DEVELOPMENT AND CHARACTERIZATION OF BRAIN TARGETED NIOSOMAL FORMULATIONS OF EMTRICITABINE TO TREAT HIV ASSOCIATED CNS DISORDERS

A THESIS

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1. INTRODUCTION

Over the past two decades, acquired immunodeficiency syndrome (AIDS) which is caused by HIV (Human immunodeficiency virus) has posed a serious life threatening problem for patients suffering from the disease. Upon invading the human body, the HIV attacks the CD4 (cluster of differentiation 4) lymphocytes of the human body and depletes the body's immune system. This increases the body's susceptibility to other infections called opportunistic infections like tuberculosis, toxoplasmosis, Kaposi's sarcoma and encephalitis. With the advent of antiretroviral therapy, though mortality as a result of HIV is significantly reduced, survival with increased chances of opportunistic infections and HIV-associated neurocognitive disorders have been observed, as most of these infections develop before the detection of HIV and commencement of antiretroviral therapy.

1.1. ACQUIRED IMMUNODEFICIENCY SYNDROME (AIDS)

1.1.1. STRUCTURE OF HIV

HIV is a retrovirus, a class of virus with subgroup lentivirus. The genes of retroviruses compose of RNA (Ribonucleic acid), unlike others that compose of DNA (Deoxyribonucleic acid). HIV occurs as particles (virions) outside the host cell (human cell) with about 0.1µ diameter.

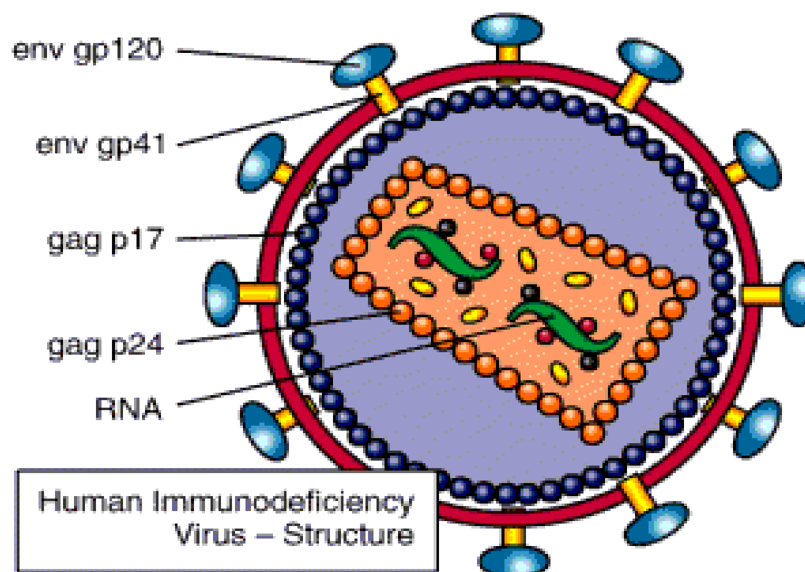


Figure 1: Structure of Human immunodeficiency virus

The HIV structure consists of the following:

1. Viral envelope (membrane), a fatty coat.
2. Around 72 spikes made up of gp120 (glycoprotein 120) and gp41 (glycoprotein 41) that protrude from the viral envelope.
3. Matrix made up of the protein p17 (protein 17), just below the viral envelope.
4. Capsid (viral core) which is bullet-shaped and made up of the protein p24 (protein 24).
5. The enzymes responsible for viral replication like reverse transcriptase, protease and integrase.
6. Two identical strands of RNA (Ribonucleic acid) within the core which constitutes the genetic material for HIV.
7. HIV has 9 genes, three of which, i.e., gag (group-specific antigen), pol (a polymerase that contains genetic coding for enzyme reverse transcriptase, protease, and integrase) and env (envelope) are structural proteins necessary for producing new virus particles. The remaining six, i.e., tat (HIV trans-activator), rev (regulator of expression of virion), nef (negative factor), vif (viral infectivity factor), vpr (viral protein R) and vpu (viral protein U). These contain proteins in a particular code that are responsible for the virus to infect, copy and cause disease.
8. HIV replication is controlled by a sequence that is present at the long terminal end of each strand of RNA.

1.1.2. LIFE CYCLE OF HIV

HIV can replicate only inside human cells. HIV particles upon invading the host get attached to a host containing a special surface protein called CD4. The spikes of the virus get attached to the CD4 occurs, followed by the fusion of the viral envelope with the human cell membrane. This results in the release of the contents of HIV particles in the human cell with the envelope left behind.

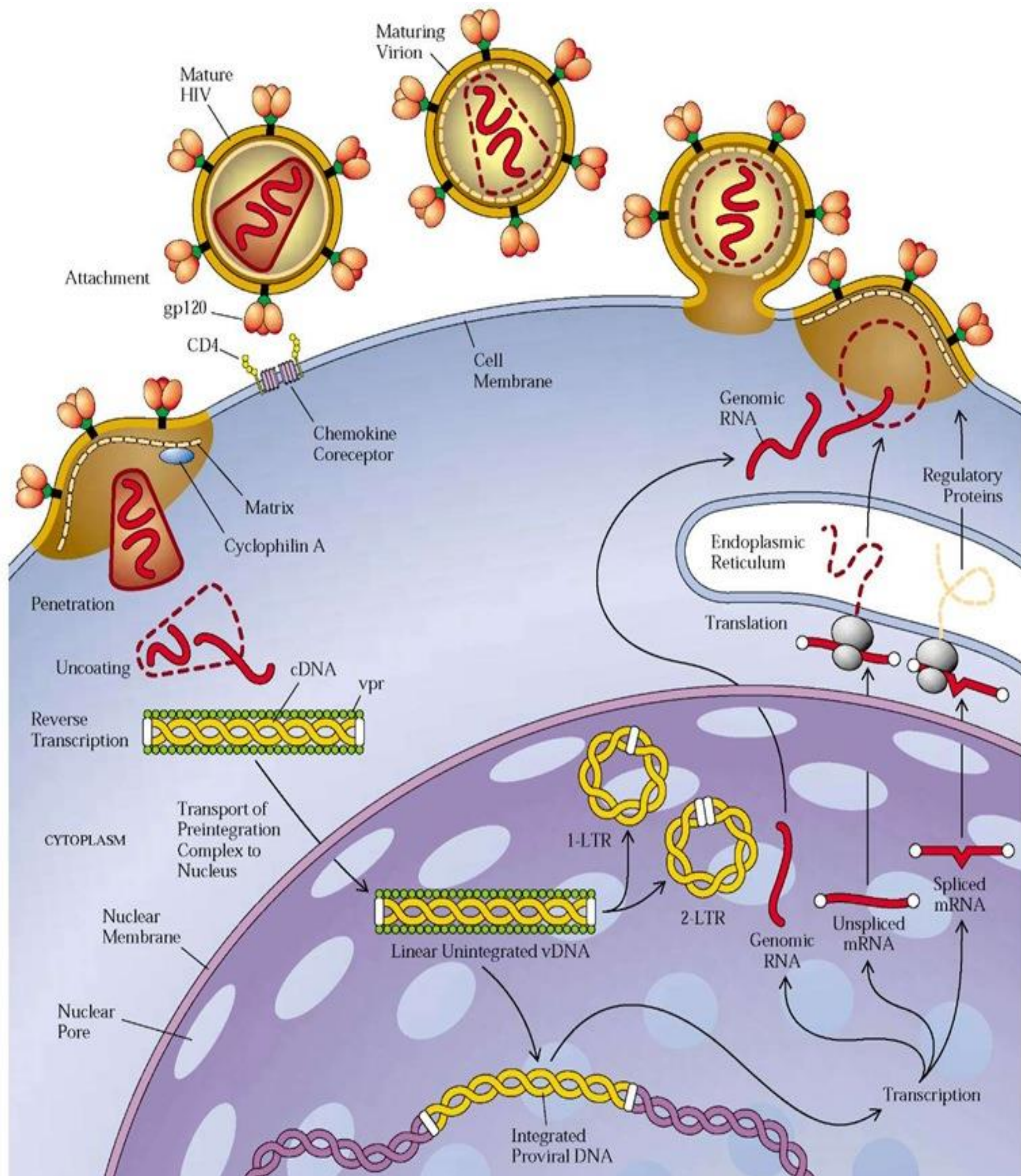


Figure 2: Life cycle of HIV

After the entry of HIV, the following changes take place in the human cell.

1. Reverse transcription and integration

The HIV enzyme reverse transcriptase converts the viral RNA into DNA which is compatible with the genetic material of host. The DNA is transported into the cell's nucleus and integrated into human DNA by the HIV enzyme integrase. This integrated HIV DNA is known as provirus.

2. Transcription and translation

The HIV provirus remains dormant within the cell for a long time. Upon activation of the cells, the HIV genes are recognized as human genes and converted to messenger RNA with the help of human enzymes. This messenger RNA gets transported out of the nucleus and this acts as a blueprint for producing HIV enzymes and proteins.

3. Assembly, budding, and maturation:

The new strands of messenger RNA contain the genetic sequence of HIV which combines with the newly formed proteins and enzymes to form HIV viral particles. These are released from the cell by budding which is chopped into smaller pieces by the enzyme protease. These are used to form the mature viral cores which are capable of infecting another cell and further continuation of viral replication takes place. Thus the virus spreads quickly and the person is capable of infecting others through body fluids.[1]

1.1.3. MODE OF TRANSMISSION

HIV is transmitted from person to person in the following ways:

1. Unprotected sexual intercourse.
2. From infected person by oral sex.
3. Blood transfusion of contaminated blood.
4. Sharing of needles with infected person during drug abuse.
5. Occupational exposure (sticking of needles with infected blood).
6. Insemination of infected semen artificially.
7. Organ transplantation from HIV infected donor.
8. Transmission from mother to newborn before birth or by breastfeeding.
9. HIV does not spread through kissing, touching, sharing and usage of food utensils, beds, towels, toilet seats or telephones. It does not spread by insect or mosquito bites.

1.1.4. SYMPTOMS

Symptoms of early stages include flu-like symptoms with

1. Fever
2. Sore throat
3. Rash
4. Nausea
5. Vomiting
6. Fatigue
7. Muscle aches
8. Diarrhoea
9. Swollen lymph nodes
10. Headaches
11. Joint pain and
12. Meningitis (in some cases)

With progression of the disease, a CD4 drop to below normal level (800-1,200 cells/mm^3 of blood) is observed with the following symptoms:

1. Swollen lymph nodes
2. Seborrheic dermatitis
3. Psoriasis
4. Minor infections
5. Ulcers around the mouth
6. Herpes infection
7. Varicella-zoster infection
8. Fever
9. Diarrhoea
10. Joint and muscle pain
11. Weight loss
12. Fatigue and
13. Reactivation of old tuberculosis

With further drop in CD4 levels, the person develops AIDS (Acquired immunodeficiency syndrome) with the following symptoms:

1. Decrease in CD4 levels to less than 200 cells/mm^3 of blood.
2. Pneumonia, diarrhoea, eye infections or meningitis
3. Cancers like cervical cancer, Kaposi's sarcoma, certain types of non-Hodgkin's lymphoma or brain lymphoma.
4. HIV encephalopathy also known as AIDS dementia or progressive multifocal leukoencephalopathy.
5. HIV wasting syndrome.
6. Pulmonary lymphoid hyperplasia.
7. Lymphoid interstitial pneumonia (only in children).

1.1.5. PREVENTION

The risk of HIV infection can be minimized by:

1. Having sex with single partner who also has sex with the same single partner or using condoms.
2. Avoiding the usage of shared needles in case of intravenous drug administration.
3. Strictly following universal precautions to prevent contact with bodily fluids, if the person is a health worker involved with HIV patients.
4. Woman in the fertile period planning to become pregnant must have a prior test for HIV, especially if more prone for HIV infection.
5. Providing special prenatal care and medications to HIV-positive pregnant women such that the risk that HIV will pass to their newborn babies will be minimized.

1.1.6. DIAGNOSIS

HIV is diagnosed by:

1. Evaluation of previous exposure to risk factors.
2. Checking for symptoms of HIV. Kaposi's sarcoma or recurrent infections also suggest the diagnosis of AIDS.
3. Blood tests:

Enzyme linked immunosorbent assay (ELISA) which detects the antibodies specific to HIV as initial screening test and Western blot test, which also measures the body's response to HIV as confirmation test. Both these do not give accurate result after immediate exposure to HIV and give positive results after few months of exposure.

The time period between exposure to HIV and obtaining positive results with the tests is called the window period. If the results are positive, the HIV RNA blood test is performed, which measures the viral load (amount of HIV virus in the blood). Confirmation of AIDS is done by determining the CD4 cell count in blood when the count is less than 200 cells/mm^3 of blood.

Other tests used to detect AIDS-related opportunistic infections like brain illness, lung illness, tumour or body wasting.

1.1.7. EXPECTED DURATION

HIV is a life-long process.

1.1.8. TREATMENT

A combination of anti-retroviral drugs is used for the treatment of AIDS. With an effective anti-retroviral therapy, a decrease in viral load in the body with increase in CD4 count can be attained but complete elimination of HIV virus from the body is questionable.

The anti-retroviral drugs that are currently available includes:

i. **Nucleoside reverse transcriptase inhibitors (NRTI)**
   Zidovudine (Retrovir, AZT), abacavir (ABC), didanosine (Videx, ddl), lamivudine (Epivir, 3TC), stavudine (Zerit, d4T), tenofovir (Viread) and Combivir, a combination of lamivudine and zidovudine.
ii. **Protease inhibitors (PI)**
   Saquinavir (Invirase, Fortovase), indinavir (Crixivan), ritonavir (Norvir), nelfinavir (Viracept) and combination of lopinavir and ritonavir (Kaletra).
iii. **Non-nucleoside reverse transcriptase inhibitors (NNRTI)**
   Nevirapine (Viramune) and efavirenz (Sustiva).
iv. **Fusion inhibitors (FI)**
   T-20 (Fuzeon)

The highly active antiretroviral therapy (HAART), which combines three nucleoside analogues, two nucleoside analogues and one protease inhibitor, or two nucleoside analogues and one non-nucleoside reverse transcriptase inhibitor, is used nowadays. Variations of these combinations are also available. Fusion inhibitors are the preferred drug of choice for patients infected with HIV strain that is drug resistant.

In addition to these medications, people with low CD4 counts may be prescribed with medications for the prevention and treatment of opportunistic infections.

1.1.9. PROGNOSIS

On an average, the progression time of HIV infection to AIDS is about 10 to 11 years in the absence of antiviral medication or with the use of one antiviral drug (monotherapy). AIDS develops sooner in about 20% of patients with high viral load (5 years after infection). In about 2% of cases, it takes greater than 12 years followed by infection.

Progression of HIV infection to AIDS and subsequently to death varies interpersonally (immediate death after diagnoses to survival for 12 years or more).[2]

1.2. HIV AND BRAIN

It is observed that, suppression of viral load in the body with the advent of highly active antiretroviral therapy (HAART) has increased the morbidity and mortality rates as a result of infection but increased the survival rates with mild to moderate neurocognitive impairment like memory loss and learning challenges.

The infection of the CNS with HIV-1 virus is clinically manifested as HIV-1 associated dementia (HAD) and HIV-associated neurocognitive disorders (HAND). HAND is associated with sub cortical events like cognition (thinking), behavior and motor dysfunctional changes.

1.2.1 SYMPTOMS OF NEUROCOGNITIVE IMPAIRMENT

The symptoms of neurocognition include:

1. Diminished concentration or attention
2. Altered mental status or confusion
3. Short-term or long-term memory impairment
4. Reduced problem-solving or calculation ability
5. Decreased ability in future planning
6. Reduced ability in learning new things
7. Speech and language comprehension changes
8. Changes in vision
9. Slowing in psychomotor activities
10. Impaired movement
11. Reduced fine motor skills
12. Decreased coordination
13. Mood changes
14. Changes in personality
15. Changes in behaviour

1.2.2. RISK FACTORS INVOLVED WITH NEUROCOGNITIVE IMPAIRMENT

The risk factors involved with neurocognitive impairment are:

1. Immune suppression
2. Older age
3. Metabolic risk factors
4. HIV viral factors
5. Hepatitis co infection

   Others like poor education level, depression, use of recreational drugs (methamphetamine) and high level of alcohol intake.

1.2.3. EFFECT OF HIV INFECTION ON NEURODEGENERATION OF BRAIN

Neurodegeneration occurs due to effect of HIV virus on the brain and neuronal cells which may have a direct and indirect impact.

1. **Direct methods**

- Increased levels of calcium, increased reactive oxygen species and caspase activation of the apoptotic pathway by HIV-1 tat.
- Greater permeability of the blood brain barrier resulting in increased infiltration of infected cells into the CNS by tat.
- Arresting of G2/M cell cycle phase leading to neuronal death by HIV-1 vpr.
- Activation of protein kinase, a stress kinase by the double-stranded RNA leads to decreased signaling effect on the NMDA receptor that results in neurotoxicity.

2. **Indirect methods**

Persistant infection of monocytes, lymphocytes and microglia in the brain leads to release of cytokines, reactive oxygen species and other neurotoxins (TNF-α, arachidonic acid, quinolinic acid and nitric oxide) resulting in neuronal apoptosis. These cascade a series of changes that lead to glial inflammatory responses and ultimate neurodegeneration.[4]

1.2.4. THE SIGNIFICANCE OF EFFECTIVE PENETRATION OF ANTI-RETROVIRAL DRUGS TO THE BRAIN TISSUE

The pharmacological and physicochemical variations of antiretroviral drugs alter the penetration effectiveness of such drugs into the brain. This explain the fact that the effective concentration of these drugs is not attained to suppress the viral load in the central nervous system (CNS) as compared to plasma with added challenge of the evolution of mutant and drug resistant strains in the CNS. Various studies have suggested that a regimen containing more CSF penetrating drugs be included to suppress the CSF viral load and thereby decrease the neurocognitive impairment.[4]

1.2.5. CNS PENETRATION EFFECTIVENESS (CPE)

The antiretroviral drugs are sorted based on their ability to traverse the blood-brain barrier using CNS Penetration Effectiveness (CPE) score as low (0), intermediate (0.5) and high (1)

I. Nucleoside/nucleotide reverse transcriptase inhibitors

1. **Low Penetration:** Zalcitabine (ddC), didanosine (ddI), tenofovir
2. **Intermediate Penetration:** Stavudine (d4T), lamivudine (3TC), emtricitabine (FTC)
3. **Higher Penetration:** Zidovudine (AZT), abacavir

II. Non-nucleoside reverse transcriptase inhibitors

1. **Intermediate Penetration:** Efavirenz
2. **Higher Penetration:** Nevirapine, delavirdine

III. Protease inhibitors

1. **Low Penetration:** Nelfinavir, ritonavir, saquinavir (boosted or unboosted), tipranavir/ritonavir
2. **Intermediate Penetration:** Unboosted amprenavir, unboosted fosamprenavir, atazanavir (boosted or unboosted), unboosted indinavir
3. **Higher Penetration:** Amprenavir/ritonavir, fosamprenavir/ritonavir, indinavir/ritonavir, lopinavir/ritonavir

IV. Entry inhibitors

1. **Low Penetration:** Enfuvirtide (T-20) [4]

1.2.6. OPPORTUNISTIC ILLNESS THAT AFFECT THE BRAIN

HIV patients upon impairment of immune system are susceptible to other infections that affect the brain known as Opportunistic illnesses. They are:

1. Cryptococcal meningitis
2. Cytomegalovirus
3. *Mycobacterium avium complex* and tuberculosis
4. Primary CNS lymphoma
5. Progressive multifocal leukoencephalopathy
6. Toxoplasmosis

7. Others like histoplasmomosis, coccidioidomycosis, aspergillosis and human herpes virus 6 (HHV-6).[4]

1.3 DRUG TARGETING TO THE CENTRAL NERVOUS SYSTEM (CNS)

Improvement in delivery of drugs to the CNS to treat CNS disorders and diseases has been an important field of interest as most of these drugs do not attain the required CSF concentration as compared to blood plasma. This is due to the presence of barriers of the brain like the blood-brain barrier and the blood-cerebrospinal fluid barrier.

I. Blood-brain barrier (BBB)

The BBB is a single layer of tightly held capillary endothelial cells of the brain. This forms a continuous cellular barrier that is impermeable to most drugs.[5]

The ependymal cells that lines the cerebral ventricles are of 3 types:

1. **Astrocytes** that form the structural framework and control their biochemical environment.
2. **Oligodendrocytes** that helps in the formation of myelin sheath and in the action potential transmission and
3. **Microglias** that are macrophages derived from blood.[6]

The influx or efflux of substances such as electrolytes, nucleosides, amino acids and glucose are regulated by the endothelial cell membrane transporters of the BBB.

The membrane permeation mechanisms include:

1. Passive diffusion
2. Carrier-mediated transport (facilitated)
3. ATP-dependent (active) transport (D-glucose, L-aminoacid, Na^{+}/K^{+}-APTase). A facilitative glucose transporter, GLUT-1 is highly expressed by the abluminal membranes compared to luminal side of BBB endothelial cells. The APT ase and the A-system amino transporters are located in the abluminal side and the Ca2+-APTases are expressed on both the luminal and abluminal endothelial membranes and in the plasmalemmal vesicles of the endothelium
4. Transcytosis

5. Receptor-mediated and adsorptive endocytosis like endothelial barrier antigen, OX-47 and endothelial glycocalyx.
6. Metabolic enzymes like alkaline hosphatise, peptidases, several cytochrome P450 isoenzymes, UDP-glucuronosyl transferase and GSH S-transferase.[5]

II. Blood-cerebrospinal fluid barrier (BCB)

The exchange of molecules between the blood and the interstitial fluid of the brain parenchyma is regulated by the BCB. The barrier between the blood and CSF includes the arachnoid membrane and the choroid plexus. The capillaries of the choroid plexus are fenestrated, non-continuous with gaps between the cerebral endothelial cells and allow the passage of small molecules. The passage of macromolecules is prevented by the adjacent choroidal epithelial cells that form tight junctions.

Furthermore, an organic transport system present in the choroid plexus drives CSF borne organic acids to blood.[6]

1.3.1 DRUG TRANSPORTERS IN THE CNS

The transport of drug across the brain barriers is found to be dependent on the physicochemical characteristics of the drug. The influx and efflux of drugs is influenced by passive diffusion of drugs and the involvement of many drug transporters. These play a key role in the pharmacokinetic properties of drugs.

A. Organic cation transport systems (OCT system)

The transport for molecules that include endogenous bioactive amines, therapeutic agents and xenobiotics is regulated by the OCT system. The net positive charge due to the nitrogen moiety is determined by the physiological pH and the compound's pKa.

The OCT system consists of:

1. Influx of organic cations which is regulated by the potential-sensitive transporter and
2. Efflux of organic cations which is regulated by the H^+ gradient-dependent transporter.

B. Organic anion transport systems (OAT systems)

This has two main families, i.e., the organic anion transporter polypeptide (oatp), and the organic anion transporter OAT will be discussed which consists of seven isoforms [oatp1, oatp2, oatp3, OAT-K1, OAT-K2, OATP, prostaglandin transporter (PGT), and the liver-specific transporter-1 (LST-1)] in the oatp family.

C. Nucleoside transport systems

The purine and pyrimidine nucleosides are the main nucleoside transporters. These nucleosides are produced by the endogenous de novo synthetic pathways which is absent in brain. Thus, the brain is dependent on purine and pyrimidine nucleoside constituents from both the endogenous synthesis *in situ* and the blood.

Concentrative transporters mediate the nucleoside flux against their concentration gradient by Na or K co-transport mechanisms.

D. Efflux transport systems

1. P-glycoprotein

P-gp is a plasma membrane energy-dependent efflux pump that belongs to the ABC superfamily of transporters. P-gp is a product of the multidrug resistance (MDR) gene which is cloned as two MDR genes, MDR1 and MDR2. The transport mechanism resembles a typical substrate-enzyme interaction resulting in the drug transportation to the outer plasma membrane.

2. Multidrug resistance protein family (MRP)

It belongs to the ABC superfamily of transporters. The MRP 1,-2,-3 and -6 contain up to six additional membrane-spanning helices at the NH_2 terminus which is necessary for protein transport. The proposed importance of MRP5 in drug resistance to nucleoside analog drugs and the transportation of antiretroviral drugs by MRP1 and/or -5 within the brain needs further research.

1.3.2 METHODS TO QUANTIFY DRUG TRANSPORT ACROSS THE CNS

A. *In vivo* methods to study the drug transport across the BBB and choroid plexus

In vivo BBB models of drug transport is broadly categorized based on methodological approach as

1. Single passage techniques

This method measures the uptake of substances into the CNS following a single passage through the brain upon injection into the blood stream. It is further classified as:

a) Indicator diffusion/dilution
b) Brain uptake index and
c) External registration

Disadvantage: The transport estimates of drugs or solutes obtained with extremely slow uptake may be inaccurate due to the short solute exposure times.

2. Multi passage techniques

It is used to allow the test substance for longer circulation times. It is further classified as:

a) Intravenous administration and
b) Microdialysis methods

These techniques are model-dependent, and the method of data analysis (i.e., two-compartment model, three-compartment model, etc.) is normally chosen prior to the experiment. Therefore, the results are model specific and may not necessarily be indicative of the actual transport and metabolic processes within the tissue.

3. Perfusion techniques

It is an *in situ* perfusion method, that exposes the brain tissue to the test substance by perfusion with a physiological buffer and is developed to provide further control over the experimental conditions (pH, temperature, etc.) and to avoid metabolism of the test substance during transfer across the BBB.

Compared with single or multi passage methods, permeability coefficients can be measured accurately over a 104-fold range making this method 100-fold more

sensitive. Therefore, measurements of brain uptake of poorly penetrating compounds (P = 10^{-8} to 10^{-7} $cm.s^{-1}$) or rapidly penetrating compounds (P = 10^{-4} $cm.s^{-1}$) can be determined allowing for the characterization of carrier-mediated transport at the BBB.

Disadvantages:

a) Involves complex surgery and
b) Requires mathematical models.

The more common used methodologies include:

a) CNS deconvolution technique

The CNS deconvolution technique is based on serial sampling of the CSF and numerical deconvolution of data to determine a transport profile of the drug in a single living animal.

b) *In situ* CP model

The *in situ* CP model replaces the endogenous CSF with oil such as ethyl iodophenylundecylate, which allows the CP to be easily visualized. The fluid droplets that are formed on the surface are collected and a steady-state clearance fraction of the drug is determined.

Disadvantages of *in vivo* methods

a) Expensive.
b) Control of environmental factors such as pH, temperature, osmotic pressure, oxygen, carbon dioxide, as well as physiological responses (metabolism, tissue distribution, excretion) that occur in the animal under normal and experimental conditions is difficult.

B. *In vitro* models to study drug transport in the brain

The *in vitro* models include:

1. Cell culture

2. Tissue culture

Tissue culture techniques are used for studying the behavior of a specific population of cells free of systemic variations that may arise in the animal both during normal homeostasis and under stress of an experiment.

Advantages

Tissue culture experiments provide

a) a level of control over the environment and various physiological responses.
b) specific information on the type of transporter(s) involved and
c) information on relative pharmacokinetic parameters (eg., carrier affinity and specificity).

Limitations:

Its limitations are:

a) many of the phenotypic and functional characteristics of the original tissue may be lost (i.e., tight junctions in brain endothelial cells, production of specific factors by cells, expression and activity of various transporters) due to culture conditions and
b) the absence of endogenous factors and signals.

Tissue culture methods are used for studying *in vitro* drug transport of nonpolarized cells and polarized cells (epithelial and endothelial cells), characterization of transport properties of substrates in isolated brain capillaries, *in vitro* BBB model (consisting of cocultures of endothelial and astrocyte cells which provides a more physiologically accurate representation of the BBB that provides information of drug transport, metabolism, and drug-drug interactions at the cellular level).[5]

1.3.3. APPROACHES FOR BRAIN TARGETED DRUG DELIVERY

The various approaches that are available are:

1. Molecular approach

The physicochemical properties are altered at molecular level.

a) Lipidization

The method involves the enhancement of molecular lipophilicity, to enhance the diffusion through the BBB by enhancement of membrane permeability of the molecule. This is achieved by chemical modification of the parent molecule or by non-polar addition.

b) Prodrugs

The drug permeability across the BBB can be enhanced by masking the ionic groups with some protecting groups, to convert it to bioreversible drug molecules called prodrugs.

c) Transport processes

The various transporters present in the cerebral endothelial cells are used as drug transporters across the BBB. The method uses the concept of coupling of drug molecules to endogenous substances which can cross the BBB and release the drug.

The various methods that utilizes the transport processes are:

- LAT1
- GLUT1
- Kynurenic anologues
- Molecular trojan horse technology that utilizes genitically engineered protein or monoclonal antibody
- P-gp modulating agents or efflux transport inhibitors (using formulation or coadministration strategy with polymeric surfactants like pluronic, polysorbates and cremophor).

2. BBB opening

The method involves the opening of the tight junctions of the BBB. The method enhances the CNS delivery of large molecules without drug modification but suffers a drawback that the BBB permeability to pathogens and toxins to brain. Thus to provide an effective therapy, the opening of tight junctions should be controlled and reversible. It is a non-invasive method that does not require surgery to produce a localized action to the brain.

a) Physical approach

i. Ultrasound and microbubbles

The ultrasound technology uses ultrasound energy focused on the target tissues which opens the BBB to allow penetration of drugs. Preformed microbubbles (small gas bubbles) are delivered (cavitation) before the exposure to ultrasound, so that opening of the BBB occurs without significant damage to the neurons. Magnetic

resonance imaging (MRI)-guided focused ultrasound methods are used to enhance the delivery of small molecules, large proteins and nanoparticulate delivery systems.

ii. Convection enhanced delivery

The convection enhanced delivery uses the continuous infusion of drug into the brain under external pressure gradient with the help of a syringe pump. The method is suitable for delivery of nanoparticles, microspheres and liposomes without using any surgery.

iii. Electromagnetic radiation

The electromagnetic radiations (EMR) emitted by GSM mobile phones are found to increase the BBB permeability. However due to the harmfulness of the EMR, necessitates a controlled and optimized use.

b) Chemical approach

The chemical approach involves the drug and inflammatory mediators that open the tight junctions of BBB are α-adrenergic agents, bradykinin, platelet activating factor, amitriptyline, prostaglandins and the substances that decreases BBB permeability by closing of tight junctions are β-adrenergic agonists, angiotensin, 2-deoxyglucose and dexamethasone.

Intracarotid arterial injection of inert hypertonic solution of osmotically active agents like mannitol, arabinose, dextrose, etc can also be used to increase the BBB permeability by the endothelial cell shrinkage due to the removal of water.

3. Alternative route

a) Intraventricular, intracerebral and intrathecal

These routes bypass the BBB but are not much popular due to its invasive method. Intracerebral implants are most popular for drug delivery at the tumor site after surgery of the tumor as they provide a controlled delivery of drugs.

b) Intranasal

The nasal route is used for delivery of drugs to the brain due the presence of the olfactory and trigeminal nerve system. Further, the nasal mucosal vasculature, cerebrospinal fluid and lymphatic system play a vital role for drug transport to the

brain. The non-invasiveness, ease of administration and safeness are considered as advantages of this method for drug delivery to the brain.

4. Novel drug delivery systems

a) Polymeric nanoparticles

Polymeric nanoparticles have polymers in the formultion. Examples of polymers include natural polymers like chitosan, alginate and gelatin and synthetic polymers like polylactic acid (PLA), polyglycolic acid (PGA), polylactide-co-glycolide (PLGA), poly-e-caprolactone (PCL) and poly (methyl methacrylate). Brain targeting of drug is enhanced by crosslinking with positively charged materials for negatively charged materials, forming coated nanoparticles with polysorbate 80 or conjugating nanoparticles with metal chelators like desferioxamine or D-penicillamine.

b) Micelles:

The polymeric micelles form self assembling macroscopic or nanoscopic systems when dispersed in water. They are also known as polymerosomes and are capable of encapsulating both hydrophobic and amphillic drugs. Pluronics®, a copolymer used in micellar drug delivery has enhanced the drug transport in BBMEC and Caco-2 monolayers.

c) Solid lipid nanoparticles

Solid lipid nanoparticles contain a hydrophobic core having a monolayer of phospholipid coating. The core consisting of dissolved or dispersed drug in the solid lipid matrix. Lipids that are used in the formulation are tripalmitin, glyceryl monostearate and steric acid. The nanosize and narrow size range enables these carriers to diffuse across the tight junctions in the endothelial cells. Moreover, these systems escape the reticuloendothelial system (RES) and bypass liver providing a prolonged action.

d) Liposomes

Liposomes are vesicular systems composed of unilamellar or multilamellar phospholipid or lipid bilayers with cholesterol in the bilayer. The limitations of liposomes are rapid elimination from circulation by macrophages of reticuloendothelial system (RES), quick metabolic degradation of the phospholipids, instability on long storage and their inability to provide controlled or sustained release of drugs. These can

be overcome by use of small sized liposomes (10 nm) and PEGylation, a method of surface coating of liposomes with PEG using PEG-conjugated phospholipids. Liposomes can also be conjugated to specific ligands to target specific receptors such as mannose, Tf and insulin receptors present in brain.

Liposomes conjugated with mAbs called immunoliposomes are used to target antigens expressed on the surface of astrocytes.

Niosomes are non-ionic surfactant vesicles similar to liposomes.[7] Niosomes are used for brain targeting of drugs such as temozolamide,[8] vasoactive intestinal peptide,[9] doxorubicin,[10] folic acid[11] and methotrexate.[12] Liposomes and niosomes are anticipated for future use in brain targeted drug delivery.

e) Nanogels

Nanogels are networks of cross-linked polymers in nanosize formed by interaction of ionic and non-ionic chains. They are prepared in two steps. Cross-linked network of polymer without drug was prepared by using emulsification solvent evaporation method. Loading of drug is done by allowing the prepared nonogels to swell in drug solution after which, the solvent volume decreases and the gel collapse to form nanoparticles. Although, nanogels are promising carriers for brain targeted delivery, more intensive research is required to further evaluate its potential.

f) Dentrimers

Dentrimers are hyperbranched, monodisperse, and uniformly distributed polymeric macromolecules. They are synthesised by polymerization reaction and are highly branched structures, unlike linear polymers. They are suitable carriers for delivering drugs to the brain as the size, molecular weight and branching can be controlled. pH sensitive dentrimers are used for drug delivery to brain tumors through an ion exchange mechanism in the brain microenvironment.

Polyamidoamine dentrimers are the commonly used dentrimers. Others include poly (propylene imine), polyether-copolyester, PEGylated, peptide and pH dentrimers. Cationic dentrimers disrupt the tight junctions but are toxic. The toxicity can be reduced without altering its properties by surface modification using carboxylic

entities. PEGylated dentrimers and Tf conjugated PEGylated dentrimers are used in drug delivery to the brain.

g) Miscellaneous

Other approaches for brain delivery of drugs include carbon nanotubes and nanofibres.

Carbon nanotubes are derivatives of carbon vapor, where carbon atoms are assembled in the form tube or cylinder. Nanofibers are electrospun and exhibits properties that are similar to nanoparticles two dimensionally and macroscopic properties in another dimension. These are safer compared with nanotubes and cause less air pollution.[7]

1.4 NIOSOMES: A NOVEL APPROACH FOR BRAIN TARGETED DRUG DELIVERY

Since the advent of non-ionic surfactants, its application in pharmaceutical research on improving drug delivery has found an enormous growth. Handjani-Vila *et al* were the first to hydrate a mixture of cholesterol and a singly alkyl chain, non-ionic surfactant to result in the formation of vesicular system.[13] L'Oreal was the first to market and patent a niosomal formulation in 1975.

Niosomes or non-ionic surfactant vesicles or surfactant membrane vesicles are osmotically active and stable unilamellar or multilamellar vesicles wherein an aqueous solution is enclosed in highly ordered bilayer made up of non-ionic surfactant with or without cholesterol and dicetyl phosphate. They are vesicular systems similar to liposomes. Niosomes can be used as carriers of amphiphilic and lipophilic drugs.[14]

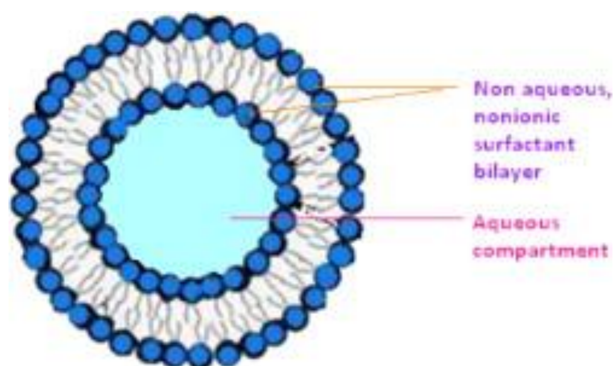


Figure 3: Structure of niosomes

1.4.1 ADVANTAGES OF NIOSOMES[15, 16, 17]

Niosomes have the following advantages over liposomes

1. They act as potential carriers for delivery of drugs, antigens, hormones and other bioactive agents.
2. They have high chemical stability as they are less susceptible to oxidation.
3. They are relatively less toxic.
4. They have greater ease of production.
5. The production is economical.
6. They have wider formulation versatility.
7. Surfactants used for forming niosomes are biodegradable, non-immunogenic and biocompatible.
8. These vesicles improve the therapeutic efficacy of drugs by reducing the clearance rate, targeting to the specific site and by protecting the encapsulated drug.
9. Drug targeting reduces the dose which leads to subsequent decrease in side effects.
10. In some instances, encapsulation of the drug by niosomes is found to reduce the toxicity as demonstrated in the study on preparation of niosomes containing vincristine. It is also found to decrease the neurological toxicity, diarrhoea and alopecia following the intravenous administration of vincristine and increased vincristine anti-tumor activity in S-180 sarcoma and Erlich ascites mouse models.[17]

1.4.2 DISADVANTAGES[18]

1. Physical instability
2. Aggregation
3. Fusion
4. Leaking of entrapped drug
5. Hydrolysis of the encapsulated drug which limits the shelf life of the dispersion.

1.4.3 COMPOSITION OF NIOSOMES

Niosomes are made up of the following components:

1. Non-ionic surfactants

It is the main composition of niosomes which forms the bilayer called as lamella that is made up of hydrophobic and hydrophilic moiety. Various non-ionic surfactants have been investigated in the formulation of niosomes which includes crown ethers, perfluro alkyl group surfactants, sorbitan esters (span 20,40,60,65,80 and 85), polysorbates (Tween 20,40,60 and 80), poly-24-oxyethylene cholesteryl ether (solulan C24), polyoxyethylene alkyl ethers (brij 30,35,52,58,72,76,92 and 97).[19]

2. Cholesterol

Cholesterol, a steroid is included in the formulation of niosomes to stabilise them by providing rigidity due to alteration of steroidal rigid skeleton with surfactant molecules in the bilayer and thereby restricting the movement of carbons of hydrocarbon.

The bilayers of the vesicles are either in the liquid state or in the gel state. This depends on the temperature, the type of lipid or surfactant used and the presence of other components such as cholesterol. The alkyl chains are observed to be in a well-ordered structure in the gel state and a more disordered bilayer structure in the liquid state.

The surfactants are characterized by the gel-liquid transition temperature, T_c. Cholesterol which is an amphiphilic molecule, orients itself by hydroxyl (-OH) group facing towards the aqueous phase and aliphatic chain towards the surfactant's hydrocarbon chain. In general the action of cholesterol is two folds; on one hand, cholesterol increases the chain order of liquid-state bilayers and on the other cholesterol decreases the chain orders of gel-state bilayers. At a high cholesterol concentration, the gel state gets transformed to a liquid-ordered phase.[20, 21]

3. Charge inducing agents

Charge inducing agents stabilises the niosomes by increasing the surface charge density thereby preventing vesicle flocculation, aggregation and fusion. Stearyl amine and cetyl pyridiium chloride are used as positive charge inducers and dicetyl phosphate,

dihexadecyl phosphate and lipoamine acid are used to provide negative charge to the vesicles. These act by increasing the interlamellar distance and thus increasing the entrapped volume of the vesicles.[22, 23]

1.4.4 PREPARATION OF NIOSOMES

Non-ionic surfactant vesicles can be prepared using the following methods:

1. Sonication method

The lipophilic components are dissolved in an apolar solvent, such as a mixture of chloroform and methanol. The solvent evaporates overnight under vacuum, resulting in a surfactant film. Hydration of the film is performed with aqueous solution and the mixture is sonicated at 60^0C for 3 min using a probe sonicator. The method results in the formation of both large unilamellar vesicles (LUVs) and multilamellar vesicles (MLVs).[24, 25, 26]

2. Ether injection method

The oil soluble components are dissolved in diethyl ether and injected slowly through a needle into the aqueous phase maintained at 60^0C. The method results in the formation of mainly multilamellar vesicles. The size of the vesicles can be made uniform by using suitable size needle but it has its limitations of limited solubility of materials in the ether and difficulty of removal of ether from the final formulation.[27, 28]

3. Hand shaking method

The apolar ingredients are dissolved in a mixture of chloroform and methanol. The solvent evaporates overnight under vacuum. The resulting surfactant film is hydrated with an aqueous solution and gently shaken at 50-60^0C to allow the formation of vesicles. If the drug is hydrophilic it can be added to the aqueous phase and if it is hydrophobic to the organic phase. The preparation method yields multilamellar vesicles.[29]

4. Reversed phase evaporation

The oil soluble components are dissolved in an apolar solvent such as chloroform. The aqueous phase is added and the mixture, sonicated to form an emulsion. The solvent evaporates overnight under vacuum. The resulting suspension is shaken to form vesicles. Mainly large multilamellar vesicles are formed.[28]

5. Method as described by Handjani-Vila *et al*

The surfactants are added to the aqueous phase and the mixture is agitated to yield a homogenous lamellar phase. The suspensions are then homogenised by means of agitation or ultracentrifugation.[30]

6. Microfluidization method

This is based on submerged jet principle wherein fluidized streams interact at ultrahigh velocities (up to 1700 ft/sec) in precisely defined micro channels within the interaction chamber to form niosomes. The method gives greater uniformity and smaller size vesicles.[31]

7. Active Trapping techniques

In this method the niosomes are prepared and then loaded with the drug maintaining the pH gradient or ion gradient to facilitate the uptake of drug by the niosomes. The resultant niosomes are found to have good entrapment, high drug lipid ratios and absence of leakage. The method is cost effective and suitable for labile drugs.[32]

8. The bubble method

This method is performed in the absence of organic solvents. All the components are dispersed in buffer in a round bottomed flask immersed in a water bath with controlled temperature. The flask is fitted with a homogenizer, thermometer and nitrogen supply, and is attached to a water cooled reflux. The dispersion is mixed with a shear homogenizer for 15 sec and then nitrogen is passed through it to form niosomes.[33]

9. Trans-mebrane pH gradient (inside acidic) drug uptake process or remote loading method

Multilamellar vesicles prepared at acidic pH by hand shaking method are then subjected to freeze-thaw cycle and later sonicated. Remote loading of drug is by adding aqueous solution of drug, pH adjusted to 7.0-7.2 and then the mixture is heated. Niosomes so formed shows greater entrapment efficiency and better retention of drug.[34, 35]

1.4.5 CHARACTERIZATION OF NIOSOMES[36, 37, 38, 39]

S.No.	Characterization parameters	Method of analysis
PHYSICAL CHARACTERIZATION		
1	Vesicle shape and morphology	TEM or freeze fracture microscopy technique.
2	Mean vesicle size and Size distribution	Dynamic light scattering, zetasizer, photon correlation spectroscopy, laser light scattering, gel permeation and size exclusion chromatography.
3	Formation of bilayer	Formation of X-cross under light polarization microscopy.
4	Number of lamellae	NMR spectroscopy, small angle X-ray scattering and electron microscopy.
5	Membrane Rigidity	Mobility of fluorescence probe as function of temperature.
6	Membrane thickness	X-ray scattering analysis.
7	Viscosity	Ostwald's viscometer at room temperature.
8	Turbidity	UV-Visible diode array spectrophotometer.
9	Thermal analysis	Differential Scanning Calorimetry.
10	Osmotic shrinkage	Incubating in hypertonic salt solution and determining the reduction in vesicle size using optical microscopy.
11	Surface charge	Free-flow electrophoresis.
12	Phase behaviour	Freeze-fracture electron microscopy, differential scanning calorimetry.
13	Electrical surface potential and surface pH	Zeta potential measurements and pH sensitive probes.

S.No.	Characterization parameters	Method of analysis
CHEMICAL CHARACTERIZATION		
1	Cholesterol concentration	Cholesterol oxidase assay and HPLC
2	Cholesterol auto-oxidation	HPLC andTLC
3	Osmolarity	Osmometer
BIOLOGICAL CHARACTERIZATION		
1	Sterility	Aerobic and anaerobic cultures
2	Pyrogenicity	Limulus Amoebocyte Lysate (LAL) test
3	Animal toxicity	Monitoring survival rates, histology and pathology.

1. Free drug removal

The unentrapped drug can be removed by

a) Gel filtration using Sephadex G-50 column and elusion with phosphate buffered saline.[40, 41]

b) Centrifugation of the sample at 4000 rotations per minute and determining the amount of drug present in the surfactant after suitable dilutions.[42, 43]

c) Dialysis using a dialysis bag containing the niosomal suspension immersed in phosphate buffered saline and determining the amount of drug dialysed.[33]

2. Entrapment efficiency

Entrapment efficiency can be determined by disrupting the drug loaded vesicles after separation from unentrapped drug using Triton X-100[44] or n-propranolol[45] or 2.5% sodium lauryl sulphate[38] and determining the drug content after suitable dilutions.

3. *In vitro* release study

The *in vitro* release study of drug from the niosomal suspension is determined by determining the amount of drug released through a dialyzing membrane placed in phosphate buffer saline under magnetic stirring.[46]

$$\text{Percentage of drug released} = \frac{\text{Amount of drug released}}{\text{Total amount of encapsulated drug}} \times 100$$

4. Permeation study

In vitro permeation study is performed using Franz diffusion cell through dehaired rat skin. In this 1 ml of the suspension is taken in the donor compartment and phosphate buffer in the receptor compartment which is stirred at 100 rpm and maintained at 37±0.5^{0}C. Samples are withdrawn at periodic intervals and the amount of drug permeated is determined spectrophotometrically.[47]

1.4.6 FACTORS AFFECTING THE CHARACTERIZATION OF NIOSOMES

1. Nature of the encapsulated drug

Entrapment of drug in the niosomes resulted in increase in vesicle size due to the interaction of the solute with the surfactant bilayer.[48] Among a series of Spans and Tweens, the entrapment of water soluble drug (diclofenac sodium) is reported to be more with hydrophilic surfactants like Tween 60.[49] Maximum entrapment is reported for slightly water soluble drugs (methotrexate) in lipophilic surfactant Span 60.[50]

2. Amount and type of non-ionic surfactant

The mean size of niosomes increases with increase in the hydrophilic-lipophilic balance (HLB value) from span 85 (HLB 1.8) to span 20 (HLB 8.6) due to decrease in surface free energy as a result of increase in hydrophobicity of surfactant. [51] A linear correlation is observed between concentration of lipid and entrapment efficiency.[52]

3. Membrane additives

Cholesterol when used in the molar ratio of 1:1 prevents the aggregation of niosomes by repulsive steric or electrostatic effects and leads to less leaking vesicles.

Increase in cholesterol increases the hydrodynamic diameter and thus its entrapment efficiency.[21]

Presence of charge tends to increase the interlamellar distance between the successive bilayers of the multilamellar vesicle structure and leads to overall entrapped volume.[25] Dicetyl phosphate provides negative charge to the vesicles and is used to prevent the aggregation of hexadecyl diglycerol ether niosomes.

Stearic acid provides positive charge to the niosomes and thus used to prepare cationic niosomes.[53]

4. Method of preparation

Niosomes prepared with Spans and cholesterol by lipid film hydration are found to give multilamellar vesicles whereas niosomes prepared by ether injection method are found to yield unilamellar vesicles or oligolamellar vesicles.[53]

5. Surfactant and lipid levels

The surfactant/lipid ratio is generally 10-30 mM (1-2.5%w/w). Increasing the surfactant/lipid ratio increases encapsulation efficiency but results in a highly viscous system.[53]

6. Hydration temperature

The surfactants and lipids are characterized by the gel-liquid phase transition temperature (T_c). The hydration temperature should be above the T_c of the system. Span 60 has a high phase transition temperature and low HLB value and thus forms stable vesicles without the formation of micelles.[53]

1.4.7 PHARMACEUTICAL APPLICATIONS OF NIOSOMES

1. Niosomes containing non-steroidal anti-inflammatory drugs

Niosomal delivery of a number of anti-inflammatory drugs are studied and they show better extended release profile with reduced dose when compared with the plain drug leading to reduced side effects.

Stable, multilamellar, non-ionic surfactant vesicles (multilamellar vesicles) are prepared using interfacial polymerization by providing a polymer coat of poly (phthaloyl-L-lysine) for each niosome and thereby providing a rigid but diffusible

multiple double barrier which controlled the release of diclofenac sodium. This is proved by *in vivo* studies in inflammed rat model compared with placebo multilamellar vesicles (MLVs).[54]

Diclofenac sodium niosomes are prepared by thin film hydration method and with *in vitro* drug release profile best fitted to Peppas equation. Niosomal vesicles acted as depot for diclofenac sodium exhibiting controlled release.[55]

Diclofenac sodium niosomes are prepared by lipid hydration method using three-level three-factor Box-Behnken experimental design to optimize the formulation. The optimized formulation prepared according to computer-determined levels provided an entrapment efficiency and controlled release profile, which approximated to the predicted values. The study proved the efficient applicability of experimental design methodology for characterization and optimization of formulation parameters affecting entrapment efficiency and drug release from diclofenac sodium niosomes.[56]

Aceclofenac niosomes are developed and optimized to improve its bioavailability that showed an extended release of the drug over a period of 72 h in all formulations. The best formulation containing Span 20 fitted to Peppas model.[57]

Ketoprofen niosomes are formulated and evaluated using *in vitro* everted rat intestine and *in vivo* anti-inflammatory activity in rats. It concluded that polysorbate 40 formulation as the best when compared with other formulations.[58]

Indomethacin loaded niosomes are formulated and evaluated. The results showed that the therapeutic effectiveness increased with niosomal encapsulation compared with free indomethacin in paw edema bearing rats.[59]

2. Topical and transdermal delivery

Flurbiprofen proniosomal transdermal carrier systems are formulated and evaluated using rabbit skin and cellophane membrane. The results concluded that diffusion through rabbit skin is slower than that through cellophane membrane due to slow diffusion properties. The proniosomal formulations controlled the diffusion rates to be faster compared with drug dispersed in HPMC gel and in distilled water respectively.[60]

Ketoprofen niosomes prepared with Span 60 showed slow and sustained release of the drug.[61]

Tretinoin loaded vesicles prepared by film hydration, extrution technique and sonication are evaluated for the influence of vesicle structure on the photostability of tretinoin compared with drug in methanol.[62]

In another study the influence of vesicle composition and preparation method on the vesicle structure (multilamellar, large unilamellar and small unilamellar vesicles), size distribution, entrapment efficiency and *in vitro* release of incorporated tretinoin are studied.[63]

Niosomal transdermal delivery of nimesulide in a 1% carbopol base is found to localize the drug to the skin for a prolonged period of time and enhance its anti-inflammatory activity compared with plain drug gel and marketed formulation.[64]

Dithranol loaded liposomes and niosomes are formulated and stabilized. Enhanced permeation is observed with dithranol in vesicles compared to cream base by *in vitro* permeation study using mouse skin.[65]

Proniosome gels of estradiol are formulated using sorbitans and polysorbates and the results concluded that two of the sorbitans showed increased estradiol permeation with rat skin.[66]

Ketoconazole niosomes are prepared by thin film hydration using different ratios of Tween 40 and 80 in a FAPG base (stearyl alcohol -20%, stearic acid – 5% and propylene glycol – 75%) and concluded that niosomal formulation offers advantage over plain drug formulation.[67]

Ketoconazole niosomes prepared by ether injection technique are evaluated for *in vitro* (cup-plate method) and *in vivo* antifungal activity in rabbits compared to free ketoconazole. The results indicated that the niosomes have the potential to reduce the therapeutic dose of ketoconazole by improving its performance.[68]

Baclofen niosomal formulations are found to have improved bioavailability and muscle relaxant activity than the conventional topical vehicle.[69]

Gallidermin, an anionic drug showed increased stability by protecting the drug from oxidation environments when entrapped in niosomes. The drug loaded niosomes when incorporated in topical gel showed high accumulation of drug in the skin with no systemic side-effects.[70]

Enoxacin niosomal formulations are formulated and evaluated for physicochemical properties, stability and percutaneous absorption compared to liposomes. The results concluded that enoxacin niosomes can be used to modulate the delivery of drug without significant toxicity.[71]

Clotrimazole niosomes showed sustained and controlled release of the drug for local vaginal therapy. Evaluation of the vesicle gel system for antifungal activity and tolerability on tissue level in rat showed sustained and controlled release of the drug.[72]

Fluconazole niosomal formulations accumulated and formed localized drug depots for sustained release with enhanced cutaneous retention of the drug.[73]

The detrimental effects on repeated exposure of the marketed alcoholic gel of naftifine hydrochloride are overcome by formulating it as non-alcoholic niosomal gel formulations.[74]

Formulation of neutral vesicles of lidocaine with Tween 20 and cholesterol are better than liposomes and Tween 20 micelles.[75]

Niosomes formed from Brij and Spans are reported as a possible approach to improve the low skin penetration and bioavailability characteristics shown by conventional topical vehicle for minoxidil. The enhanced percentage of dose accumulated in the skin compared with commercial and control suggested that, niosomal topical minoxidil as a suitable alternative for hair loss treatment.[76]

Niosomes of rofecoxib are prepared by lipid film hydration technique, incorporated in a gel base and compared with plain drug gel. The lower flux value of niosomal gel as compared to plain drug gel across pig skin assured the prolonged drug release behaviour with sustained action.[77]

Niosomes of ciclopirox olamine (CPO), a broad spectrum antifungal drug are prepared by ethanol injection method to improve the poor skin penetration and residence that account for the long treatment regimes in cutaneous mycosis.[78]

3. Antiplatelet activity

Indomethacin niosomes showed sustained antiplatelet effect due to greater quantity of drug reaching the specific site of inhibition in the interior of the platelets and direct action on the cyclo-oxygenase system to prevent thromboxane formation.[79]

4. Occular delivery

Chitosan or carbopol coated timolol maleate niosomes showed an extended release of the drug compared with marketed *in situ* gel forming solution of timolol.[80]

Prolonged effect on intraocular pressure is observed with acetazolamide niosomes formulated with Span 60 and cholesterol.[81]

Cyclopentolate niosomes prepared by sonication are found to promote absorption of cyclopentolate by preferentially modifying the permeability characteristics of the conjunctival and scleral membranes.[82]

Promising controlled release of the drug is observed from niosomal formulations of gentamicin for topical ophthalmic drug delivery.[83]

5. Respiratory drug delivery

Development of optimized formulation of all-trans retinoic acid is carried out for delivering the drug as inhaled aerosol formulation. The results concluded that, this will be an alternative approach for respiratory delivery of drug by aerolization.[84]

6. Anti-tumor activity

Niosomal formulations of the anti-cancer drug daunorubicin hydrochloride prepared by modified reverse evaporation process are studied to increase its therapeutic efficacy in Dalton's ascetic lymphoma.[38]

Methotrexate niosomes subjected for pharmacokinetic evaluation in tumour bearing mice showed that unilamellar vesicles prepared with Span 60 has maximum

entrapment and a marked difference in pharmacokinetics compared with the unentrapped drug.[85]

5-Fluorouracil niosomes are prepared with Spans using different methods and the pharmacokinetic calculations concluded that niosomes can act as promising carriers of 5-fluorouracil.[86]

Intratumoral modified-release chemotherapy with fluorouracil/epinephrine injectable gel are prepared and evaluated to provide a non-surgical treatment alternative in selected patients with superficial squamous cell carcinoma.[87]

Vincristine niosomes prepared by different techniques using different surfactants are evaluated and concluded that the transmembrane pH gradient drug uptake process as the most satisfactory method yielding stable niosomes with Span 40-cholesterol (1:1).[88]

Negatively charged paclitaxel (PCT) niosomes are prepared using different surfactants (Tween 20, 60, Span 20, 40, 60, Brij 76, 78, 72) with dicetyl phosphate by film hydration method. High surface charges showed that niosomes can be suspended in water well and this is beneficial for their storage and administration. PCT released from niosomes by a diffusion controlled mechanism. The slow release observed from these formulations might be advantageous for decreasing the toxic side effects of PCT.

Reproducible niosomal formulations are produced in terms of size distribution, zeta potential and percentage drug loading values. The efficiency of niosomes to protect PCT against gastrointestinal enzymes (trypsin, chymotrypsin, and pepsin) for oral delivery is shown by the increased gastrointestinal stability of PCT prepared with Span 40 niosomes.[89]

7. CNS targeting

Improved efficacy against cerebral tumours has been observed with Temozolamide loaded niosomes which on pharmacokinetic evaluation proved its *in vivo* brain targeting in rats.[8]

Encapsulation of vasoactive intestinal peptide (VIP) within glucose-bearing niosomes mainly allowed a significantly higher VIP brain uptake compared with

control niosomes (up to 86%, 5min after treatment). Brain distribution of intact VIP after injection of glucose-bearing niosomes, indicated preferential location of radioactivity in the posterior and the anterior parts of the brain and homogeneous distribution in the whole brain after the administration of control vesicles. Thus, the novel vesicular formulation of VIP delivers intact VIP to particular brain regions in mice and the glucose-bearing vesicles might be therefore a novel tool to deliver drugs across the blood-brain barrier (BBB).[9]

N-palmitoyl glucosamine bearing doxorubicin niosomal formulations are developed to target the drug to the brain. Stable, nano-sized vesicles with improved brain delivery are produced with preliminary *in vivo* studies.[10]

The low blood levels of folates are the prime cause for causing depression in Alzheimer's disease. Folic acid is a water soluble vitamin showing difficulty in crossing the blood brain barrier and thus is formulated as niosomal nasal drug delivery system to target the brain. The release of drug from niosomes follows anomalous diffusion with first order release kinetics. About 48.15% of drug is to be absorbed through nasal cavity at the end of 6 h in *ex-vivo* perfusion studies using rat model.[11]

8. Hormone delivery

Luteinising releasing hormone (LRH) niosomes are formed by varying the composition of the vesicle membrane consisting of hexadecyl diglycerol ether (C16G2), cholesterol, and poly-24-oxyethylene cholesteryl ether (Solulan C24) to give polyhedral, spherical and tubular niosomes. Polyhedral niosomes released more radiolabeled LHRH ([^{125}I] LHRH) than spherical/tubular niosomes in both muscle homogenate and plasma. In clearance experiments in the rat, following intramuscular injection, both polyhedral and spherical/tubular niosomes gradually released [^{125}I] LHRH into the blood, but some radioactivity remained at the injection site for 25 and 49 h, respectively. In contrast, [^{125}I] LHRH in phosphate buffered saline is completely cleared from the injection site at 2 h. The release of drug is sustained by both niosome formulations, but spherical/tubular niosomes possess more stable membranes than polyhedral niosomes due to the presence of cholesterol.[90]

9. Vaccine delivery

An adjuvant for tetanus toxoid is prepared as an aqueous dispersion of niosomes emulsified in an external oil phase forming the vesicle-in-water-in-oil (V/W/O) system using cottonseed oil as the external oil phase. Initial studies of the system *in vivo*, showed enhanced immunological activity over the free antigen or vesicles.[91]

Non-ionic surfactant vesicular carrier, i.e. niosomes, is evaluated for topical delivery of vaccines using hepatitis B surface protein as an antigen and cholera toxin B as an adjuvant. The study suggests that topical immunization with cholera toxin B as potential adjuvant for cutaneous immune responses when coadministered with the HBsAg encapsulated niosomes and thus can be effective as topical delivery of vaccines.[92]

10. Diagnostic markers

Iopromide, a radiocontrast agent, is prepared and characterized in different gel and liquid crystalline formulations of liposomes and niosomes. Niosomes showed greater physical stability compared with liposomes. *Ex vivo* leakage of iopromide from vesicles is observed in human plasma and *in vitro* release fitted Higuchi model.[93]

Polymeric vesicles with encapsulated carboxyfluorescein are developed and evaluated using palmitoyl glycol chitosan and cholesterol (2:1) by weight, and then encapsulating within egg phosphatidylcholine, cholesterol (2:1) by weight, to yield liposomes or hexadecyl diglycerol ether to yield niosomes in vesicle system.[94]

11. Anti-fertility activity

An inclusion complex of plumbagin with hydroxypropyl beta-cyclodextrin entrapped in an aqueous layer of niosomes is prepared and evaluated. It concluded that an increase in stability and anti-fertility activity of the complex is observed, compared with control and niosomes with lipid layer entrapment.[95]

The anti-fertility activity of centchroman in niosomes is increased showing 83.3% protection against pregnancy with histopathological studies showing no side effects and toxic effects.[96]

12. Anti-leishmanial therapy

Liver and serum concentrations of antimony in mouse are determined after administration of sodium stibogluconate in the free, liposomal and niosomal form. High liver and low serum values are attained by the use of both vesicular formulations. Niosomal sodium stibogluconate is shown to be more effective than free drug in treating experimental murine visceral leishmaniasis, an effect apparently dependent on maintaining high drug levels in the infected reticuloendothelial system.[97]

13. As drug carriers

a) Anti-viral drugs

Distribution of formulated zidovudine niosomes in lungs, kidney, heart, liver and spleen of mice are studied after i.v bolus injection. The formulation with Tween 80 is optimized with increased half-life, mean residence time and reduced leakage of drug at 4^{0}C.[98]

Tenofovir niosomes are formulated using different compositions and evaluated. The results concluded that microfluidization can be used for further scale-up of the niosomes with very small mean vesicular sizes.[99]

Ribavirin niosomes enhanced up to 6 folds of liver targeting property, thereby proving to be effective at low doses compared with high doses of plain drug which have toxic side-effects.[100]

b) Anti-fungal drugs

The oral bioavailability of griseofulvin is increased by sustained release of drug from niosomal formulation containing Span 60.[38]

Nystatin niosomes demonstrated less nephrotoxicity and hepatotoxicity with higher level of drug in vital organs and pronounced efficacy in elimination of fungal burden in experimental animals compared with those treated with free nystatin.[101]

c) Anti-Tubercular drugs

Niosomal formulations of rifampicin are developed with various Spans and cholesterol for sustained release of the drug. Formulation with Span 20 is found to have maximum drug release and that of Span 85 with minimum drug release.[102]

The cellular uptake by macrophage cells of isoniazid loaded niosomes is 61.80% which is sufficient to achieve effective treatment of tuberculosis. The formulations showed reduced dose, reduced toxicity, reduced dosing frequency and increased patient compliance.[103]

Isoniazid niosomal formulations showed sustained *in vitro* release of drug and lesser toxicity than the free drug *in vivo.*[104]

Niosomal encapsulated pyrazinamide showed maximum concentration of drug in the lungs, with reduced side effects, toxicity and drug resistance.[105]

The release pattern of anti-tubercular drugs like rifampicin and isoniazid showed fickian/diffusional release whereas pyrazinamide had non-fickian release mechanism from Triton-X 100 niosomes.[106]

d) Antibiotics

Cefuroxime axetil bioavailability and stability in bile salts are improved by formulating it as niosomes.[107]

Cefpodoxime proxetil niosomes showed controlled release of the drug with zero- order release kinetics, thus reduces the chances of dose dumping during usage.[108]

e) Anti-Diabetic

Gliclazide oral bioavailability is improved by formulation as niosomes with sustained release of the drug. The stability of the niosomes is attributed to the electrostatic repulsive forces which is indicated by high zeta potential.[109]

Niosomal formulations of metformin are developed with the aim of sustaining the release of metformin so as to decrease its side effects and also reduce its dosing frequency.[110]

f) Haemoglobin

Niosomal encapsulated haemoglobin is permeable to oxygen and hence can act as a carrier for haemoglobin in anaemic patients.[111]

14. Gene therapy

In this study neutral and negatively charged niosomes are used for DNA (PUC18 supercoiled plasmid) complexation. Different proportions of Span/Tween/Cholesterol with or without dicetylphosphate are utilized for niosome preparation by the film hydration method. The complexation percent of DNA-niosomes is as high as 80% in some formulations and the DNA is stable during complexation and extraction processes. The results concluded that negatively-charged niosomal systems can be used as a gene-delivery vector in the presence of Ca^{2+}.[112]

Polysorbate cationic niosomes (PCNs) comprising of nonionic surfactants (i.e., polysorbates) and a cationic cholesterol are synthesized using film hydration method and developed as gene carriers. The binding capacity of PCNs toward oligodeoxynucleotides (ODN) is assessed by a gel retardation approach, which demonstrated that the ionic complexes are formed when 6 charge ratio reached to 4 or greater. Gene transfer study showed that the PCNs exhibited a high efficiency in mediating cellular uptake and transferred DNA expression. Based on these findings, PCNs may offer the potential to function as an effective gene delivery system.[113]

Disturbance in the synthesis of tyrosinase might be one of the major causes of vitiligo. The enhancement of tyrosinase gene expression and melanin production by loading the plasmid in elastic cationic niosomes are investigated in tyrosinase gene knocked out human melanoma (M5) cells and in tyrosine-producing mouse melanoma ($B_{16}F_{10}$) cells. The study demonstrated not only the enhancement of the expression of human tyrosinase gene by loading in elastic cationic niosomes, but also the potential application of this gene delivery system for the further development of vitiligo gene therapy.[114]

1.5 DRUG PROFILE

EMTRICITABINE (FTC) [115, 116]

Synonym:

(-)-(2R,5S)-5-Fluoro-1-[2-(hydroxymethyl)-1,3-oxathiolan-5-yl]cytosine;

(-)-2'-Deoxy-5-fluoro-3'-thiacytidine;

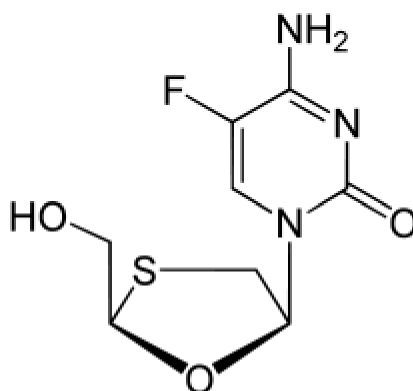
(-)-2'-Deoxy-5-fluoro-3'-thiacytidine;

(-)-beta-2',3'-Dideoxy-5-fluoro-3'-thiacytidine;

(-)-FTC;

(-)-cis-4-amino-5-Fluoro-1-(2-hydroxymethyl-1,3-oxathiolan-5-yl)-(1H)-pyrimidin-2-one.

Structure:



CAS number	**:**	143491-57-0
Molecular weight	**:**	247.247 g/mol
Chemical name	**:**	$C_8H_{10}FN_3O_3S$

IUPAC name:

4-amino-5-fluoro-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-1,2-dihydropyrimidin-2-one.

Mechanism of action:

Emtricitabine acts by inhibiting the enzyme, reverse transcriptase that copies HIV RNA into new viral DNA. Emtricitabine is a synthetic nucleoside analogue of cytidine. It undergoes phosphorylation by the enzymes present in the cells to form

emtricitabine 5'-triphosphate, which is responsible for the inhibition of HIV-1 reverse transcriptase. Emtricitabine inhibits the activity of HIV-1 reverse transcriptase (RT) by two mechanisms. It competes with the natural substrate deoxycytidine 5'-triphosphate and also acts by its incorporation into viral DNA.

Physicochemical properties

Appearance	:	White to off white crystalline powder.
Density	:	1.82 g/cm^3
Melting Point	:	136-140°C
Boiling Point	:	443.3°C at 760 mmHg
Refractive Index	:	1.731
Flash Point	:	221.9°C
Solubility	:	About 112 mg/mL in water at 25°C.

Pharmacokinetics

1) Absorption

t_{max}	:	1–2 hours.
C_{max}	:	1.8 ± 0.7 µg/mL
Half-life	:	10 hours

Area-under the plasma concentration-time curve over a 24-hour dosing interval (AUC): 10.0 ± 3.1 µg·h/mL.

Mean absolute bioavailability:

93% for EMTRIVA® capsules and 75% for EMTRIVA® oral solution (manufactured by Gilead Sciences, Inc.).

Relative bioavailability of EMTRIVA® oral solution:

Approximately 80% of EMTRIVA® capsules.

2) Distribution

Plasma protein binding: Less than 4% is protein bound. It is independent of concentration over the range of 0.02–200 µg/mL.

Metabolism

- It does not inhibit human CYP450 enzymes.

- Following administration, ~86% of the dose administerd was excreated in urine and ~14% in feces.
- Three putative metabolites were recovered in urine which constituted 13% of the dose.
- Emtricitabine undergoes biotransformation by oxidation of the thiol moiety (~9% of dose) to form the 3'-sulfoxide diastereomers. It also undergoes conjugation by reaction with glucuronic acid (~4% of dose) to form 2'-O-glucuronide.

3) Elimination

The renal clearance of emtricitabine is higher than the estimated creatinine clearance. This may be due to both active tubular secretion and glomerular filtration.

4) Effects of food on oral absorption

EMTRIVA® capsules and oral solution (Manufactured by Gilead Sciences, Inc.) may be taken with or without food.

Special Populations

1) Race, Gender

No significant differences in pharmacokinetics due to race and gender have been identified.

2) Paediatric Patients

A daily dose of 6 mg/kg up to a maximum of 240 mg oral solution or a 200 mg in paediatric subjects was found to be similar to adult subjects receiving a once-daily dose of 200 mg.

3) Patients with Hepatic Impairment

Impact of liver impairment is limited as emtricitabine is not metabolized by liver enzymes.

Assessment of Drug Interactions

Antiviral Activity

EC_{50} (50% effective concentration) value is 0.0013 – 0.64 μM (0.0003–0.158 μg/mL).

Resistance

Isolates of emtricitabine-resistant HIV-1 have been recovered from some subjects treated with emtricitabine alone or in combination the drug with other antiretroviral agents.

Cross Resistance

Cross-resistant Emtricitabine-resistant isolates (M184V/I) to lamivudine and zalcitabine were observed. These retained sensitivity in cell culture to didanosine, stavudine, tenofovir, zidovudine, and NNRTIs (delavirdine, efavirenz, and nevirapine).

Indication:

It is used for the treatment of HIV-1 infection in adults in combination with other antiretroviral agents. It is also used in the prophylactic treatment of post exposure HIV infection in health care workers, occupational and non-occupational workers.

1.6 EXCIPIENT PROFILE

SPAN® 40 (SORBITAN MONOPALMITATE) [117]

Nonproprietary Names:

BP	**:**	Sorbitan Palmitate
PhEur	**:**	Sorbitan Palmitate
USP-NF	**:**	Sorbitan Monopalmitate

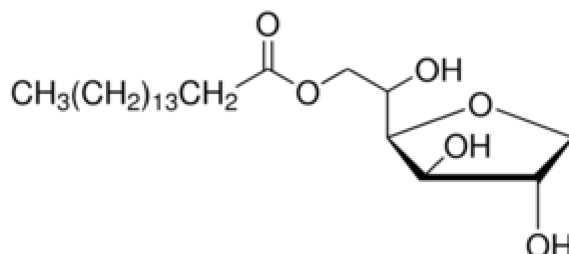
Synonym:

1,4-Anhydro-D-glucitol, 6-hexadecanoate; Ablunol S-40; Arlacel 40; Armotan MP; Crill 2; Dehymuls SMP; E495; Glycomul P; Hodag SMP; Lamesorb SMP; Liposorb P; Montane 40; Nikkol SP-10; Nissan Nonion PP-40R; Protachem SMP; Proto-sorb SMP; Sorbester P16; Sorbirol P; sorbitan palmitate; sorbitani palmitas; Span 40.

Chemical name : Sorbitan monohexadecanoate

CAS Registry number: [26266-57-9]

Structure:



Chemical formula : $C_{22}H_{42}O_6$

Molecular weight : 403 g/mol

Category : Nonionic surfactant; solubilizing agent; dispersing agent; emulsifying agent; suspending agent; wetting agent.

Appearance : Cream solid

Viscosity at 25^{0}C (mPas) : Solid

Melting point : 44 – 51^{0}C

Solubility : Soluble or dispersible in oils; soluble in most organic solvents and are dispersible in water.

HLB value : 6.7

Storage : Should be stored in a well-closed container in a cool, dry place.

SPAN® 60 (SORBITAN MONOSTEARATE) [117]

Nonproprietary Names

BP : Sorbitan Stearate

PhEur : Sorbitan Stearate

USP-NF : Sorbitan Monostearate

Synonym:

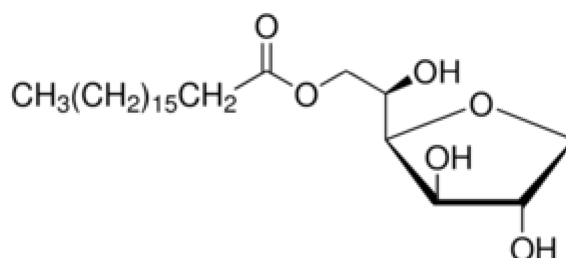
Ablunol S-60; Alkamuls SMS; 1,4-Anhydro-D-glucitol, 6-octadecanoate; anhydrosorbitol monostearate; Arlacel 60; Armotan MS; Atlas 110K; Capmul S; Crill 3; PolyconS60K;ProtachemSMS;Prote-sorbSMS;S-Maz60K;SMaz 60KHS; Sorbester

P18; Sorbirol S; sorbitan stearate; sorbitani stearas; Sorgen 50; Span 60; Span 60K; Span 60 VS; Tego SMS.

Chemical name : Sorbitan monooctadecanoate

CAS Registry number: [1338-41-6]

Structure:



Chemical formula : $C_{24}H_{46}O_6$

Molecular weight : 431 g/mol

Category : Nonionic surfactant; dispersing agent; emulsifying agent; solubilizing agent; suspending agent; wetting agent.

Appearance : Cream solid

Viscosity at 25^{0}C (mPas): Solid

Melting point : 50 – 60^{0}C

Solubility : Soluble or dispersible in oils; soluble in most organic solvents and are dispersible in water.

HLB Value : 4.7

Storage : Should be stored in a well-closed container in a cool, dry place.

CHOLESTEROL[117]

Nonproprietary Names:

BP : Cholesterol

JP : Cholesterol

PhEur : Cholesterol

USP-NF : Cholesterol

Synonyms : Cholesterin; cholesterolum.

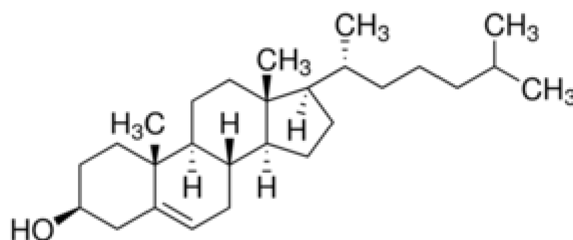
Chemical Name : Cholest-5-en-3b-ol

CAS Registry Number : [57-88-5]

Empirical Formula : $C_{27}H_{46}O$

Molecular Weight : 386.67 g/mol

Structural Formula:



Functional Category : Emollient; emulsifying agent.

Applications in Pharmaceutical Formulation or Technology:

At concentrations of 0.3–5.0% w/w it is used as an emulsifying agent in cosmetics and topical pharmaceutical formulations.

Description:

White or faintly yellow. Odorless. It occurs as pearly leaflets, needles, powder, or granules. It changes a yellow to tan color on prolonged exposure to light and air.

Melting point : 147 – 150^{0}C

Solubility:

Soluble in acetone, ethanol, ethanol (95%), ether, hexane, benzene, chloroform, isopropyl myristate, methanol and vegetable oils. Practically insoluble in water.

Stability and Storage Conditions:

Cholesterol is stable and should be stored inawell-closed container, protected from light.

SOLULAN™ C-24[118]

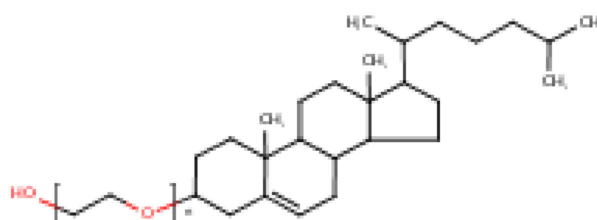
INCI name:

Choleth-24, Ceteth-24, POE-24-cholesteryl ether, polyoxyethylene-24-cholesterol ether, polyoxyethylene-24-cholesteryl ether, Solulan C 24, Aquasol.

IUPAC name:

2-[[10,13-dimethyl-17-(6-methylheptan-2-yl)-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-yl]oxy]ethanol

Structure:



Molecular formula	**:**	$C_{29}H_{50}O_2$
Molecular weight	**:**	430.717 g/mol

Description:

Solulan™ C-24 lanolin derivative is a complex of ethoxylated cholesterol and ethoxylated vegetable fatty alcohol.

Appearance **:** Off-white waxy solid

Uses:

It in oil in water emulsifier helps to prevent excessive viscosity build-up in fluid emulsions and is a source of water-soluble cholesterol for conditioning.

2. AIM AND OBJECTIVE OF STUDY

AIM OF STUDY

The aim of the present work is to develop an effective niosomal formulation based on sorbitan esters (Span) as main surfactant and bearing N-palmitoyl glucosamine, in view of its possible application in brain targeted delivery of emtricitabine.

OBJECTIVE OF STUDY

The objectives of the study are:

1. To formulate and optimize the molar concentration and the formulation method for empty niosomes prepared using Span 60 and Span 40 with cholesterol as membrane stabilizer and Solulan C24 as steric stabilizer in the molar ratios of 100 and 50:40:10 by Thin Layer Evaporation-Paddle, Thin Layer Evaporation-Vortex, Reverse phase evaporation and Proniosome method.
2. To select the best surfactant among Span 60 and Span 40 in producing niosomes with maximum drug entrapment and stability.
3. To determine the total lipid concentration of the surfactant lipid mixture to achieve formation of niosomes.
4. To determine the effect of cholesterol and Solulan in the formulation of niosomes as membrane stabilizer and steric stabilizer respectively.
5. To choose the best size reduction method among sonication and size extrusion methods in reducing the particle size of the prepared niosomes from multilamellar vesicles to homogenous dispersion of small unilamellar vesicles.
6. To synthesize glucose-derivatized surfactant N-palmitoyl glucosamine (NPG).
7. To optimize the formulation parameters of empty NPG-niosomal formulations prepared at two different hydration temperatures of 65 ± 5^0C and 90 ± 5^0C by the optimized formulation methods obtained in the preparation of empty niosomal formulations considering the potential benefits of increase in hydration temperature on entrapment efficiency of niosomes.

8. To prepare emtricitabine loaded-NPG-niosomes of the optimized formulation.
9. To evaluate the particle size and percentage encapsulation efficiency (by size exclusion chromatography and dialysis method) of the prepared emtricitabine loaded-NPG-niosomes.
10. To perform stability studies of empty NPG-niosomes and emtricitabine loaded-NPG-niosomes stored at 4^0C and 25^0C for a period of 6 months to determine the physical stability of the formulated niosomes since niosomes have instability problems such as change in particle size due to aggregate formation and fusion, phase separation of bilayer components and leakage of encapsulated drug post formulation.
11. To perform *in vitro* blood-brain barrier penetration of emtricitabine from optimized niosomal formulation using immobilized artificial membrane phosphatidylcholine column chromatography with the optimized emtricitabine loaded-NPG-niosomal formulation.

3. REVIEW OF LITERATURE

Abdelbary *et al.,*[83] has developed ophthalmic controlled delivery of gentamicin sulphate using various surfactants (Tween 60, Tween 80 or Brij 35), in the presence of cholesterol and a negative charge inducer dicetyl phosphate (DCP) in different molar ratios and by employing a thin film hydration technique. Evaluation on the ability of these vesicles to entrap the studied drug has shown that the preparation with the ratio of 1:1:0.1 molar ratio of Tween 60, cholesterol and DCP as the best formulation. Further it was concluded that ocular irritancy studies using albino rabbits showed no sign of irritation for all tested niosomal formulations.

Adams *et al.,*[119] has compared concentrations of tenofovir-diphosphate (TFV-DP) and FTC-triphosphate (FTC-TP) in the upper layer packed cells (ULPCs) obtained after whole blood centrifugation to active metabolites of tenofovir (TFV) and emtricitabine (FTC) in isolated peripheral blood mononuclear cells (PBMCs) as a possible alternative marker of adherence. The results showed that the ULPC concentrations significantly correlated with PBMC concentrations. Preliminary single-dose data has suggested some discrimination between intermittent versus consistent dosing. Thus ULPC concentrations of TFV-DP and FTC-TP may be further investigated as a measure of ARV adherence.

Agarwal *et al.,*[120] has studied the effect of cholesterol on entrapment efficiency and HLB value of surfactant on the vesicle size using primaquine phosphate niosomes prepared from sorbitan esters by hand shaking technique and has concluded that entrapment efficiency increased with increasing cholesterol content and mean size increased with increasing HLB value.

Agarwal *et al.,*[121] has synthesized and evaluated three fatty acyl conjugates of (-)-2',3'-dideoxy-5-fluoro-3'-thiacytidine (FTC, emtricitabine) against HIV-1 cell-free and cell-associated virus and compared with the corresponding parent nucleoside and physical mixtures of FTC and fatty acids. Among all the compounds, the myristoylated conjugate of FTC displayed the highest potency with higher activity against multidrug resistant viruses B-NNRTI and B-K65R, indicating that FTC conjugation with myristic

acid generates a more potent analogue with a better resistance profile than its parent compound.

Akhilesh ***et al.,***[122] has systemically reviewed on preparation methods, characterization, factors affecting release kinetics, advantages and applications of niosomes. The advantage of Span 60 as surfactant in the formulation of niosomes has been reviewed.

Akhter ***et al.,***[123] has prepared nanosized niosomal dispersion of ganciclovir from Span40, Span60, and cholesterol in the molar ratio of 1:1, 2:1, 3:1 and 3:2 using reverse evaporation method. The developed niosomal dispersions were characterized for entrapment efficiency, size, shape, *in vitro* drug release, release kinetic study, and *in vivo* performance. The release of the drug from the formulations showed a sustained release profile for more than 24 h with zero-order release kinetics. Thus it has been concluded that the developed niosomal formulation of ganciclovir may act as a potential oral drug delivery.

Attia ***et al.,***[124] has prepared acyclovir niosomes by thin film hydration method using cholesterol, Span 60 and dicetyl phosphate in the molar ratio of 65:60:5. Niosomes produced were unilamellar spherical shape. The *in vitro* release for free drug and niosomal formulation followed Higuchi's equation with significant retard in the release from the niosomal formulation. Following oral administration, the relative bioavailability of niosomal dispersion in relation to free solution increased to 2-fold with increased mean residence time (MRT) showing a sustained release.

Babita ***et al.,***[125] has reviewed on advantages, disadvantages, methods of preparation and applications of niosomes as promising carriers for drug delivery.

Balakrishnan ***et al.,***[126] has formulated and evaluated minoxidil niosomes using polyoxyethylene alkyl ethers (Brij) or sorbitan monoesters (Span) with cholesterol molar ratios of 0, 1 and 1.5 by thin film-hydration method. The prepared niosomes were compared with control minoxidil solution (propylene glycol-water-ethanol at 20:30:50, v/v/v) or a leading topical minoxidil commercial formulation (Minoxyl). The results showed that the type of surfactant, cholesterol and incorporated amount of drug altered the entrapment efficiency of niosomes. Greater accumulation was observed with

the niosomal formulation, thus suggesting the potential use of niosomal topical delivery of minoxidil in hair loss treatment.

Balasubramaniam *et al*.,[38] has formulated daunorubicin hydrochloride niosomes by modified reverse evaporation process to increase the therapeutic efficacy of Dalton's ascetic lymphoma.

Basiri *et al.,*[112] has studied the use of neutral and negatively charged niosomes for DNA (PUC18 supercoiled plasmid) complexation. Different proportions of Span/Tween/ Cholesterol with or without dicetylphosphate were utilized for niosomes preparation by the film hydration method. The complexation percent of DNA-niosomes was as high as 80% in some formulations and the DNA was stable during complexation and extraction processes. The results concluded that the negatively-charged niosomal systems can be used as a gene-delivery vector in the presence of Ca2+.

Bayindir *et al.,*[127] has formulated paclitaxel (PCT) niosomes using different surfactants (Tween 20, 60, Span 20, 40, 60, Brij 76, 78, 72) by film hydration method. PCT has been successfully entrapped in all of the formulations. Depending on the incorporation of negatively charged dicetyl phosphate to the formulations negative zeta potential values were obtained. High surface charges showed that niosomes can be suspended in water well and this is beneficial for their storage and administration. The release of PCT from niosomal preparations was found to be slow with reduced side effects and greater gastrointestinal stability.

Bhardwaj *et al*.,[128] have reviewed on niosome as a novel prospect of drug delivery.

Bhaskar *et al*.,[129] has reviewed on the use of multifunctional nanocarriers for diagnostic purpose, drug delivery and drug targeting across blood-brain barrier with perspectives on tracking and neuroimaging.

Bisson *et al.,*[130] has tested the hypothesis that initiation of ART during initial treatment of human immunodeficiency virus (HIV)/cryptococcal meningitis (CM) would improve CSF clearance of C.neoformans. The results concluded that early ART was not associated with improved CSF fungal clearance, but resulted in a high risk of

CM immune reconstitution inflammatory syndrome (CM-IRIS) and further research on optimal incorporation of ART into CM care is needed.

Budhiraja ***et al.,***[131] has formulated niosomes of rosemarinic acid (ROA), a naturally occurring ester of caffeic acid by reverse phase evaporation method using different ratios of Span 85 and cholesterol and performed the *in vitro* antimicrobial evaluation against P. acne and S. aureus. Niosomal gel formulations of rosemarinic acid for sustained delivery were developed and *in vivo* study of developed formulation on Swiss albino mice in comparison with solution of plain drug and a marketed formulation of benzoyl peroxide has proved that niosomes may act as novel carriers for delivery rosemarinic acid to deeper tissues of skin in the treatment of acne vulgaris.

Bulteel ***et al.,***[132] has analysed linked data from the observational UK Collaborative HIV Cohort (CHIC) Study and UK HIV Drug Resistance Database (HDRD) to investigate the rate of development of K65R or M184V resistance mutations in patients failing on combinations containing tenofovir (TDF) and efavirenz (EFV) with either Lamivudine (3TC) or emtricitabine (FTC). Virological failure was defined as 1 viral load >400 copies/ml. Rates were stratified by demographic variables, baseline viral load, current CD4 count, current viral load and year of starting regimen. Logistic regression was used to determine whether there were any significant associations between type of regimen and detection of resistance mutation. The results concluded no evidence of an increased risk of development of M184V and K65R in patients exposed to 3TC.

Das ***et al.,***[133] has formulated and evaluated rofecoxib niosomes by lipid film hydration technique. Niosomal vesicles were then incorporated into blank carbopol gel to form niosomal gel. The *in vitro* permeation through pig skin showed a prolong drug release behavior with sustained action compared to plain drug gel.

Dufes ***et al.,***[9] has studied the encapsulation of vasoactive intestinal peptide (VIP) within glucose-bearing niosomes significantly allows higher VIP brain uptake compared to control niosomes (up to 86%, 5min after treatment). Brain distribution of intact VIP after injection of glucose-bearing niosomes indicated preferential location of radioactivity in the posterior and the anterior parts of the brain. Homogeneous distribution was observed in the whole brain after the administration of control vesicles.

The results showed that this novel vesicular formulation of VIP delivers intact VIP to particular brain regions in mice. Thus glucose-bearing vesicles might be a novel tool to deliver drugs across the blood-brain barrier (BBB).

Dumond ***et al.,***[134] has studied the steady-state pharmacokinetics (PK) of two common ARV regimens [tenofovir (TFV)/emtricitabine (FTC)/efavirenz (EFV) and TFV/FTC/atazanavir (ATV)/ritonavir (RTV)] in older nonfrail HIV-infected patients using non-compartmental pharmacokinetic analysis to estimate PK parameters [area under the concentration-time curve over 24 h (AUC_{0-24} h) and maximal concentration (C_{max})]. The results concluded that the PK of these ARVs is altered by 5-78% in an older HIV-infected population. Implications of PK differences form the basis for further study of ARV PK, efficacy, and toxicity in older HIV-infected patients.

El-Ridy ***et al.,***[135] has developed niosomal formulations of silymarin which is a purified extract isolated from seeds of milk thistle *Silybum marianum* to enhance its activity with reduced side effects. Niosomes were prepared using different non-ionic surfactants, cholesterol and charge inducing agents. The niosomes were characterized by transmission electron microscopy (TEM), differential scaning colorimetry (DSC), particle size analysis and *in vitro* release profiles. The hepatoprotective activity has been evaluated against carbon tetrachloride induced oxidative stress in albino rats by measuring the degree of liver protection using biochemical parameters like like serum glutamate oxaloacetic transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT) and serum alkaline phosphatase (SALP). Silymarin niosomal formulations showed reduced transaminase levels and SALP level compared to silymarin suspension which has been further proved histopathologically.

El-Say ***et al.,***[136] has formulated diclofenac sodium niosomes by lipid film hydration method and optimized the best formulation using response surface methodology by a three-level three-factor Box-Behnken experimental design. The variables selected were HLB, cholesterol ratio and lipid ratio as formulation variables and dependent variables as drug entrapment efficiency and the cumulative % release of DS from entrapped niosomes after 1, 6, and 12 h. The shape of the predicted response surfaces and model optimization was done by applying canonical analysis. The optimized formulation prepared according to computer-determined levels provided an entrapped efficiency and controlled release profile, which were close to the predicted

values and thus proved that experimental design methodology could efficiently be applied for characterization and optimization of formulation parameters affecting entrapment efficiency and drug release from DS niosomes.

Friden *et al*., [137] has studied on use of drug-specific correction for residual blood for measuring the brain drug exposure and has found that the results highlight the value of determining unbound brain-to-plasma concentration ratio of a drug with statistical precision to enable appropriate interpretation of brain exposure for drugs that appear to be restricted to the brain vascular spaces.

Gish *et al*.,[138] has performed the dose range study of pharmacokinetics, safety and preliminary antiviral activity of emtricitabine (FTC) in adults with hepatitis B virus infection as phase I-II, open-label dose range study of FTC in patients with HBV infection receiving FTC monotherapy at one of five doses ranging from 25-300 mg once daily (q.d) for 8 weeks. The results concluded that the optimal dose of FTC for the treatment of HIV infection is the same as the optimal dose for HBV infection and thus supporting further clinical development in chronic active hepatitis B.

Guinedi *et al.,*[139] has formulated acetazolamide niosomes with Span 40 or Span 60 and cholesterol in the molar ratios of 7:4, 7:6 and 7:7 using reverse-phase evaporation and thin film hydration methods. The niosomes were characterized for entrapment efficiency, size, shape and *in vitro* drug release. The results showed that the type of surfactant, cholesterol content and the method of preparation altered the entrapment efficiency and drug release rate from niosomes. Higher entrapment efficiency has been obtained with multilamellar niosomes prepared from Span 60 and cholesterol in a 7:6 molar ratio. Stability studies showed a fairly high retention of acetazolamide inside the vesicles (approximately 75%) at a refrigerated temperature up to a period of 3 months. Significant decrease in intraocular pressure (IOP) has been observed with niosomal formulations prepared by either method compared to free drug and plain solution in rabbits which was measured using ShiØtz tonometer. Effective and prolonged decrease in IOP has been observed with multilamellar acetazolamide niosomes formulated with Span 60 and cholesterol in a 7:4 molar ratio. Histological examination of corneal tissues after instillation of niosomal formulation for 40 days showed slight irritation in the substantia propria of the eye which is reversible. No major changes in tissues were observed.

Hamarapurkar ***et al.,***[140] has developed a new high-performance liquid chromatography method for a stability-indicating assay for emtricitabine and the quantification of its related substances. The degradation behaviour has been determined by subjecting the drug to stress conditions of hydrolysis, oxidation, photolysis and thermal decomposition. Extensive degradation was found under acid, alkaline and oxidative stress and 5 related substances were consistently monitored under stress conditions. Thus it has been concluded that as the method separates the degradation products effectively, it can be used as a stability indicating method and for purity control of emtricitabine.

Hashim ***et al.,***[141] has prepared ribavirin niosomes by the thin film hydration method using Span 60, cholesterol, and dicetyl phosphate in molar ratios of (1:1:0), (4:2:0), (1:1:0.1), and (4:2:1). The optimized formulation with the molar ratio (4:2:1) was selected for *in vivo* liver targeting study and compared to free ribavirin solution. The results showed a 6-fold increase in ribavirin liver concentration for niosomal formulation in comparison with ribavirin-free solution thus showing improved efficacy of low doses of ribavirin and minimized toxic side-effects at higher doses.

Huang ***et al.,***[113] has synthesized and developed polysorbate cationic niosomes (PCNs) comprising of nonionic surfactants (i.e., polysorbates) and a cationic cholesterol using a film hydration method as gene carriers. The binding capacity of PCNs toward oligodeoxynucleotides (ODN) was assessed by a gel retardation approach, which demonstrated that the ionic complexes were formed when ± charge ratio reached to 4 or greater. Gene transfer study showed that the PCNs exhibited a high efficiency in mediating cellular uptake and transferred DNA expression. Based on these findings, PCNs may offer the potential to function as an effective gene delivery system.

Ibrahim ***et al.,***[60] has formulated and evaluated proniosomal transdermal carrier systems for flurbiprofen using span 20 (Sp 20), span 40 (Sp 40), span 60 (Sp 60) and span 80 (Sp 80) as surfactants with and without cholesterol at various concentrations from 0% to 50%. The effect of surfactant type and cholesterol content on drug release was tested by diffusion through cellophane membrane and rabbit skin. Drug release from niosomal preparations was compared to flurbiprofen suspensions in distilled water

and HPMC (hydroxyl propyl methyl cellulose) gels. Niosomes prepared with Sp 40 and Sp 60 produced gel systems with and without cholesterol. In case of Sp 20 and Sp 80, addition of cholesterol resulted in proniosomal alcoholic solutions or liquid crystalline gel systems. On the other hand, both Sp 40 and Sp 60 produced gel systems in presence or absence of cholesterol. Lower drug diffusion rates were observed with rabbit skin compared to cellophane membrane. The proniosomal composition controlled drug diffusion rates to be either faster or slower than the prepared flurbiprofen suspensions in HPMC gels or distilled water, respectively and thus concluded that proniosomal formulation to be a better alternative for transdermal drug delivery.

Jackson *et al.,*[142] has investigated the pharmacokinetics (PK) of plasma tenofovir (TFV)/emtricitabine (FTC), their intracellular (IC) anabolites, TFV-diphosphate (DP) and FTC-triphosphate (TP), and plasma efavirenz (EFV) over 10 days after intake cessation in HIV-negative volunteers. The results fully characterized the PK of TFV and TFV-DP, FTC and FTC-TP, and EFV after stopping the drug combination. Thus, it has been concluded that although decay in concentrations can be related to a target for EFV, it is more difficult for the IC phosphates.

Jadon *et al.,*[44] developed and evaluated niosomal formulations of griseofulvin by using different non-ionic surfactants like Span 20, Span 40, and Span 60. The lipid mixture consisted of surfactant, cholesterol, and dicetyl phosphate in the molar ratio of 125:25:1.5, 100:50:1.5, and 75:75:1.5, respectively and were prepared by thin film method and ether injection method. The *in vitro* and *in vivo* studies have shown a sustained release profile and thus it has been concluded that niosomal formulations of griseofulvin could be a better delivery with improved bioavailability.

Jain *et al.,*[143] developed mannosylated niosomes as oral DNA vaccine carriers using Span 60, cholesterol and stearylamine by reverse phase evaporation method and coating with a modified polysaccharide o-palmitoyl mannan (OPM) in order to protect them from bile salt.

Jatav *et al.,*[144] has prepared rifampicin niosomes using various non-ionic surfactants of sorbitan ester class and cholesterol in 50:60 (1:1.2) percent mol fraction ratios by hand shaking method. The percent of drug estimated to be entrapped decreased progressively for various sorbitan esters used in the order of Span-85>Span-80>Span-

60>Span-40>Span-20. *In vitro* release rate studies revealed that the cumulative percent rifampicin released was maximum for Span-20-based niosomes and minimum for Span-85-based niosomes.

Josserand *et al*.,[145] has evaluated drug penetration into the brain using a double study by *in vivo* imaging with positron emission tomography and *in vitro* model of the human blood-brain barrier and has found a remarkable correlation between the *in vitro* human model and *in vivo* permeability coefficients (r = 0.99) using rats.

Khositsuntiwong *et al.,*[114] has studied the enhancement of tyrosinase gene expression and melanin production by loading the plasmid in elastic cationic niosomes by investigating in tyrosinase gene knocked out human melanoma (M5) cells and in tyrosine-producing mouse melanoma ($B_{16}F_{10}$) cells. The study has demonstrated not only the enhancement of the expression of human tyrosinase gene by loading in elastic cationic niosomes, but also the potential application of this gene delivery system for the further development of vitiligo gene therapy.

Kromdijk *et al.,*[146] has developed and validated an assay for the simultaneous quantification of the antiviral and antiretroviral drugs zidovudine, abacavir, emtricitabine, lamivudine, tenofovir and ribavirin in human plasma using liquid chromatography coupled to tandem mass spectrometry and it has been to found to be a suitable method for the determination of zidovudine, abacavir, emtricitabine, lamivudine, tenofovir and ribavirin in human plasma in clinical practice to monitor plasma concentrations in selected cases to optimize therapy.

Lee *et al*.,[6] have reviewed on the drug transporters in the central nervous system: brain barriers and brain parenchyma considerations and has focused on the molecular characteristics, localization, and substrate specificities of several classes of well-known membrane drug transporters in the brain.

Liu *et al*.,[147] has reviewed on the surrogate measurement for brain free concentration by using cerebrospinal fluid concentration and plasma free concentration of the drug. It has been hypothesized that concentration in CSF is equivalent to or better than unbound drug in plasma to predict unbound concentration in the brain.

Maheshwari ***et al.,*** [92] has evaluated niosomes for topical delivery of vaccines using hepatitis B surface protein as an antigen and cholera toxin B as an adjuvant. The characterized niosomes were evaluated for skin deposition studies of antigen using human cadaver skin and skin penetration efficiency of niosome by confocal laser scanning microscopy. The immune stimulating activity of these vesicles was studied by measuring the serum IgG titer, isotype ratio IgG2a/IgG1 and mucosal immune responses following transcutaneous immunization in Balb/c mice and results were compared with the alum adsorbed HBsAg given intramuscularly and topically administered plain HBsAg solution. The results concluded that topical immunization with cholera toxin B is potential adjuvant for cutaneous immune responses when coadministered with the HBsAg encapsulated niosomes and thus to be an effective topical vaccine delivery.

Manconi ***et al.,*** [148] has studied the influence of vesicle composition and preparation method on the vesicle structure (MLV, LUV, and SUV), size distribution, entrapment efficiency and *in vitro* release of incorporated tretinoin.

Manconi ***et al.,*** [149] has prepared tretinoin loaded vesicles by film hydration, extrution technique and sonication, and evaluated for the influence of vesicle structure on the photostability of tretinoin compared to drug in methanol.

Manikandan ***et al.,*** [150] has formulated and evaluated emtricitabine and tenofovir disproxil fumarate tablets and has concluded that the prepared tablets could perform therapeutically, with improved efficacy and better patient compliance like that of marketed product.

McPhail ***et al.,*** [94] has developed and evaluated polymeric vesicles using palmitoyl glycol chitosan and cholesterol (2:1) by weight, and then encapsulating within egg phosphatidylcholine, cholesterol (2:1) by weight, to yield liposomes or hexadecyl diglycerol ether to yield niosomes in vesicle system with encapsulated carboxyfluorescein.

Misra ***et al.,*** [6] has reviewed on the drug delivery to the central nervous system which includes recent advances in brain targeting, rational drug design approach and drug delivery to the CNS with a brief intercellular characterization of the blood-barrier.

Mujoriya *et al.,*[151] has reviewed on niosomes as carriers for site specific drug delivery system. A detailed review was presented on their merits, demerits, factors affecting vesicle size, entrapment efficiency and release characteristics, stability of niosomes, interactions of niosomes with the cells, *in vivo* behaviour of niosomes, characterization of niosomes and niosomally entrapped bioactive agents.

Nagaraju *et al.,*[152] has formulated folic acid, a water soluble vitamin with low blood brain barrier penetrability as niosomal nasal delivery to target the brain. Folic acid niosomes were prepared using different non-ionic surfactants like Span 20, Span 60, Span 80, Tween 20 and Tween 80 with cholesterol by lipid layer hydration technique. The niosomes were evaluated for particle size, viscosity, osmotic shock, entrapment efficiency and *in vitro* drug release. The prepared folic acid niosomes were found to be stable and followed anomalous diffusion with first order release kinetics. *Ex-vivo* perfusion studies using rat model, showed that about 48.15% of drug has been absorbed through nasal cavity at the end of 6 hrs.

Pardakhty *et al.,*[153] has reviewed on niosomes as vectors for gene transfer. The methods of niosome preparation, the vesicle stability related aspects, routes of administration and pharmaceutical applications are discussed.

Pardakhty *et al.,*[154] has developed niosomal formulations of recombinant human insulin using Brij 52, Brij 92 or Span 60 and cholesterol. The studies were performed to evaluate the *in vitro* release of insulin in simulated intestinal fluid (SIF) and simulated gastric fluid (SGF), protection of entrapped insulin compared to free insulin solution against pepsin, α-chymotrypin and trypsin and its effect on diabetes induced by IP injection of streptozotocin (65mg/kg) in male wistar rats after oral and subcutaneous administration. The results concluded that Brij 52 niosomes showed better release rate than Brij 92 and Span 60 vesicles. Protection against proteolytic enzymes was more pronounced with niosomally encapsulated vesicles compared to free insulin solution. Niosomes formulated with Brij 92 showed lower blood glucose and elevated serum levels. Thus niosomal insulin may be a better alternative to oral insulin delivery provided further studies on improving oral bioavailability by encapsulating with protease inhibitors are carried out.

Portegies *et al*.,[155] have studied the CSF/CNS penetration and efficacy of antiretroviral drugs in HIV associated CNS disease. They have classified the drugs as low, intermediate and high CNS penetration effectiveness rank and have correlated it with the neurocognitive disorders. Emtricitabine is classified as a drug with intermediate rank.

Priprem *et al*.,[156] has formulated and evaluated intranasal delivery of nanosized melatonin-encapsulated niosomes in rats and has concluded that melatonin niosomes of about 100 nm, intranasaly administered, could distribute melatonin to the liver, hypothalamus and testis.

Reichel.,[157] has reviewed the role of blood-brain barrier studies in the pharmaceutical industry with emphasis on target identification and validation, lead generation and optimization, candidate selection and profiling, preclinical development and clinical studies.

Ruckmani *et al.,*[158] has formulated zidovudine (ZDV) niosomes for intravenous administration by a thin-film hydration technique. Proniosomes were prepared in the form of a slurry using beta-cyclodextrin as carrier. The distribution of ZDV in lungs, kidney, heart, liver and spleen of mice after intravenous bolus injection was higher in Tween 80 niosomes without DCP than either niosomes with DCP or Tween 80 proniosomes. The amount of ZDV in plasma was low in Tween 80 niosomes without DCP. Pharmacokinetic study in rabbits showed that Tween 80 formulations with DCP were cleared from the circulation within five hours. An increased half-life and mean residence time was observed in Tween 80 formulation. Greater stability was observed with Tween 80 formulations stored at 4^0C. The results concluded that niosomes as promising vehicle for targeted delivery of ZDV to macrophages in spleen and liver.

Ruckmani *et al.,*[159] has formulated zidovudine niosomes and evaluated process-related variables like hydration and sonication time, rotation speed of evaporation flask, and the effects of charge-inducing agent and centrifugation on zidovudine entrapment and release from niosomes. Formulation of zidovudine niosomes was optimized by altering the proportions of Tween, Span and cholesterol. The results showed that zidovudine niosomes formulated with Tween 80 entrapped high amounts of drug and the addition of DCP enhanced drug release for a longer time (88.72% over 12 h). The

mechanism of release from Tween 80 formulation was the Fickian type and obeyed first-order release kinetics. Thus it has been concluded that by proper adjustment of process parameters a sustained delivery of zidovudine niosomes may be obtained.

Sankhyan ***et al.,***[16] has reviewed on niosomes as drug delivery system for various drugs for their maximum therapeutic u[1]tilization in management and treatment of various dreadful diseases.

Sankhyan ***et al.,***[110] has formulated and evaluated metformin loaded niosomes by thin film hydration technique using various surfactants. The *in vitro* release studies concluded that metformin loaded niosomes to be effective in sustaining the drug release leading to decreased side effects and increased patient compliance.

Shaikh ***et al.,***[78] has formulated and evaluated ciclopiroxolamine (CPO), a broad spectrum antifungal drug as niosomes by ethanol injection method using Span 60, cholesterol and dicetyl phosphate by 3(2) factorial design. The niosomes obtained were unilamellar with entrapment efficiency of 38-68%. The results showed that increasing the concentration of span 60 and cholesterol increased the vesicle size and thus its entrapment efficiency. *In vitro* percent drug deposition was found to be poor with increase in surfactant concentration. Deposition of CPO into rat skin from niosomal dispersion and gel was significantly higher than plain CPO solution. It was concluded that niosomes may be a promising tool for cutaneous retention of CPO.

Shirsand ***et al.,***[160] has developed and evaluated ketoconazole niosomes for topical administration to target the drug to the superficial areas of the skin. Niosomes were prepared by a thin film hydration method using different ratios of non-ionic surfactants (Span 40, 60 and Tween 60) along with cholesterol (CHO). The optimized formulation containing Span 60 and CHO in the ratio of 1:0.2 was incorporated into 1% Carbopol gel. The niosomal gel was evaluated for various physicochemical parameters and antifungal activity. The results concluded that niosomal formulation has better antifungal activity compared to the non-niosomal formulation.

Singh ***et al.,***[161] has developed niosomal encapsulated nimesulide using span 20, 40, 60 and cholesterol in different molar ratios. The niosomes prepared by lipid film hydration method were multilamellar vesicles (MLVS) and niosomes prepared by ether

injection technique were unilamellar vesicles (ULVS) or oligolamellar vesicles. The higher entrapment efficiency was observed with MLVS prepared from span 60 and cholesterol in an 80:70 molar ratio. The *in vitro* release kinetics was found to follow zero order and Higuchi diffusion controlled mechanism. Niosomal preparation stored at refrigerated temperature for 60 days was found to be more stable compared to niosomes stored at room temperature and elevated temperature conditions. Thus niosomal encapsulation of nimesulide may prolong the drug release at a constant and controlled rate.

Srinivas *et al.,*[162] has developed and optimized niosomal formulation of aceclofenac to improve its bioavailability. By varying the composition of non-ionic surfactant and cholesterol in the formulations, various evaluations such as encapsulation efficiency, particle size and drug release were studied. The release showed an extended release up to 72 h in all formulations. The release of the optimized formulation containing drug:surfactant:cholesterol in the ratio of 1:2:1 with Span 20 as surfactant was found to follow Peppas kinetics.

Tangri *et al.,*[163] has reviewed on the preparation methods, characterizations, factors affecting release kinetic, advantages, and applications of niosomes.

Uglietti *et al.,*[164] has studied on the PK/PD features of Emtricitabine (FTC) and tenofovir disoproxil fumarate (TDF), when given as single agent or when administered as FDC. The results concluded that toxicity issues (kidney, bone) are still to be entirely elucidated and the drug-induced component well separated from patient- and HIV-related ones.

Valli *et al.,*[55] has formulated and evaluated diclofenac sodium suspension prepared by thin film hydration method using different ratios of cholesterol and surfactant (Span 60) and optimized the formulation with 1:2:1 as the best formulation with highest entrapment efficiency. The release was found to follow Korsmeyer-Peppas equation with considerable stability for 2 weeks. Thus has been concluded that diclofenac sodium niosomal formulation may act as depot and offer a controlled release of drug compared to conventional drug delivery.

Venkatesh *et al*.,[8] have formulated and evaluated temozolamide loaded niosomes for improved efficacy against cerebral tumours and has performed pharmacokinetic evaluation to prove its *in vivo* brain targeting in rats.

Yoon *et al*.,[165] has used immobilized artificial membrane phosphatidyl choline column chromatography for rapid screening of blood-brain penetration of twenty-three structurally diverse reference drugs and has concluded that *in vitro* IAM capacity factors (k_{IAM}/MW^4) may be used to classify drug as CNS+ and CNS- with a high rate of success.

6. MATERIALS AND METHODS

S.No.	Name of the chemical	Name of manufacturer
1	Emtricitabine	Abbott Laboratories as gift sample
2	Span 60	S.D.Fine-Chem Limited, India
3	Span 40	S.D.Fine-Chem Limited, India
4	Solulan C24	Lubrizol
5	Cholesterol Extrapure	S.D.Fine-Chem Limited, India
6	Glucosamine	Sigma-Aldrich Company
7	Palmitic acid N-hydroxy succinimide	Sigma-Aldrich Company
8	Di-sodium hydrogen O-phosphate anhydrous AR	S.D.Fine-Chem Limited, India
9	Sodium hydroxide pellets	S.D.Fine-Chem Limited, India
10	Choloroform AR	S.D.Fine-Chem Limited, India
11	Triethanolamine AR	S.D.Fine-Chem Limited, India
12	Dimethyl sulfoxide AR	S.D.Fine-Chem Limited, India
13	D-Sorbitol Extrapure	S.D.Fine-Chem Limited, India
14	Ethanol	S.D.Fine-Chem Limited, India
15	Sephadex G-50 (Granulometry 20-80 m)	Sigma-Aldrich Company
16	Triton X-100	S.D.Fine-Chem Limited, India
17	Acetronitrile	S.D.Fine-Chem Limited, India
18	Dulbecco’s phosphate buffered saline	Sigma-Aldrich Company
19	Hydrochloric acid	S.D.Fine-Chem Limited, India

7. EQUIPMENTS

S.No.	Name of the instrument	Name of the manufacturer
1	Rota evaporator	Labsol Enterprises, India
2	Laboratory vibrational micromill pulverisette 0	Laval Lab Inc, Canada
3	Probe sonicator	Mjl Lab Instruments and Equipments
4	Extrusor Liposo Fast-Basic	Avestin, Inc. Canada.
5	Nitrocellulose membranes (Nucleopore 100 nm)	Whatman, GE Healthcare Life Sciences
6	UV-visible spectrophotometer (Shimadzu 1601)	Shimadzu, Tokyo, Japan
7	Ultra centrifuge	Thermo Fisher Scientific
8	Dialysis bag	Spectra/Por®
9	Magnetic stirrer	Stuart Scientific
10	Magnetic bead	Sunsil
10	High-performance liquid chromatography	Shimadzu, Tokyo, Japan
11	IAM. PC. DD2 column	Regis Technologies, Morton Grove, IL.

8. METHODOLOGY

PREFORMULATION STUDIES

1. Identification of emtricitabine[169]

The infrared absorption spectrometry peaks of emtricitabine obtained were compared with that obtained with emtricitabine RS as per IP and represented in Figure 4.

2. Identification of sorbitan monopalmitate (Span 40) and sorbitan monostearate (Span 60) [170, 171]

The identification tests for sorbitan monopalmitate and sorbitan monostearate such as assay for fatty acids, iodine value, acid value, hydroxyl value, saponification value, water, residue on ignition, heavy metals, organic volatile impurities, residual solvents and assay for polyols were performed as per USP25 and the results are reported in Table .Excipient materials complies with USP standards.

S.No.	Identification tests	Standard limits	
		Span 40	Span 60
1	Assay for fatty acids	210-225 of palmitic acid	200-215 of stearic acid
2	Iodine value	NMT 4	NMT 4
3	Acid value	NMT 8	NMT 10
4	Hydroxyl value	275-305	235-260
5	Saponification value	140-150	147-157
6	Water	NMT 1.5%	NMT 1.5%
7	Residue on ignition	NMT 0.5%	NMT 0.5%
8	Heavy metals	NMT 0.001%	NMT 0.001%
9	Organic volatile impurities	Meets the requirements	Meets the requirements
10	Residual solvents	Meets the requirements	Meets the requirements
11	Assay for polyols	NLT 32.0% and NMT 38.0% of polyols w/w	NLT 27.0% and NMT 34.0% of polyols w/w

3. Identification of cholesterol[172]

Solubility in Alcohol: Accurately weighed 500 mg of cholesterol was dissolved in 50 mL of warm alcohol in a stoppered flask or cylinder, and allowed to stand at room temperature for two hours: no deposit or turbidity should be formed.

Identification tests

A. One mL of sulfuric acid was added to a solution of 10 mg in 1 mL of chloroform. The chloroform acquires a blood red colour and the sulfuric acid shows a green fluorescence.
B. About 5 mg was dissolved in 2 mL of chloroform. To this 1 mL of acetic anhydride was added, followed by addition of one drop of sulfuric acid: a pink colour that rapidly changes to red, then to blue, and finally to a brilliant green should be produced.

The sample passed the identification tests.

Melting Range: Between 140^0 C and 150^0 C. Sample melted at 143^0 C.

Table 1: Formulation of drug-free niosomes[10]

Formulation code	Method of preparation	Composition	Mole percent	Total concentration (mM)
Formulations containing Span 60				
F1A1	(TLE) – Vortex	Span 60	100	0.1
F1A2	(TLE) – Paddle			
F1A3	RPEM			
F1A4	Proniosome			
F1B1	(TLE) – Vortex	Span 60:CHL:SOL	50:40:10	38
F1B2	(TLE) – Paddle			
F1B3	RPEM			
F1B4	Proniosome			
F1C1	(TLE) – Vortex	Span 60:CHL:SOL	50:40:10	9.5
F1C2	(TLE) – Paddle			
F1C3	RPEM			
F1C4	Proniosome			
Formulations containing Span 40				
F2A1	(TLE) – Vortex	Span 40	100	0.1
F2A2	(TLE) – Paddle			
F2A3	RPEM			
F2A4	Proniosome			
F2B1	(TLE) – Vortex	Span 40:CHL:SOL	50:40:10	38
F2B2	(TLE) – Paddle			
F2B3	RPEM			
F2B4	Proniosome			
F2C1	(TLE) – Vortex	Span 40:CHL:SOL	50:40:10	9.5
F2C2	(TLE) – Paddle			
F2C3	RPEM			
F2C4	Proniosome			

(TLE) – Vortex	**:**	Thin layer evaporation-vortex method
(TLE) – Paddle	**:**	Thin layer evaporation-paddle method
RPEM	**:**	Reverse phase evaporation method
CHL	**:**	Cholesterol
SOL	**:**	Solulan C24
mM	**:**	Millimolar

PREPARATION OF DRUG-FREE NIOSOMES[10]

Niosomal suspensions were prepared by four different methods (thin layer evaporation (TLE)-vortex method, thin layer evaporation (TLE)-paddle stirring method, reverse phase evaporation method and proniosome method) with Span 40 and Span 60 as the main surfactant. Cholesterol (CHL) and Solulan C24 (SOL) were also included in the niosomal formulations. Variations in compositions of the surfactant or surfactant-lipid mixture were made by varying the molar ratios of Span:CHL:SOL as 100:0:0 and 50:40:10. The total concentration of components ranged from 0.1 to 38 mM. The compositions of the various formulations are represented in Table 1.[10]

The total concentration of components was fixed to 38 mM, since higher lipid amounts produce more viscous samples.[173]

Studies reported show that SOL when included in niosomal formulation has reduced haemolytic effect but when the concentration is increased beyond 10 mol% has increased haemolytic effect due to the soluble surfactant SOL not incorporated into the membrane of niosomes and is thus present in solution as monomers or micelles. So, the concentration of SOL was kept at 10 mol%, since high levels of SOL above this are potential haemolytic.[174, 175]

Method of Preparation[10]

1) Thin layer evaporation (TLE)-vortex method

The surfactant or surfactant-lipid mixture was dissolved in chloroform and introduced into a round-bottom flask. The solvent was evaporated under vacuum to form a thin layer on the flask wall. After hydration of the thin layer with 5 mL of

phosphate buffer saline, 4 cycles of heating (3 min at 65±5 °C) and vortex mixing (3 min) were performed. The temperature of 65°C was selected since it is above the phase transition temperature of Span 60,[176] which is the component of the lipid mixture with the highest value of transition temperature (50°C).

2) Thin layer evaporation (TLE)-paddle stirring method

The surfactant or surfactant-lipid thin layer obtained as above was hydrated with 25 mL of phosphate buffer saline. A paddle was then introduced into the flask and the suspension was stirred for 30 min at 65°C.

3) Reverse phase evaporation method

Ten mL of the surfactant or surfactant-lipid mixture dissolved in chloroform was added to 5 mL of phosphate buffer saline. The resulted dispersion was sonicated for 3 h to form water in oil emulsion. Chloroform was then removed by evaporation of the solvent overnight under vaccuum.

4) Proniosome method

Sorbitol was ground for 30 min at 24 Hz in a high-energy vibrational micro mill. The 150-300 µm granulometric fractions were isolated and 1 g of this was added to a round-bottom flask connected to a rotary evaporator. The surfactant or surfactant-lipid mixture dissolved in chloroform (3 mL) was sequentially sprayed onto the powder surface by alternating with solvent evaporation. After addition of the last aliquot, the solvent was removed completely to obtain proniosomes. Hydration of proniosomes was carried out with 20 ml of phosphate buffer saline.

SIZE REDUCTION OF NIOSOMES[5]

The formulated niosomal suspensions were centrifuged for 15 min at 4000 rpm. The supernatant was collected and divided into two portions of which one portion was subjected to sonication and the other for extrusion method for size reduction.

1. **Sonication method:** The niosomal suspensions were sonicated for 5 min with the instrument set at 80 % of its maximum power.

2. **Extrusion method:** The niosomal suspensions were extruded 11 times through nitrocellulose membranes (Nucleopore 100 nm, Whatman) using Extrusor Liposo Fast-Basic.

All the size reduced samples were stored at 4° C protected from the light.[10]

SYNTHESIS OF GLUCOSE-DERIVATIZED SURFACTANT N-PALMITOYL GLUCOSAMINE (NPG) [9]

N-palmitoyl glucosamine (NPG) was synthesized by the slightly modified method of Dufes *et al*.[9] Glucosamine (1.27 g) was added to a mixture of 1.5 mL of triethanolamine and 220 mL of DMSO. The mixture was stirred for 30 min at room temperature. Palmitic acid N-hydroxysuccinimide (2.5 g) dissolved in chloroform was then added to the mixture, left stirring for 72 h at room temperature, protected from the light. This was immersed in an ice bath and 100 mL of cold water was added. The precipitated product was collected by filtration, washed with water, DMSO and ethanol. Then it was air dried for 48 h and stored at 4°C protected from the light.

STANDARD CALIBRATION OF EMTRICITABINE IN DISTILLED WATER AT 281 nm

Accurately 100 mg of the drug emtricitabine was weighed and dissolved in distilled water in a 100 mL standard volumetric flask. The volume was made up to the mark with distilled water. From this stock solution containing 1 mg/mL, solutions containing 5, 10, 15, 20 and 25 µg/mL concentrations of drug were prepared and the absorbance of these solutions were determined spectrophotometrically at 281 nm.[177] The values are tabulated in Table 3. A graph was plot by taking concentration of drug on X-axis and absorbance on Y-axis and is represented in Figure 5.

PREPARATION OF NPG-NIOSOMES AND EMTRICITABINE (FTC) LOADED-NPG-NIOSOMES[10]

Based on the results obtained on the evaluation of the niosomal dispersions, the total concentration of components chosen, method of preparation and their composition by Span:CHL:SOL:NPG were optimized. The drug free-NPG niosomes were prepared by these optimized conditions with different hydration temperatures of 65° C and 90° C. Literature also suggests that increasing the hydration temperature may have a

positive effect on the entrapment efficiency.[178, 179] After preparation, all suspensions were centrifuged and sonicated as described above.[10]

The suitability of these techniques for drug-loaded vesicles prepared at different hydration temperatures was assessed by determining the stability of emtricitabine under these experimental conditions. Studies reported on stability of emtricitabine show that emtricitabine is stable to forced degradation by acid hydrolysis, alkali hydrolysis and thermal degradation.[178, 179] Stability studies of emtricitabine was carried out under the experimental conditions by maintaining the drug solution containing 50 mg of emtricitabine in 50 mL of distilled water under stirring for 1 h at 65±5° C and 90±5° C, protected from the light. The residual unaltered drug was quantified by UV-visible spectrophotometric analysis at 281 nm. The results are tabulated in Table 4.

Drug (FTC)-loaded NPG-niosomes were prepared according to the optimized method, using the same concentrations and component ratios previously used for empty NPG-niosomes. The aqueous phase consisted of 20 mL emtricitabine solution (2 mg/mL).[10]

CHARACTERIZATION OF NIOSOMAL SUSPENSIONS[10, 159]

1) Particle size of vesicles

Particle size analysis was carried out using optical microscopy with calibrated eyepiece micrometer. About 200 niosomes were measured individually and the mean diameter was calculated.[159] The results are tabulated in Table 2 for drug-free niosomes, Table 5 for NPG-niosomes and Table 6 for FTC-NPG-niosomes.

2) Formation of vesicles:

The effective formation of the vesicles was investigated by scanning electron microscopy (SEM). The SEM images are shown in Figure 6.

3) Drug encapsulation efficiency

Encapsulation efficiency of the niosomal dispersions were indirectly determined by using the following two methods:

i) Size exclusion chromatography method:

From each sample of emtricitabine-NPG-niosomal dispersion, 0.5 mL of the dispersion was loaded onto a Sephadex G50 column (granulometry 20-80 m) and eluted with water. Loaded-niosomes eluted as a liquid, while free emtricitabine remained bound to the column. Vesicles were disrupted by addition of 0.05 mL Triton X-100 followed by ultra-centrifugation at 120000 g for 1 h. The total drug amount in the niosomal dispersion and the drug amount actually encapsulated were determined by UV-visible spectrophotometric analysis at 281 nm in the supernatant after disrupting the vesicles before and after gel chromatography, respectively.[10] The encapsulation efficiency was calculated according to the following equation:

$$EE\% = \frac{[\text{Encapsulated drug}]}{[\text{Total drug}]} \times 100$$

All the data are the mean of five separate experiments. The results are tabulated in Table 7.

ii) Dialysis method:

From each of the emtricitabine-NPG-niosomal dispersion, 3 mL of the dispersion was dropped into a cellulose acetate dialysis bag (Molecular weight cut-off 12000), immersed in 150 mL of distilled water and magnetically stirred at 30 rpm. The unencapsulated drug was separated by replacing the receiving medium every 30 min, until the level of dialyzed drug was undetectable. The collected samples after suitable dilutions were analyzed spectrophotometrically at 281 nm using UV-visible spectrophotometer from which the amount of drug dialyzed were determined. The total amount of drug initially present in each niosomal suspension was determined by disrupting the vesicles by the addition of TritonX-100 followed by ultra-centrifugation (as described in size exclusion chromatography method) of 1 mL of non-dialyzed sample (to disrupt the vesicles) followed by emtricitabine assay in the supernatant by UV-visible spectrophotometric analysis at 281 nm. The percent of encapsulation efficiency (EE%) was determined by the following equation:

$$EE\% = \frac{[\text{Total drug}] - [\text{Diffused drug}]}{[\text{Total drug}]} \times 100$$

Each result is the mean of three separate experiments. The results are tabulated in Table 8.

STABILITY STUDIES OF NIOSOMAL DISPERSIONS

Stability studies of drug-loaded and unloaded niosomal dispersions were carried out by storing the niosomal suspensions protected from light and sealed at 4° C and 25° C. This was done to determine the physical stability of the formulated niosomes since niosomes have instability problems such as change in particle size due to aggregate formation and fusion, phase separation of bilayer components and leakage of encapsulated drug post formulation.[151] The characterization parameters like particle size and encapsulation efficiency (determined after separation of loaded from un-loaded drug by size exclusion chromatography) were evaluated immediately after the preparation and periodically with 3 months interval for a period of six months. The results are tabulated in Table 9.[10]

IN VITRO BLOOD-BRAIN BARRIER PENETRATION OF EMTRICITABINE USING IMMOBILIZED ARTIFICIAL MEMBRANE PHOSPHATIDYL-CHOLINE COLUMN CHROMATOGRAPHY[180]

High-performance liquid chromatography conditions

System	:	Shimadzu high-performance liquid chromatography
UV-Visible detector	:	SPD-10A
System controller	:	SCL-10A
Pump	:	LC-10AT
Auto sampler	:	SIL-10A
Column oven	:	CTO-10A

Analytical column:

IAM. PC. DD column (4.6 mm i.d.×10 cm length, particle size 5 µm, pore size 300 Å) purchased from Regis Technologies (Morton Grove, IL).

Mobile phase:

Mixture of acetonitrile and DPBS (20:80 v/v) with pH adjusted to 5.5 and 7.0 using hydrochloric acid.

Flow rate	:	0.5 ml/min at 37°C
λ_{max}	:	281 nm.

Determination of IAM partition coefficients

The CNS penetration of the formulation was evaluated by the method as described by Yoon *et al.*[165] Emtricitabine niosomal preparation was dissolved in a mixture of acetonitrile:distilled water (50:50, v/v) at a concentration of 100 µg/ml, and a portion (5 µl) was injected into the chromatograph (n = 3 each). The IAM capacity factor (kIAM) was calculated as

$k_{IAM} = t_r - t_o / t_o$ .... (1)

Where, t_r = the retention time of the drug and t_0 = the holdup time of the column. The membrane permeability (P_m) of a drug following passive diffusion may be expressed based on correction by the molecular size:

$$P_m \propto \frac{k_{IAM}}{MW^n} \quad \ldots\ldots\ldots\ldots\ldots\ldots \text{(2)}$$

Prediction of CNS penetration potential based on IAM partition coefficients The kIAM values was determined at the mobile phase pH of 5.5 and 7.0. The differentiation between CNS+ and CNS- drugs were made based on kIAM corrected by the molecular size with the power function set at n = 4 as at this power function of the capacity factors (kIAM/MWn), the values separated into two ranges.

The values for human CNS penetration and physicochemical parameters (pK_a, Clog P, and PSA) of emtricitabine obtained from the literature are tabulated in Table 10.[115, 181, 182, 183]

The classification of blood-brain barrier (BBB) penetration of drugs based on immobilized artificial membrane (IAM) capacity factors (k_{IAM}/MW^4 x 1010) using IAM chromatography is tabulated in Table 11.[165]

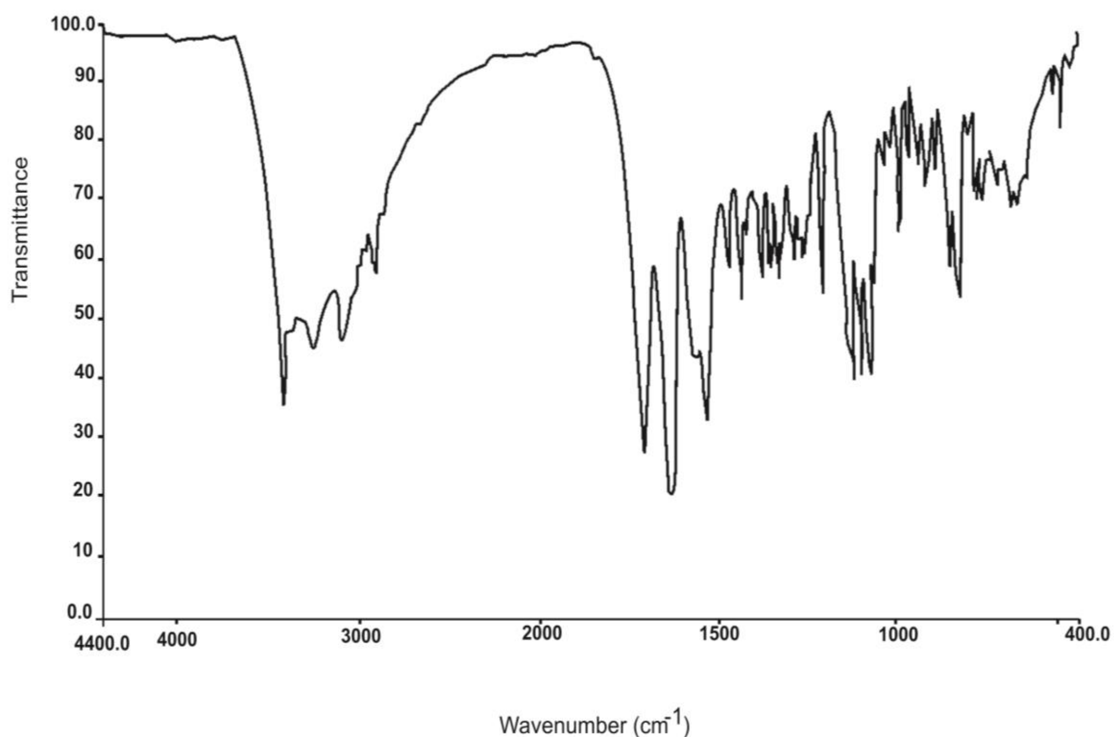
In vitro blood-brain barrier penetration values of emtricitabine from the niosomal formulation using immobilized artificial membrane phosphatidylcholine, column chromatography is tabulated in Table 12.

9. RESULTS AND ANALYSIS

PREFORMULATION STUDIES

1. Identification of emtricitabine

Figure 4: FTIR spectra of emtricitabine



2. Identification of sorbitan monopalmitate (Span 40) and sorbitan monostearate (Span 60)

The identification tests for sorbitan monopalmitate and sorbitan monostearate such as assay for fatty acids, iodine value, acid value, hydroxyl value, saponification value, water, residue on ignition, heavy metals, organic volatile impurities, residual solvents and assay for polyols were performed and the values were found to be within the limits as per USP25 standards.

3. Identification tests for cholesterol

The identification tests comply with the USP25 standards.

Table 2: Particle size of drug-free niosomes

S.No.	Formulation code	Mean particle size ± S.D (μm)	
		Sonication method	Size extrusion method
1	F1A1	5.81 ± 3.02	10.42 ± 3.41
2	F1A2	6.01 ± 3.41	10.12 ± 3.46
3	F1A3	5.07 ± 1.48	10.28 ± 1.04
4	F1A4	8.25 ± 1.30	9.22 ± 1.30
5	F1B1	3.13 ± 0.62	6.43 ± 0.61
6	F1B2	3.15 ± 0.65	6.34 ± 0.66
7	F1B3	3.24 ± 0.81	6.52 ± 0.86
8	F1B4	5.94 ± 2.92	11.13 ± 3.11
9	F1C1	5.75 ± 0.84	10.95 ± 0.89
10	F1C2	5.90 ± 0.81	11.23 ± 0.82
11	F1C3	5.85 ± 0.65	11.32 ± 0.76
12	F1C4	6.28 ± 2.52	12.45 ± 2.60
13	F2A1	8.91 ± 2.89	16.60 ± 3.01
14	F2A2	9.06 ± 2.97	17.42 ± 2.42
15	F2A3	7.43 ± 1.50	15.03 ± 1.63
16	F2A4	10.43 ± 1.99	19.21 ± 2.01
17	F2B1	3.46 ± 1.09	7.65 ± 1.46
18	F2B2	3.52 ± 1.14	7.64 ± 1.10
19	F2B3	3.10 ± 0.55	6.24 ± 0.57
20	F2B4	9.22 ± 1.23	17.67 ± 1.35
21	F2C1	5.87 ± 1.13	119 ± 1.24
22	F2C2	7.015 ± 1.45	14.00 ± 1.56
23	F2C3	8.09 ± 1.41	16.23 ± 1.42
24	F2C4	10.64 ± 2.05	19.89 ± 2.11

n = 200

Table 3: Standard calibration Emtricitabine (FTC) in distilled water at 281 nm[176]

S.No.	Concentration (µg/mL)	Absorbance
1	5	0.198
2	10	0.398
3	15	0.594
4	20	0.778
5	25	0.980

Figure 5: Standard calibration graph of Emtricitabine (FTC) in distilled water at 281 nm

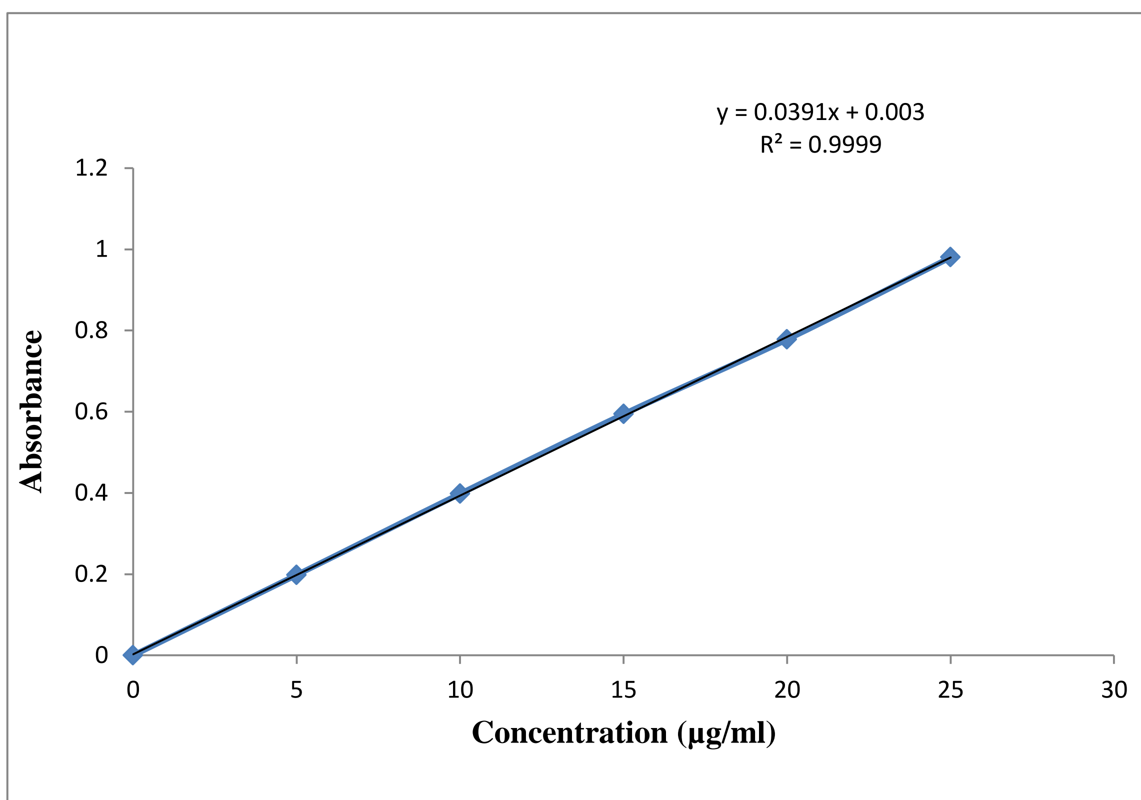


Table 4: Stability of Emtricitabine (FTC) at different experimental temperatures

S.No.	Temperature (^{0}C)	Absorbance	Concentration (µg/mL)	Amount of drug present (mg)
1	65±5	0.382	9.9	49.5
2	90±5	0.360	9.8	49

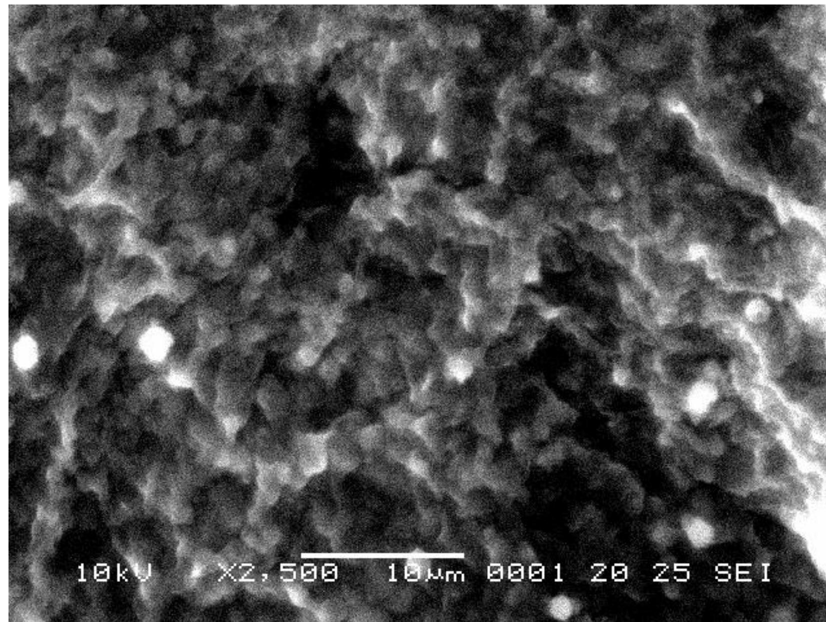
Table 5: Particle size of drug-free NPG niosomes prepared at different temperatures

S.No.	Formulation code	Temperature (^{0}C)	Mean particle size ± S.D by Sonication method (µm)
1	NPG-F1B1	65±5	3.13 ± 0.62
		90±5	4.22 ± 0.82
2	NPG-F1B2	65±5	3.15 ± 0.65
		90±5	4.20 ± 0.94
3	NPG-F2B1	65±5	3.46 ± 1.09
		90±5	4.43 ± 0.99
4	NPG-F2B2	65±5	3.52 ± 1.14
		90±5	4.40 ± 0.72

n = 200

Figure 6: Scanning electron microscopic images of formulations prepared by TLE-paddle method with

A) Span 60



B) Span 40

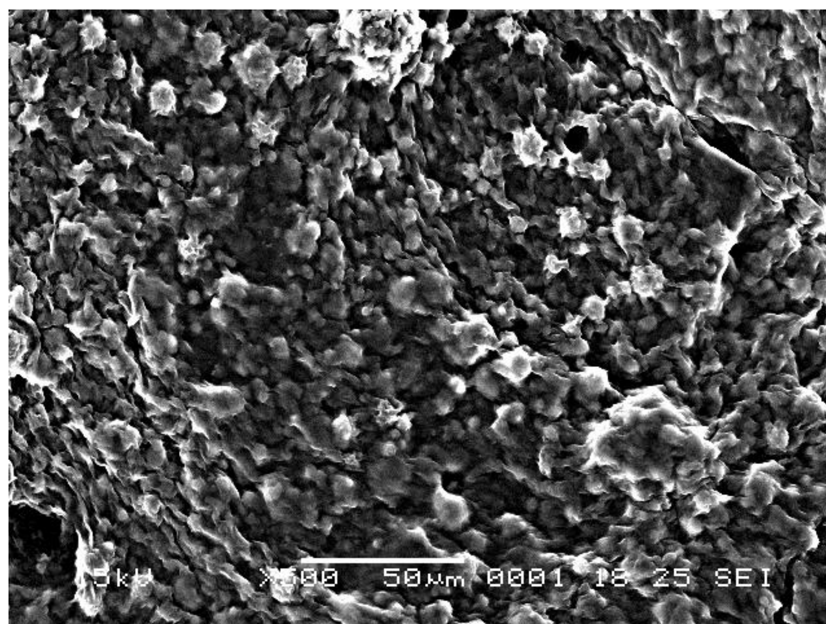


Table 6: Particle size of FTC-NPG-niosomes

S.No.	Formulation code	Mean particle size ± S.D by Sonication method (nm)
1	FTC-NPG-F1B2	154 ± 4
2	FTC-NPG-F2B2	169 ± 4

n = 200

Table 7: Entrapment efficiency of FTC-NPG-niosomes by size exclusion chromatographic method

S.No.	Formu-lation code		Absorbance	Concen-tration (µg/mL)	Amount of drug (mg)	Encapsulation efficiency ± S.D (%)
1	FTC-NPG-F1B2	Total drug	0.0343	0.9	0.9	66.26 ± 1.32
		Encapsulated drug	0.0249	0.6	0.6	
2	FTC-NPG-F2B2	Total drug	0.0343	0.9	0.9	27.37 ± 0.71
		Encapsulated drug	0.982	25	0.25	

n = 5

Table 8: Entrapment efficiency of FTC-NPG-niosomes by dialysis method

S.No.	Formulation code	Absorbance	Concen-tration (µg/mL)	Total amount of encapsulated drug (mg)	Amount of drug diffused (mg)	Encapsulation efficiency ± S.D (%)
1	FTC-NPG-F1B2	0.768	19	5.7	2.0122	64.45 ± 1.14
2	FTC-NPG-F2B2	0.768	19	5.7	4.4318	22.46 ± 0.91

n = 3

Theoretical total amount of drug present in the formulation = 6 mg

Table 9: Stability of NPG-niosomal dispersion and FTC-NPG-niosomal dispersion of formulation F1B2

S.No	Formulation code	Temperature (^{0}C)	Parameter	0 day	3 months	6 months
1	NPG-F1B2	4	Mean particle size ± SD	157 ± 4	158 ± 4	158 ± 4
2	NPG-F1B2	25	Mean particle size ± SD	157 ± 4	157 ± 4	158 ± 4
3	FTC-NPG-F1B2	4	Mean particle size ± SD	153 ± 3	153 ± 3	155 ± 3
			Encapsulation efficiency	64.45 ± 1.14	64.93 ± 1.83	64.52 ± 0.58
4	FTC-NPG-F1B2	25	Mean particle size ± SD	153 ± 3	154 ± 3	154 ± 3
			Encapsulation efficiency	22.46 ± 0.91	22.61 ± 0.75	22.28 ± 0.87

n = 200 (Mean particle size)
n = 3 (Encapsulation efficiency)

Table 10: Values for the human CNS penetration and physicochemical parameters (pK_a, Clog P, and PSA) of emtricitabine obtained from literature[115, 181, 182, 183]

Drug	MW (g/mol)	CNS penetration	pK_a	Clog P	PSA (Å^2)
Emtricitabine	247.248	Intermediate	2.65	-0.43	88.15

PSA: Polar surface area

Table 11: Classification of blood-brain barrier (BBB) penetration of drugs based on immobilized artificial membrane (IAM) capacity factors ($k_{IAM}/MW^4 \times 10^{10}$) using IAM chromatography[165]

BBB penetration	IAM capacity factor ($k_{IAM}/MW^4 \times 10^{10}$)	
	pH 5.5	pH 7.0
High	>1.01	>0.85
Uncertain	0.65-1.00	--
Low	<0.64	<0.84

Table 12: *In vitro* blood-brain barrier penetration of emtricitabine from FTC-NPG-F1B2 using immobilized artificial membrane phosphatidylcholine column chromatography

20% Acetonitile, $(k_{IAM}/MW^4) \times 10^{10}$			
pH 5.5		**pH 7.0**	
$\bar{X} \pm SD$	**CNS Penetration**	$\bar{X} \pm SD$	**CNS Penetration**
2.79 ± 0.05	CNS+	8.48 ± 0.18	CNS+

10. DISCUSSION

Emtricitabine is a nucleoside reverse transcriptase inhibitor (NRTI) used in treating HIV infected adults and children. It is marketed as oral solution and capsules.[183] It is classified as a drug with intermediate CNS penetration with a CNS Penetration-Effectiveness (CPE) score of 0.5.[153] The CSF/plasma ratio was reported to be about 36% and that concentration of emtricitabine exceeds the wild-type IC_{50} in most individuals and may inhibit viral replication in the nervous system.[168]

The HIV replication in the brain at sub-therapeutic concentrations of the drug in the CSF, subjects the patients to the risk for HIV Associated Neurocognitive Disorders (HAND). So, in the present study an attempt was made to formulate emtricitabine as functionalized niosomes by parenteral administration to improve its delivery to the brain. Optimization of various parameters such as the molar ratio of lipids, total lipid composition, method of preparation, hydration temperature, size reduction method and encapsulation efficiency determination method were carried out.

Formulations of drug-free niosomes were prepared by four different methods (Thin layer evaporation-vortex, Thin layer evaporation-paddle, Reverse phase evaporation method and Proniosome method) using two surfactants, Span 60 and Span 40 with cholesterol and Solulan C24. Variations were made in the molar ratios and the total concentration of lipid components (Table 1).

The total concentration of components was fixed to 38 mM, since higher lipid amounts produce more viscous samples.[10, 173]

Studies reported show that Solulan C24 when included in niosomal formulation has reduced haemolytic effect but when the concentration is increased beyond 10 mol% has increased haemolytic effect due to the soluble surfactant Solulan C24 not incorporated into the membrane of niosomes and is thus present in solution as monomers or micelles. So, the concentration of SOL was kept at 10 mol%, since high levels of SOL above this are potential haemolytic.[174, 175]

The formulated niosomes were evaluated for mean particle size. Formulations prepared by proniosome method were found to have considerably larger sized vesicles

(8.25 ± 1.30, 5.94 ± 2.92, 6.28 ± 2.52, 10.43 ± 1.99, 9.22 ± 1.23 and 10.64 ± 2.05 μm for formulations F1A4, F1B4, F1C4, F2A4, F2B4, and F2C4 respectively) compared with formulations prepared by thin layer evaporation-vortex, thin layer evaporation-paddle and reverse phase evaporation method indicating the aggregation of vesicles as determined by dynamic light scattering analysis.

Formulations prepared by reverse phase evaporation posed problems in ensuring complete removal of chloroform in the final niosomal dispersion as it required a longer duration for its complete removal.[186] Moreover; results of dynamic light scattering studies revealed that the vesicle sizes of these formulations were greater than other niosomal formulations indicating aggregation of vesicles. The values were found to be 5.07 ± 1.48, 3.24 ± 0.81, 5.85 ± 0.65, 7.43 ± 1.50, 3.10 ± 0.55 and 8.09 ± 1.41 μm for formulations F1A3, F1B3, F1C3, F2A3, F2B3 and F2C3 respectively.

Among all the formulations niosomes prepared by TLE-vortex (5.81 ± 3.02, 3.13 ± 0.62, 5.75 ± 0.84, 8.91 ± 2.89, 3.46 ± 1.09 and 5.87 ± 1.13 μm for formulations F1A1, F1B1, F1C1, F2A1, F2B1 and F2C1 respectively) and TLE-paddle method (6.01 ± 3.41, 3.15 ± 0.65, 5.75 ± 0.84, 9.06 ± 2.97, 3.52 ± 1.14 and 7.015 ± 1.45 μm for formulations F1A2, F1B2, F1C2, F2A2, F2B2 and F2C2 respectively) produced narrow sized niosomal dispersions. Moreover, vesicles formulated with Span alone (F1A1, F1A2, F1A3, F1A4, F2A1, F2A2, F2A3 and F2A4) yielded comparatively larger niosomes indicating the aggregation of vesicles than those with cholesterol and Solulan added to it (F1B1, F1B2, F1B3, F1B4, F1C1, F1C2, F1C3, F1C4, F2B1, F2B2, F2B3, F2B4, F2C1, F2C2, F2C3 and F2C4). Thus, inclusion of cholesterol showed improved stability by its effect on gel-liquid phase transition temperature and inclusion of Solulan showed improved steric stabilization effects by preventing the aggregation of vesicles.[187, 188] It has also been suggested that when cholesterol is included in the formulation of vesicular systems at a concentration of 30-50 mol%, an interaction occurs between the ester bond of the surfactant and the 3 -OH group of cholesterol.[189, 190, 191] Cholesterol also has destabilizing effects of plasma and serum proteins and decreases the permeability of vesicles to entrapped solute, preventing leakage.[97, 192]

Unilamellar vesicles are preferred to multilamellar vesicles due to their larger entrapped aqueous volume. Large unilamellar vesicles (LUVs) are more effective in passive loading as the volume of the encapsulated water is higher when compared to

small unilamellar vesicles (SUVs). The circulation of niosomes in plasma is prolonged with decrease in vesicle size. Thus encapsulation efficiency and circulation half-life are inter related.

To obtain LUVs (80-200 nm) from the heterogeneous multilamellar vesicles (MLVs), the two most common procedures for downsizing were selected - sonication and extrusion method. It has been reported that extrusion method is suitable for a dispersion of 100 mM lipid with a possibility to generate 100 nm niosomes by passing the dispersion through the filter 10 or more times and this can be accomplished in less than 30 min.[193]

Among these two methods of size reduction employed, probe sonication was found to be more satisfactory in reducing the vesicle size by approximately two times than that of size extrusion method for all formulations. Thus, the probe sonication method was selected as the optimized method of size reduction for further studies.

Based on the results obtained on the evaluation of the niosomal dispersions, the total concentration of components chosen, method of preparation and their composition by Span:CHL:SOL:NPG were optimized. The drug free-NPG niosomes were prepared by these optimized conditions with different hydration temperatures of 65° C and 90° C. Literature also suggests that increasing the hydration temperature may have a positive effect on the entrapment efficiency.[178, 179] After preparation, all suspensions were centrifuged and sonicated as described above.[10]

The suitability of these techniques for drug-loaded vesicles prepared at different hydration temperatures was assessed by determining the stability of emtricitabine under these experimental conditions. Studies reported on stability of emtricitabine show that emtricitabine is stable to forced degradation by acid hydrolysis, alkali hydrolysis and thermal degradation.[180, 181] So, the suitability of the drug to be subjected at different hydration temperatures was evaluated by subjecting the drug solution to 65^0 and 90^0C.[10] The results showed that the drug is stable and can be subjected to such temperatures.

N-palmitoyl glucosamine was synthesized by the method described by Dufes *et al*[9] and was obtained as white powder.

Based on the results of mean particle size, formulations containing Span 60 and Span 40 with a total molar concentration of 38 mM in the molar ratio of Span:CHL:SOL:NPG as 50:40:10:10 prepared by TLE-vortex (F1B1 and F2B1) and TLE-paddle (F1B2 and F2B2) were selected as the optimized formulations.

The NPG drug-free niosomes (NPG-F1B1, NPG-F1B2, NPG-F2B1, and NPG-F2B2) were prepared by varying the hydration temperatures of 65 ± 5^0 and 90 ± 5^0C. The mean particle size of the formulations prepared at the hydration temperature of 90 ± 5^0C did not show considerable increase in particle size compared with that prepared at 65±5°C. Moreover, the phase transition temperature of Span 60, which is the component of the lipid mixture with the highest value of phase transition temperature is 50°C.[166] Hence, in order to avoid unnecessary exposure of the formulations to higher temperatures, the temperature of 65°C was selected as the optimized phase transition temperature.

Emtricitabine loaded NPG niosomes were prepared with Span 60 (FTC-NPG-F1B2) and Span 40 (FTC-NPG-F2B2) with a total molar concentration of 38 mM in the molar ratio of Span:CHL:SOL:NPG as 50:40:10:10 TLE-paddle method at 65 ± 5^0C. The drug-loaded niosomes were characterized for formation of niosomes by scanning electron microscopy. Scanning electron microscopic images confirmed the formation of vesicles with spherical shape (Figure 6). The mean particle size and encapsulation efficiency were evaluated by size exclusion method and dialysis method.

The results of particle size of niosomes were greater after probe sonication at 60% of its maximum power and so probe sonication was carried out at 80% of its maximum power to reduce the particle size. The resultant particle size analysis showed that the mean particle size of formulation containing Span 40 (FTC-NPG-F2B2) was considerably greater than Span 60 (FTC-NPG-F1B2) whereas drug encapsulation was found to be more for Span 60 (FTC-NPG-F1B2) compared with a formulation containing Span 40 (FTC-NPG-F1B2) by both size exclusion methods (66.67% and 27.78% respectively) and dialysis method (64.70% and 22.25% respectively). Thus, suggesting that both size exclusion and dialysis method are efficient in determining the encapsulation efficiency of the drug. It also confirms the effect of chain length and size of the hydrophilic head group of the non-ionic surfactant on the entrapment efficiency

of drug showing that non-ionic surfactants with stearyl (C18) chains have greater entrapment efficiency than those with palmityl (C16) chains.[187, 188]

The entrapment efficiency is affected by the phase transition temperature (Tc) of the surfactant. Sorbitan monopalmitate (Span 40) and sorbitan monostearate (Span 60) has a gel transition temperatures of 46 to 47 °C and 56 to 58 °C respectively. Thus Span 60 with a high Tc exhibits the highest entrapment efficiency.[188] Vesicles made with these higher molecular weight Spans are less leaky and more stable to osmotic gradients.[166]

The optimized drug-free NPG niosomal formulations and drug-loaded formulations (NPG-F1B2, NPG-F2B2, FTC-NPG-F1B2, and FTC-NPG-F2B2) were subjected to stability studies for a period of 6 months at 4^0C and 25^0C.[181] The results of mean vesicle size showed that the niosomes were considerably stable at both the temperatures.

Among all the formulations, the emtricitabine loaded NPG niosomal formulation (FTC-NPG-F1B2) was selected for further studies.

The various methods reported on the evaluation of the blood brain barrier (BBB) penetration potential of drug candidates are ***in silico, in vivo,*** **and** ***in vitro*** **methods.**

The ***in silico*** approaches include the determination of physicochemical properties such as **octanol-water partition coefficient (log P),**[167] **hydrogen-bonding potential (Δ log P),**[194] **molecular polar surface area (PSA),**[195] **linear free energy,**[196, 197] **and surface tension**[198, 199] (many current models are based on data, which may not represent BBB permeability as such (log BB which describes brain to plasma ratio; CNS activity); still very limited data bases for BBB transport (log PS models which describes BBB permeability surface area product)).[200]

In vivo biological approaches include **microdialysis**[201, 202] (technically involved; *in vivo* probe calibration required for valid quantitative evaluation; local damage to BBB integrity)[200] **cerebrospinal fluid sampling**[201, 203] (reflects permeability of blood-cerebrospinal fluid-blood and cerebrospinal fluid dynamics rather than blood brain barrier),[200] **intravenous injection/brain sampling**[204] (may require good

analytical tools to exclude metabolite uptake and careful pharmacokinetic analysis to discriminate unidirectional uptake versus bidirectional transfer)[200] **autoradiography**[205] (time-consuming evaluation; no proof of integrity of tracer),[200] **nuclear magnetic resonance,**[206] **and positron emission spectroscopy**[207] (expensive equipment in magnetic resonance imaging (MRI) and positron emission tomography (PET) and tracers (PET); limited sensitivity (MRI) and availability of labelled tracers (MRI, PET); poor spatial resolution for small animals in single-photon emission computed tomography (SPECT)), **brain perfusion**[208] (technically more difficult than intravenous experiments and brain uptake index (BUI)).[200]

In vitro methods use **isolated brain capillaries**[209] (transcellular passage cannot be measured)[200] and **cultured cells such as bovine brain micro vessel endothelial cells,**[210] **brain capillary endothelial cells,**[211] **mouse brain endothelial cells**[212, 213] **and Caco-2 cells**[214] (no system yet able to represent *in vivo* condition with respect to barrier tightness and blood brain barrier specific transporter expression; multitude of models makes comparison of results between studies difficult).[200]

Other *in vitro* approaches of the parallel artificial membrane permeability assay (PAMPA)[215] and the immobilized artificial membrane (IAM) chromatography.[216, 217] These latter approaches are based on artificial membrane-mimic systems and may offer the advantages of experimental simplicity over the cultured cell permeability test, without requiring cell culture cycles. The PAMPA requires a membrane permeability experiment through artificial membranes,[218] whereas the IAM chromatographic assay is based on the solute interaction with IAMs.[219]

The IAM chromatography consists of columns made up of a monolayer of phospholipid covalently bonded to silica packed columns namely; immobilized artificial membranes.[210, 221] The surface of the columns emulates the lipid surface and drug interaction as liposomes and cell membranes.[221, 222] The IAM chromatography has been used in predicting the drug transport across biological barriers.[217, 218, 223] Chromatographic partitioning has several advantages over other partitioning systems for its simplicity, reproducibility, and being qualitative rather than quantitative. It also needs small quantity of the solute and the use of high purity of the compound is not necessary.[224] The correlation between k_{IAM}/MW^n and CNS penetration was found to be highest when the power function (n) was set at 4 and indicates that *in vitro* IAM

capacity factors (k_{IAM}/MW^4) may be used to classify drugs as CNS+ and CNS– with a high rate of success.[165, 225] Considering the potential advantages of the use IAM chromatography in determining the penetration of drugs across the blood brain barrier, a novel attempt was made to prove the *in vitro* blood-brain barrier penetration of emtricitabine from the niosomal formulation using immobilized artificial membrane phosphatidylcholine column chromatography.

In vitro blood-brain barrier penetration of emtricitabine from drug loaded NPG niosomes (FTC-NPG-F1B2) using immobilized artificial membrane phosphatidylcholine column chromatography showed an improved CNS penetration of the drug with (k_{IAM}/MW^4) X 10^{10} values of 2.79 ± 0.05 at pH 5.5 and 8.48 ± 0.18 at pH 7.0 (Table 12). This might be attributed to the increased penetration efficacy of the surfactants Span 60 and Solulan included in the formulation.[10, 175, 226]

11. SUMMARY AND CONCLUSION

Formulations of drug-free niosomes were prepared by four different methods (Thin layer evaporation-vortex, Thin layer evaporation-paddle, Reverse phase evaporation method and Proniosome method) using two surfactants, Span 60 and Span 40 with cholesterol and Solulan C24. Variations were made in the molar ratios and the total concentration of lipid components.

Among all the formulations niosomes prepared by TLE-vortex and TLE-paddle methods produced narrow sized niosomal dispersions.

It was found that vesicles formulated with Span alone yielded comparatively larger niosomes than those with cholesterol and Solulan added to it.

Since, small unilamellar vesicles exhibit prolonged plasma concentrations with more entrapment of aqueous phase compared with that of large unilamellar and multilamellar vesicles, two widely used size reduction methods (sonication and size extrusion) were carried out. Among the two methods of size reduction employed, probe sonication was found to be more satisfactory in reducing the vesicle size by approximately two times than that of size extrusion method for all formulations.

The suitability of the drug at different hydration temperatures was evaluated by subjecting the drug solution to 65 ± 5^0 and 90 ± 5^0C. The results showed that the drug is stable and can be subjected to such temperatures.

Based on the results of mean particle size, formulations containing Span 60 and Span 40 with a total molar concentration of 38mM in the molar ratio of Span:CHL:SOL:NPG as 50:40:10:10 prepared by TLE-vortex and TLE-paddle were selected as the optimized formulations.

The NPG drug-free niosomes were prepared by varying the hydration temperatures of 65 ± 5^0 and 90 ± 5^0C and the hydration temperature was optimized to 65 ± 5^0C.

12. IMPACT OF STUDY

Emtricitabine is nucleoside reverse transcriptase inhibitor used in the treatment of HIV patients. The drug is an intermediate CNS penetrating drug. So, the effective concentration of the drug required to reduce the viral load in the CSF of HIV patients is not attained.

In this regard, considering the potential benefits of niosomal formulations with glucose analogues for brain-targeted drug delivery, emtricitabine loaded N-palmitoyl glucosamine niosomal formulations were developed and optimized for various formulation parameters.

The optimized emtricitabine loaded N-palmitoyl glucosamine niosomal formulation (FTC-NPG-F1B2) with good drug entrapment and stability showed improved CNS penetration as determined by the *in vitro* blood-brain barrier penetration of emtricitabine from drug loaded NPG niosomes using immobilized artificial membrane phosphatidylcholine column chromatography.

Thus, this formulation may be a boon for HIV patients suffering from HIV-associated neurocognitive disorders by improving their living during the survival period.

Emtricitabine loaded NPG niosomes were prepared with Span 60 (FTC-NPG-F1B2) and Span 40 (FTC-NPG-F2B2) with a total molar concentration of 38 mM in the molar ratio of Span:CHL:SOL:NPG as 50:40:10:10 by the TLE-paddle method at 65±5^{0}C. The drug-loaded niosomes were characterized for mean particle size and evaluated for encapsulation efficiency by size exclusion method and dialysis method.

The results of particle size of niosomes were greater after probe sonication at 60% of its maximum power and so probe sonication was carried out at 80% of its maximum power to reduce the particle size. The resultant particle size analysis showed that mean particle size of formulation containing Span 40 (FTC-NPG-F2B2) was considerably greater than Span 60 (FTC-NPG-F1B2) whereas drug encapsulation was found to be more for Span 60 (FTC-NPG-F1B2) compared with formulation containing Span 40 (FTC-NPG-F1B2) by both size exclusion method (66.67% and 27.78% respectively) and dialysis method (64.70% and 22.25% respectively).

The optimized drug-free NPG niosomal formulations and drug-loaded formulations (NPG-F1B2, NPG-F2B2, FTC-NPG-F1B2, and FTC-NPG-F2B2) were subjected to stability studies for a period of 6 months at 4^{0}C and 25^{0}C. The results of mean vesicle size and entrapment efficiency showed that the niosomes were considerably stable at both the temperatures.

In vitro blood-brain barrier penetration of emtricitabine from drug loaded NPG niosomes using immobilized artificial membrane phosphatidylcholine column chromatography showed an improved CNS penetration of the drug when encapsulated in niosomal formulation compared with that of the plain drug.

To conclude, by considering the potential benefits of the drug formulated as niosomal dispersion, the optimized emtricitabine loaded NPG niosomal formulation (FTC-NPG-F1B2) with improved CNS penetration may be considered as a potential alternative to improve brain targeting of emtricitabine and thus minimize HIV Associated Neurocognitive Disorders. Thus, this formulation may be considered for further *in vivo* studies.

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