liposome preparation and that which demonstrated the best zeta potential (the best negatively surface charged formulation) and the polydispersity index. This method was chosen due to its easy facility accessibility and low cost that enabled encapsulation of hydrophobic drugs (Deniz *et al.*, 2010). The development of 27 possible efavirenz loaded liposomes were passively performed in order to select optimal formulations with the best encapsulation efficiency for further analysis, and also to be employed a solvent media for the preparation of liposomal buccal films.

Briefly, appropriate amount of lipid components, a total (100 mg) was dissolved alongside 25 mg, 50 mg and 100 mg of efavirenz respectively in 1 ml clean round bottom flask. The organic solvent was dried up using a (Buchi Rotavapor R-205 Switzerland) rotary evaporator at 60 °C under vaccum at 400 rpm for 5 minutes. The flask was detached from the rotary evaporator and stored in a vacuum desiccator overnight. Millipore water (2 ml, 4 ml and 8 ml) depending on the drug to water mass ratio was added into the thin film lipid for hydration at 60 °C under stirring at 400 rpm for 60 minutes. The resulting milky suspension was transferred to 50 ml centrifuge tube and Millipore water was used to rinse the flask to provide a final volume (10 ml) for centrifugation.

2.3.2.2. Determination of encapsulation efficiency.

The liposomal suspension obtained from the encapsulation of efavirenz in **section 2.3.2.2** above was centrifuged at 4000 rpm for 10 minutes using a low speed MSE-Mistral-1000 centrifuge at room temperature. The supernatant was decanted and subjected to ultrasonic agitation for homogenized mixture using a bath sonicator (Digital Cleaner Spellbound, 909) at 60 °C. The resultant homogenized liposomes were freeze-dried using (Apolo scientific Lyo lab-3000) and stored for further analysis. On the other hand, the isolated pellets (viz., non-encapsulated efavirenz particles) were dissolved in 30 ml of the mobile phase, and the resultant solution was subjected to HPLC analysis of efavirenz. For the assessment of encapsulation efficiency (EE), a positive control dispersion of efavirenz (without lipids) was produced following the liposome's preparation procedure (except addition of lipids). The resultant control sample was used for theoretical estimation of the total amount of efavirenz used in the formulation (Nkanga and Krause, 2018). The validated HPLC method described in **section 2.3.1.3** was used for EFV quantification of the prepared pellet solution and the corresponding control. Prior to HPLC analysis, samples were diluted 1/100 using mobile phase and filtered through PVDF 0.45 µm Millipore filters. The encapsulation efficiency (%EE) was calculated using the following equation **2.1** (Panwar *et al.*, 2010; Costa *et al.*, 2014). The formulation with the best %EE efficiency was selected for further extensive characterization.

$$\%EE = \frac{Efavirenz\ total\ amount - Efavirenz\ pellet\ amount}{Efavirenz\ total\ amount} X\ 100$$ …. Equation 2.1

2.3.3. Characterization of EFV Loaded Liposomes

2.3.3.1. Characterization of Particle size and zeta Potential

The obtained samples that were freeze-dried were dispersed into de-ionized water at room temperature to obtain a homogeneous mixture. The experiment for particle size and surface charge was performed using (Zetasizer nano ZEN MAL1043132 Malvern instrument) as described in **section 2.3.1.2.2.**

2.3.3.2. Morphology

The obtained liposomal dispersion from the particle size and zeta potential analysis were used for TEM evaluation. The evaluation of the particle shape was carried out using the described TEM method in **section 2.3.1.2.2.**

2.3.3.3. Differential scanning calorimetry

The thermal behaviour of the pure efavirenz, the freeze dried EFV loaded liposomes having the best %EE, and the empty liposomes were evaluated and compared using (Perkin Elmer DSC 6000 instrument) using an aluminium pan along with the standard reference to obtain endothermic peaks as described in **section 2.3.1.1** of the compatibility study.

2.3.3.4. X- ray diffraction (XRD)

X- ray diffraction of the pure efavirenz, freeze-dried EFV loaded liposomes, and empty liposomes were conducted to evaluate the crystalline nature of EFV in liposomes compared with the pure drug and empty liposomes. The analysis was performed using the described method and instrument in **section 2.3.1.1**.

2.3.3.5. Fourier transform infrared

The presence of the drug and its functional group in the pure drug**,** EFV loaded liposomes and the empty liposomes were analysed using (FTIR) spectroscopy and also helped to evaluate the interaction of the drug in the liposomes. FT-IR spectra were recorded in transmission mode between 4000-650 cm^{-1} wave number range using 8 scans as described in **section 2.3.1.1.**

2.3.3.6. Energy dispersive X-ray spectroscopy (EDS)

The elemental composition on the surface of liposomes were conducted as part of the localization analysis for the encapsulated EFV drug. The energy dispersive x-ray spectroscopy (VEGA TESCAN connected to Oxford penta FET), in conjunction with scanning electron microscopy (SEM), which detects the x-ray emitted from the sample during bombardment by electron, was used in the study of the elemental composition of EFV pure drug, EFV loaded liposomes and the empty liposomes.

2.3.3.7. *In vitro* release study

Prior to release study, the freeze-dried EFV loaded liposomes which demonstrated the best %EE was evaluated for EFV drug content using Nkanga *et al* (2017) described method. The freeze-dried sample (5 mg), was dissolved in a 25 ml volumetric flask containing 10 ml methanol in order to disrupt the liposomal structure for EFV dissolution. HPLC water grade was used to make up to the volume. The volumetric flask was then subjected to a bath sonication using (Digital Ultrasonic Cleaner, Spellbound 909) at 60 °C for 30 minutes. These solutions were analysed in triplicate using the validated HPLC method described in **section 2.2.1.3**. The drug content (%DC) was calculated using equation 2.2

$$\%DC = \frac{Amount\ of\ recovered\ drug}{Amount\ of\ the\ formulation\ used}\ X\ 100\ \ldots . Equation\ 2.2$$

The *in vitro* release study was conducted using a dialysis method described by Dixit *et al* (2010). Liposome suspension (3 ml) (equivalent to 6 mg of EFV) was placed in a dialysis bag (DM70, Himedia, MWCO 12000- 14000 Da, with pore size of 2.4 nm) in 150 ml phosphate buffer saline (PBS) (pH 7.4 at 37 °C) and maintained under stirring at 100 rpm. At regular time intervals (0.5, 1.5, 2, 3, 4, 5, 6, 8, 10, 12, 24, 48 and 72 hours), a sample (5 ml) was ejected for quantification and the release medium was replaced with an equivalent volume of the fresh PBS. An aqueous solution of EFV raw material was treated in the same manner as the control in order to evaluate the drug diffusion rate under actual conditions, in comparison to the release profile from the liposomes. All the experiments were performed in triplicate and a release profile generated.

2.4. Results and Discussions- Preliminary Studies on drug compatibility

DSC. The recorded DSC thermograms presented in **Figure 2.2** represented the mixture of EFV with CL and cholesterol. EFV pure drug exhibited the melting peak at 137.2 °C which is within EFV onset and offset peak as has been reported in the literature(Madhavi *et al.*, 2011). However, there is a major shift in the melting peak of the physical mixtures. The presence of the EFV endothermic peak in the physical mixtures, however this suggests no major structural changes of the drug.

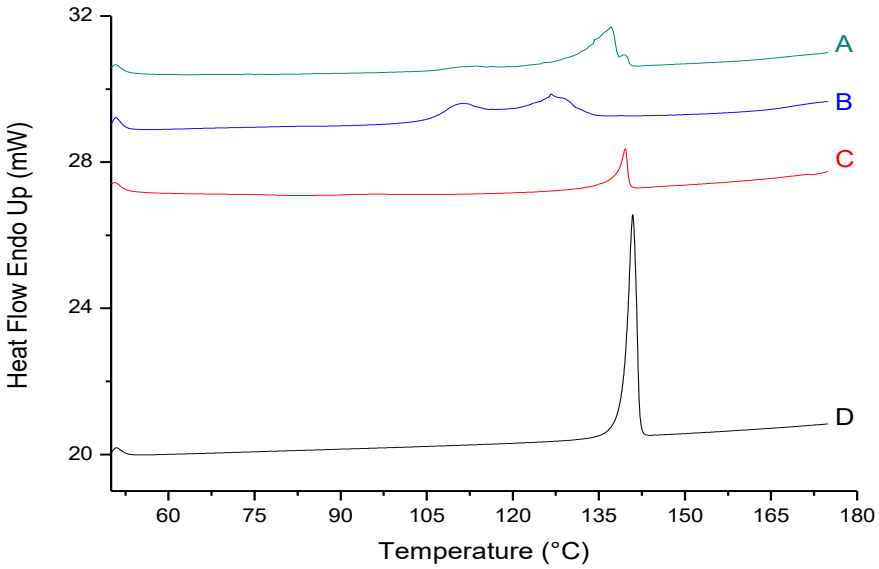


Figure 2.2. DSC thermograms of Pure EFV (D), physical mixture of EFV + Chol (C), Physical mixture of EFV + CL (B), Physical mixture of EFV + Chol + CL (A)

XRD. The XRD diffractograms of the pure EFV drug and the physical mixture of the drug with CL and cholesterol are presented in the **Figure 2.3**. The X-ray diffractogram of EFV showed the characteristic sharp multiple peaks at 12.9 °, 20.3 ° 22.65 ° and 29.6 °C, indicating the crystalline nature of the drug. However, several distinct peaks were also observed in physical mixture of EFV, lecithin and cholesterol indicating the crystalline nature of the drug in the mixture. This was also reported by (Sathigari *et al.*, 2012).

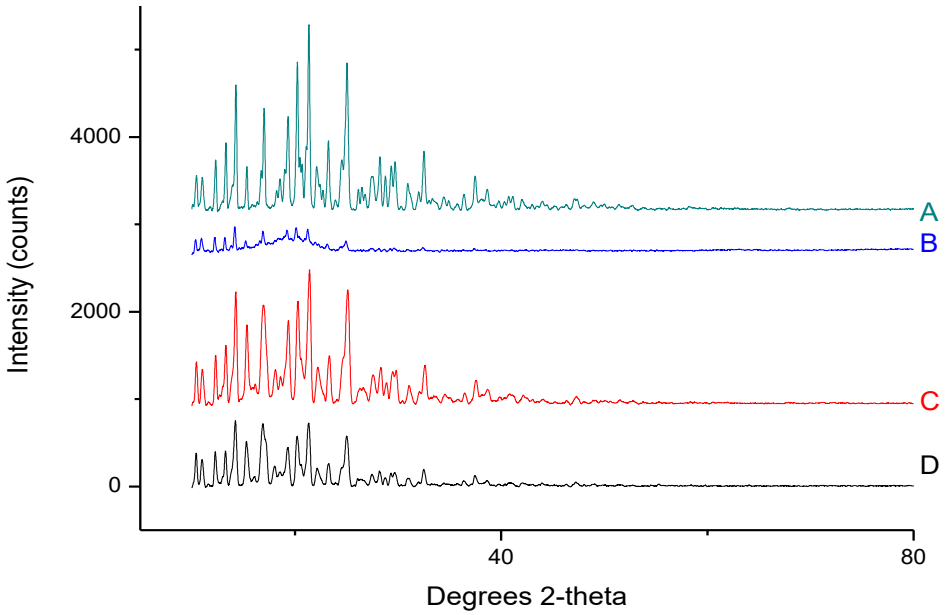


Figure 2.3. XRD diffractograms of EFV pure drug (A), EFV + Chol (B), EFV + CL (C) and EFV + Chol + CL(D).

FTIR. The FTIR spectra of the analysed physical mixtures of EFV with CL and cholesterol are presented in **Figure 2.4** and have been compared to EFV pure drug. EFV showed its characteristic band at 1743 cm^{-1} due the C=O stretching vibration and a band at 3310 because of the -NH vibration. The intense stretching vibration at 1183 cm^{-1} was due to the stretching vibration of -C-F. The stretching vibration due to triple bonds was observed at 2243 cm^{-1}. The C=C bonds in the aromatic ring was observed at 1600 and 1494 cm^{-1}, with the stretching vibration due to C-CL occurring at 1096 and 1089 cm^{-1}, was also reported by (De Gomes *et al.*, 2013). The FTIR revealed not much difference in the spectra between the characteristic absorption bands of EFV pure drug and the physical mixture with lecithin and cholesterol, therefore indicating the chemical intactness of the drug in the physical mixtures.

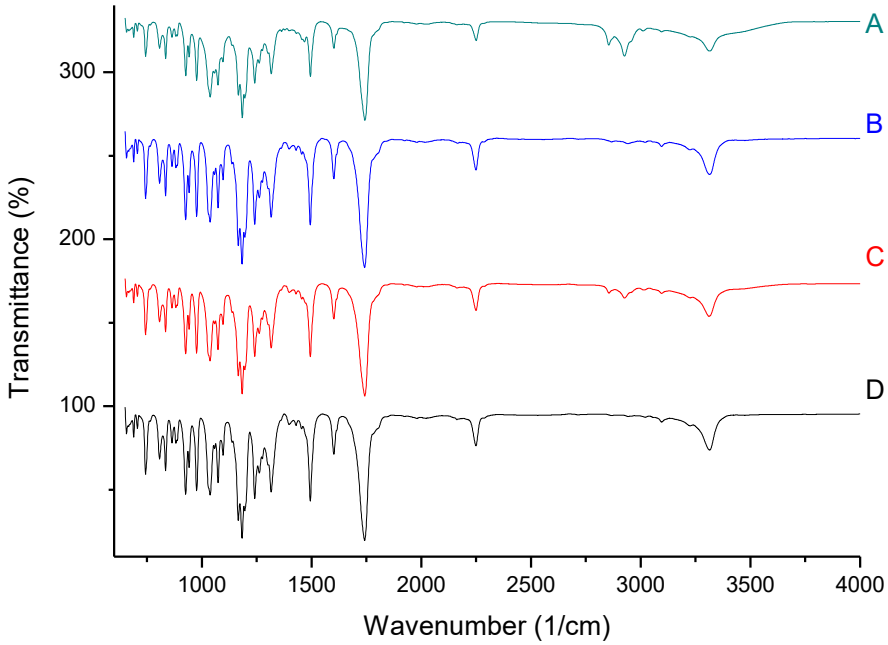


Figure 2.4. FTIR spectra of pure EFV (D), Physical mixture of EFV + Chol (C), EFV + CL (B), EFV + Chol + CL

2.5 Evaluation of Empty Liposomes

2.5.1. Particle size and zeta potential

The formulations of empty liposomes (F1-F27) with their respective particle sizes, polydispersity index (PDI) and zeta potential (ZP) are presented in **Table 2.4.** The formulations which demonstrated the most accepted (PDI) from the range of 0.5 were selected for zeta potential evaluation. Zeta potential is known to affect the stability and can be used to improve formulation of dispersions, emulsion and suspension such like lipid suspensions(Majumdar and Kansara, 2010; Pattni *et al.*, 2015). The two formulations that showed high surface liposomes were selected for encapsulation of EFV. This includes F9 and F27 with zeta potential (ZP) of -63.7 mV and -54.9 mV, with PDI value of 0.202 and 0.322 respectively. The particle size range of the two formulations F9 (174 nm), and F27 (157 nm) are significantly higher than the other formulations and acceptable for proposed encapsulation of EFV antiretroviral drug.

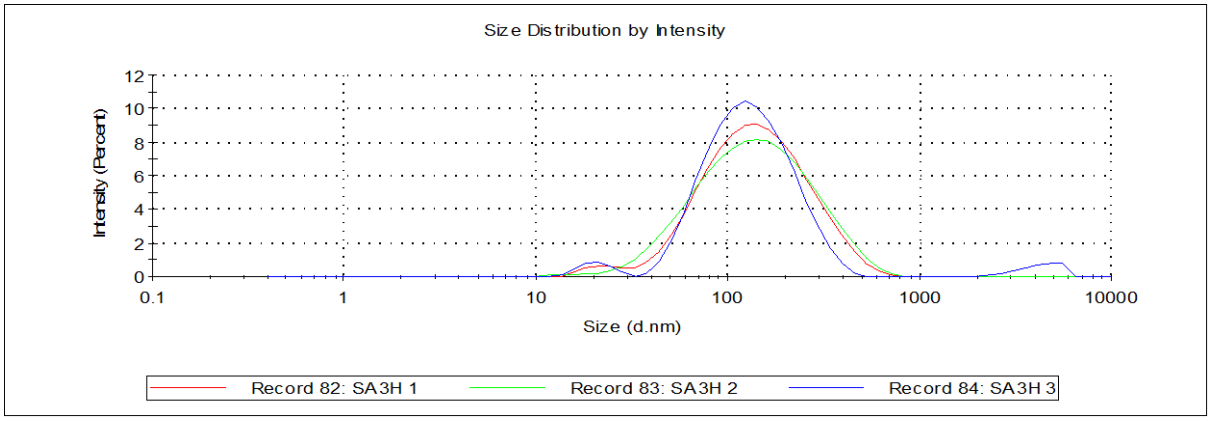
Additionally, the other parameters which included the hydration time at 60 min, vortex time at 20 minutes and sonication time at 45 minutes, that gave rise to high surface charge and acceptable PDI were selected for encapsulation of EFV loaded liposomes. The size distributions of the empty liposomes are shown in **Figure 2.5**.

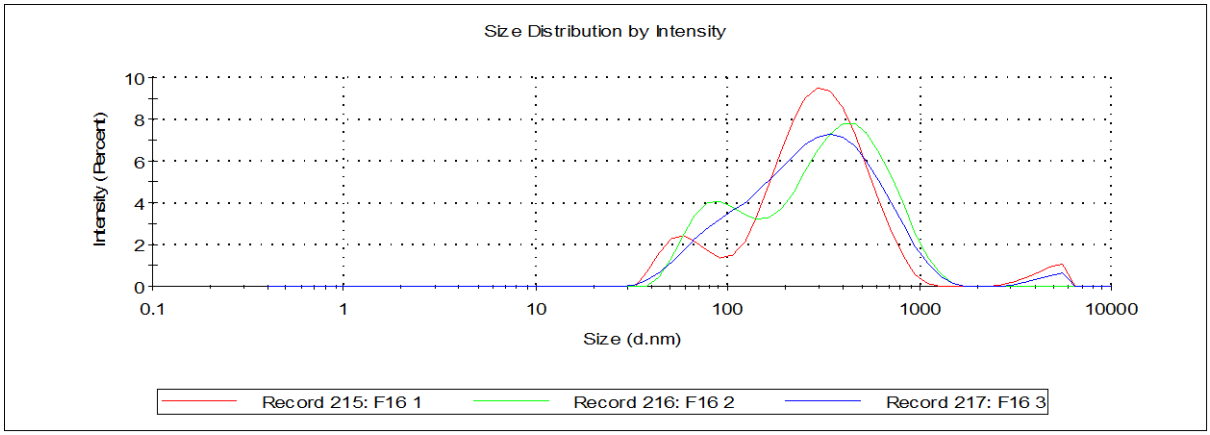
Table 2.4. Average particle size and PDI of the empty liposomes with some of the ZP

Formulation code (F1-F27)	Lecithin-Chol mass ratio	Lipid-water Mass Ratio	Hydration Time (min)	Vortex Time (min)	Sonication Time (min)	Zeta sizes (nm)	PDI	ZP (mV)
F1	3:1	1:30	20	5	15	198.2	0.528	*
F2	3:1	1:30	20	5	30	144.6	0.493	*
F3	3:1	1:30	20	5	45	73.4	0.282	-26.8
F4	3:1	1:30	40	10	15	122.3	0.433	*
F5	3:1	1:30	40	10	30	86.5	0.232	-35.7
F6	3:1	1:30	40	10	45	170.1	0.212	-43.7
F7	3:1	1:30	60	20	15	117.5	0.315	*
F8	3:1	1:30	60	20	30	144.7	0.353	*
F9	3:1	1:30	60	20	45	174.4	0.204	-65.1
F10	3:1	1:30	20	5	15	124.6	0.316	-28.5
F11	3:1	1:30	20	5	30	82.0	0.276	-27.7
F12	3:1	1:30	20	5	45	83.0	0.290	-21.7
F13	3:1	1:30	40	10	15	205.3	0.573	*
F14	3:1	1:30	40	10	30	113.3	0.285	-30.5
F15	3:1	1:30	40	10	45	169.4	0.282	-45.5
F16	3:1	1:30	60	20	15	132.9	0.382	*
F17	3:1	1:30	60	20	30	111.9	0.277	-35.4
F18	3:1	1:30	60	20	45	59.3	0.253	-22.6
F19	3:1	1:30	20	5	15	207.3	0.587	*
F20	3:1	1:30	20	5	30	86.1	0.239	-39.7
F21	3:1	1:30	20	5	45	47.2	0.272	-21.3
F22	3:1	1:30	40	10	15	163.8	0.774	*
F23	3:1	1:30	40	10	30	70.3	0.272	-41.8
F24	3:1	1:30	40	10	45	56.5	0.398	*
F25	3:1	1:30	60	20	15	74.2	0.420	*
F26	3:1	1:30	60	20	30	74.0	0.420	*

F27	3:1	1:30	60	20	45	156.6	0.322	-54.9

NA * were not analysed.





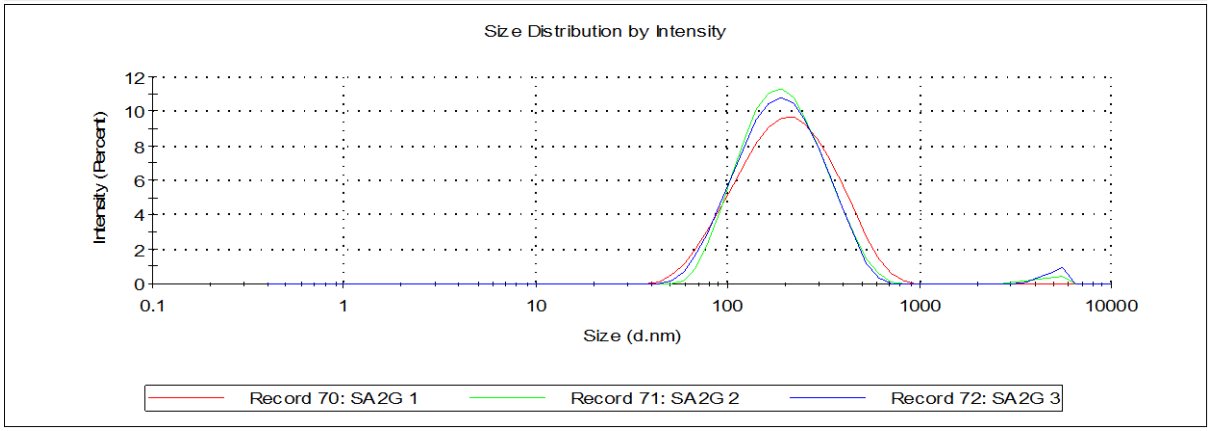


Figure 2.5. Particle size distribution by intensity of the empty liposomes.

2.5.2. Morphology analysis

The TEM micrographs of the selected empty liposomes chosen for TEM analysis which includes (F6, F9, F24 and F27) are shown in **Figure 2.6.** The empty liposomes demonstrated a typical spherical shape of liposomes. However, there was no sign of aggregation observed in the liposomes, which is significant of the high liposome surface charge observed.

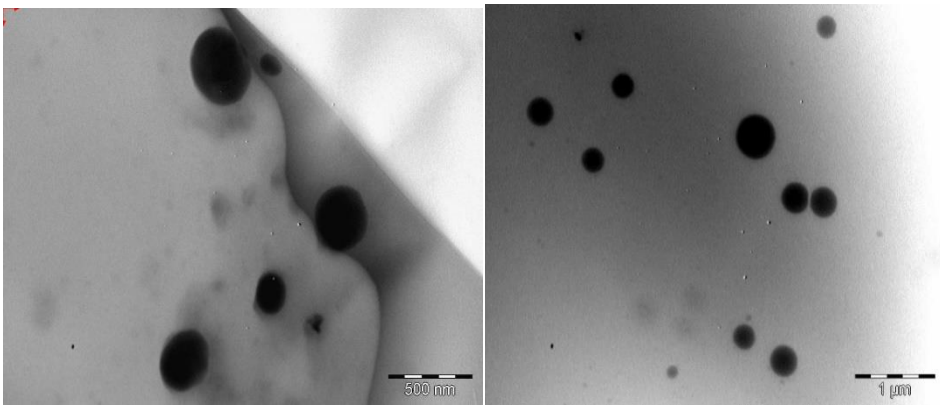


Figure 2.6. Typical liposome images of the formulated empty liposomes

2.5.3. Validation study

The calibration line of the validation study presented in **Figure 2.7**, denotes a linear correlation between EFV concentration and the corresponding peak area with regression equation $(y = 165.21x - 180.6)$ and (R^2= 0.09998) in the linear range of 10-40 µg/ml and closer to the standard value of (R^2 > 0.998), with the retention of EFV drug observed at 4.294 minutes as presented in **Figure 2.8**. The LOD and LOQ were found to be 0.017 µg/ml and 0.038 µg/ml respectively. The interday and intraday deviations (RSD) arising from various concentrations as presented in **Table 2.5** below. The RSD values for interday and intraday were found below the standard maximum 2 % and 5 % respectively. This can be concluded that the HPLC method evaluated, possesses a repeatability and intermediate precision (Ayyappan *et al.*, 2011). The percentage recoveries obtained were within the range of 92.31-103.27 %. This validates the accuracy of the method, as the recoveries are within the acceptable range of 90-110 % (Ayyapapan *et al.,* 2011). The retention time for EFV however was observed at 5.254 minutes.

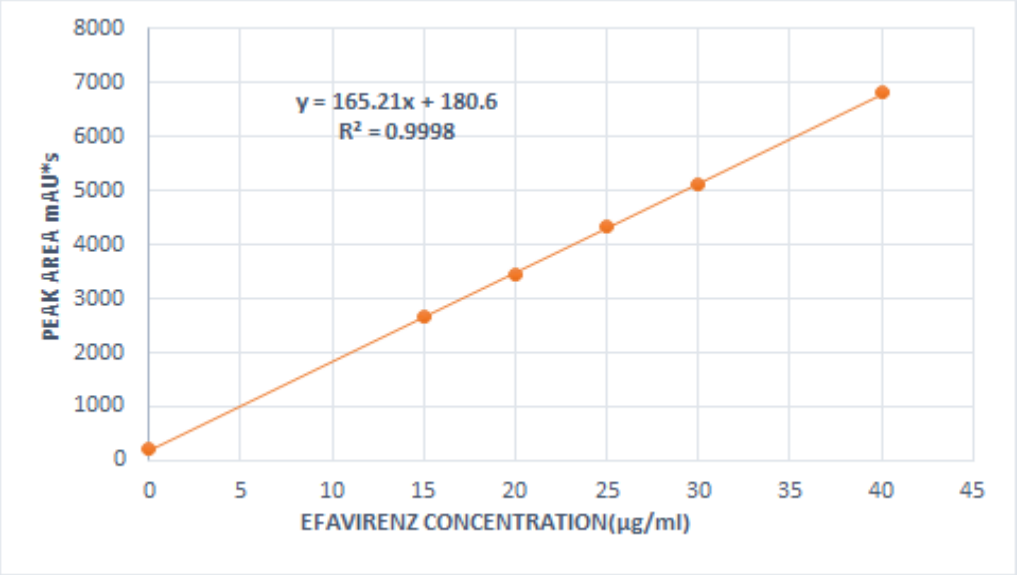


Figure 2.7. Standard calibration curve for EFV in concentration range of (10-40 µg/ml)

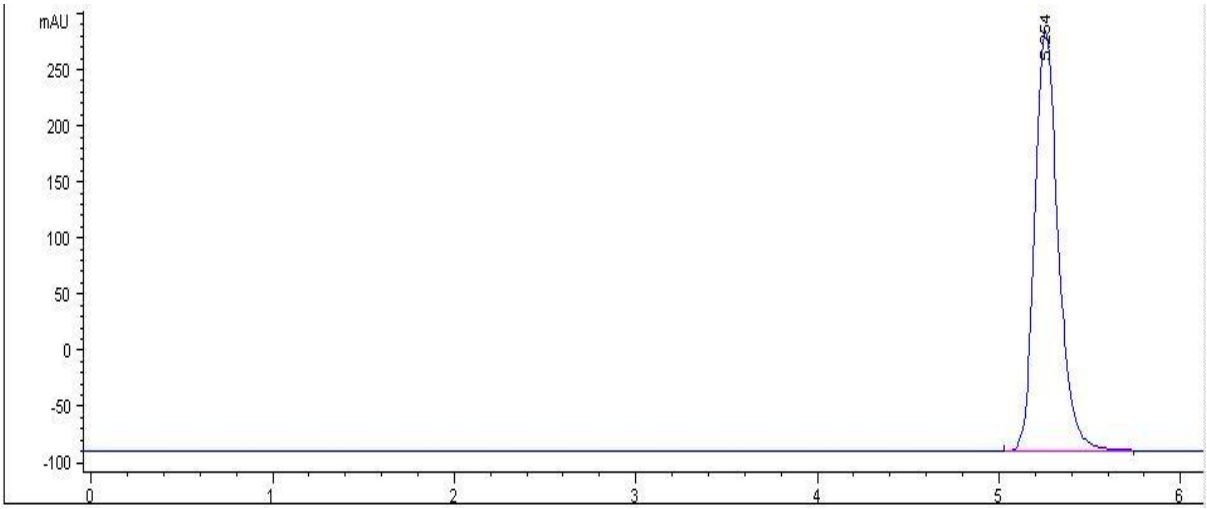


Figure 2.8. Chromatogram of EFV with the retention time for HPLC validated method

Table 2.5. RSD and recovery results for 4 days analyses

Concentration (µg/ml)	Intraday precision (%RSD)	Interday precision (%RSD)	Accuracy (% recovery)
10	0.77	2.65	102.31
15	0.48	1.14	98.52
20	0.26	0.73	95.41
25	0.16	0.52	101.34
30	0.82	1.76	96.28
40	0.14	0.66	99.24

2.6. Characterisation of Efavirenz Loaded Liposomes

2.6.1. Encapsulation efficiency

The encapsulation efficiency (EE%) of efavirenz loaded liposomes obtained are presented in **Table 2.6**. The EE% was found to increase with the amount of the cholesterol incorporated in the liposomes. The formulation containing 1:1 CL to Cholesterol exhibited the highest EE% of (98.8 ± 0.003 %). This is however comparable to the encapsulation efficiency of zidovudine myristate loaded liposomes of 99.4 % and 98.3 % reported in the literature by Jin *et al.*, (2005). The increase in EE% could be attributed to the effect of increase of cholesterol incorporation, as previously reported in the literature (Pattni *et al.,* 2015). This may suggest that cholesterol is highly embedded in lipid bilayers as a hydrophobic compound as presented in **Figure 2.9.** Cholesterol increases the hydrophobicity of liposome membranes and creates more voids as its concentration increases in the hydrophobic segment, which facilitates accommodation of more EFV molecules in the lipid bilayers(Ramana *et al.*, 2014).

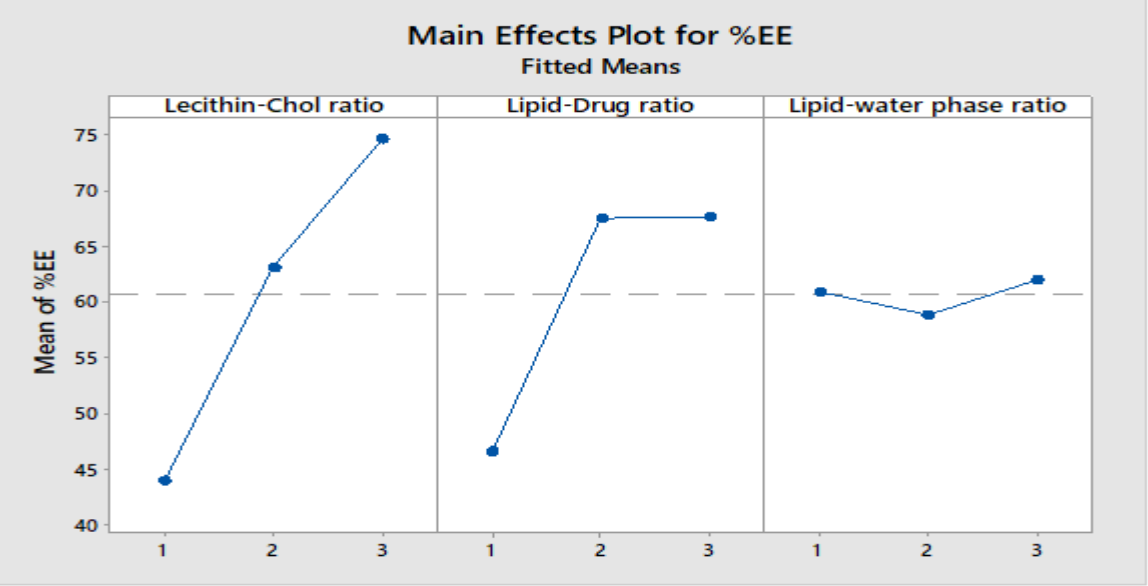


Figure 2.9. Main effect plot of Lecithin-Chol, Lipid-drug and Lipid-water ratio on EE% of EFV loaded liposomes

However, there was no significant correlation between lipid to drug ratio and drug water ratio as lipid to water showed not impacted so high the EE% of the encapsulated drug. The high EE% is quite interesting as EE% has been reported to have an enhanced cost effectiveness of drug carriers and economic potentials of CL made liposomes (Yanamandra *et al.*, 2014).

Table 2.6. Encapsulation efficiency of Efavirenz loaded crude lecithin Liposomes with their particle size, PDI and ZP

Formulation (F1-F27)	PS±SD (nm)	PDI±SD	ZP±SD (mV)	EE±SD (%)
F1	172.72 ± 2.12	0.30 ± 0.02	-49.77 ± 1.75	20.13 ± 2.23
F2	194.70 ± 0.43	0.26 ± 0.02	-48.76 ± 0.88	18.10 ± 2.02
F3	154.30 ± 2.95	0.31 ± 0.05	-48.11 ± 0.53	20.31 ± 8.66
F4	153.53 ± 2.91	0.36 ± 0.05	-45.55 ± 1.25	44.35 ± 1.15
F5	198.97 ± 1.24	0.36 ± 0.03	-40.52 ± 3.21	44.33 ± 1.20
F6	148.71 ± 0.86	0.34 ± 0.04	-41.44 ± 3.58	43.11 ± 0.94
F7	222.64 ± 4.07	0.65 ± 0.03	-46.77 ± 1.79	66.12 ± 1.67
F8	147.22 ± 2.72	0.35 ± 0.05	-44.55 ± 2.15	65.27 ± 1.93
F9	196.24 ± 1.66	0.33 ± 0.04	-49.53 ± 0.25	61.86 ± 1.35
F10	184.26 ± 0.69	0.30 ± 0.03	-56.74 ± 1.18	57.85 ± 1.27
F11	124.57 ± 0.42	0.26 ± 0.01	-55.26 ± 1.48	62.28 ± 1.53
F12	132.54 ± 0.67	0.27 ± 0.01	-46.92 ± 1.22	74.05 ± 0.57
F13	187.06 ± 0.93	0.30 ± 0.02	-66.66 ± 1.46	55.51 ± 1.08
F14	332.32 ± 2.04	0.57 ± 0.12	-58.82 ± 1.28	56.82 ± 1.65
F15	144.54 ± 2.12	0.33 ± 0.02	-54.64 ± 0.02	54.27 ± 0.61
F16	106.72 ± 1.96	0.42 ± 0.03	-42.73 ± 1.26	69.44 ± 1.91
F17	107.55 ± 0.35	0.40 ± 0.05	-41.27 ± 2.36	69.56 ± 1.93
F18	188.13 ± 1.18	0.32 ± 0.04	-38.02 ± 1.34	68.54 ± 1.35
F19	305.10 ± 1.65	0.46 ± 0.02	-44.33 ± 0.85	46.12 ± 1.48
F21	209.11 ± 4.26	0.56 ± 0.11	-50.42 ± 1.12	55.30 ± 1.42
F22	411.10 ± 7.40	0.40 ± 0.06	-53.53 ± 0.06	98.86 ± 0.01
F23	274.20 ± 1.42	0.52 ± 0.02	-45.88 ± 1.46	98.84 ± 0.03
F24	104.82 ± 2.29	0.51 ± 0.02	-50.33 ± 0.95	98.88 ± 0.03
F25	166.64 ± 1.57	0.45 ± 0.02	52.82 ± 0.46	69.42 ± 1.86
F26	174.20 ± 1.87	0.37 ± 0.029	-50.36 ± 1.55	69.50 ± 1.79
F27	149.82 ± 0.89	0.47 ± 0.014	-59.24 ± 1.42	69.71 ± 1.97

2.6.2. Particle size and zeta potential analysis

The particle size, ZP and PDI values obtained for the drug loaded liposomes are shown in **Table 2.6**. All the liposomal formulations demonstrated average particle sizes between the range of 104 to 411 nm**.** The average particle sizes are small enough to cross the leaky vasculature and preferentially target the infected blood cell, thereby enhancing the therapeutic efficacy of EFV encapsulated drug and minimizing the adverse side effects (Limasale *et al.*, 2015). The Zeta potential obtained in the range of -38.1 to -66.6 mV proved to have an impact on the liposome stability towards the storage(Nagayama *et al.*, 2007; Pattni *et al.*, 2015). There is little or no difference in the particle sizes and zeta potential values for EFV loaded liposomes, when compared to the empty liposomes. This suggests that the encapsulation of efavirenz did not significantly affect the particle size and the surface charges of the both liposomes. More also, the obtained PDI values of 0.20 and 0.26 for empty liposomes and efavirenz loaded liposomes indicates homogeneous liposome population.

The incorporation of cholesterol however, slightly increased the particle sizes of the EFV loaded liposomes at lecithin to cholesterol mass ratio, with increase in drug ratio not really initiating an increase in particle size. Increase of water ratio tend to decrease the particle size, though the decrease in sizes was not so significant as presented in **Figure 2.10.**

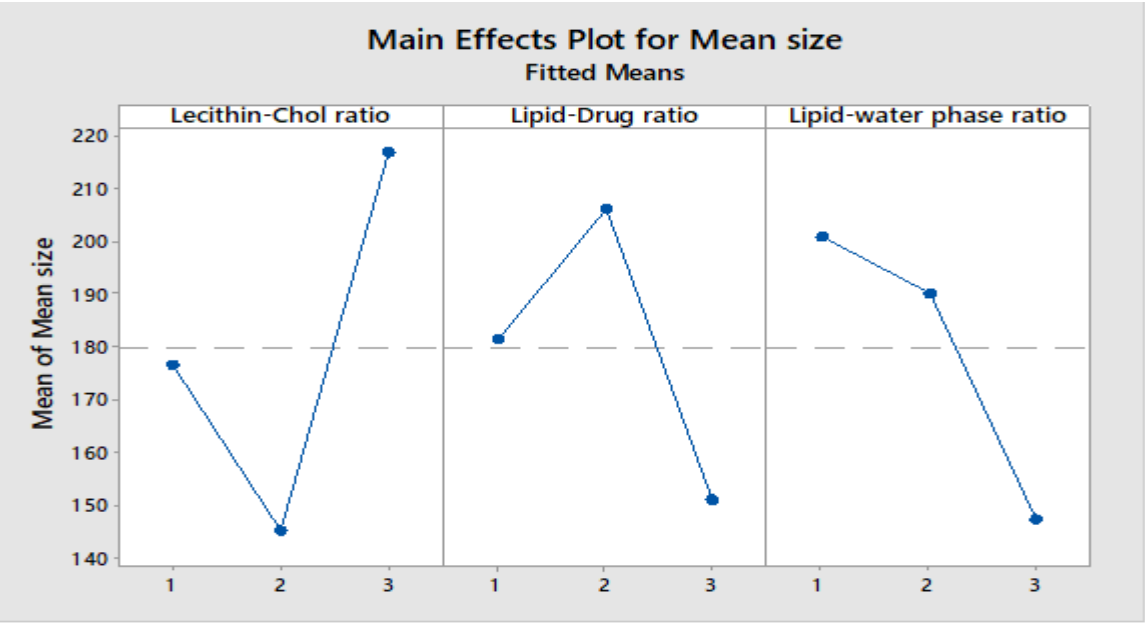


Figure 2.10. Main effects plot for CL – Chol, Lipid – drug and Lipid – water ratio on EFV loaded liposomes

However, the obtained particle sizes are quite acceptable for the delivery of an antiretroviral drug carrier as small particle sizes of liposomes easily penetrate the anatomical and cellular reservoir that cannot be accessed, when the drug is administered without a delivery system(Ramana *et al.*, 2012).

The effect plot of CL, Cholesterol, and water ratio on ZP presented in **Figure 2.11,** showed no significant impact or correlation between CL to Cholesterol, lipid to drug and lipid to water mass ratios on the zeta potentials. However, it is important to note that an increase in Cholesterol concentration in the liposomes may have likely given rise to higher zeta potentials which implies high stability of the liposomes thereby indicating more rigid and stable liposomes(Haeri *et al.*, 2014). Also, high increase of the drug does not generally imply high ZP, as excessive use of the drug could affect the stability of the liposomes by reducing the surface charges.

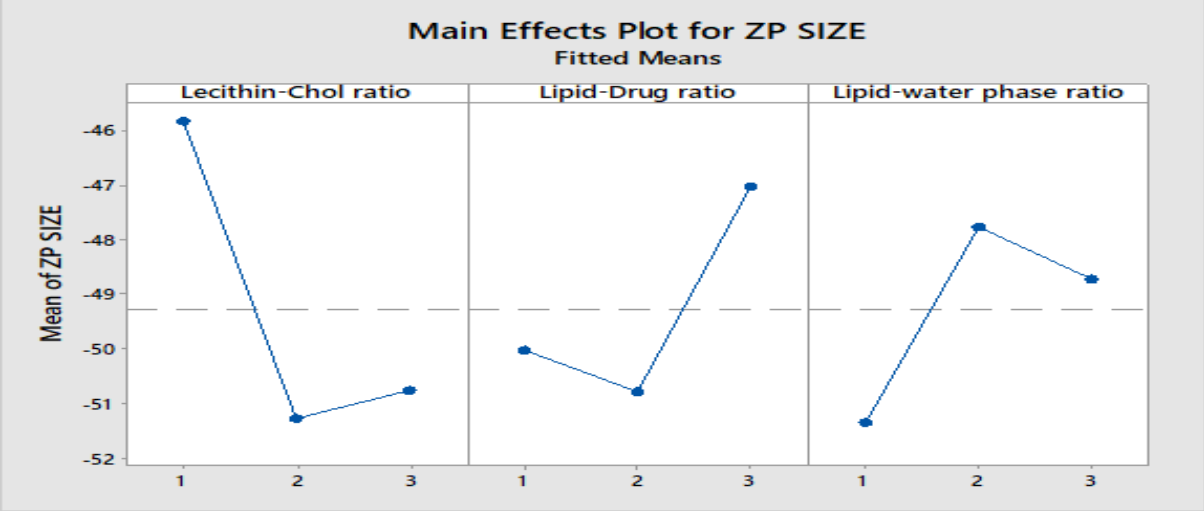


Figure 2.11. Plot effect of CL – Chol, Lipid -drug and Lipid – water ratio on EFV loaded liposomes

Increase in cholesterol concentration however did not have much significant impact in obtaining the best PDI values, as there was no correlation in the effect of cholesterol to lipid as shown in **Figure 2.12.** Drug increase, however tended to increase the PDI values. This suggests that the amount of drug to be used in encapsulation should be considered not to be emphatically high, in order not to affect the distribution of the liposome population. However, there was a correlation between lipid to water mass ratio as the PDI decreases with an increase in the amount of water. Low PDI therefore is an ideal PDI and recommended for

delivery of antiretroviral drugs, as it makes the lipids to be more homogenized to provide release residence time in the body (Yanamandra *et al.*, 2014).

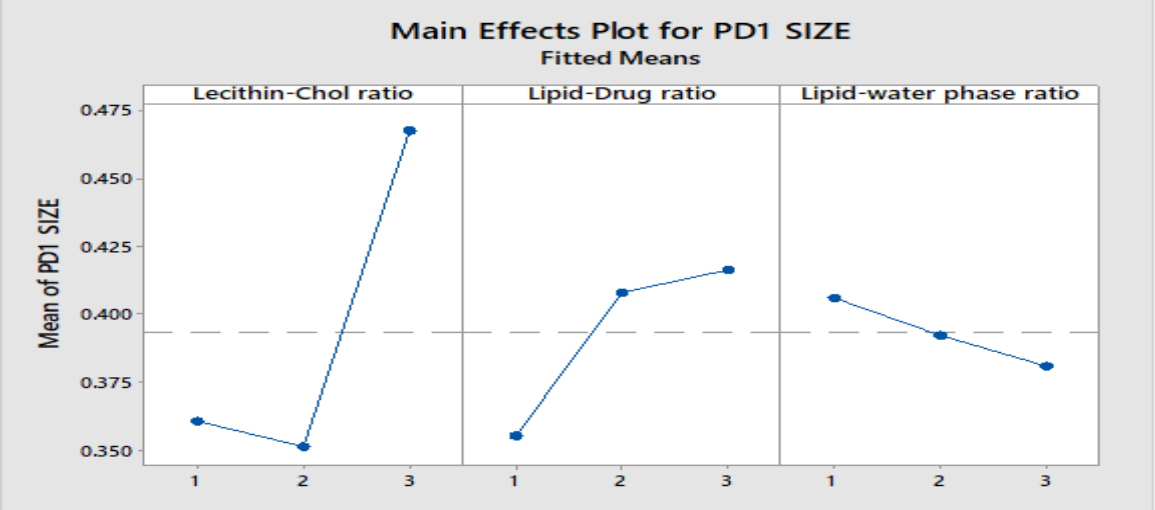


Figure 2.12. Effect plot of CL - cholesterol lipid – drug, and Lipid - water mass ratio on the PDI

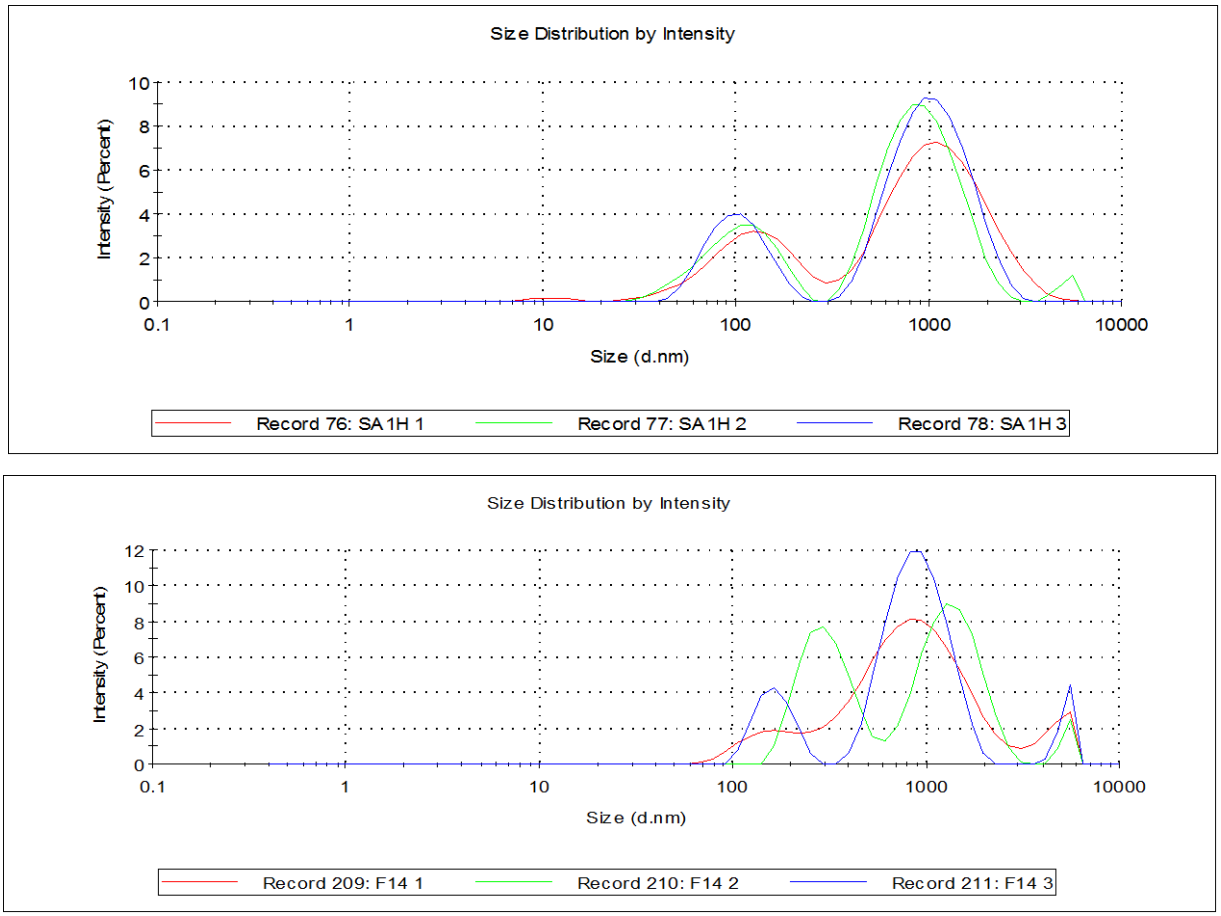


Figure 2.13. Particle size distribution of Efavirenz Loaded Liposomes

2.6.3. Morphology analysis

The TEM analyses revealed the presence of nanosized particles with spherical shape as depicted in **Figure 2.14**. The spherical feature suggests the presence of liposomal vesicles that are endowed with high surface charges. More also, the presence of well dispersed individual particles could be attributed to strong repulsive forces between particles as also demonstrated by ZP measurement.

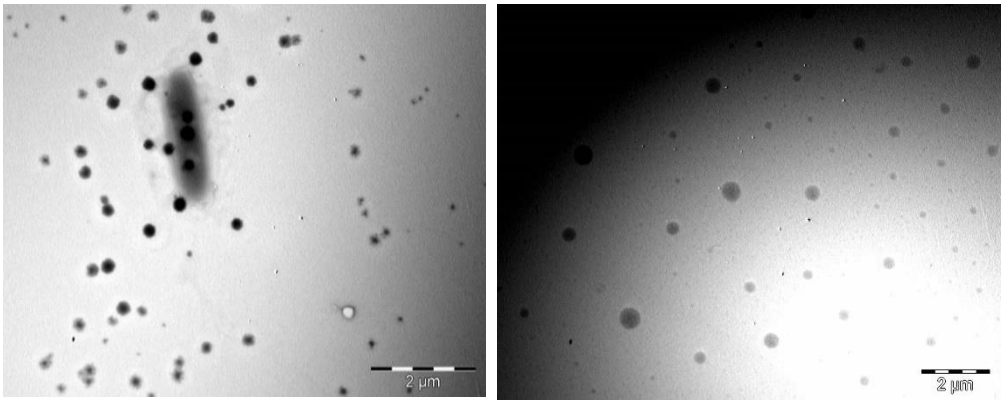


Figure 2.14. Typical TEM images of Efavirenz Loaded Liposomes

2.6.4. Differential scanning calorimetry

DSC studies were carried out to investigate the thermal behaviour of the drug encapsulated in liposomes upon continuous heating. The DSC data of EFV pure drug, EFV loaded liposomes (F27) and the empty liposomes are presented in **Figure 2.15.** The presence of the sharp peak endothermic peak at 138 °C in EFV pure drug indicates its crystalline nature. The absence of the endothermic peak in the DSC curve of EFV loaded liposomes suggests that all the components present in the formulation were in an amorphous state, since the melting peak found in the DSC thermogram did not appear.

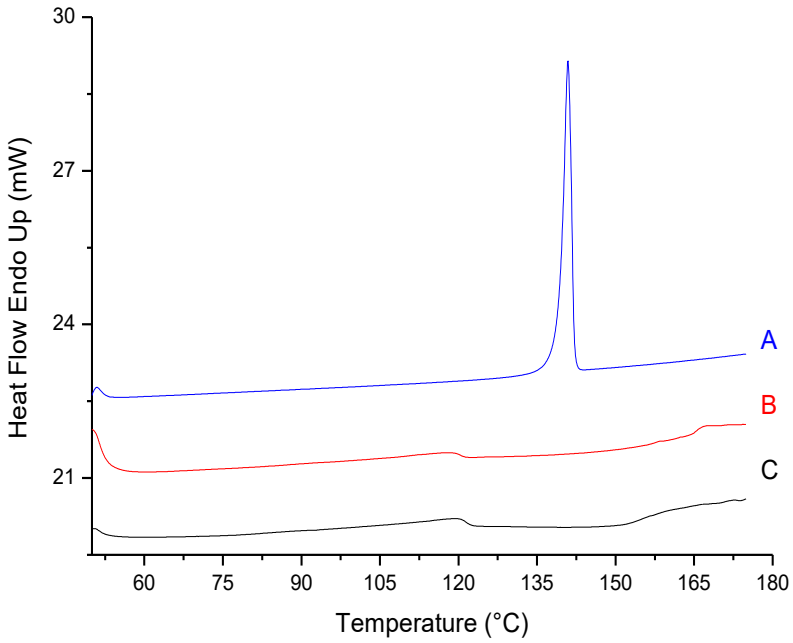


Figure 2.15. DSC thermograms of pure EFV (A), EFV Loaded Liposomes (B) and Empty Liposomes (C)

2.6.5. Crystallinity evaluation.

The XRD results of EFV pure drug, EFV loaded liposomes and empty liposomes are presented in **Figure 2.16.** From the XRD data, it appears that the empty liposomes are of amorphous nature, while both EFV loaded liposomes and EFV pure drug exhibited some sharp peaks indicating the presence of crystalline structure. Despite the presence of the crystalline peaks of EFV in liposomes, the disappearance of most EFV peaks remained evident. This denotes clear changes in the physical state of EFV and supports the DSC data, regarding loss of drug crystallinity upon liposomal encapsulation. The loss of crystalline structure due to liposomal encapsulation has been reported as an evidence of molecular interactions within the lipid bilayers where the cargo got trapped(Nkanga and Krause, 2018).

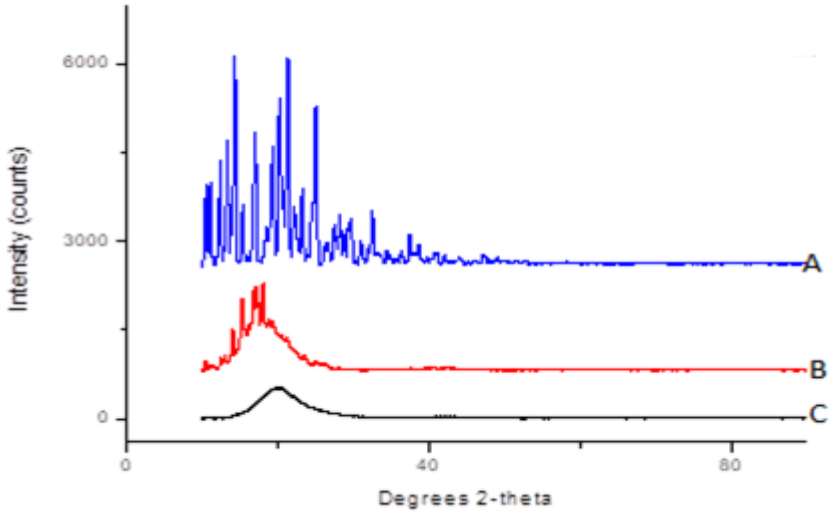


Figure 2.16. XRD thermograms of EFV pure drug (A), EFV loaded Liposome (B)and Empty Liposome (C)

2.6.6. Fourier transform infrared

The FTIR spectra of EFV raw material, EFV loaded liposomes and empty liposomes are shown in **Figure 2.17**. The spectrum for pure EFV drug showed the characteristics peak at 689 and 652 cm^{-1} for (-CF stretch), 1096 – 1057 and 1074 cm^{-1} for (-C-O-C stretch), 1492 cm^{-1} for (C≡C) of benzene ring stretching vibration, 1742 cm^{-1} for (C=O stretching vibration) and 3314 cm^{-1} for the (-NH stretching vibration) (Gaur *et al.*, 2014). The spectrum of EFV loaded liposomes however exhibited some of the characteristic peaks of EFV at the same spectral regions, in addition to lipid peaks appearing at 2911-2931, and 1455- 1468 cm^{-1} for alkane (C-H stretches) and at 1103 – 1110 cm^{-1} for the ether region(Senapati *et al.*, 2016).

In conjunction with the FT-IR spectrum empty liposomes, it appears that there is no major drug-lipid interaction in EFV loaded liposomes, suggesting that liposomal encapsulation has not affected the chemical structure of EFV drug.

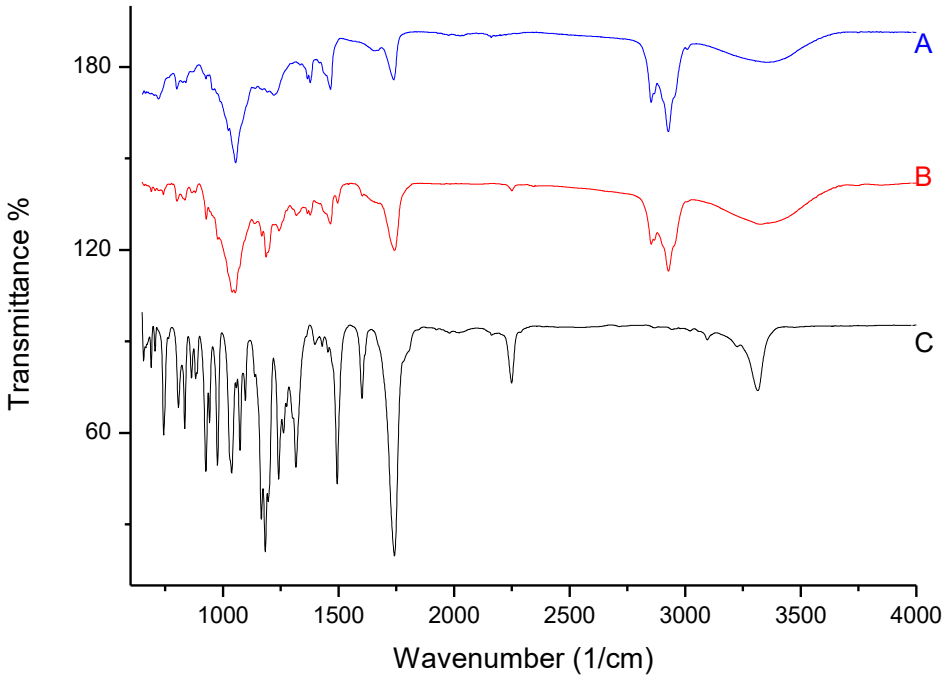


Figure 2.17. FTIR Spectra of Pure EFV (C), EFV loaded liposomes (B) and Empty liposomes (A)

2.6.7. Energy dispersive x-ray spectroscopy (EDS)

The elemental surface composition of pure EFV, EFV loaded liposomes and empty liposomes are shown in **Figure 2.18**. The elemental composition of EFV raw material was found to be (C, O, CL and F). However, none of these elements was found on the surface of EFV loaded liposomes, which exhibited similar elemental composition with empty liposomes (C, O and P). This indicates the absence of EFV in the liposome surface, probably due to its hydrophobic nature which might have caused its embedding within the lipid bilayers (Nkanga and Krause, 2018).

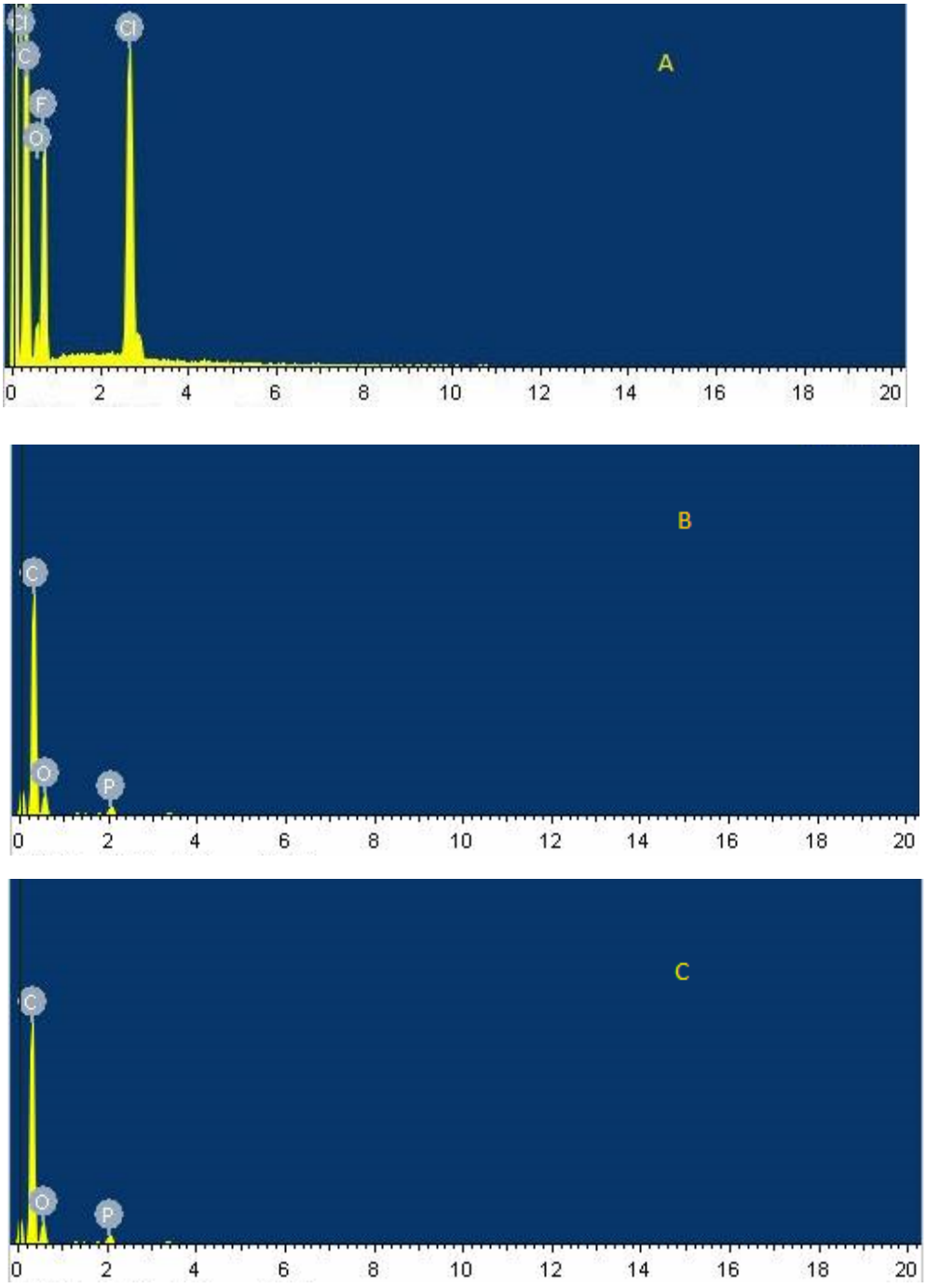


Figure 2.18. Elemental characterisation of pure EFV (A), EFV loaded liposome (B) and empty liposome (C)

2.7. *In vitro* release study

Table 2.7 presents the results obtained from drug content evaluation and the *in vitro* release study carried out using the dialysis bag method under simulated physiological conditions at (pH 7.4 phosphate buffer and temperature 37 °C) as was described by Dixit *et al.*, (2010).

Table 2.7. In vitro release profile result for EFV loaded liposome and the free drug

Time (hour)	EFV loaded Liposome (%)	% Free EFV
0.0	0.00	0.00
0.5	24.56	33.36
1	28.43	35.46
1.5	29.24	37.15
2	31.46	39.26
3	34.24	48.34
4	37.33	52.26
5	42.44	55.25
6	45.43	59.64
8	48.26	61.36
10	51.36	66.23
12	52.44	70.26
24	59.63	74.24
48	70.46	79.36
72	78.62	87.25

EFV loaded liposomes exhibited about 63 % of the EFV release, while the dissolution profile of raw EFV showed 79 % of EFV diffusion from the dialysis bag. The two dissolution profiles (**Figure 2.19**) tend to describe a biphasic pattern of release in which a sudden increase in percentage release over the first 10 hours followed by slower or sustained release behaviour over the remainder of the time. However, it appears mind-blowing that the release data showed no significant difference between the EFV-loaded liposomes and free EFV. This behaviour could be due to the structural specificity of EFV as a hydrophobic compound and has been previously observed by other authors who investigated EFV release from microemulsion (Hemal et al. 2015). There is no other report discussing efavirenz release from liposomes, but the recent work by Nkanga et al. (2019) revealed similar release profiles of some hydrophobic drug (rifampicin) as a liposomal formulation and as free-drug.

Nevertheless, the potential benefit of the liposomal EFV would include the possibility for surface modification of the resultant nano-system (EFV-loaded liposomes). EFV loaded liposomes may be a promising technology that may exhibit advantages of achieving surface modification for targeting infected cells while providing almost similar drug diffusion profiles with non-encapsulated EFV.

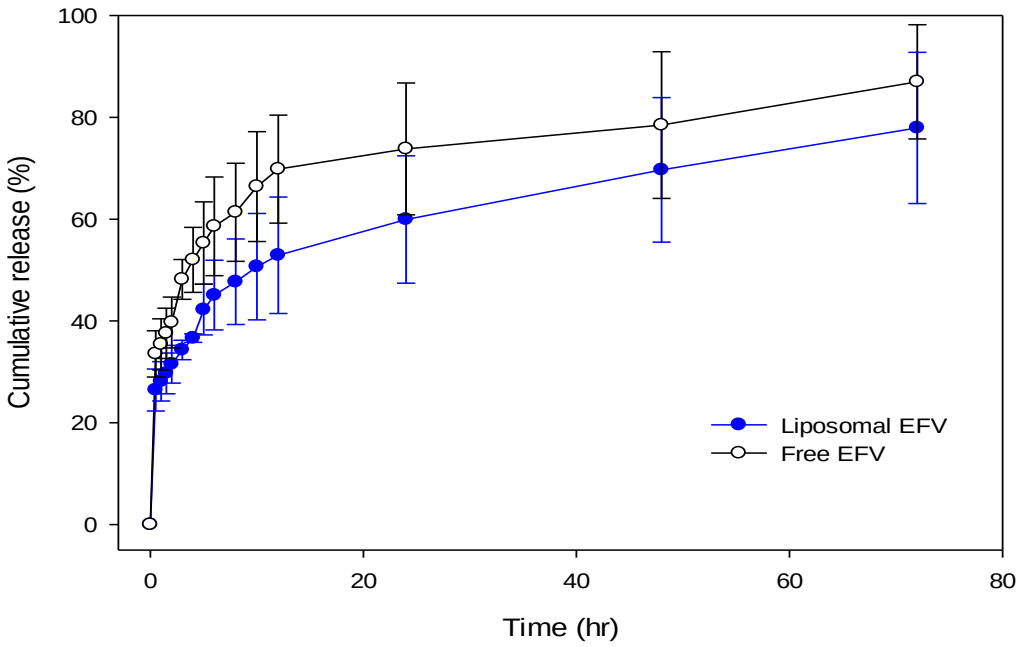


Figure 2.19. Drug release profile for EFV loaded liposome and the control at pH7.4

2.8. Conclusion.

This chapter was aimed on encapsulation of EFV (a non-nucleoside transcriptase inhibitor highly hydrophobic in liposomes), using CL phospholipid, which is less expensive when compared to synthetic lipids. The successful encapsulation of efavirenz in liposomes demonstrated the best encapsulation efficiency of 98.8 %. Furthermore, efavirenz loaded liposomes also showed an interesting particle size of 104.82 ± 2.29 nm and Zeta potential of -50.33 ± 0.95 mV, which is attractive for liposomal delivery of antiretroviral drugs and targeting of the infected blood cells with their prolonged release up to 2 hours at pH 7.4. Incorporation of cholesterol in the liposomes proved to enhance the stability and rigidity of the liposomes against the action of the protein. The above results prove that the properties

of efavirenz loaded CL liposomes could provide interesting parameters in reducing the adverse side effects, frequent dosing of antiretroviral drugs, and enhancing the poor solubility of the drug while also improving their bioavailability at a specific targeted site. The novelty of this approach is in that there has not been any work done currently or any literature reported on ''Liposome encapsulation of efavirenz drug''. On this note, I encourage use of CL, a less expensive phospholipid when compared to synthetic lipid in encapsulation for antiretroviral drugs for HIV treatment.

CHAPTER 3:

FORMULATION OF MUCOADHESIVE LIPOSOMAL FILMS FOR BUCCAL DELIVERY OF EFAVIRENZ (EFV)

Part of this work has been presented orally in the chemistry departmental conference/presentation 2018.

3.1. Introduction

The buccal mucosa has been identified as an attractive and favourable site for both local and systemic delivery of therapeutic agents since the mucosal lining of the buccal tissues provides a milder environment for drug absorption, unlike oral delivery which presents hostile environment due to acid hydrolysis and hepatic first-pass effect (Donnelly *et al*., 2011; Abd El Azim *et al*., 2015; Qidra *et al.*, 2018). The emergence of the buccal film (polymeric matrices) is not a recent formulation, this was introduced in late 1970s to overcome the swallowing difficulties exhibited by tablets and capsules, and is regarded as an ideal candidate for targeting sensitive sites of the central nervous system such as the CD4T lymphocytes, dendritic cells, monocytes and the macrophages that are not possible with tablets or liquid formulation in the oral delivery (Karki *et al*., 2016).

The use of buccal films has shown the potential to improve the onset of drug action, minimize the dose frequency of the drugs and enhance their efficacy. However, the accidental swallowing of the delivery system and the continuous dilution of the released drug by the saliva could cause the low resident time of the formulation in the buccal cavity and consequently, a low bioavailability (Shojaei *et al*., 1998; Abruzzo *et al.,* 2012). EFV in combination with other antiretroviral drugs has shown to undoubtedly reduce greater than 50 % HIV viral load, retarding and preventing damage to the immune system. However, it suffers significantly from the problem of bioavailability, due to the first-pass metabolism, and poor solubility thereby resulting to severe side effects (Gaur *et al*., 2014; Hemal *et al*.,2015)

Hydrophobic drugs like EFV, are poorly water soluble and cannot be successfully delivered via the buccal delivery unless they are modified first (Q.Xu *et al*., 2007). Hence, encapsulating the drug in a hydrophilic lipid-based system (like liposomes), within which the drug will be dispersed at the molecular level and further incorporate the drug-loaded liposomes into the polymeric matrices using bio-adhesive polymers, which becomes adhesive on hydration to form a chain interaction with the mucus. If these polymers are incorporated into the buccal formulations, the absorption of the drug by buccal mucosa layers would be enhanced, and drug released for extended period of time (Akhter *et al.*, 2012; Shojaei *et al.*, 1998; Abruzzo *et al.*, 2012). Like this, the drug though hydrophobic could easily get absorbed since dispersed at the molecular level as it is dissolved in the lipid system (Chen *et al.,* 2014). Few similar

strategies has been adopted in the assessment of tenofovir-loaded nanoparticles in films (Machado *et al*., 2016), and monolayered multi-polymeric films embedded with didanosine-loaded solid lipid nanoparticles for potential buccal drug delivery system for ARV therapy (Jones *et al*., 2014).

Therefore, the aim of this study was to formulate and evaluate EFV loaded liposomes incorporated in polymeric mucoadhesive films (PMFs), using bio-adhesive polymers (BAP) for the easy and effective administering of EFV through the buccal route. Using the formulated liposome delivery system, this will enhance the solubility and promote permeation of the drug across the membrane and offer prolonged drug resident time.

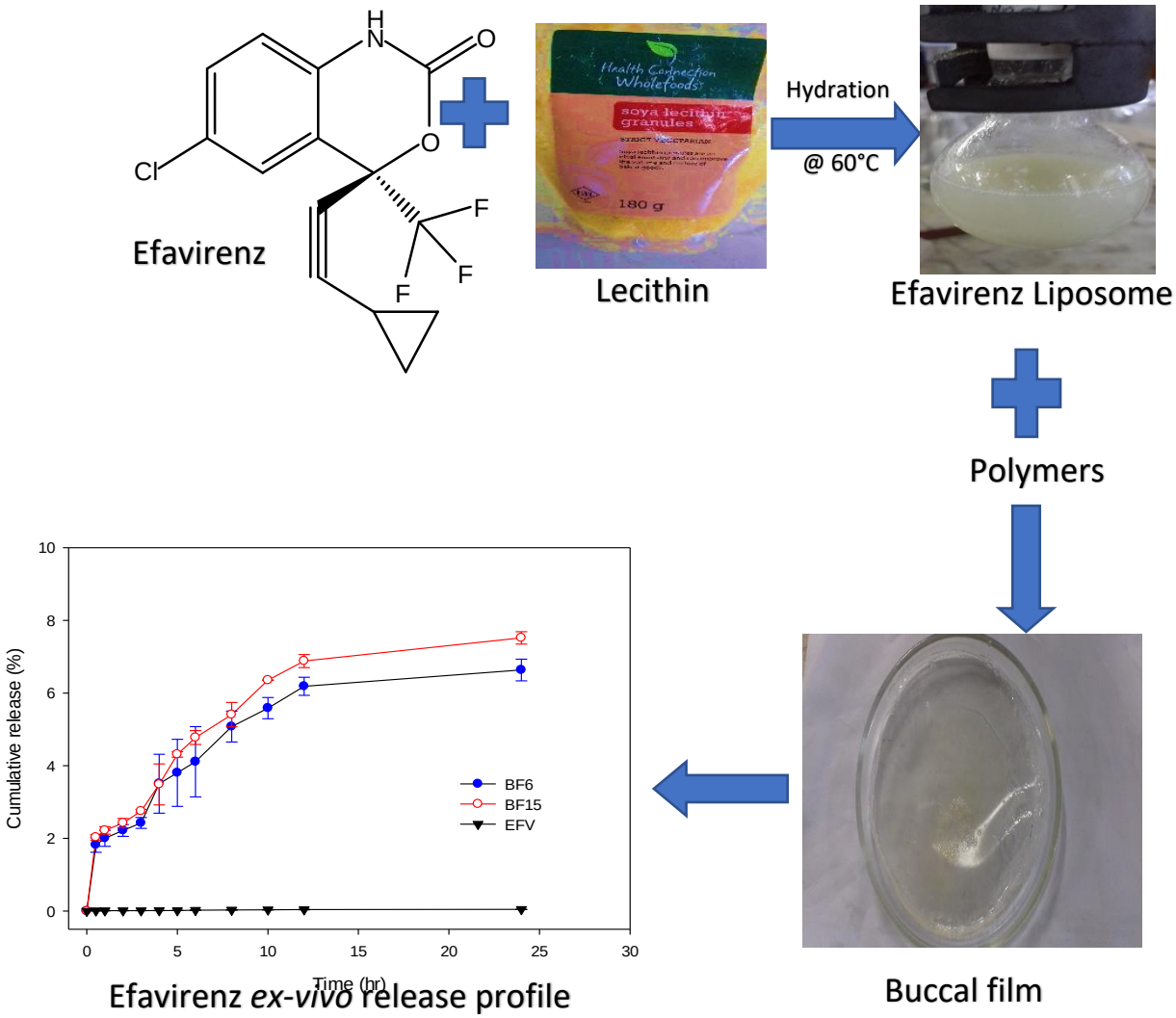


Figure 3.1. Graphical illustration of buccal film formulation

3.2. Experimentals- Materials and Methods

Soybean lecithin granules, hydroxyl propyl methyl cellulose (HPMC, K4M), Carbopol (CP,1120P), Pluronic (PF127) were purchased from (Sigma Aldrich, USA). Cholesterol was procured from Carlotiba Division Chemical (Italy). EFV, monobasic and dibasic sodium phosphate, chloroform, methanol, acetonitrile (Chromatography grade), ethylene glycol, glycerine, propylene glycol orthorphosphoric acid were purchased from Merck, South Africa and were used as such without further purification.

The equipments used includes; Buchi rotary evaporator, digital spellbound, 909 sonicator, low speed centrifuge machine (MSE-Mistral 1000),dynamic light scattering (zetasizer nanoZEN Malvern), digital Vernier caliper, RM- Leica microtome, franz diffusion cell, differential scanning calorimeter (DSC), x- ray diffractometer (XRD), energy dispersive spectroscopy (VEGA TESCAN), fourier transform infrared spectroscopy (FTIR), transmission electron microscopy (TEM), scanning electron microscopy (SEM), Ultimate 3000 ultra-high pressure liquid chromatography (UHPLC) 5u XB-C18 100A 150 x 4.6 mm coupled to mass spectrometry (LC-MS).

3.3. Methods- Preliminary Study

3.3.1. Optimization of the Plasticizers and Concentrations

The software Minitab (version.12) was used for general statistics and for factorial design of the experiment to generate nine possible formulations for 3 different plasticizers. The plasticizers were evaluated for particle size, PDI, zeta potential and encapsulation efficiency. The best plasticizer and the concentration were selected based on their evaluation for formulation of LBFs.

Table 3.1. Different types of plasticizers and their different concentrations

Formulation code	Plasticizer type	Plasticizer Concentration % (w/v)
P1	Ethylene glycol	2.5
P2	Ethylene glycol	5
P3	Ethylene glycol	10
P4	Glycerol	2.5
P5	Glycerol	5
P6	Glycerol	10
P7	Propylene glycol	2.5
P8	propylene glycol	5
P9	propylene glycol	10

3.3.2. Preparation of liposomes containing the plasticizers and their concentrations

The liposome formulation F24 had the best encapsulation efficiency as shown in **Table 2.7.** They were freshly prepared using the hydration technique described in **section 2.3.2.2** for encapsulation of EFV loaded liposomes. Upon hydration, Millipore water (8 ml) containing the different concentrations of the plasticizers was added to the lipid film and hydrated for 1 hour at 60 °C under stirring at 400 rpm. The resulting vesicle suspension was transferred into a centrifuge tube; made up to the centrifugation level using Millipore water and subjected to low speed centrifugation using low speed MSE-Mistral-1000 centrifuge tubes. The formulations were treated as described in **section 2.3.2.2**, and characterized for particle size, zeta potential, PDI and %EE.

3.3.3. Evaluation of the plasticizers and the concentrations

3.3.3.1. Particle size

The effect of the plasticizers and their various concentrations are shown in **Table 3.1,** the nine pre-formulations were evaluated for particle size, PDI and zeta potential using the zeta sizer nano Zen 3600 MAL1043132 Malvern instrument.

3.3.3.2. Determination of encapsulation efficiency

The encapsulation efficiencies of the nine pre-formulated liposomes for determination of the best plasticizer with suitable concentration are shown in **Table 3.1** was evaluated as described in (**section 2.3.2.3).** The encapsulation efficiency was calculated as;

$$\%EE = \frac{Efavirenz\ total\ amount - Efavirenz\ pellent\ amount}{Efavirenz\ toal\ amount}\ X\ 100 \ldots . \text{Equation 3.1}$$

3.4. Optimization of the polymers

The full factorial design of experiment was generated using Minitab software (v.12) for general statistics to generate 18 possible formulations containing three different polymers and different quantities of polymers for formulation of muco-adhesive liposomal buccal films. This preliminary screening for optimization of the polymers was carried out for selection of good bio-adhesive polymers with ideal mass for buccal delivery of EFV as represented in **Table 3.2.**

Table 3.2. Different types of polymers and different quantities of polymers for buccal film formulation

Formulation Code	Polymer type	Amount of Polymers (mg)
BF1	HPMC	200
BF2	HPMC	300
BF3	HPMC	600
BF4	CP	200
BF5	CP	300
BF6	CP	600
BF7	PF127	200
BF8	PF127	300
BF9	PF127	600
BF10	HPMC + CP	200
BF11	HPMC + CP	300
BF12	HPMC + CP	600
BF13	HPMC + PF127	200
BF14	HPMC + PF127	300
BF15	HPMC + PF-127	600
BF16	CP + PF-127	200
BF17	CP + PF-127	300
BF18	CP + PF-127	600

3.5. Preparation of liposomes for buccal film formulation- Method

Liposomal suspensions were newly prepared using the thin film hydration method, discussed in **section 2.3.2.1** with slight modifications. Millipore water (8 ml) containing 10 % (w/v) of propylene glycol (the best concentration of plasticizer) was added to the lipid film upon hydration at 60 °C, under stirring at 400 rpm for 60 minutes. The milky lipid suspension obtained was treated in the same manner as described in **section 2.3.2.2** and characterized before use for film formulation.

3.6. Characterization of the Liposomes for Buccal Film Formulation

3.6.1. Particle size, PDI and zeta potential

The freshly prepared formulations for liposomal buccal film preparation was evaluated for particle size, PDI and zeta potential using the ZEN-360 MAL1043132 Nano Malvern instrument at dynamic angle scattering of 173 °.

3.6.2. Shape analysis

The formulations were checked for spherical shape of liposomes using the Zeiss Libra 120 KV transmission electron microscopy instrument (TEM).

3.6.3. Encapsulation efficiency

Encapsulation efficiency of the freshly prepared liposome formulations for liposomal buccal film preparation was obtained using the HPLC validated method for efavirenz quantification described in **section 2.3.1.3**. The encapsulation efficiency was calculated using the following equation;

$$\%EE = \frac{Efavirenz\ total\ amount - Efavirenz\ pellet\ amount}{Efavirenz\ total\ amount}\ X\ 100 \ldots.$$ Equation 3.2

3.7. Preparation of Liposomal Buccal Films- Method

For the preparation, solvent casting method was adopted in preparing the buccal films. The polymers (hydroxyl propyl methyl cellulose, Carbopol) were used alone and in combination with Pluronic at three different masses as shown in **Table 3.2**. The polymers were added to the freshly prepared lipid dispersions containing 10 % (w/v) of propylene glycol, while stirring for proper and complete dissolution of the powders. Propylene glycol was utilized as a stabilizer. The gel formed was stored in a desiccator overnight at room temperature for clear bubble free gel (Abd El Azim *et al.,* 2015). The gels were cast into petri dish and allowed to dry at 40 °C. The liposomal films formed, were wrapped in an aluminium foil and stored in glass container at 4 °C for longer stability of the films. This was performed in triplicate.

3.8. Physicochemical characterization of the liposomal buccal films

The physical evaluation of the liposomal buccal films was carried out to examine the bio-adhesive properties of the formulated buccal films containing the EFV drug. The selected buccal films were further characterized for chemical interaction of the drug with the excipients using DSC, XRD, FTIR and EDS.

3.8.1. Weight uniformity

Weight uniformity of the formulated buccal films was investigated using the digital weighing balance. The films were weighed individually and collectively, and the weight variation of the films was calculated.

3.8.2. Thickness

The film thickness of the buccal films (BFs) was evaluated as one of the parameters to determining and selecting the buccal films, with an ideal thickness for buccal delivery of EFV. The thickness of the BFs was evaluated using an (In-size 150 mm, Vernier digital electronic calliper ruler). The thickness of the film was assessed at different points of the films and the average thickness calculated.

3.8.3. Folding endurance

The formulated buccal films were further evaluated for flexibility study. The folding endurance of the selected buccal films, with ideal thickness was determined by repeatedly folding the film at the same place at an angle of 180 ° till it is broken or folded up to 300 times or more. The breaking time was taken as the end point (Karki *et al.,* 2016).

3.8.4. Swelling study

The selected buccal films (BF-6 and BF-18), with interesting flexibility values were evaluated and compared for their swelling behaviour as shown in **Table 3.2**. The two selected BFs were weighed individually and immersed in the petri dishes containing 10 ml phosphate buffer (pH 7.4). At time respective intervals (10, 20, 40, 60, 120, 180 and 360 minutes), the BFs were removed from the petri dishes and excess surface water removed carefully using filter paper. The swollen films were re-weighed. The analyses was performed in triplicate and the percentage swelling index was calculated (Kumria *et al.*, 2016).

$$\%\ Swelling\ index = \frac{W2-W1}{W1}\ X\ 100 \quad \text{…. Equation 3.3}$$

Where W1 = Initial weight of the film

W2 = Weight of film at particular interval

3.8.5. Surface pH

The selected EFV loaded buccal films (BF-6 and BF-18) containing Carbopol alone and combination of Carbopol and Pluronic, with ideal flexibility value were further analysed for their surface pH. The films were placed in 5 ml distilled water for 1 hour at room temperature. The pH was recorded by mounting the electrode on the surface of the swollen films and allowing to equilibrate for 1 min. The experiment was done in triplicate and the average of the three determinations was calculated (Samanthula *et al*., 2014).

3.8.6. XRD evaluation

The pure drug, the selected EFV buccal films (BF-6 and BF-18) were examined for their crystallinity in comparison to their respective empty BFs prepared in water and in lipid suspension containing no drug. The study was carried out using Bruker D8 Discover instrument in the range 2- theta of 10 to 80 °C, using cu-kα radiation set at 1.5404 angstrom, equipped with lynx Eye detector and a slit width of 6.0 mm and a size of 1 °/min.

3.8.7. Differential scanning calorimetry (DSC)

The thermal behaviour of the pure drug and the selected EFV buccal films (BF-6 and BF-18) along with the empty buccal films was evaluated using the Perkin Elmer - 600 R DSC instrument. The samples were sealed in aluminium sample pans and heated at a speed of 1 ° C/min, from 50 to 170 °C in nitrogen environment. The control empty pan was also subjected to same condition.

3.8.8. Fourier infrared spectroscopy (FT-IR)

The FTIR spectrum of the pure drug, the selected EFV buccal films (BF-6 and BF-18) with the empty buccal Films was evaluated using a FTIR spectrometer (PerkinElmer Spectrum 100). The scans were run at 8 scans at frequency of 4000 – 650 cm^{-1} respectively. The data were processed using the FTIR spectrum software.

3.8.9. Energy – dispersive X – ray spectroscopy (EDX)

The elemental composition analysis of the pure drug, the selected EFV loaded buccal films (BF-6 and BF-18) with the empty films were examined and compared using (An INCAPENTA

FET, connected to the VAGA TESCAM), which relied on the interaction of some source of X-ray excitation of the samples.

3.8.10. Surface morphology

The surface morphology of the selected films (BF-6 and BF-18) was examined along with the buccal films respectively using the SEM instrument. The cross section of the selected buccal films was placed in an argon atmospheric condition, using gold sputter.

3.9. *Ex-vivo* permeation studies

The selected buccal films (BF-6 and BF-18) were tested for drug permeation through chicken pouch mucosa while the pure drug was used as the control. The study was carried out using Franz diffusion cell system adopted from PermeGear VDC system with 12 ml volume and 1.76 cm diameter placed on six station magnetic stirring unit having two compartments of donor and receptor compartment (El-Samaligy *et al.*, 2006; Avachat *et al.*, 2013).

Briefly, the chicken heads were procured from his Majesty local slaughter house (Grahamstown, South Africa), as a barrier membrane. The chicken membrane was used because of its similarity with the human non-keratinized mucosa. Chicken pouch was surgically removed from the underlying muscular layer, cleaned and washed with isotonic phosphate buffer (pH 7.4) from the surface fats and used immediately (Amira *et al*., 2017). The surface of the mucosa was spread over with 1 ml of the isotonic buffer pH 6.8 to simulate saliva. The receptor medium was filled with isotonic phosphate buffer IPB (pH 7.4), and the study was performed at 37 °C with the help of circulating water under stirring at 150 rpm. The simulated chicken mucosa was mounted over the donor compartment and the film (2 x 2 cm) was placed on the mucosa, with the back layer facing the donor compartment and tightly covered with the lid collar. The pure drug (10 mg) was used as the control along with chicken mucosa and was treated in the same manner (Patel *et al.*, 2012; Raghavendra Rao *et al.*, 2012).

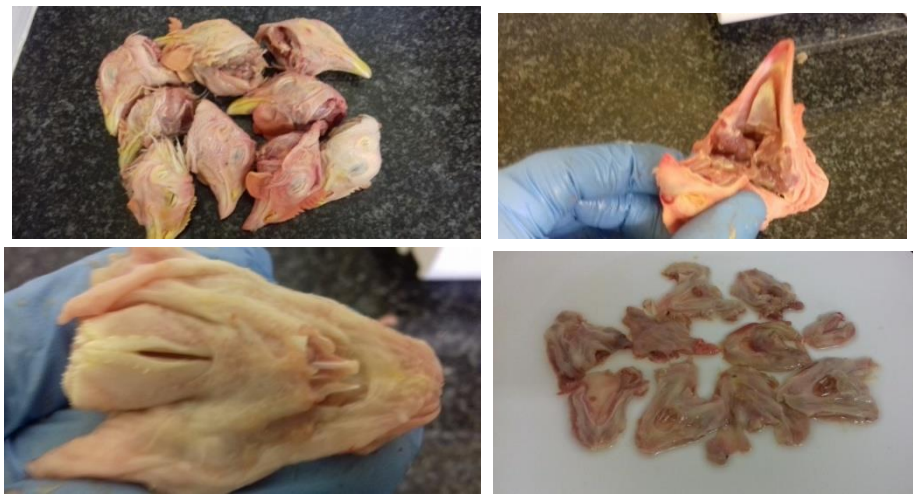


Figure 3.2. Surgically processed chicken mucosa for *ex-vivo* permeation study

At different time intervals (0.5, 1, 2, 3, 4, 5, 6, 8, 10, 12 and 24 hours), 1 ml of the samples was withdrawn and replaced with fresh buffer for 24 hours. Aliquots of samples were filtered using 0.45 µm syringe filters and were analysed using ultra high-pressure liquid chromatography (UHPLC) with C18 100A column, size 150 x 4.6 mm coupled to Ultimate 3000, Liquid chromatography mass spectrometry LC-MS Quadrupole time of flight (Q-TOF, Bruker compact). The HPLC chromatographic condition for EFV validation as described in **section 2.3.1.3** was used with minor modification. The injection volume was changed from 20 to 5 µg/ml for quantification of the permeated drug in both selected formulations and control. This experiment was done in duplicate and the drug content (%DC) was calculated using the following equation.

$$\%DC = \frac{Amount\ of\ recovered\ drug}{Amount\ of\ the\ formulation\ used}\ X\ 100 \ldots . Equation\ 3.4$$

3.10. Histological evaluation

The histological analyses of the chicken mucosal samples used in the permeation study was carried out to examine and investigate the pathological changes in the morphology of the chicken mucosa, as the result of any possible damage from the used BFs. The results were compared with fresh untreated membrane control as illustrated by (El-Nahas *et al.*, 2017) .

Briefly, the used mucosal samples were carefully removed from the Franz diffusion cells, cleaned from the buccal films residuals and properly washed with the buffer solution. The mucosal membranes were preserved using Bouin's fixatives containing solution of (picric acid, formalin and glacial acetic acid) to avoid autolysis or bacterial attack.

Prior to use, chicken mucosal membranes and the freshly processed positive control were dehydrated each in 50, 70, 90 and 100 % alcohol and xylene. The membranes were all embedded in melted paraffin wax and were left over night for proper drying before sectioning. The waxed mucosal membranes with the positive control were sectioned using Leica RM 2035 Microtome equipment with a sharp sectioning knife. The sectioned mucosal membranes were transferred into warm water bath for expansion of the wax containing the mucosa. The expanded waxed membranes were transferred onto microscope slides wet with Haupt's adhesive and left in an oven for 48 hours for complete drying of water. The membranes were dewaxed using xylene and absolute alcohol and were stained with haematoxylin and eosin. Microscopy mounting medium (enthalen) was spread on the surface of the slides and were covered with the cover slip and left to dry before viewing with microscope. The stained chicken membranes were all viewed and analysed using (Olympus B X 50 with analysis software).

3.11. Integrity of the liposomes

The integrity of the liposome carrier in the formulated BFs was evaluated to examine the effect of the polymers on the particle size; PDI, zeta potential and shape of liposomes after the films were stored for 3 months. They were placed in foil wrapped air tight vials at 4 °C and placed in glass tight 4 °C air environment. The liposomal BFs were dissolved in distilled water and the integrity of the restored liposomes was examined for the particle size, PDI, zeta potential using the nano Malvern zeta instrument. The spherical shape of the liposomes was also re-examined using TEM to confirm the structural integrity of liposomes after storage.

3.12. Results and Discussion- Optimization of the Plasticizers and Concentrations

3.12.1. Evaluation of particle size, PDI and zeta potential

The nine pre-formulated liposomes containing different plasticizer of varying concentrations were examined for their particle size, PDI and zeta potential to help select the best plasticizer and concentration suitable for the formulation of liposomal films for buccal delivery of EFV.

The result for particle size, PDI, zeta potential and %EE of the formulations containing different plasticizers with varying concentrations is presented in **Table 3.3**. The data revealed interesting and ideal particle sizes within liposomal nano range pivotal in prolonging the circulation of liposomes in the blood, with suitable PDI and high surface charges for buccal

drug delivery. However, formulation (P7-P9) containing propylene glycol showed regular correlation both in particle sizes, PDI and zeta potential compared to the six other formulations (P1-P6) containing ethylene glycol and glycerine, respectively. This evaluation showed that formulation (P9) containing 10 % (w/v) of propylene glycol, demonstrated higher particle size, PDI and the highest surface charge, compared to other formulations.

Table 3.3. Particle size, PDI, ZP and %EE of the pre-formulated liposomes

Formulation Code	Particle size	PDI	ZP (mV)	%EE
P1	455.33 ± 21.21	0.540 ± 0.06	-31.72 ± 1.71	13.11 ± 2.33
P2	420.26 ± 45.74	0.653 ± 0.07	-37.84 ± 1.71	54.06 ± 1.43
P3	481.93 ± 38.29	0.631 ± 0.12	-42.14 ± 3.42	57.1 1± 1.32
P4	310.9 3± 6.71	0.550 ± 0.01	-33.56 ± 0.59	61.01 ± 0.22
P5	371.82 ± 2.98	0.460 ± 0.06	-35.90 ± 1.21	63.72 ± 1.21
P6	388.8 4± 16.89	0.527 ± 0.06	-38.72 ± 2.18	66.46 ± 1.23
P7	471.3 2± 61.28	0.620 ± 0.15	-40.74± 2.48	62.44 ± 2.22
P8	473.40 ± 42.40	0.575 ± 0.04	-43.71 ± 2.43	79.42 ± 1.34
P9	533.23 ± 86.76	0.458 ± 0.02	-47.5 ± 1.75	92.33 ± 1.16

3.12.2. Encapsulation efficiency

The encapsulation efficiency of the nine pre-formulated liposomes containing different plasticizers with varying concentrations is presented in **Table 3.3**. The result showed that formulation (P9) containing propylene glycol in the concentration 10 % (w/v), demonstrated the highest encapsulation efficiency compared to other formulations. High encapsulation efficiency witnessed in the formulations (P7-P9), containing propylene glycol could be attributed to the hydrophobic nature of propylene glycol as its incorporation improved the entrapment of the EFV drug as a result of improved solubility of the drug in the liposomes (Elsayed *et al.*, 2007). Hence formulation (P9) containing 10 % (w/v) of propylene, was chosen based on its high particle size, ZP and %EE for formulation of liposomes in preparation of buccal films.

3.13. Characterization of Efavirenz Loaded Liposomes for Preparation of Buccal Films

3.13.1. Particle size and zeta potential

The result for particle size, ZP and PDI for the freshly prepared liposomes containing 10 % (w/v) of propylene glycol for preparation of buccal films is shown in **Table 3.4**. The result demonstrated that the formulated liposomes revealed an acceptable and ideal particle size, PDI and zeta potential for buccal drug delivery, hence they were all considered for use in preparing the liposomal films for buccal delivery of EFV drug. This experiment was done in triplicate and were stored for further use.

3.13.2. Encapsulation efficiency

The encapsulation efficiency result of liposome formulations for preparation of buccal films are presented in **Table 3.4**. The result exhibited high encapsulation efficiency in the range 72.82 ± 3.20 to 92.32 ± 2.43 in all the formulations. The %EE could be attributed to the interaction of solubilized drug in liposome, with the hydrophobic propylene glycol used, thereby leading to more entrapped drug in the liposomes. Increased %EE of drugs has also been reported for propylene glycol use in encapsulation of diclofenac in liposomes containing glycol (Manconi *et al.*, 2009).

Table 3.4. Particle size, PDI, ZP and %EE of the formulated liposomes for buccal film formulation

Formulation Code	particle size (nm)	PDI	ZP (mV)	%EE
L1	164.11 ± 24.73	0.609 ± 0.03	-42.41 ± 2.52	89.24 ± 2.28
L2	293.93 ± 1.41	0.509 ± 0.06	-45.62 ± 1.21	72.42± 2.32
L3	180.52 ± 8.18	0.665 ± 0.09	-49.11 ± 2.81	70.82 ± 3.20
L4	279.20 ± 17.60	0.654 ± 1.19	-40.62 ± 3.20	74.33 ± 1.65
L5	289.33 ± 3.40	0.531 ± 0.03	-41.04 ± 1.12	76.22 ± 0.34
L6	205.31 ± 16.83	0.750 ± 0.06	-46.46 ± 2.07	71.33 ± 1.65
L7	312.43 ± 24.47	0.639 ± 0.11	-40.01 ± 1.07	73.01 ± 0.86
L8	295.81 ± 4.15	0.802 ± 0.09	-37.22 ± 0.64	78.11 ± 1.13
L9	265.42 ± 17.23	0.829 ± 0.02	-48.06 ± 1.54	74.63 ± 1.65
L10	186.95 ± 2.25	0.564 ± 0.01	-50.30 ± 0.80	87.24 ± 2.72
L11	269.11 ± 32.21	0.751 ± 0.22	-44.12 ± 1.10	77.48 ± 2.12
L12	194.72 ± 14.30	0.772 ± 0.16	-45.84 ± 0.55	78.32 ± 0.64
L13	205.74 ± 5.12	0.380 ± 0.08	- 37.95 ± 3.17	92.30 ± 2.43
L14	391.43 ± 44.89	0.646 ± 0.03	-35.92 ± 0.99	86.42 ± 0.86
L15	241.52 ± 25.07	0.517 ± 0.07	-46.82 ± 0.80	88.66 ± 2.11
L16	196.62 ± 0.404	0.549 ± 0.05	-39.44 ± 0.89	74.11 ± 3.22
L17	269.62 ± 2.406	0.457 ± 0.11	-51.82± 1.36	79.33 ± 0.41
L18	212.22 ± 3.421	0.475 ± 0.03	-44.7 ± 0.23	81.55 ± 0.82

3.13.3. TEM analysis

TEM result for the formulated liposomes for buccal film formulation is displayed in **Figure 3.2**. The result showed that all the formulations all demonstrated spherical and dispersed liposomes shapes, thereby confirming the presence of liposomes.

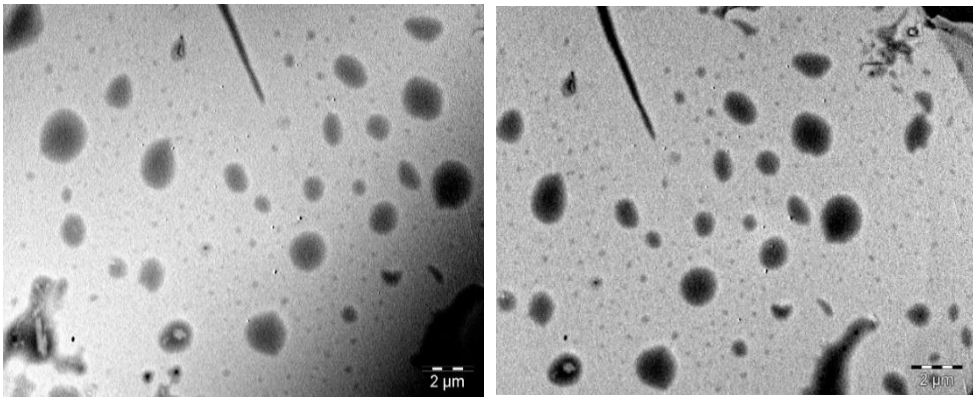


Figure 3.3. TEM Micrographs of liposome formulations containing PEG for buccal film preparation

3.14. Characterization of the Liposomal Buccal Films

3.14.1. Weight uniformity

The result for weight uniformity of the formulated buccal films is presented in **Table 3.5**. Weight variation values (mg) of the films was found to be between 24 – 86 mg. The proportional gain in weight of the films was observed as the amount of the polymers increased in the formulations. However, the variation values revealed that the buccal films all demonstrated uniform weight.

3.14.2. Thickness

The film thickness of the formulated films is shown in **Table 3.5**. The thickness of the films was found between the range of 0.22 to 1.23 mm. The thickness of the films was found to increase with increased amounts of polymers in the formulations. The result agreed with the increased weight variation values ranging from 27.82 ± 1.04 – 86.28 ± 2.16 mg. The increased film thickness has also been reported by (Karki *et al*., 2016). However, most of the film formulations containing 200 and 300 mg of various polymers, showed tears and slits within the films, which is not ideal for buccal drug delivery. The tear and slits in the formulated buccal films could be attributed to less amounts of polymers in the lipid suspension used. Therefore, the formulations (BF3, BF6, BF12, BF15 and BF18) with highest film thicknesses and no tear, were selected for further analysis. The formulations (BF7-BF9) containing Pluronic (PF127) as single polymer, were not analysed because of their inability to form films, as PF127 can only be used as co-polymer.

Table 3.5. Weight uniformity, thickness and folding endurance of the BFs

Formulation Code	Weight uniformity (mg) (n=3±SD)	Thickness (mm) (n=3±SD)	Folding Endurance (no. of time)
BF1	27.82 ± 1.04	0.42 ± 0.02	*
BF2	44.62 ± 1.22	0.64 ± 0.08	*
BF3	75.33 ± 2.03	0.87 ± 0.12	86
BF4	24.46 ± 0.16	0.49 ± 0.05	*
BF5	52.71 ± 1.02	0.53 ± 0.04	*
BF6	68.22 ± 1.04	0.88 ± 0.10	258
BF7	*	*	*
BF8	*	*	*
BF9	*	*	*
BF10	31.24 ± 1.02	0.46 ± 0.04	*
BF11	56.56 ± 1.18	0.49 ± 0.06	*
BF12	78.33 ± 1.21	1.23 ± 0.12	88
BF13	24.82 ± 0.26	0.17 ± 0.05	*
BF14	48.64 ± 2.16	0.47 ± 0.04	*
BF15	65.42 ± 1.14	0.75 ± 0.06	74
BF16	26.42 ± 1.06	0.22 ± 0.02	*
BF17	47.32 ± 1.14	0.24 ± 0.04	*
BF18	86.28 ± 2.16	0.76 ± 0.14	321

NA: * were not analysed

3.14.3. Folding endurance

The result of the folding endurance for the selected buccal films (BF3, BF6, BF12, BF15 and BF18) is presented in **Table 3.5**. The folding endurance result revealed that the formulation (BF6 and BF18), containing Carbopol (CP) alone and a combination of CP and Pluronic (PF127) respectively, demonstrated the best folding flexibility. The flexibility of the buccal film is highly important, considering the films can be administered without breakage. Hence the two

selected formulations from the folding endurance analysis were further evaluated for comparison between CP as single polymer and CP blended with PF127 co-polymer.

3.14.4. Swelling study

The result of the swelling study of the selected formulation of the films (BF6 and BF18) is shown in **Table 3.6**. The swelling index results of the formulations were compared, to investigate the effect of using Carbopol (CP) alone and in combination with Pluronic (PF127) as swelling of the polymers allows mechanical entanglement by opening the bio-adhesive sites for hydrogen bonding between the polymer and the mucosa (Roy and Prabhakar, 2010). The swelling study of the two formulations (**Figure 3.3**) revealed adequate swelling behaviour. However, the swelling was more pronounced in formulation (BF18), containing combination of CP and PF127 with 78 % swelling index compared to formulation (BF6) containing only CP, having a 50 % swelling index. The pronounced swelling in (BF18) could be attributed to increased (-OH) interaction between CP to PF127 polymer blend, compared to CP alone. The maximum swelling of the two formulations was seen after 6 hours with an increase in swelling within the period with no excessive hydration of the films.

Table 3.6. Swelling index of the formulations (BF6 and BF18)

Time (min)	BF6 (Carbopol) %	BF18 (Carbopol-Pluronic F127) %
10	19.94 ± 0.24	39.93 ± 0.12
20	20.77 ± 0.14	46.94 ± 0.22
40	20.73 ± 0.46	59.78 ± 0.18
60	34.64 ± 0.12	63.34 ± 0.22
120	48.33 ± 0.44	69.32 ± 0.14
180	49.62 ± 0.23	77.62 ± 0.24
360	50.46 ± 0.32	78.33 ± 0.16

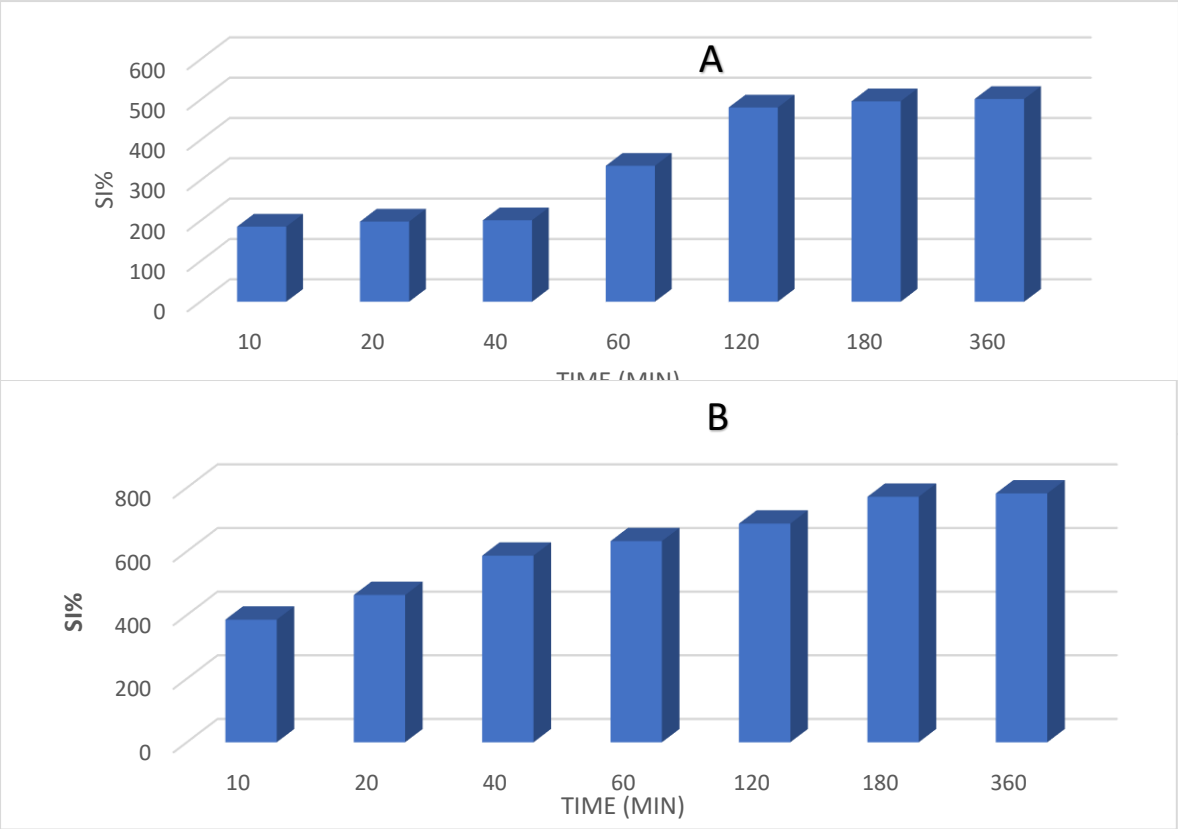


Figure 3.4. Swelling index of BF6-EFV loaded liposomes in CP polymeric BF (A), BF18-EFV loaded liposomes in CP to PF127 polymeric BF (B)

3.14.5. Surface pH

The surface pH result of the two selected formulated buccal films (BF6 and BF18) showed surface pH within the salivary range of 6.43 ± 0.016 and 6.32 ± 0.012, respectively. This suggested that the films would show no irritation within the surface of the mucosa.

3.14.6. XRD

The result of the X-ray analysis of the two selected film formulations (BF6 and BF18) is presented in **Figure 3.4.** The XRD results were compared with pure EFV drug, empty Carbopol (CP) film in water, empty CP film in liposomes, empty CP to Pluronic (PF127) film in water and empty CP to PF127 film in liposomes.

EFV pure drug demonstrated its characteristic sharp XRD pattern with a sharp peak at 2θ and range of 10.43 °, 10.98 °, 12.27 °, 13.25 °, 14.20 °, 16.92 °, 20.14 ° and 21.25 °. The presence of the peak indicates the crystalline nature of the EFV raw material. However, the disappearance of the characteristic peaks of EFV drug in the CP polymeric BFs, confirmed the

amorphous nature of EFV drug in the polymeric materials used with the empty buccal films in water and liposome respectively, all showing to be amorphous.

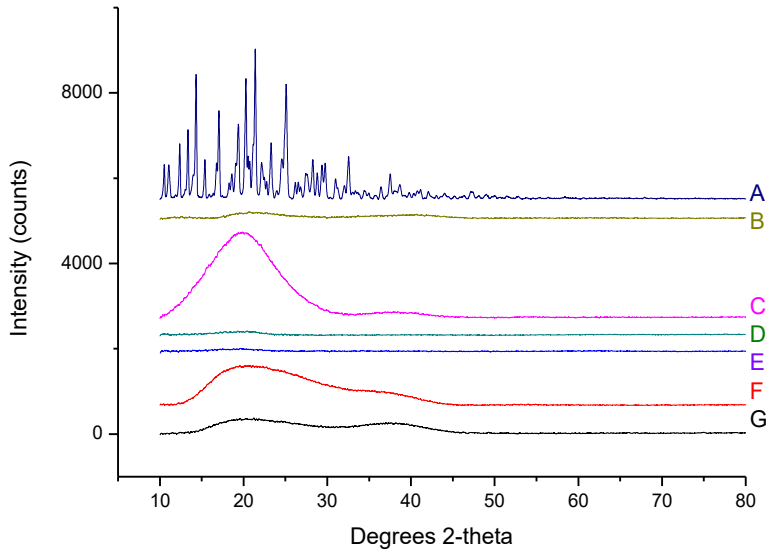


Figure 3.5. XRD diffractogram of pure EFV drug (**A**), BF6-EFV loaded liposomes in CP polymeric BF (**B**), BF18-EFV loaded liposomes in CP to PF127 polymeric BF (**C**), empty CP film in water (**D**), empty CP film in liposomes (**E**), empty CP to PF127 in water (**F**) and empty CP to PF127 in liposomes (**G**)

3.14.7. Differential scanning calorimetry (DSC)

The DSC result of the of the selected films is depicted in **Figure 3.5,** in comparison to the EFV pure drug and the respective empty film formulation in water and in liposomes. The DSC study revealed a single sharp endothermic melting peak for EFV pure drug at 139.60 °C, indicating the crystalline nature of EFV drug. The drug showed complete amorphous state in Carbopol (CP) and CP to Pluronic polymeric films with the empty polymeric films all showing amorphous nature of the polymers. The DSC result however confirmed, the XRD analysis results above.

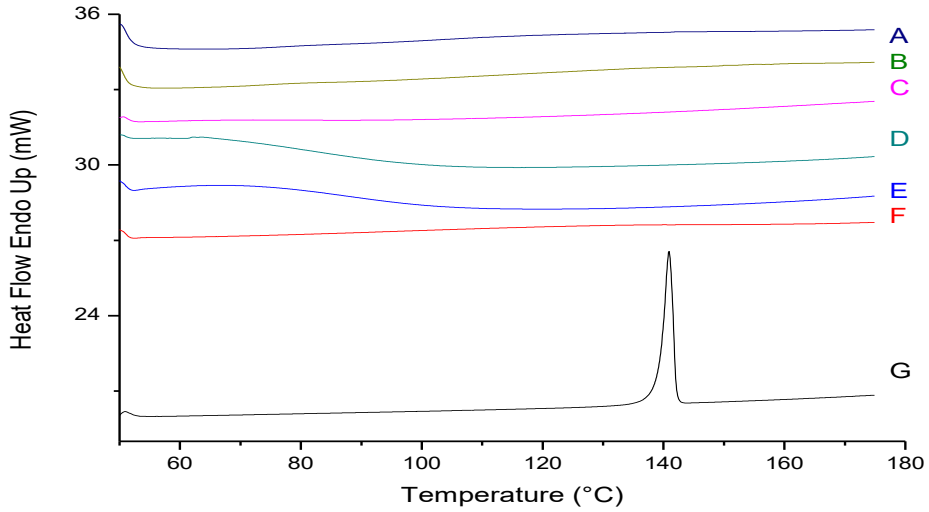


Figure 3.6. DSC pattern of Pure EFV drug (**G**), BF6-EFV loaded liposomes in CP polymeric BF (**F**), BF18-EFV loaded liposomes in CP to PF127 polymeric buccal film (**E**), empty CP film in water (D), empty CP film in liposomes (**C**), empty CP to PF127 in water (**B**) and empty CP to PF127 in liposomes (**A**)

3.14.8. Fourier infrared spectroscopy

The FTIR spectra of EFV drug, the formulated buccal films BFs (BF6 and BF18) and the respective empty films in water and in liposomes are presented in **Figure 3.6**. The FTIR spectra of EFV pure drug revealed the characteristic feature of the drug at stretching band;

-NH stretching of the benzoxazine -2-one ring at 3314 cm^{-1}

C-C stretching due to alkyne (C≡C) at 2251 cm^{-1}

C=O stretching of the amide group at 1745 cm^{-1} and

CF3 stretching band at 1240cm^{-1}, 1160 cm^{-1}.

However, the formulation (BF6) exhibited the presence of the characteristic peaks of EFV at the same spectral regions, in addition to polymer peaks appearing at 2911-2931 cm^{-1}, and 1455-1468 cm^{-1} for alkane (C-H stretches) and at 1103-1110 cm^{-1} for the ether region, revealing the presence of EFV drug in the formulated BFs.

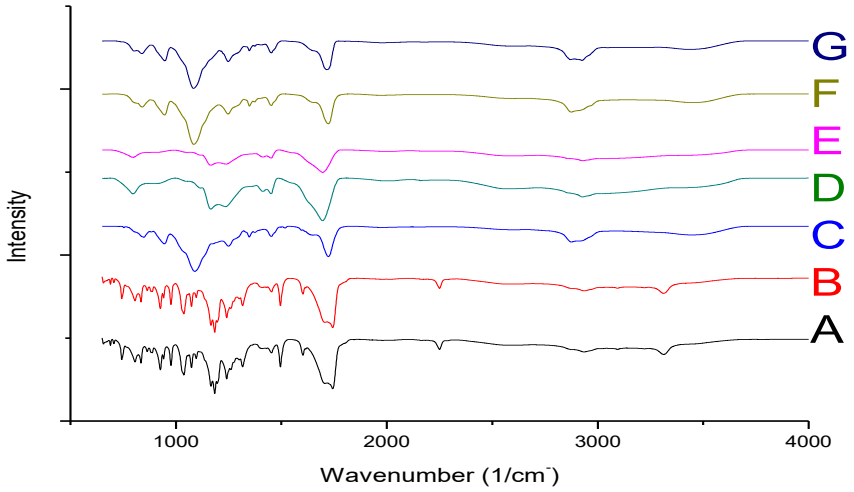
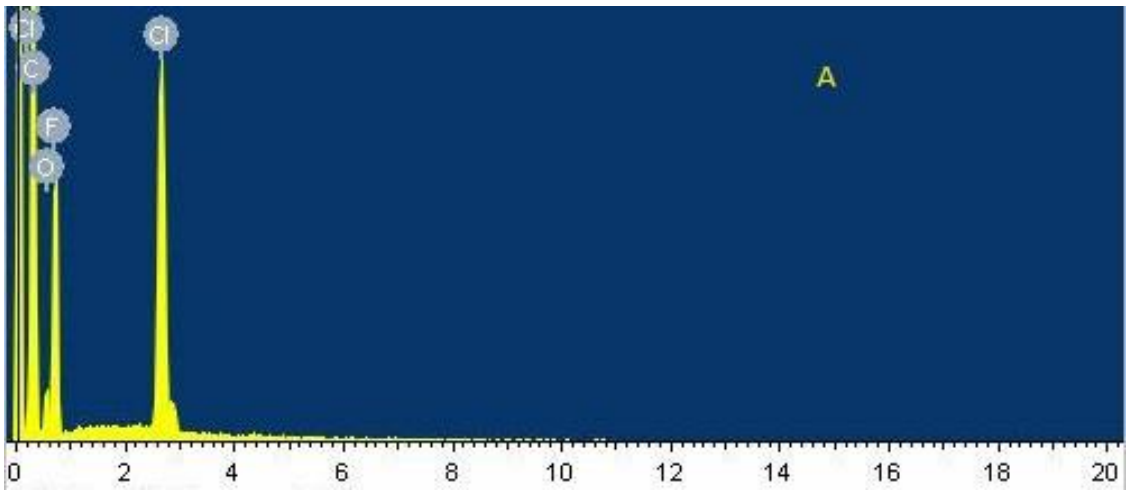
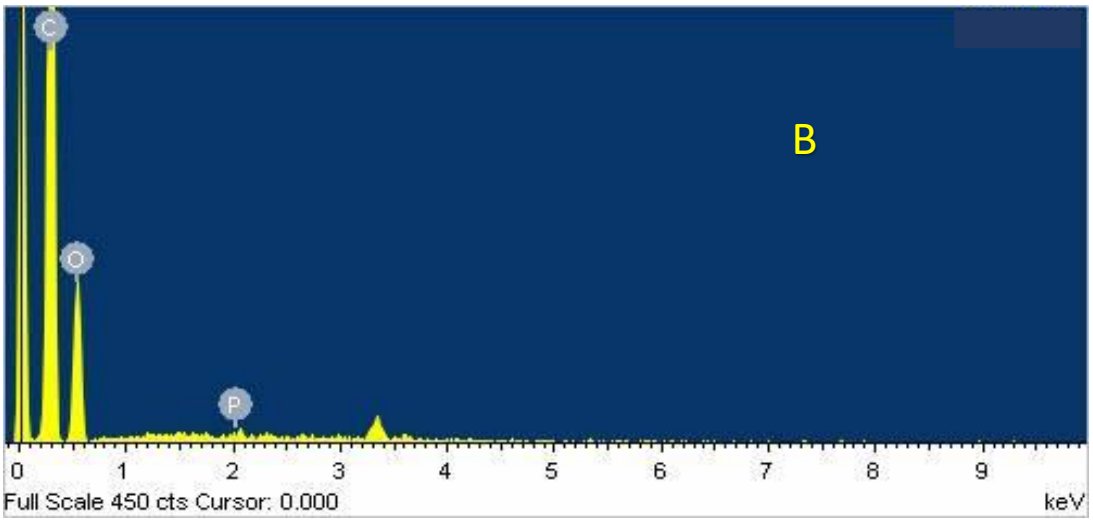


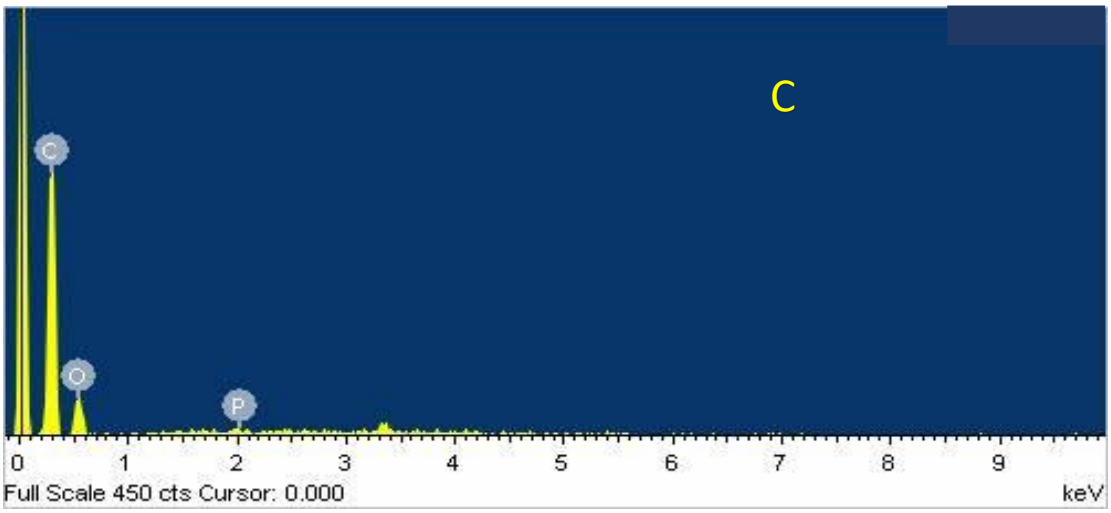
Figure 3.7. FTIR spectra of Pure EFV drug (**A**), BF6-EFV loaded liposomes in Cp polymeric BF (**B**), BF18-EFV loaded liposomes in CP to PF127 polymeric BF (**C**), empty CP film in water (**D**), empty CP film in liposomes (E), empty CP to PF127 in water (**F**) and empty CP to PF127 in liposomes (**G**)

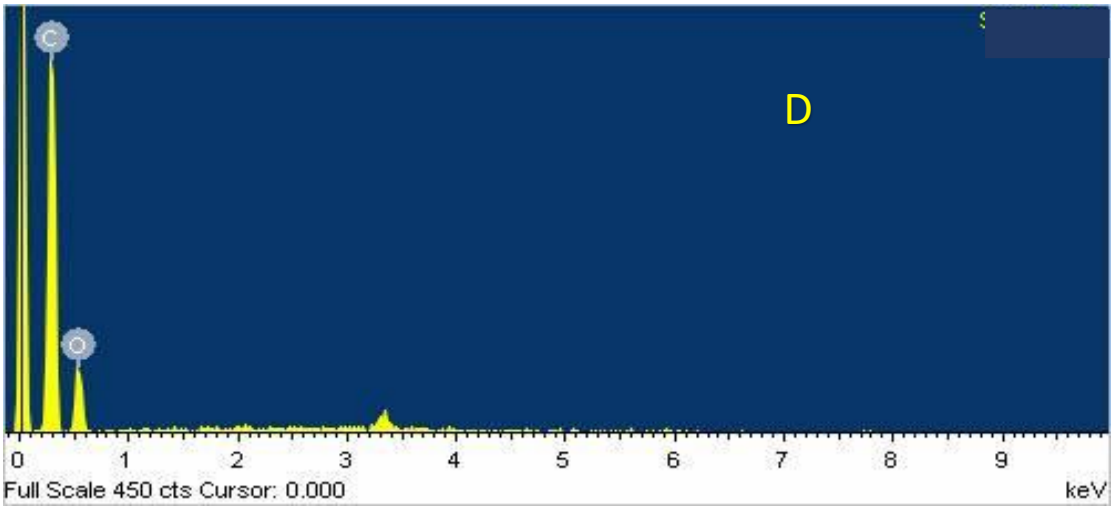
3.14.9. Energy- dispersity X-ray spectroscopy (EDS)

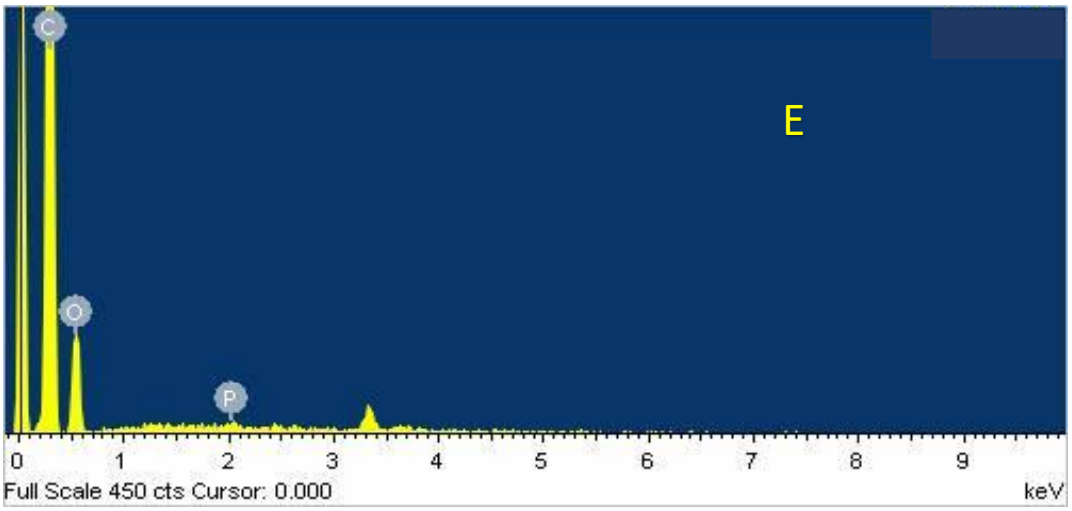
The EDS result of the selected films is shown in **Figure 3.7**. The EDS results of the formulated films (BF6 and BF18) were compared with EFV pure drug and the respective empty polymeric buccal films in water and liposomes. The result showed all the elemental surface composition features of the EFV pure drug. However, the disappearance of these elements in the formulated films (BF6 and BF18) suggested total embedding of the drug within the liposomes, coated with Carbopol and Pluronic polymers with the drug loaded films.

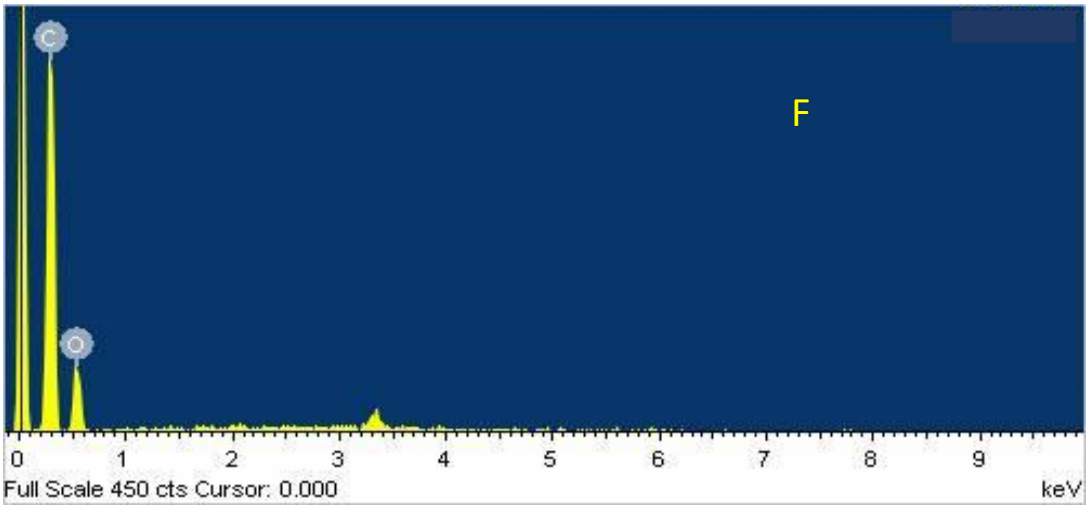



B
C
O
P
0 1 2 3 4 5 6 7 8 9
Full Scale 450 cts Cursor: 0.000
keV


C
C
O
P
0 1 2 3 4 5 6 7 8 9
Full Scale 450 cts Cursor: 0.000
keV


D
C
O
0 1 2 3 4 5 6 7 8 9
Full Scale 450 cts Cursor: 0.000
keV





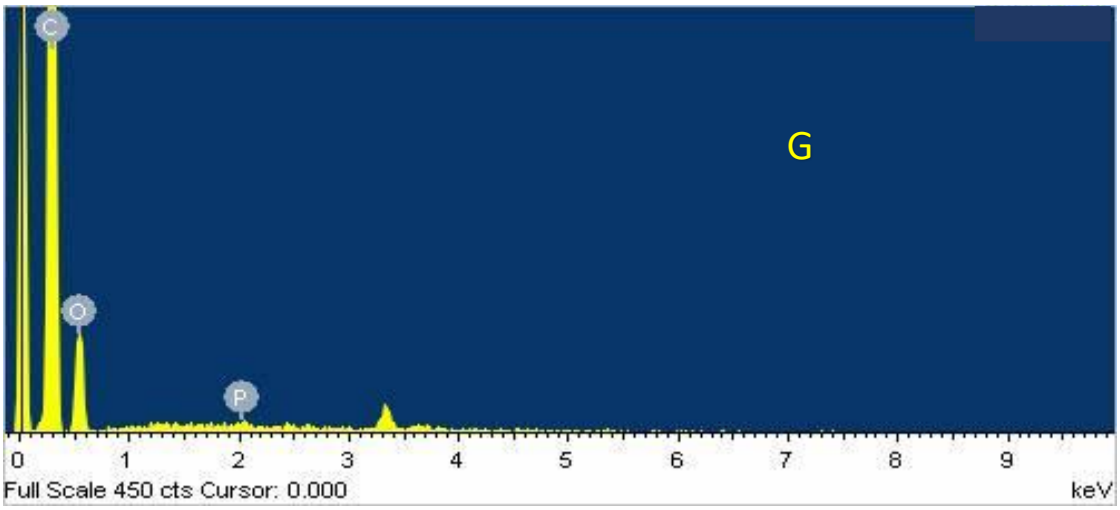


Figure 3.8. Elemental composition of Pure EFV drug (**A**), BF6-EFV loaded liposomes in CP polymeric BF (**B**), BF18-EFV loaded liposomes in CP to PF127 polymeric BF (**C**), empty CP film in water (**D**), empty CP film in liposomes (**E**), empty CP to PF127 in water (**F**) and empty CP to PF127 in liposomes (**G**)

3.14.10. Surface morphology

The surface morphology of the selected buccal films BFs (BF6 and BF18) and the empty BFs in water and liposomes is presented in **Figure 3.8**. The formulated BFs (A-BF6, and B-BF18) all demonstrated a rough surface morphology. The rough surface could be attributed to the polymer interaction with the drug. However, empty BFs in water showed smooth surface morphology. There was sign of the liposomes embedded in the BFs (D and F) in the empty BFs prepared using drug free liposome suspension. The embedded liposomes in the empty BF confirmed the presence of liposomes, as the drug carrier in the formulated drug loaded liposomes BFs of Carbopol and the combination with Pluronic, respectively while the empty BFs in water (C and E) showed smooth surface morphology.

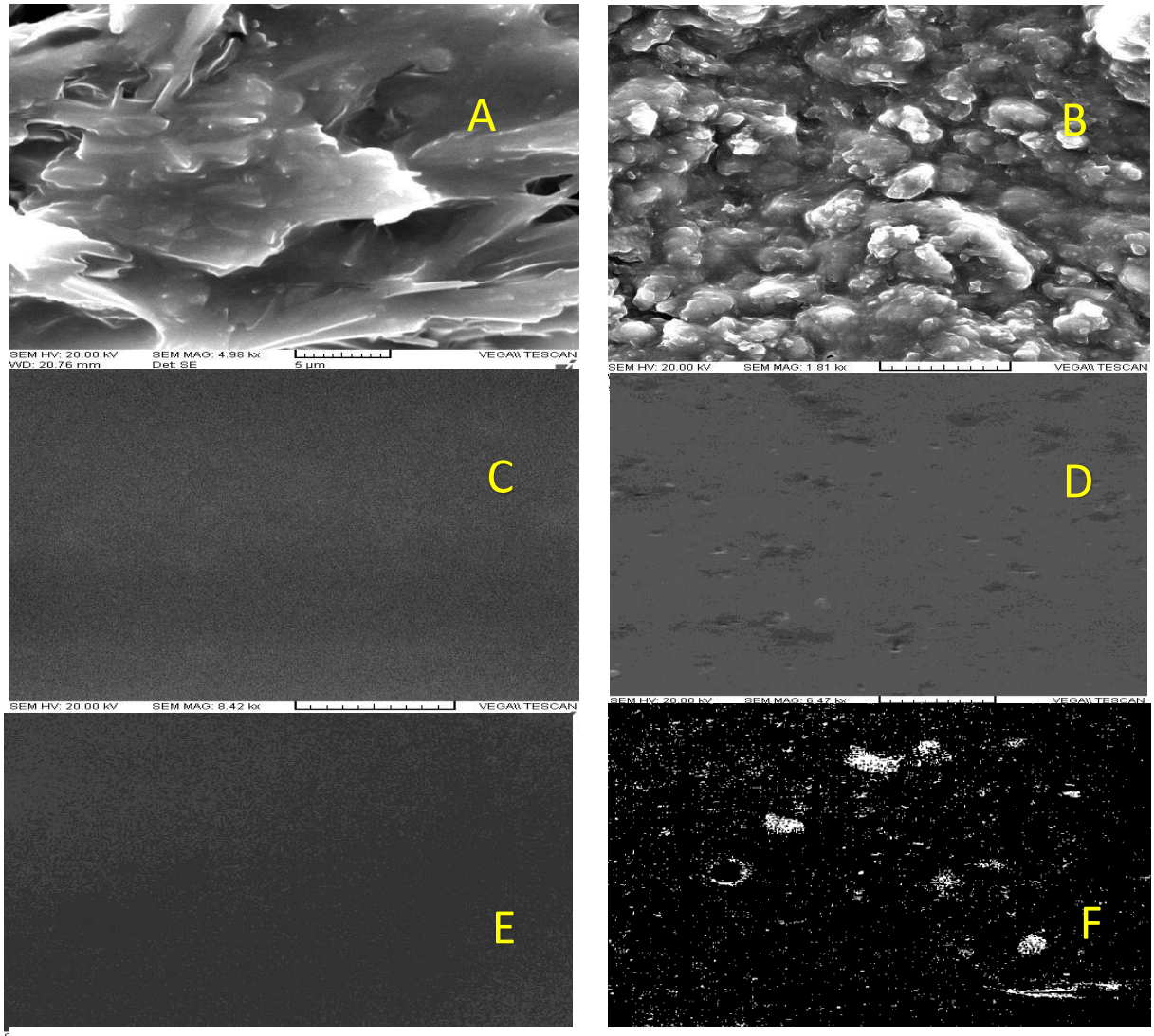


Figure 3.9. SEM images of BF6- EFV loaded liposomes in CP BF (**A**), BF18- EFV loaded in CP to PF127 BF (**B**), Empty CP BF in water (**C**), Empty CP BF in liposomes (**D**), Empty CP to PF127 BF in water (**E**), Empty CP to PF127 BF in liposomes (**F**)

3.15. *Ex- vivo* permeation studies

The *ex- vivo* permeation results of the selected buccal formulations (BF6 and BF18) made of Carbopol (CP) alone and the combination of CP and Pluronic (PF127) containing equivalent of 726 µg and 914 µg with the control 10 mg (raw EFV) conducted in chicken mucosa using Franz diffusion cell is presented in **Table 3.7** and **Figure 3.10** respectively. The high-resolution electrospray ionization HR – ESI – MS spectrum and the total ion chromatography spectrum (A, and B) of the quantified drug showing the retention time and mass of the drug are also presented in **Figure 3.9**. The results revealed maximum cumulative drug permeation of 66.42 ± 0.30 % for BF6, 75.26 ± 0.17 % for BF18, and 1.0 % ± 0.04 % for the control (raw EFV) through the epithelium after 24 hours. The pure drug (EFV) showed little permeation of the drug through the mucosa after 24 hours. This could be attributed to the high poor solubility of the EFV crystalline drug in water. The rapid permeation of the drug through the mucosal membrane from the films suggests the effect of the phospholipid and the polymers used which permits permeation of the solubilized EFV drug. However, the permeation was higher in formulation (BF18), combination of CP and PF127 suggesting high swelling ability of the combined polymers as a result of increased (OH) group which allowed more permeation and release of the drug over period of time. The cumulative permeation of the drug through the membrane was rapid in the two BFs formulations after 30 minutes followed by a steady and sustained release for 24 hours. The overall cumulative permeation of the drug from the buccal films is an indication of convincing performance of the polymers in the buccal delivery of antiviral drugs with formulation BF18 a combination of CP and PF127 suggesting more profitable for longer release of the drug.

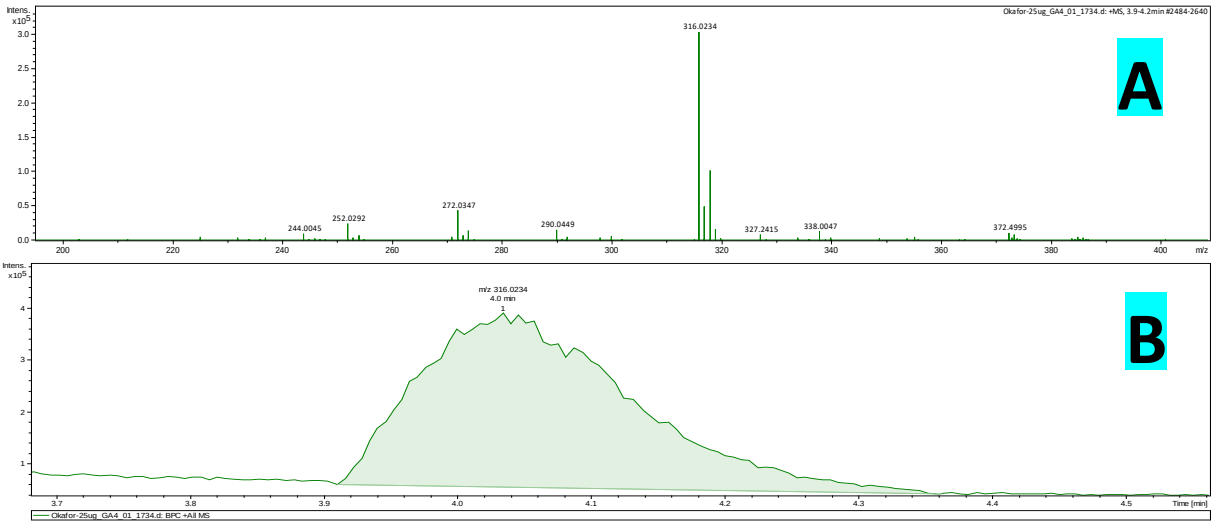


Figure 3.10. HR-ESI$^+$ MS (A), TIC (B) spectra of the quantified EFV drug from the BFs with the retention time and the molecular mass

Table 3.7. Cumulative drug release of EFV from the formulated BFs (BF6, BF18) and EFV pure drug

Time (hour)	Bf6 (%)	BF18 (%)	Pure (EFV) Drug (%)
0.5	18.22 ± 0.20	20.33 ± 0.08	0.11 ± 0.02
1	20.03 ± 0.22	22.24 ± 0.10	0.14 ± 0.04
2	22.53 ± 0.16	24.22 ± 0.13	0.15 ± 0.06
3	24.77 ± 0.15	27.55 ± 0.05	0.16 ± 0.01
4	35.13 ± 0.81	34.82 ± 0.56	0.22 ± 0.02
5	38.02 ± 0.93	43.11 ± 0.08	0.24 ± 0.01
6	41.11 ± 0.97	47.83 ± 0.19	0.27 ± 0.01
8	50.86 ± 0.43	54.11 ± 0.34	0.31 ± 0.02
10	55.93 ± 0.29	63.66 ± 0.12	0.42 ± 0.01
12	61.90 ± 0.25	68.84 ± 0.18	0.44 ± 0.01
24	66.44 ± 0.32	75.22 ± 0.17	1.12 ± 0. 01

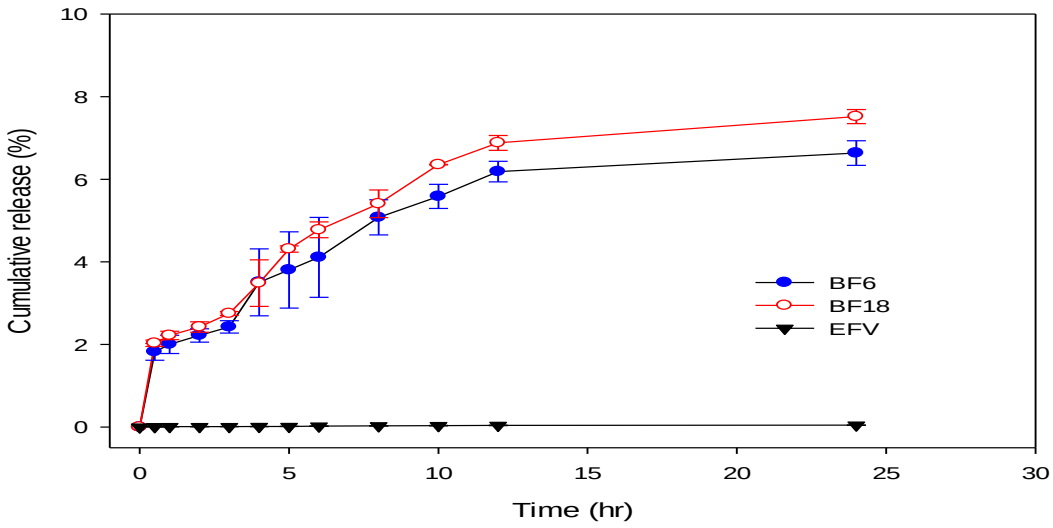


Figure 3.11.. Cumulative ex- vivo release profile of EFV drug from selected buccal films BF6, BF18 with pure EFV drug

3.16. Histological analysis

The histological result of the chicken mucosal membranes along with the respective controls is presented in **Figure 3.11**. The microscopical evaluation of the mucosal membranes revealed that there was no change in the integrity of the cell morphology and the tissue organization of the chicken pouch. This indicated that the formulated buccal films caused no damage to the mucosal membranes used in the permeation study.

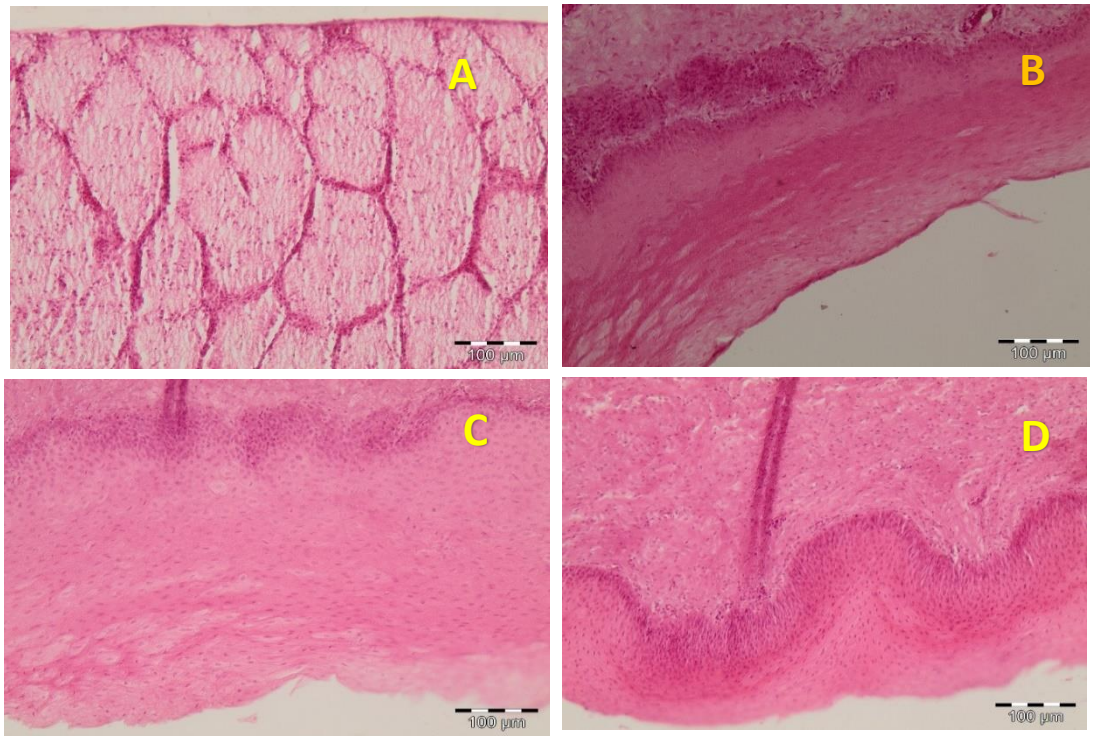


Figure 3.12. Histologically evaluated chicken mucosal membranes used against (**A**) BF6 EFV loaded liposomes in CP BF, (**B**) BF18 EFV loaded liposomes in CP to PF127, (**C**) Drug control, (**D**) Positive control Chicken mucosa

3.17. Evaluation of Liposomes Integrity

3.17.1. Particle size, PDI and surface charge integrity

The result for the integrity of the liposomes particle size, PDI and surface charge in the formulated buccal films (BFS), after 3 months of storage is presented in **Table 3.7**. The result revealed huge increases in the particle size of liposomes and PDI. The increase could be attributed to the effect of the polymers on the lipid bilayers. However, most of the formulation retained the ideal sizes within nano range, suitable for penetrating the mucous layer, while also showing acceptable PDI. On the other hand, the surface charges of the liposomes in the BFs after 3 months drastically decreased. Since the liposome surface was

coated with polymers, there is an indication of the anionic surface charge consumption by ion complex formation between the polyethylene glycol and carboxyl groups of the polymers. However, liposomes were still found to be stable as they still possess a negatively charged surface, which could not be attacked by the positively charged enzyme (lysozyme), that is highly concentrated in the mucosa.

Table 3.8. Particle size, PDI and ZP integrity of the liposomes after 3 months

Formulation code	Particle size (nm)	PDI	ZP (mV)
BF1	519 ± 146.96	0.53 ± 0.09	-6.86 ± 0.20
BF2	544 ± 29.21	0.36 ± 0.17	2.49 ± 0.04
BF3	747 ± 68.64	0.89 ± 0.06	-1.63 ± 0.12
BF4	618 ± 15.50	0.89 ± 0.04	-12.82 ± 5.41
BF5	888 ± 120.62	0.95 ± 0.08	-5.88 ± 0.02
BF6	639 ± 166.73	0.85 ± 0.10	-6.64 ± 11.7
BF7	*	*	*
BF8	*	*	*
BF9	*	*	*
BF10	1438 ± 12.34	0.38 ± 0.10	-2.13 ± 0.72
BF11	684 ± 82.79	0.54 ± 0.29	-4.14 ± 0.35
BF12	362 ± 13.96	0.59 ± 0.05	-3.46 ± 0.02
BF13	1202 ± 66.29	0.98 ± 0.04	-16.71 ± 1.57
BF14	682 ± 67.66	0.62 ± 0.11	-30.42 ± 1.59
BF15	373 ± 32.43	0.41 ± 0.01	-5.26 ± 0.87
BF16	484 ± 80.39	0.92 ± 0.08	-7.84 ± 0.53
BF17	159 ± 7.605	0.45 ± 0.05	-33.46 ± 3.63
BF18	441 ± 53.79	0.93 ± 0.03	-29.56 ± 0.85

NA: * were not examined.

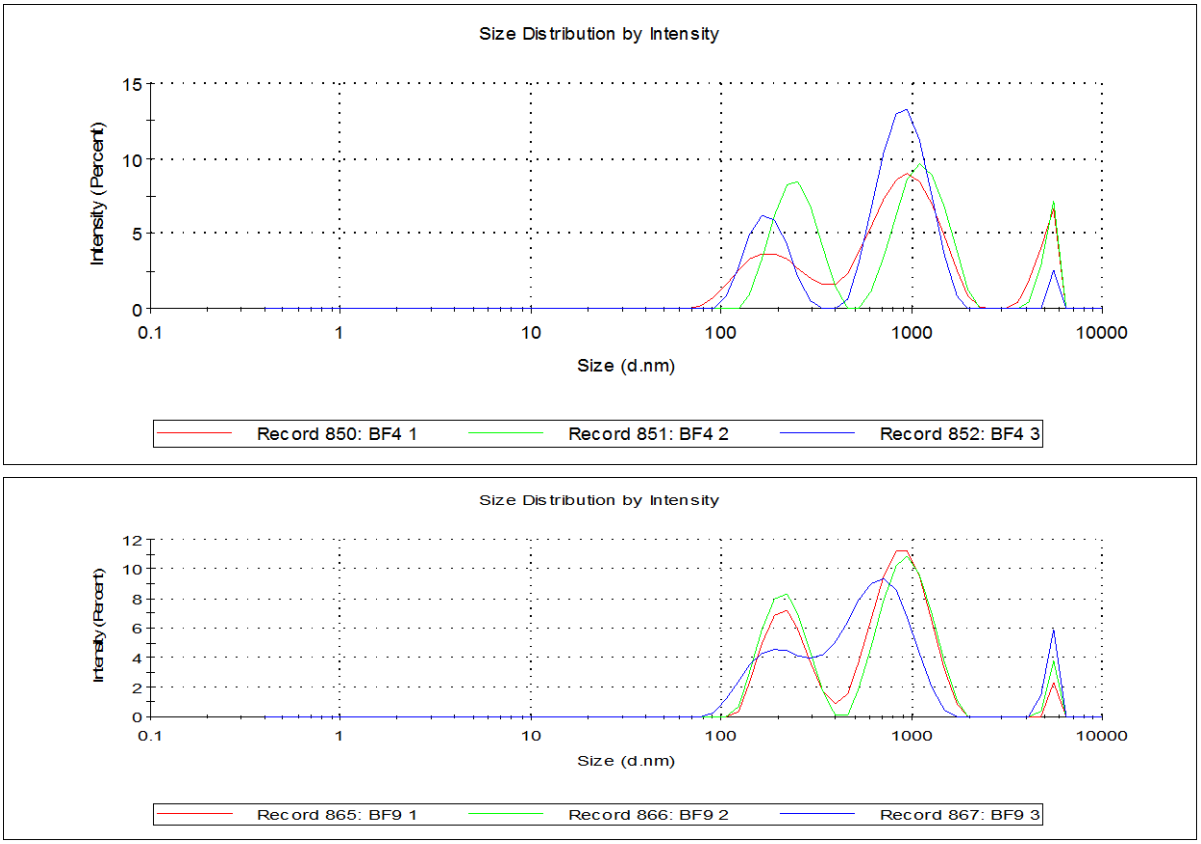


Figure 3.13. Particle size distribution of liposomes in the buccal films after 3 months storage

3.17.2. Shape analysis

The TEM result for the confirmation of liposome presence in the buccal films after 3 months storage is presented in **Figure 3.13**. The result showed that the liposomes retained their spherical shape in the films, after several months of storage. This suggests that the liposome shape was not affected by the polymeric materials used and confirmed absolute integrity of the liposome carrier in the formulated films.

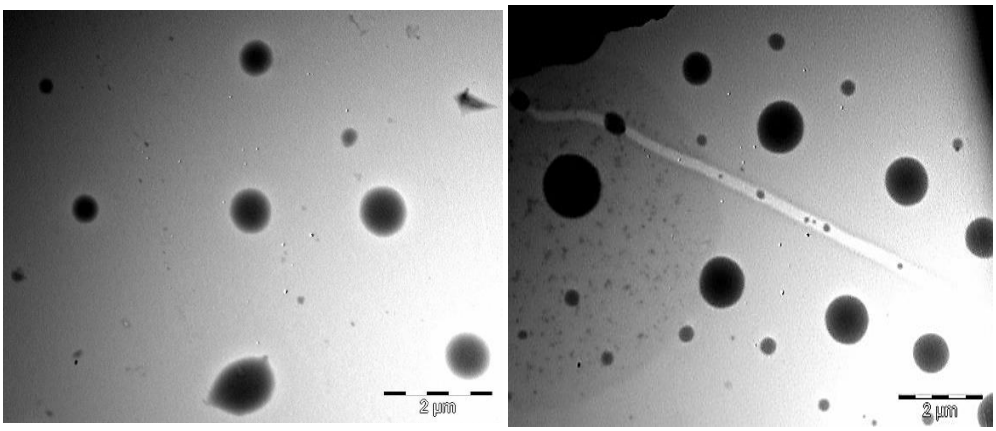


Figure 3.14. TEM micrographs of the EFV loaded liposomes in polymeric BFs after 3 months storage

3.18. Concluding Remarks

This chapter investigated the formulation of liposomal mucoadhesive buccal films, for the potential buccal delivery of EFV antiviral drug, using polymers that have shown bio-adhesive strength and features with propylene glycol to enhance the permeation of the drug through the mucosa. The buccal films (BFs) were prepared with solvent casting method using liposome suspension loaded with the EFV drug as the solvent. The satisfactory data obtained throughout the formulation, revealed that the liposomal BFs (BF6 and BF18) containing Carbopol (CP 600 mg) and mixture of CP to Pluronic (PF127 600 mg) were the best compared to the other formulations. Based on the physicochemical characterization, the selected BFs (BF6 and BF18), demonstrated better thickness of 0.88 ± 0.10, 0.76 ± 0.14 mm, better flexibility values of 258 and 321 respectively, compared to the other formulations containing HPMC and mixture with CP and PF127. The two selected formulations, however demonstrated promising swelling behaviour of 58 % and 76 % swelling ability, respectively with no excessive hydration of the polymers as they showed significant enhancement in gel strength during formulation and in the physiological conditions thereby indicating their mucoadhesive behaviour in the release of the encapsulated drug.

The *ex-vivo* permeation profile of the two selected formulations (BF6 and BF18) showed that the use of polymeric excipients (i.e. CP, combination of CP to PF127 and the lipid) ensured slower release and enhanced the permeability of the drug through the chicken mucosa with the help of propylene glycol (permeation enhancer), compared to the low permeation of the poor soluble EFV drug in its pure form (control). However, combination of CP and PF127 polymer showed greater permeation of the drug than the single polymer. This could be attributed to the increased hydroxyl groups from the two combined polymers, thereby leading to longer hydration of the films which allowed more drug release. The histological examination revealed no damage to the integrity of the membrane treated with the EFV BFs. Hence the controlled release with the enhanced permeation effects of the BFs, with reported mucoadhesive features of the BFs make them an ideal buccal delivery system for antiretroviral drugs in minimizing the high dosing and severe side effects of the drugs, while bypassing the first pass metabolism in achieving total patient compliance in treatment of HIV/AIDS.

CHAPTER 4

GENERAL CONCLUSION

4.1. Conclusion

Encapsulation of antiretroviral drugs (ARVDs) in suitable delivery system like liposomes and buccal films (BFs) can help overcome several limitations attributed to the use of the drugs, both in buccal route thereby improving the drug therapy and patient compliance. Due to high cost of the liposomes formulation resulting from use of the expensive synthetic or purified natural phospholipids, strategies to adopt use of locally and affordable crude soybean lecithin phospholipid and polymers in the research field for the delivery of ARVDs would be highly encouraging especially for poor patients in the developing countries who live with HIV/AIDs The following conclusions were ascertained from various investigations in this work.

The average particle size of the drug loaded liposome demonstrated an interesting size within the nano dimensions 104.82 ± 2.29 nm and zeta potential -50.33 ± 0.95 mV, which can easily penetrate the leaky vasculature and target the infected cell. The high %EE of 98.88 ± 0.03 is comparable to the reported %EE of 99.4 % in the literature by (Jin *et al.*, 2005) in the encapsulation of zidovudine. This is encouraging in minimizing frequent dosage of ARVDs and reducing their adverse side effects. The release profile of EFV from liposomes showed signs of sustained release, compared to the release of the free drug.

The characterized BFs obtained after the optimization of EFV loaded liposomes coated with various polymeric excipients, showed that the selected BFs made of Carbopol (CP) alone and mixture of CP and Pluronic (PF127) revealed good film thickness 0.88 ± 0.10, 0.76 ± 0.14 mm with outstanding flexibility values 258 and 321 which are highly important for buccal delivery of ARVDs. The polymeric excipients in the selected BFs both demonstrated promising swelling behaviour that enhanced the release of the drug through the chicken mucosa. Incorporation of the polymers in the formulation enhanced the permeability of the drug across the mucosa. However, combination of two different polymers (CP to PF127) in the formulation revealed high permeability of the drug compared to the single polymer (CP). The histological examinations showed no loss in the integrity of the mucosa used in the permeation studies treated with EFV pure drug and EFV coated BFs. These findings demonstrated the importance in evaluating the mucosal integrity and understanding of the drug permeation pathways through the mucosa.

It is pertinent to say that this study has shown several interesting results that can help eradicate the several deficiencies associated with the use of ARVDs while improving patient compliance by encapsulation of the drugs using locally available and affordable soybean lecithin and developing of liposomal buccal films with less expensive and easily accessed polymers with mucoadhesive behaviour for buccal delivery of ARVDs. These findings will provide researchers with worthwhile strategies for optimizing ARV delivery system for the effective treatment of HIV/AIDS and other viruses via the buccal route. It is interesting to report that this work on encapsulation of EFV in liposomes and formulation of EFV liposomal BFs using CP and PF127 for buccal delivery in treatment of HIV, has not been reported in literature and thus highlights the novelty of this approach.

REFERENCES

Abd El Azim H. (2015) 'Liposomal buccal mucoadhesive film for improved delivery and permeation of water-soluble vitamins', *International Journal of Pharmaceutics*. 488:78–85.

Abruzzo A. (2012) 'Mucoadhesive chitosan/gelatin films for buccal delivery of propranolol hydrochloride, *Carbohydrate Polymers*. 87: 581–588.

Adkins JC, Noble S. (1998) 'Efavirenz' *Drugs*, 56:1055–1064.

Adriana, RM. (2014) 'Importance of lecithin for encapsulation processes', *African Journal of Food Science*, 8(4), 176–183.

Akbarzadeh A. (2013) 'Liposome: Classification, preparation, and applications', *Nanoscale Research Letters* 8:1-9

Akhter MH. (2012) 'A Comprehensive Review On Buccal Delivery System' *International Journal Of Pharmaceutical Research and Development*, 3:59–77.

Albuquerque TG. (2016)'Cholesterol determination in foods:Comparison between high performance and ultra-high performance liquid chromatography',*Food Chemistry*,193:18–25.

Amin PM, Gangurde, AB, Alai, PV (2015) 'Oral Film Technology : Challenges and Future Scope for Pharmaceutical Industry', *International Journal of Pharmacy & Pharmaceutical Research*, 3:183–203.

Anderson RM. (1986) 'A preliminary study of the transmission dynamics of the Human Immunodeficiency Virus (HIV), the causative agent of AIDS', *Mathematical Medicine and Biology*, 3:229–263.

Andhale VA. (2016) 'Liposome: An emerging tool in drug carrier system' *International Journal of Pharmacy and Technology*, 8:10982–11011.

Andrews GP, Laverty, TP, Jones, DS. (2009) 'Mucoadhesive polymeric platforms for controlled drug delivery', *European Journal of Pharmaceutics and Biopharmaceutics*, 71:505–518.

Antonio, P, Xu X, Burgess, DJ. (2014) 'Freeze-Anneal-Thaw Cycling of Unilamellar Liposomes : Effect on Encapsulation Efficiency', *Pharmaceutical Research*, 31:97–103.

ArkendunChatterjee, Bhowmik BB and Awasthi D. (2010) 'Prolong Release Bioadhesive Vaginal Film of Anti-HIV Drug (Zidovudine): Arkendu Chatterjee *, Benoy Brata Bhowmik and Deepak Awasthi', *International Journal of Pharmaceutical Sciences and Research*, 1:28–37.

Avachat AM, Gujar KN, Wagh KV. (2013) 'Development and evaluation of tamarind seed xyloglucan-based mucoadhesive buccal films of rizatriptan benzoate', *Carbohydrate Polymers*. Elsevier Ltd., 91:537–542.

Ayyappan J. (2011) 'Development and validation of a stability indicating high-performance liquid chromatography (HPLC) method for the estimation of isoniazid and its related substances in fixed dose combination of isoniazid and ethambutol hydrochloride tablets', *African Journal of Pharmacy and Pharmacology*, 5:1513–1521.

Banerjee R. (2001) 'Liposomes: Applications in medicine', *Journal of Biomaterials Applications*, 16:3–21.

Barenholz Y. (2001) 'Liposome application: Problems and prospects', *Current Opinion in Colloid and Interface Science*, 6:66–77.

Barre-SINOUSSI F. (1983) 'Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS)', *Science*, 220:868–871.

Bhattarai M, Gupta AK. (2015) 'Baharya paper.pdf', *Sunsari Technical College Journal*, 2:58–68.

Bhyan B. (2011) 'Orally Fast Dissolving Films: Innovations in Formulation and Technology', *International Journal of pharmaceutical Sciences Review and Research*, 9:50–57.

Boateng JS. (2010) 'Characterisation of freeze-dried wafers and solvent evaporated films as potential drug delivery systems to mucosal surfaces', *International Journal of Pharmaceutics*, 389:24–31.

Bobade NN. (2013) ' A Review on Buccal Drug Delivery System', International Journal of Pharmacy and Pharmaceutical Science Research 3:35–40.

Boddupalli B. (2010) 'Mucoadhesive drug delivery system: An overview', *Journal of Advanced Pharmaceutical Technology & Research*, 1:381-387

Bohorquez M. (1999) 'A Study of the Temperature-Dependent Micellization of Pluronic F127', *Journal of colloid and interface science*, 216:34–40.

Bond L. (2002) 'Differential scanning calorimetry and scanning thermal microscopy analysis of pharmaceutical materials', *International Journal of Pharmaceutics*, 243:71–82.

Borges AF. (2015) 'Oral films: Current status and future perspectives: I-Galenical development and quality attributes', *Journal of Controlled Release*, 206:1–19.

Bozzuto G, Molinari A. (2015) 'Liposomes as nanomedical devices', *International Journal of Nanomedicine*, 10:975–999.

Brannon-Peppas L. (1990) 'Preparation and Characterization of Crosslinked Hydrophilic Networks', *Studies in Polymer Science*, 8:45–66.

Brown SP, Cornforth DM, Mideo N. (2012) 'Evolution of virulence in opportunistic pathogens: Generalism, plasticity, and control', *Trends in Microbiology*, 20:336–342.

Cammack, N. (2001) 'THE POTENTIAL FOR HIV FUSION INHIBITION', *CURRENT OPINION IN INFECTIOUS DISEASES*, 14:13–16.

Carne CA. (1982) 'by pyrexia', *Lancet (London, England)*, 5:1206–1208.

Carr A, Cooper DA. (2000) 'Adverse effects of antiretroviral therapy.', *The Lancet*, 356:1423–1430. .

Carr A, Cooper DA, Penny R. (1991) 'Allergic manifestations of human immunodeficiency virus (HIV) infection', *Journal of Clinical Immunology*, 11:55–64.

Charman HP. (1976) 'Equine infectious anemia virus: evidence favoring classification as a retravirus', *Journal of Virology* 19:1073–1079.

Charrois GJR, Allen TM. (2003) 'Rate of biodistribution of STEALTH® liposomes to tumor and

skin: Influence of liposome diameter and implications for toxicity and therapeutic activity', *Biochimica et Biophysica Acta - Biomembranes*, 1609:102–108.

Chaturvedi A. (2011) 'Fast dissolving films: a review.', *Current drug delivery*, 8(4), 373–80.

Chauhan, A. (2014) 'GC-MS Technique and its Analytical Applications in Science and Technology', *Journal of Analytical & Bioanalytical Techniques*, 5:2-5.

Chawla G. (2003) 'Gastroretention A Means to Address Intestinal Drug Absorption', *Pharmaceutical Technology*, 50:1–12.

Chen J. (2014) 'Drug-in-Cyclodextrin-in-liposomes: a promising delivery system for hydrophobic drugs', *Drug Delivery*, 11:565–577.

Chowdary KPR, Enturi V. (2013) 'Preclinical pharmacokinetic evaluation of efavirenz solid dispersions in two new modified starches', *Journal of Applied Pharmaceutical Science* 3:89–92.

Chowdary KPR, Naresh, A. (2011) 'Formulation development of efavirenz tablets employing β cyclodextrin- PVP K30- SLS: A factorial study', *Journal of Applied Pharmaceutical Science*, 1:130–134.

Christian IN, Rui WMK (2018) 'Conjugation of isoniazid to a zinc phthalocyanine via hydrazone linkage for pH-dependent liposomal controlled release', *Applied Nanoscience 8: 1313-1323*.

Chudasama A. (2015) 'Role of lipid-based excipients and their composition on the bioavailability of antiretroviral self-emulsifying formulations', *Drug Delivery*, 22:531–540.

Chun TW, Fauci AS. (2012) 'HIV reservoirs: Pathogenesis and obstacles to viral eradication and cure', *Aids*, 26:1261–1268.

Cichero JAY. (2013) 'Thickening agents used for dysphagia management: Effect on bioavailability of water, medication and feelings of satiety', *Nutrition Journal*, 12(1), 1–9.

Cilurzo F. (2008) 'Fast dissolving films made of maltodextrins', *European Journal of Pharmaceutics and Biopharmaceutics*, 70:895–900.

Clayton R. (2009) 'Sustained and specific in vitro inhibition of HIV-1 replication by a protease inhibitor encapsulated in gp120-targeted liposomes', *Antiviral Research*, 84:142–149.

Coffin JM. (1986) 'Genetic variation in AIDS viruses', *Cell*, 46:1–4.

Coffin JM. (1992) *genetic diversity and evolution of retroviruses*, *Springer, Berlin, Heidelberg*.

Connolly G. (1989) 'Non-cryptosporidial diarrhoea in human immunodeficiency virus (HIV) infected patients', *GUT*, 30:195–200.

Cooley LA, Lewin SR. (2003) 'HIV-1 cell entry and advances in viral entry inhibitor therapy', *Journal of Clinical Virology*, 26:121–132.

Cooper DA. (1985) 'ACUTE AIDS RETROVIRUS INFECTION. Definition of a Clinical Illness Associated with Seroconversion', *The Lancet*, 325:537–540.

Daraee H. (2016) 'Application of liposomes in medicine and drug delivery', *Artificial Cells, Nanomedicine and Biotechnology*, 44:381–391.

Deniz A. (2010) 'Celecoxib-loaded liposomes: effect of cholesterol on encapsulation and *in vitro* release characteristics', *Bioscience Reports*, 30:365–373.

DeSesso JM, Jacobson CF. (2001) 'Anatomical and physiological parameters affecting gastrointestinal absorption in humans and rats', *Food and Chemical Toxicology*, 39:209–228.

Dinh TTN. (2011) 'Cholesterol content and methods for cholesterol determination in meat and poultry', *Comprehensive Reviews in Food Science and Food Safety*, 10:69–289.

Dixit AR, Rajput SJ, Patel S. (2010) 'Preparation and Bioavailability Assessment of SMEDDS Containing Valsartan', *American Association of Pharmaceutical Scientists*, 11:314–321.

Dixit RP, Puthli SP. (2009) 'Oral strip technology: Overview and future potential', *Journal of Controlled Release*, 139:94–107.

Donnelly R. (2011) 'Mucoadhesive drug delivery systems', *Journal of Pharmacy and Bioallied Sciences*, 3:89–100.

Dubey V. (2010) 'Enhanced transdermal delivery of an anti-HIV agent via ethanolic liposomes', *Nanomedicine: Nanotechnology, Biology, and Medicine*, 6:590–596.

Dwivedi C, Verma S. (2013) 'Review on Preparation and Characterization of Liposomes with Application', *Journal of Scientific & Innovative Research Review*, 2:2320–4818.

Eckert DM, Kim PS. (2001) 'M Echanisms of V Iral M Embrane F Usion and I Ts I Nhibition', *Annual Review of Biochemistry*, 70:777–810.

El-Maghraby, G. M. and Abdelzaher, M. M. (2015) 'Formulation and evaluation of simvastatin buccal film', *Journal of Applied Pharmaceutical Science*, 5:70–77.

El-Nahas AE, Allam AN, El-Kamel AH. (2017) 'Mucoadhesive buccal tablets containing silymarin Eudragit-loaded nanoparticles: formulation, characterisation and ex vivo permeation', *Journal of Microencapsulation*, 34:463–474.

El-Samaligy MS, Afifi NN, Mahmoud EA. (2006) 'Increasing bioavailability of silymarin using a buccal liposomal delivery system: Preparation and experimental design investigation', *International Journal of Pharmaceutics*, 308:140–148.

Elsayed MMA. (2007) 'Lipid vesicles for skin delivery of drugs: Reviewing three decades of research', *International Journal of Pharmaceutics*, 332:1–16.

Eron J. (2006) 'The KLEAN study of fosamprenavir-ritonavir versus lopinavir-ritonavir, each in combination with abacavir-lamivudine, for initial treatment of HIV infection over 48 weeks: a randomised non-inferiority trial', *Lancet*, 368:476–482.

Escape H. (2018) 'HIV-1 escape from a small molecule , CCR5-specific entry inhibitor does not involve CXCR4 use' PNAS 99:395-400.

Escobar-Chavez JJ. (2006) 'Article Reference Guide', *Journal of Pharmacy and Pharmaceutical Sciences*, 9:339–58.

Fauci AS. (2003) 'HIV SPECIAL HIV and AIDS : 20 years of science', *Nature Medicine*, 9(7), 839–843.

Fettig J. (2014) 'Global epidemiology of HIV', *Infectious Disease Clinics of North America*.

28:323–337.

Fröhlich E. (2012) ‘The role of surface charge in cellular uptake and cytotoxicity of medical nanoparticles’, *International Journal of Nanomedicine*, 7:5577–5591.

Gao F. (1999) ‘Origin of HIV-1 in the chimpanzee Pan troglodytes troglodytes’, *Nature*, 397:436–441.

Garg M. (2006) ‘Stavudine-loaded mannosylated liposomes: in-vitro anti-HIV-I activity, tissue distribution and pharmacokinetics’, *Journal of Pharmacy and Pharmacology*, 58(5),605–616.

Garg M. (2008) ‘Radiolabeling, pharmacoscintigraphic evaluation and antiretroviral efficacy of stavudine loaded99mTc labeled galactosylated liposomes’, *European Journal of Pharmaceutical Sciences*, 33:271–281.

Gaur PK. (2014) ‘Enhanced Oral Bioavailability of Efavirenz by Solid Lipid Nanoparticles: In vitro Drug Release and Pharmacokinetics Studies’, *BioMedical Research International*, 2014:1-9.

Ghosal K. (2016) ‘Formulation Development, Physicochemical Characterization and’, *Current HIV Research*, 14:295–306.

Gibbs BF. (1999) ‘Encapsulation in the food industry: a review.’, *International journal of food sciences and nutrition*, 50:213–224.

Gill P, Moghadam TT, Ranjbar B. (2010) ‘Differential scanning calorimetry techniques: applications in biology and nanoscience.’, *Journal of biomolecular techniques,* 21(4),167–93.

Giron D. (2002) ‘Applications of thermal analysis and coupled techniques in pharmaceutical industry’, *Journal of Thermal Analysis and Calorimetry*, 68:335–357.

De Gomes ECL. (2013) ‘Chemical interactions study of antiretroviral drugs efavirenz and lamivudine concerning the development of stable fixed-dose combination formulations for AIDS treatment’, *Journal of the Brazilian Chemical Society*, 24:573–579.

Gomez C, Hope TJ. (2005) ‘The ins and outs of HIV replication’, *Cellular Microbiology*, 7:621–626.

Goyal U. (2012) ‘Self microemulsifying drug delivery system: A method for enhancement of bioavailability’, *International Journal of Pharmaceutical Sciences*, 3:66–79.

Greene WC. (2007) ‘A history of AIDS: Looking back to see ahead’, *European Journal of Immunology*, 37:94–102.

Gupta H, Bhandari D, Sharma A. (2009) ‘Recent trends in oral drug delivery: A Review, *Drug Delivery Formulation* 3:162–173.

Gupta U, Jain NK. (2010) ‘Non-polymeric nano-carriers in HIV/AIDS drug delivery and targeting’, *Advanced Drug Delivery Reviews*, 62:478–490

Haeri A.(2014) ‘Preparation and characterization of stable nanoliposomal formulation of fluoxetine as a potential adjuvant therapy for drug-resistant tumors’, *Iranian Journal of Pharmaceutical Research*, 13:3–14.

Hanif M, Zaman M, Chaurasiya V. (2015) ‘Polymers used in buccal film: A review’, *Designed*

Monomers and Polymers, 18:105–111.

Hansen DL, Tulinius D, Hansen EH. (2008) 'Adolescents' struggles with swallowing tablets: Barriers, strategies and learning', *Pharmacy World and Science*, 30:65–69.

Hawkins T. (2010) 'Understanding and managing the adverse effects of antiretroviral therapy', *Antiviral Research*, 85:201–209.

Helal DA. (2012) 'Formulation and evaluation of fluconazole topical gel', *International Journal of Pharmacy and Pharmaceutical Sciences*, 4:176–183.

Hippalgaonkar K, Majumdar S, Kansara V. (2010) 'Injectable Lipid Emulsions—Advancements, Opportunities and Challenges', *AAPS PharmSciTech*, 11:1526–1540.

Hooper E. (2000) 'Genesis of AIDS: Mother Nature, or the Hand of Man?', *Science as Culture*, 9:73–101.

Hosmani AH, Yogesh S, Gonjari ID. (2013) "Formation and Characterization of Carbopol 971P-PVP Interpolymer complex and its application for sustained delivery of Acyclovir", *Journal of Advance Pharmaceutical Education and Research*, 3:94–101.

Ilett KF. (1990) 'Metabolism of drugs and other Xenobiotics in the gut lumen and wall', *Pharmacology and Therapeutics*, 46:67–93.

Jin SX. (2005) 'Pharmacokinetics and tissue distribution of zidovudine in rats following intravenous administration of zidovudine myristate loaded liposomes', *Pharmazie*, 60:840–843.

Jones E. (2014) 'In vitro comparative evaluation of monolayered multipolymeric films embedded with didanosine-loaded solid lipid nanoparticles: A potential buccal drug delivery system for ARV therapy', *Drug Development and Industrial Pharmacy*, 40:669–67.

Joseph M, Catanzaro MJ, Graham Smith JrMD, Augusta G. (1991) 'Propylene glycol dermatitis', *journal of the American Academy of Dermatology*, 24:90–5.

Karami N, Moghimipour E, Salimi A. (2018) 'Liposomes as a Novel Drug Delivery System: Fundamental and Pharmaceutical Application', *Asian Journal of Pharmaceutics*, 12:31–41.

Karki S. (2016) 'Thin films as an emerging platform for drug delivery', *Asian Journal of Pharmaceutical Sciences*. Elsevier B.V., 11:559–574.

Kirby C, Clarke J, Gregoriadis G. (1980) 'Effect of the cholesterol content of small unilamellar liposomes on their stability in vivo and in vitro.', *The Biochemical journal*, 186:591–598.

Kirchhoff F. (2010) 'Immune evasion and counteraction of restriction factors by HIV-1 and other primate lentiviruses', *Cell Host and Microbe*, 8:55–67.

Koyama TM. (1999) 'Characterizing the Gel to Liquid Crystal Transition in Lipid-Bilayer Model Systems', *Transition*, 4171:12–15.

Kumar S. (2013) 'Drug Carrier Systems for Solubility Enhancement of BCS Class II Drugs: A Critical Review', *Critical Reviews in Therapeutic Drug Carrier Systems*, 30:217–256.

Kumria R. (2016) 'Buccal films of prednisolone with enhanced bioavailability', *Drug Delivery*, 23:471–478.

Landova H. (2014) ‘Evaluation of the Influence of Formulation and process Variables on Mechanical properties of Oral Films Using Multivariate Data Analysis’, *BioMedical Research International*, 18:1–9.

Lasic DD. (1998) ‘Novel applications of liposomes’, *Trends in Biotechnology*, 16:307–321.

Lau ETL. (2018) ‘Dosage form modification and oral drug delivery in older people’, *Advanced Drug Delivery Reviews*.

Lavelle EC. (1995) ‘The importance of gastrointestinal uptake of particles in the design of oral delivery systems’, *Advanced Drug Delivery Reviews*, 18:5–22.

Lawn SD. (2004) ‘AIDS in Africa: The impact of coinfections on the pathogenesis of HIV-1 infection’, *Journal of Infection*, 48:1–12.

Lazzarini Z. (2009) ‘Lessons from the rise and fall of the military AIDS hypothesis: politics, evidence and persuasion’, *Lancet*, 18:436–454.

Levy JA. (1993) ‘The transmission of HIV and factors influencing progression to AIDS’, *The American Journal of Medicine*, 95:86–100.

Levy JA. (2006) ‘H I V P a t h o g e n e s i s : K n o w l e d g e G a i n e d a f t e r T w o D e c a d e s o f R e s e a r c h’, Advanced Dent Res 19:10–16.

Li J. (2014) ‘A review on phospholipids and their main applications in drug delivery systems’, *Asian Journal of Pharmaceutical Sciences*, 10:81–98.

Li SD, Huang L. (2010) ‘Stealth nanoparticles: High Density but Sheddable PEG is a Key for Tumor Targeting’, *Journal of Contolled Release*, 145:178–181.

Li X, Chan WK. (1999) ‘Transport, metabolism and elimination mechanisms of anti-HIV agents’, *Advanced Drug Delivery Reviews*, 39:81–103.

Lian T, Ho RJ. (2001) ‘Trends and developments in liposome drug delivery systems.’, *Journal of pharmaceutical sciences*, 90:667–680.

Limasale YDP. (2015) ‘Epidermal growth factor receptor-targeted immunoliposomes for delivery of celecoxib to cancer cells’, *International Journal of Pharmaceutics*, 479:364–373.

Lin JH, Chiba M, Baillie TA. (1999) ‘Is the role of the small intestine in first-pass metabolism overemphasized?’, *Pharmacological reviews*, 51:135–158.

Doelker C, Doelker E, Buri P, Waginaire L. (1986) ‘In vitro release profiles of liquid, deliquescent or unstable drugs with fusible excipients in’, Drug Development and Industrial Pharmacy 12:1553–1565.

Longmire M, Choyke PL, Kobayashi H. (2008) ‘Clearance Properties of Nano-sized Particles and Molecules as Nanomedicine’, *Nanomedicine*, 3:703–717.

Lotan M, Zysman L. (2006) ‘The digestive system and nutritional considerations for individuals with Rett syndrome’, *The Scientific World Journal*, 2:86–88.

Lulio J. di. (2009) ‘In vivo analysis of efavirenz metabolism in individuals with impaired CYP2A6 function’, *Pharmacogenetics and genomics*, 19:300–309.

Mansoori MA, Agrawal S, Jawade S, Khan MI. (2012) 'A Review on Liposome', *International Journal of Advanced Research in Pharmaceutics and Bio Sciences*, 2:453–464.

Anitha LMR, Neema A, Rinu V, Lekshmi L, Mohana MN, Nikhila MN. (2016) 'Buccal Mucoadhesive drug delivery system: A novel drug delivery technique', *European Journal of Pharmaceutical and Medical Research*, 3:129–137.

Ramya Teja M*, Ramy Reddy GV, Jhansi Lakshmi G, Sudheer B.* (2015) 'Formulation and evaluation of fast dissolving Lamivudine oral thin films : an innovation in paediatric medication', *Indian Journal of Research in Pharmacy and Biotechnology*, 3:423–428.

Mozafari RM. (2005) 'Liposomes: An overview of manufacturing techniques', Cellular and Molecular Biology Letters 10:711–719.

MacArio A. (2013) 'Pure silica nanoparticles for liposome/lipase system encapsulation: Application in biodiesel production', *Catalysis Today*, 20:148–155.

Machado A*.* (2016) 'Development and in vivo safety assessment of tenofovir-loaded nanoparticles-in-film as a novel vaginal microbicide delivery system', *Acta Biomaterialia*, 44:332–340.

Madhavi Bb. (2011) 'Dissolution enhancement of efavirenz by solid dispersion and PEGylation techniques',*International Journal of Pharmaceutical Investigation*, 1:29-33

Mahungu T, Owen A. (2011) *Current Progress in the Pharmacogenetics of Infectious Disease Therapy*. 1st *ed. Genetics and Evolution of Infectious Diseases*.

Mallipeddi R, Rohan LC. (2010) 'Progress in antiretroviral drug delivery using nanotechnology', *International Journal of Nanomedicine*, 5:533–547.

Manconi M. (2009) 'Development and characterization of liposomes containing glycols as carriers for diclofenac', *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 342:53–58.

Marimutho, J. (2016) 'Formulation and evaluation of zidovudine mucoadhesive buccal patches', *International Journal of Pharmacology and Pharmaceutical Sciences*, 3:30–40.

Marques De Assis L*.* (2014) 'Development and Characterization of Nanovesicles Containing Phenolic Compounds of Microalgae Spirulina Strain LEB-18 and Chlorella pyrenoidosa', *Advances in Materials Physics and Chemistry*, 4:6–12.

Marzolini C. (2001) ''`Efavirenz plasma levels can predict treatment failure and central nervous system side effects in HIV-1-infected patients'', *Aids*, 15:1193–1194.

Mcdonagh EM. (2015) 'PharmGKB Summary: Efavirenz Pathway, Pharmacokinetics (PK)', *HHS Public Access*, 25:363–376.

Jeffrey Greenwald L*.* (2006) 'A rapid review of rapid {HIV} antibody tests', *Current Infecteous Disease Report*, 8:125–131.

Merson MH. (2008) 'The history and challenge of HIV prevention', *The Lancet*, 372:475–488.

Mitsuya H, Broder S. (1987) 'Strategies for antiviral therapy in AIDS', *Nature*, 325, 773–778.

Morbidity and Mortality Weekly Report (2011) *Morbidity and Mortality Weekly Report*,

MMWR. Morbidity and mortality weekly report, 60:689-693.

Moghimi SM, Farhangrazi ZS. (2013) 'Nanomedicine and the complement paradigm', *Nanomedicine: Nanotechnology, Biology, and Medicine*, 9:458–460.

Moir S. (2010) 'B cells in early and chronic HIV infection: Evidence for preservation of immune function associated with early initiation of antiretroviral therapy', *Blood*, 116:5571–5579.

Morales JO, McConville JT. (2011) 'Manufacture and characterization of mucoadhesive buccal films', *European Journal of Pharmaceutics and Biopharmaceutics*, 77:187–199.

Nafee NA. (2003) 'Design and characterization of mucoadhesive buccal patches containing cetylpyridinium chloride.', *Acta pharmaceutica* 53:199–212.

Nagayama S. (2007) 'Time-dependent changes in opsonin amount associated on nanoparticles alter their hepatic uptake characteristics', *International Journal of Pharmaceutics*, 342:215–221.

Naif HM. (2013) 'Pathogenesis of HIV infection', *Infectious Disease Reports*, 5,26–30.

Nair AB. (2013) 'In vitro techniques to evaluate buccal films', *Journal of Controlled Release*, 166:10–21.

Nair AS.(2014) 'Mucoadhesive buccal patch of Cefixime trihydrate using biodegradable natural polymer', *International Journal of Pharmacy and Pharmaceutical Sciences*, 6:366–371.

Nayak AK, Malakar J, Sen KK. (2010) 'Gastroretentive drug delivery technologies: current approaches and future potential.', *Journal of Pharmaceutical Education and Research*, 1:1–12.

Nkanga CI., Rui W K. (2018) 'Conjugation of isoniazid to a zinc phthalocyanine via hydrazone linkage for pH-dependent liposomal controlled release', *Applied Nanoscience*. Springer International Publishing, 8:1313–1323.

Noble GT. (2014) 'Ligand-targeted liposome design: Challenges and fundamental considerations', *Trends in Biotechnology*, 32:32–45.

Ochubiojo ME. (2012) 'Nanotechnology in Drug Delivery', in *Recent Advances in Novel Drug Carrier Systems*, In: Ali Demir S (ed) IntecOpen 3rd ed., London P70–106.

Ogburn ET. (2010) 'Efavirenz primary and secondary metabolism in vitro and in vivo: Identification of novel metabolic pathways and cytochrome P450 2A6 as the principal catalyst of efavirenz 7-hydroxylation', *Drug Metabolism and Disposition*, 38:1218–1229.

Ogunwuyi O. (2016) 'Antiretroviral Drugs-Loaded Nanoparticles Fabricated by Dispersion Polymerization with Potential for HIV/AIDS Treatment', *Infectious Diseases: Research and Treatment*.

Ojewole E. (2008) 'Exploring the use of novel drug delivery systems for antiretroviral drugs', *European Journal of Pharmaceutics and Biopharmaceutics*, 70:697–710.

Omwoyo WN. (2014) 'Preparation, characterization, and optimization of primaquine-loaded solid lipid nanoparticles', *International Journal of Nanomedicine*, 9:3865–3874.

Oussoren C. (1999) 'Liposomes as carriers of the antiretroviral agent dideoxycytidine-5'-

triphosphate', *International Journal of Pharmaceutics*, 180:261–270.

Pai R, Devi K. (2009) 'Lamivudine liposomes for transdermal delivery-formulation, characterization, stability and in vitro evaluation', *International Journal of Pharmaceutical Science and Nanotechnology*, 1:317–326.

Pandey H, Rani R, Agarwal V. (2016) Liposome and Their Applications in Cancer Therapy Human & Animal Health', Brazian *Archieves of Biology Technology*,5:2-10.

Panwar P. (2010) 'Study of Albendazole-Encapsulated Nanosize Liposomes', *International Journal of Nanomedicine*, 5:101–108.

Patel AR, Patel DA, Chaudhry SV. (2011) 'Mucoadhesive buccal drug delivery system: A review', *International Journal Of Pharmaceutics and Life Science*, 2:848–856.

Patel DA. (2012) 'Buccal Mucosa As A Route For Systemic Drug Delivery : A Review', *International Journal of Drug Development and Research*, 4:99–116.

Patel DA. (2016) 'Formulation and Evaluation of Fast Mouth Dissolving Chewable', *International Journal of Advanced Research in Science Management and Technology*, 2:1–8.

Patel PS. (2013) 'Buccal Drug Delivery System: A Review', *International Journal of Drug Development and Research,* 5:35–48.

Patel VM, Prajapati BG, Patel MM. (2007) 'Effect of hydrophilic polymers on buccoadhesive Eudragit patches of propranolol hydrochloride using factorial design.', *AAPS PharmSciTech*, 8:1-8.

Patil P, Shrivastava, S. K. (2014) 'Fast Dissolving Oral Films : a Novel Drug Delivery System', *International Journal of Science and Research (IJSR)*, 3:2088–2093.

Patil YP, Jadhav S. (2014) 'Novel methods for liposome preparation', *Chemistry and Physics of Lipids*, 177:8–18.

Patravale VB, Mandawgade SD. (2008) 'Novel cosmetic delivery systems: An application update', *International Journal of Cosmetic Science*, 30:19–33.

Pattni BS, Chupin, VV, Torchilin VP. (2015) 'New Developments in Liposomal Drug Delivery', *Chemical Reviews*, 115:10938–10966.

Patwa S. (2011) 'An Overveiw On Fast Dissolving Tablet', *WebmedCentral Pharmaceutical Sciences*, 2:1–16.

Pillay D, Richman DD. (1995) 'MEDICAL HIV-I Protease Inhibitors : Their Development , Mechanism of Action and Clinical Potential U' 33:23–33.

Popovic M. (1984) 'Detection, Isolation, and Continuous Production of Cytopathic Retroviruses (HTLV-111) from Patients with AIDS and Pre-AIDS', *Science*, 224:497–500.

Pradhan B.(2015) 'Journal of Apllied Pharmaceutical Research', *Journal of Applied Pharmaceutical Research*, 3:2348-0335.

The Joint United Nation Programme on HIV/AIDS; Global HIV Statistics *(2018) .*

Puratchikody A. (2011) 'Mucoadhesive Patches of Salbutamol Sulphate for Unidirectional

Buccal Drug Delivery: Development and Evaluation', *Current Drug Delivery*, 8:416–425.

Rabeneck L. (1990) 'Acute HIV Infection Presenting With Painful Swallowing and Esophageal Ulcers', *The Journal of the American Medical Association*, 263:2318–2322.

Raghavendra Rao NG. (2012) 'Development and evaluation of ropinirole mucoadhesive patches for buccal drug delivery', *Drug Invention Today*, 4:519–526.

Ramana LN. (2010) 'Development of a liposomal nanodelivery system for nevirapine', *Journal of Biomedical Science*, 17:1–9.

Ramana LN. (2012) 'Investigation on the stability of saquinavir loaded liposomes: Implication on stealth, release characteristics and cytotoxicity', *International Journal of Pharmaceutics*, 431:120–129.

Ramana LN. (2014) 'Targeting strategies for delivery of anti-HIV drugs', *Journal of Controlled Release*,192:271–283.

Rani DT. (2013) 'Liposome as a potential drug delivery system: A review', *International research journal of pharmacy*, 4:6-12.

Roy SK, Prabhakar BR. (2010) 'Design and characterization of acrylate based buccoadhesive tablets of diltiazem hydrochloride', *International Journal of ChemTech Research*, 2:965–972.

Sahoo SK, Labhasetwar V. (2003) 'Nanotech approaches to drug delivery and imaging', *Drug Discovery Today*, 8:1112–1120.

Salisbury BG. (1995) 'Hypocholesterolemic activity of a novel inhibitor of cholesterol absorption, SCH 48461', *Atherosclerosis*, 115:45–63.

Sathigari S. (2009) 'Physicochemical Characterization of Efavirenz–Cyclodextrin Inclusion Complexes', *AAPS PharmSciTech*, 10:81–87.

Sathigari SK (2012) 'Amorphous -State Characterization Of Efavirenz-Polymer, Hot-Melt Extrusion Systems for Dissolution Enhancement', *Journal of pharmaceutical sciences*, 101:3456–3464.

Sellon DC, Fuller, FJ, Mcguire TC. (1994) 'V irus anemia virus', 32:111–138.

Senapati PC, Sahoo SK, Sahu AN. (2016) 'Mixed surfactant based (SNEDDS) self-nanoemulsifying drug delivery system presenting efavirenz for enhancement of oral bioavailability', *Biomedicine and Pharmacotherapy*, 80:42–51.

Şenel S. (2000) 'Chitosan films and hydrogels of chlorhexidine gluconate for oral mucosal delivery', *International Journal of Pharmaceutics*, 193:197–203.

Şenel S, Hincal AA. (2001) 'Drug permeation enhancement via buccal route: Possibilities and limitations', *Journal of Controlled Release*, 72:133–144.

Sercombe L. (2015) 'Advances and challenges of liposome assisted drug delivery', *Frontiers in Pharmacology*, 6:1–13.

Shah LK, Amiji MM. (2006) 'Intracellular delivery of saquinavir in biodegradable polymeric nanoparticles for HIV/AIDS', *Pharmaceutical Research*, 23:2638–2645.

Sharma A, Sharma US. (1997) 'Liposomes in drug delivery: Progress and limitations', *International Journal of Pharmaceutics*, 154:123–140.

Sharp PM, Hahn BH. (2011) 'Origins of HIV and the AIDS Pandemic, Cold spring Habor Perspectives in Medicine;a006841.

Shojaei AH. (1998) 'Buccal Mucosa As A Route For Systemic Drug Delivery : A Review Epithelium Lamina Propria', *J Pharm Pharmaceutical Sciences*, 1:15–30.

Siepmann J, Peppas NA. (2012) 'Modeling of drug release from delivery systems based on hydroxypropyl methylcellulose (HPMC)', *Advanced Drug Delivery Reviews*, 64:163–174.

Silva BMA. (2015) 'Mucoadhesive oral films: The potential for unmet needs', *International Journal of Pharmaceutics*. Elsevier B.V., 494:537–551.

Simon V, Ho DD, Karim QA. (2006) 'HIV/AIDS epidemiology, pathogenesis, prevention, and treatment', *Lancet*, 368(5),489–504.

Singh H, Kaur M, Verma H. (2013) 'Optimization and Evaluation of Desloratadine Oral Strip: An Innovatio in Paedatric Medication', *The scientific Worlld Journal*, 2:1–9.

Škalko N. (1998) 'Morphological observations on liposomes bearing covalently bound protein: Studies with freeze-fracture and cryo electron microscopy and small angle X-ray scattering techniques', *Biochimica et Biophysica Acta - Biomembranes*, 1370:151–160.

Solaro R, Chiellini F, Battisi A. (2010) 'Targeted Delivery of Protein Drugs by Nanocarriers', *Materials*, 3:1928–1980.

Storm G, Crommelin, D. J. . (1998) 'Liposomes: quo vadis?', *Pharmaceutical science and technology today*, 1:19–31.

Sudhakar B, Krishna MC, KVR. (2016) 'Factorial design studies of antiretroviral drug-loaded stealth liposomal injectable: PEGylation, lyophilization and pharmacokinetic studies', *Applied Nanoscience*. Springer Berlin Heidelberg, 6:43–60.

Sungkanuparph S. (2007) 'Options for a Second-Line Antiretroviral Regimen for HIV Type 1 – Infected Patients Whose Initial Regimen of a Fixed-Dose Combination of Stavudine ', *Clinical Infectious Diseases*, 44:12–15.

Van Swaay D, Demello A. (2013) 'Microfluidic methods for forming liposomes', *Lab Chip*, 13:752–767.

Swamy SK, Reddy LN, Goud BA.(2014) 'Development and in vitro evaluation of bioadhesive buccal tablets of Hydralazine hydrochloride', *International Journal of Pharmacy Education and Research*, 1:8–16.

Tarkunde SV. (2014) 'Liposome – A Novel Drug Delivery', *International Journal of Pure and Applied Bioscience*, 2:92–102.

Teixeira C. (2011) 'Viral surface glycoproteins, gp120 and gp41, as potential drug targets against HIV-1: Brief overview one quarter of a century past the approval of zidovudine, the first anti-retroviral drug', *European Journal of Medicinal Chemistry*, 46:979–992. .

Tindall B. (1988) 'Characterization of the acute clinical illness associated with HIV infection',

Arch Intern Med, 148:945–949.

Torchilin VP. (2005) 'Recent advances with liposomes as pharmaceutical carriers', *Nature Reviews Drug Discovery*, 4:145–160.

Torchilin VW. (2003) *Liposomes: A Practical Approach*. Second. Edited by V. W. Torchilin. New York: Oxford University Press.

Uhumwangho MU. (2005) 'Current trends in the production and biomedical applications of liposomes: membrane vesicles; a review.', *J. Med. Biomed. Res.*, 4: 9–21.

UNAIDS (2013) *Global HIV Report*.

UNAIDS (2015) '*AIDS by the Numbers 2015*: 1–11.

Global AIDS Update,*World Health Organization*/UNAIDS (2016).

Uziely B. (1995) 'Liposomal doxorubicin: Antitumor activity and unique toxicities during two complementary phase I studies', *Journal of Clinical Oncology*, 13:1777–1785.

Vajpayee M, Mohan T. (2011) 'Current practices in laboratory monitoring of HIV infection', *Indian J Med Res*, 134:801–822.

Vemuri S, Rhodes CT. (1994) 'Development and Characterization of a Liposome Preparation by a pH-Gradient Method', *Journal of Pharmacy and Pharmacology*, 46: 778–783.

Venkatalakshmi R. (2012) 'Buccal drug delivery using adhesive polymeric patches', *International Journal of Pharmaceutical Sciences*, 3:35–41.

Verma S. (2011) 'An overview on buccal drug delivery system', *International Journal of Pharmaceutical Sciences and Research*, 2:1303–1321.

Wang J. (2006) 'Identification of a novel specific CYP2B6 allele in Africans causing impaired metabolism of the HIV drug efavirenz.', *Pharmacogenetics and genomics*, 16:191–198.

Warnke D, Barreto J, Temesgen Z. (2007) 'Therapeutic review: Antiretroviral drugs', *Journal of Clinical Pharmacology*, 47:1570–1579.

Watson ES. (1964) 'A Differential Scanning Calorimeter for Quantitative Differential Thermal Analysis', *Analytical Chemistry*, 36:1233–1238.

Witvrouw M. (2004) 'Novel inhibitors of HIV-1 integration', *Curr Drug Metab*, 5(4), 291–304.

Wu CY. (1995) 'Differentiation of absorption and first-pass gut and hepatic metabolism in humans: Studies with cyclosporine', *Clinical Pharmacology and Therapeutics*, 58:492–497.

Xu Q, Tanaka Y, Czernuszka JT. (2007) 'Encapsulation and release of a hydrophobic drug from hydroxyapatite coated liposomes', *Biomaterials*, 28:2687–2694.

Yanamandra S. (2014) 'Proliposomes as a drug delivery system to decrease the hepatic first-pass metabolism: Case study using a model drug', *European Journal of Pharmaceutical Sciences*, 64:26–36.