PREPARATION AND EVALUATION OF TENELIGLIPTIN HYDROBROMIDE LOADED LIPOSOME FOR THE TREATMENT OF DIABETES

Submitted by

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CERTIFICATE OF APPROVAL

This is to certify that **Rima Chandra** bearing **Registration No: 160254 of 2021-22** has carried out the research work entitled **"PREPARATION AND EVALUATION OF TENELIGLIPTIN HYDROBROMIDE LOADED LIPOSOME FOR THE TREATMENT OF DIABETES"** independently with proper care and attention under my supervision and guidance in the Pharmaceutics Research Laboratory in the Department of Pharmaceutical Technology, Jadavpur University. He has incorporated his findings into this thesis of the same title, being submitted by him. in partial fulfilment of the requirements for the degree of **MASTERS OF PHARMACY** from Jadavpur University. I appreciate his endeavour to do the project and his work has reached my gratification.

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DECLARATION OF ORIGINALITY AND COMPLIANCE OF ACADEMIC ETHICS

I hereby declare that this thesis contains a literature survey and original research work by the undersigned candidate as part of his Master of Pharmaceutical Technology studies. All information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all materials, and the results are original to this work.

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Date: 14.09.23

Place: Jadavpur University

RIMA CHANDRA

Rima Chandra.

Dedicated to

My family and my guide

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CHAPTER I
INTRODUCTION

INTRODUCTION:

Nanotechnology is an advanced branch of science which involves manufacture, processing and application of substances or devices that have dimensions in nanometer range. Nanotechnology is an interdisciplinary area of research and development that provides its impact on a number of various fields, like- medicine, cosmetics, agriculture, food etc. Research and application in the field of nanoscience and nanotechnology have grown at an unparalleled rate in recent years. Growing confidence exists that the application of nanotechnology to medicine will result in significant advancements in disease diagnosis and treatment. When drugs are administered, only a very small fraction of dose reaches the receptors or sites of action, and remaining amount of dose is wasted either by being taken up by wrong tissue or removed from right tissue or quickly destroyed before reaching at the target site. For development of new drug delivery systems our aims are – increase drug activity and decrease drug toxicity.(1) As a promising delivery system liposomes are becoming more favourable due to many advantages which includes :

- Biocompatibility
- Biodegradability
- Targetability
- Non toxicity
- Carry both water and oil soluble drugs
- Decrease dose and reduce side effects
- Protein stabilization
- Reduce exposure of sensitive tissues to toxic drugs

1.1. DIABETES MELLITUS: Diabetes mellitus is a metabolic disorder in which the body's capacity to produce or respond to the hormone insulin is compromised, leading to improper carbohydrate metabolism and increased blood glucose levels. The chronic metabolic disorder diabetes mellitus is a fast-growing global problem with huge social, health, and economic consequences(2).

TYPES OF DIABETES MELLITUS: There are two types of diabetes mellitus:

TYPE I – Insulin dependent diabetes mellitus (IDDM)

It is also called as juvenile onset diabetes mellitus. This type of diabetes mellitus occurs due to β cell destruction in pancreatic islets.

Cause- Type 1A autoimmune antibodies that destroy β cells are detectable in blood

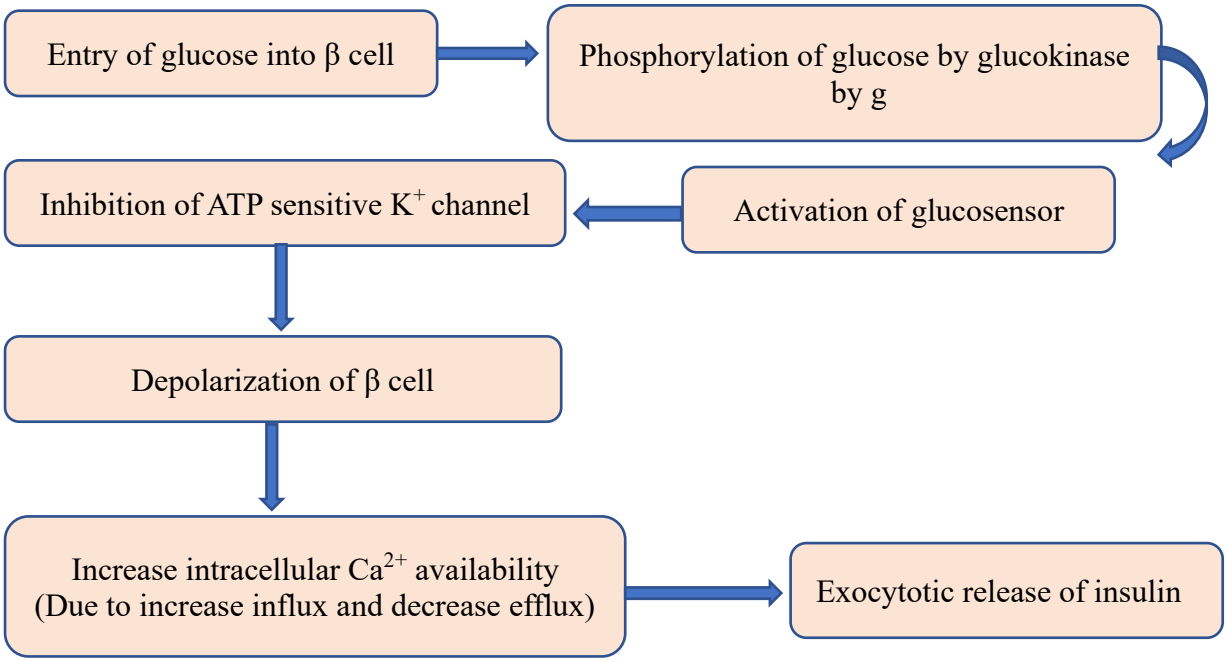
Type 1B idiopathic- no β cell antibody is found

TYPE II- Noninsulin dependent diabetes mellitus (NIDDM)

Cause – Abnormality in gluco- receptor of β cells, causing them to respond to greater glucose concentrations.

Reduced sensitivity of peripheral tissues to insulin

REGULATION OF INSULIN SECRETION:



SYMPTOMS OF DIABETES MELLITUS: The symptoms of diabetes are related to high glucose levels. They includes:

- Thrust and hunger
- Frequent urination
- Rapid weight loss
- Blurred vision

- Slow healing of cut

Type 2 diabetes affects all parts of the body. It can cause serious, life-threatening complications. These include:

- **Atherosclerosi**s- It is basically fat build up in artery walls. It can impair blood flow to all the organs. Heart, brain and legs are primarily affected(3).
- **Retinopathy**- Tiny blood vessels in the retina can become damaged by high blood sugar. The damage can block blood flow to the retina, and can lead to bleeding into the retina. Both damage the ability of the retina to see light.
- **Neuropathy**- It is basically nerve damage and most common type is peripheral neuropathy. Nerves of the legs are damaged first, causing pain and numbness in the feet(4).
- **Foot problems**- Sores and blisters on the feet occur for two reasons:
    - If peripheral neuropathy causes numbness, the person may not feel irritation in the foot. The skin can break down, form an ulcer, and the ulcer can get infected.(5)
    - Blood circulation can be poor, leading to slow healing. Left untreated, a simple sore can become infected and very large. If medical medical treatment cannot heal the sore, an amputation may be required.
- **Nephropathy**- It means damage to the kidney. This is more likely if blood sugars remain elevated and high blood pressure is not treated aggressively(4).

DIAGNOSIS : Diabetes is diagnosed by testing the blood for sugar levels. Blood is tested in the morning after you have fasted overnight.

Typically the body keeps blood sugar levels between 70 to 100 milligrams per deciliter (mg/dl), even after fasting. If a blood sugar level after fasting is greater than 125 mg/dl, diabetes is diagnosed

Laboratory tests are used routinely to evaluate diabetes. These include:

- Fasting plasma glucose test
- Oral glucose tolerance test
- Random blood glucose test
- Hemoglobin A1C (glycohemoglobin) test
- Lipid profile

PREVENTION- If a close relative- particularly a parent or sibling has type 2 diabetes, or if blood glucose test shows "pre-diabetes"- defined as blood glucose levels between 100 and 125 mg/dl, then you are at increased risk for developing type 2 diabetes. You can help to prevent type 2 diabetes by:

- Maintaining ideal body weight
- Exercising regularly
- Eating healthy diet
- Taking medication
-

If you already have type 2 diabetes, you can still delay or prevent complications:

- Keep control of blood sugar
- Lower risk of heart related complication by:
    - Consider taking low dose aspirin daily
    - Aggressively managing other risk factors for atherosclerosis, such as:
        - ✓ High blood pressure
        - ✓ High cholesterol and triglycerides
        - ✓ Cigarette smoking
        - ✓ Obesity
- Visit eye doctor and foot specialist every year to reduce eye and foot complication.

TREATMENT:

INSULIN- Type 2 diabetes develops when the pancreas cannot make enough insulin to overcome insulin resistance. In advanced type 2 diabetes, or for people who want to tightly control glucose levels, insulin may be needed more than once per day and in higher doses(6).

Treatment plans that include both very long-acting insulin and very short acting insulin are frequently the most successful for controlling blood sugar.

The most usual side effects of insulin treatment is lowering of blood sugar (hypoglycemia). The usual symptoms are weakness, sweating, feeling unwell and not thinking clearly(7).

Approaches to drug therapy:

ORAL HYPOGLYCAEMIC DRUGS

1. Enhance Insulin secretion
    - Sulfonylureas- Tolbutamide (First generation)
      Glibenclamide
      Glipizide
      Glimepiride
    - Meglitinide/ Phenylalanine- Nateglinide
      Repaglinide
    - Dipeptidyl peptidase-4 (DPP-4) inhibitors- Sitagliptine
      Teneligliptin
      Vildagliptine
      Saxagliptine
    - Glucagon like peptide-1 (GLP-1) receptor agonists-Liraglutide
      Exenatide
2. Overcome insulin resistance
    - Biguanide- Metformine
      Phenformine
    - Thiazolidinediones- Pioglitazone

3. Miscellaneous drugs
    - Dopamine-D2 receptor agonist- Bromocriptine
    - α-Glucosidase inhibitors- Acarbose, Miglitol
    - Amylin analogue- Pramlintide

Role of DPP-4 inhibitors in the management of T2DM:

The first DPP-4 inhibitor, approved in 2006 for the treatment of T2DM is sitagliptine. DPP-4 inhibitors are considered as a favourable class in the management of T2DM due to their efficacy, favourable tolerability such as low risk of hypo glycemia and weight gain and once a day dosage. Now, eight DPP-4 inhibitors, namely, alogliptin, anagliptin, gemigliptin, linagliptin, saxagliptin, sitagliptin, teneligliptin, and vildagliptin, are available for the management of T2DM. All these DPP-4 inhibitors have similar action and safety profile but differ in some important pharmacokinetic and pharmacodynamic parameters which may have clinical significance in real life scenario(8).

Adverse effects like immune dysfunction, skin reactions and impaired healing are reported due to off target inhibition of selective DPP-4 enzymes. Though these toxicities are not clinically significant(9).

Table 1: Important differences among the eight DPP-4 inhibitors

Pharmacokinetic differences	Pharmacodynamic differences
Oral bioavailability, elimination half-life, binding to plasma proteins, metabolic pathways, formation of active metabolite(s), main excretion routes, and potential drug–drug interactions	Potency, target selectivity, dosage adjustment for renal and liver insufficiency

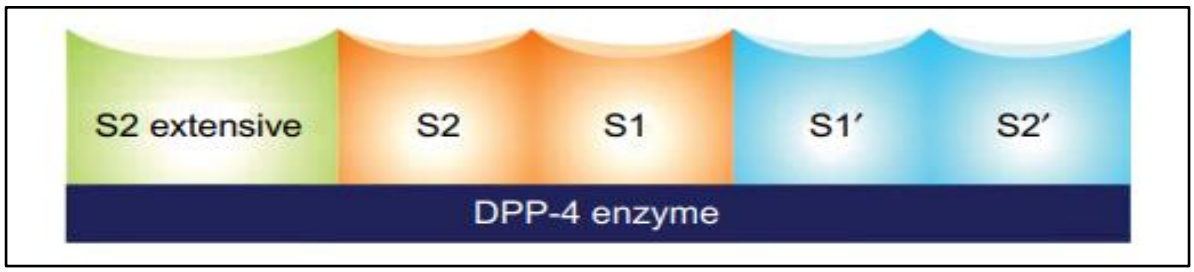
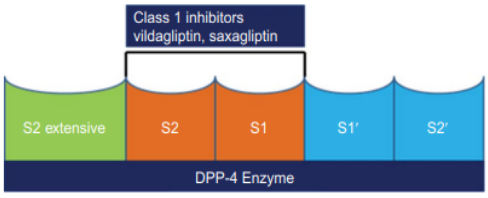
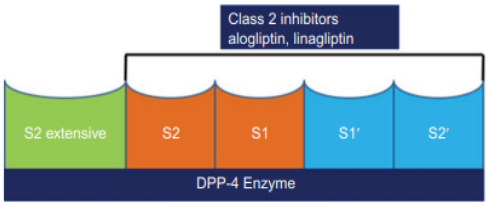
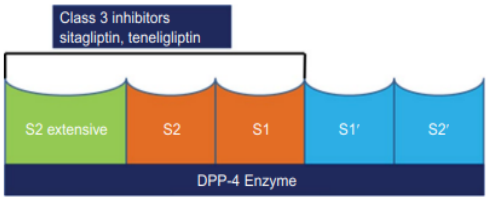


Fig 1: DPP-4 enzyme binding site(10)

Table 2: Summary of the interactions of various DPP-4 inhibitors with DPP-4 enzyme(10)

Class	DPP-4 inhibitors	Binding at DPP-4	Interaction with DPP-4 at various sites	Details
I	Vildagliptin and saxagliptin	S1 and S2 subsites		• Most fundamental level of interaction • Cyanopyrrolidine moieties bind with S1 • Hydroxy adamantly group binds with S2 • Saxagliptin has fivefold higher activity than vildagliptin
2	Alogliptin and linagliptin	S1, S2, S1′, and S2′ subsites		• Additional binding to S1′ and S2′ • Alogliptin binds to S1, S2, and S1′ • Linagliptin binds to S1, S2, S1′, and S2′ • Linagliptin had eightfold higher activity than alogliptin

3	Sitagliptin and teneligliptin	S1, S2, and S2 extensive subsites		• Binds S1, S2, and S2 extensive
				• Teneligliptin has fivefold higher activity than sitagliptin, because of:
				  • Teneligliptin has favorable (J-shaped) structure leading to small loss of energy during binding with DPP-4
				  • Teneligliptin forms hydrogen bond with DPP-4
				  • Teneligliptin has more extensive binding at "S2 extensive" site than sitagliptin

1.2 TENELIGLIPTIN HYDROBROMIDE:

A new class of compounds which revolutionized for the treatment of diabetes mellitus during the recent past is dipeptidyl peptidase-4 inhibitors (DPP-4). They are most commonly known as gliptins derivatives.

Teneligliptin hydrobromide is a drug for the treatment of Type 2 diabetes mellitus which belongs to the class of antidiabetic drugs known as dipeptidyl peptidase-4 inhibitors(11).

It was created by Mitsubishi Tanabe Pharma and launched in September 2012 by both Mitsubishi Tanabe Pharma and Daiichi Sankyo in Japan.(https://en.wikipedia.org/wiki/Teneligliptin)

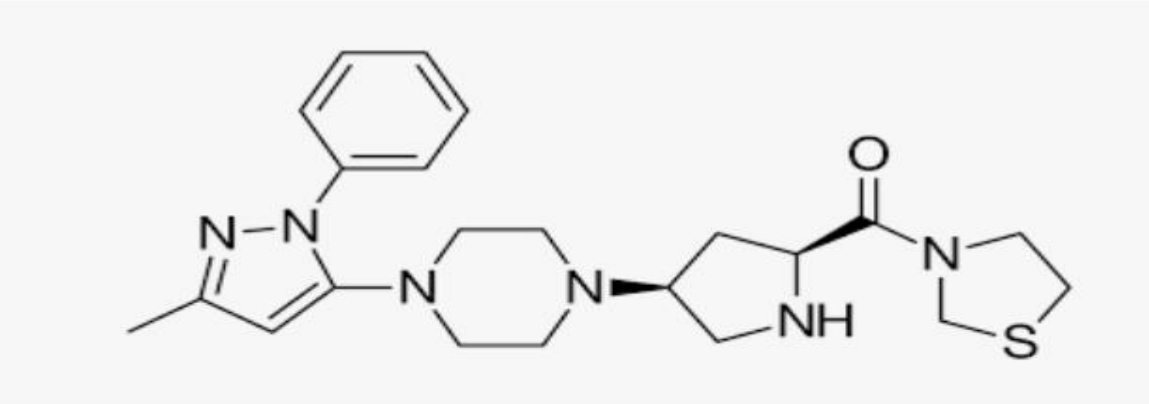


Fig 2: Teneligliptin hydrobromide {(2S,4S)-4-[4-(3-methyl-1-phenyl-1H-pyrazol-5-yl)piperazin-1yl]pyrrolidin-2-yl}(1,3-thiazolidin-3-yl)methanonehemipentahydrobromide hydrate

Mechanism of action of Teneligliptin:

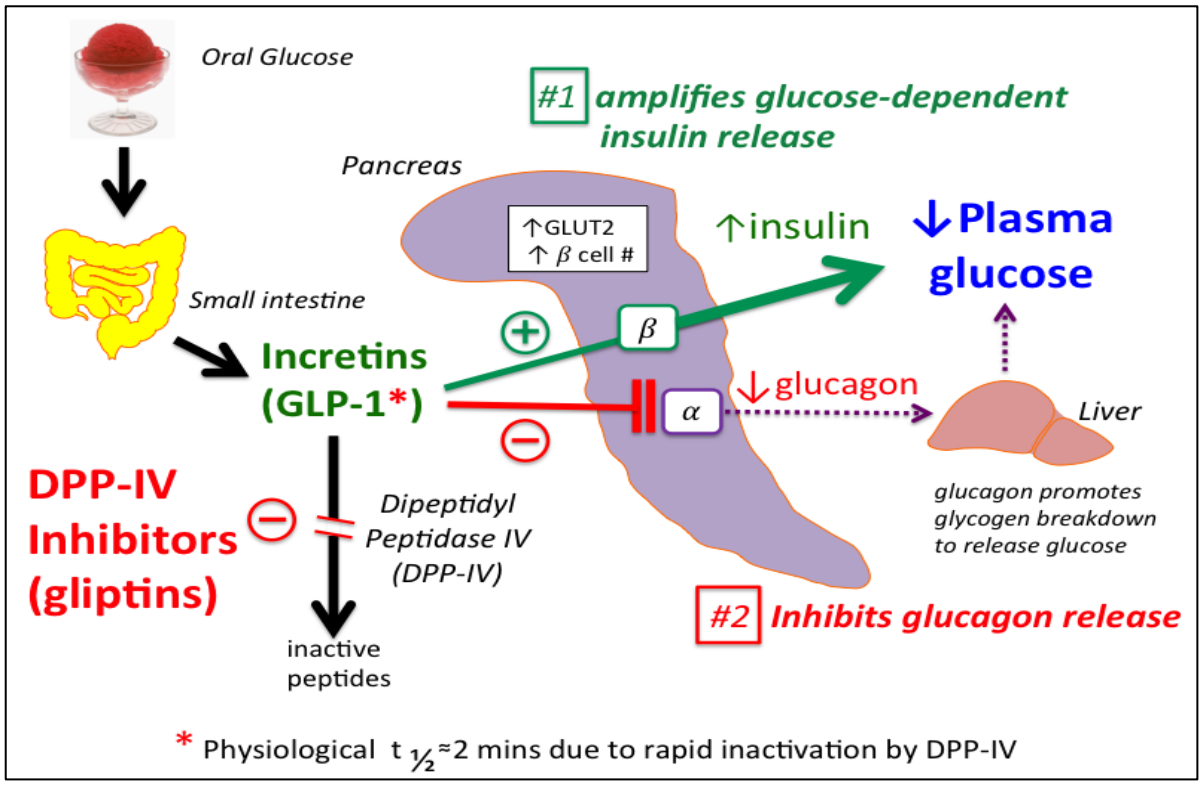
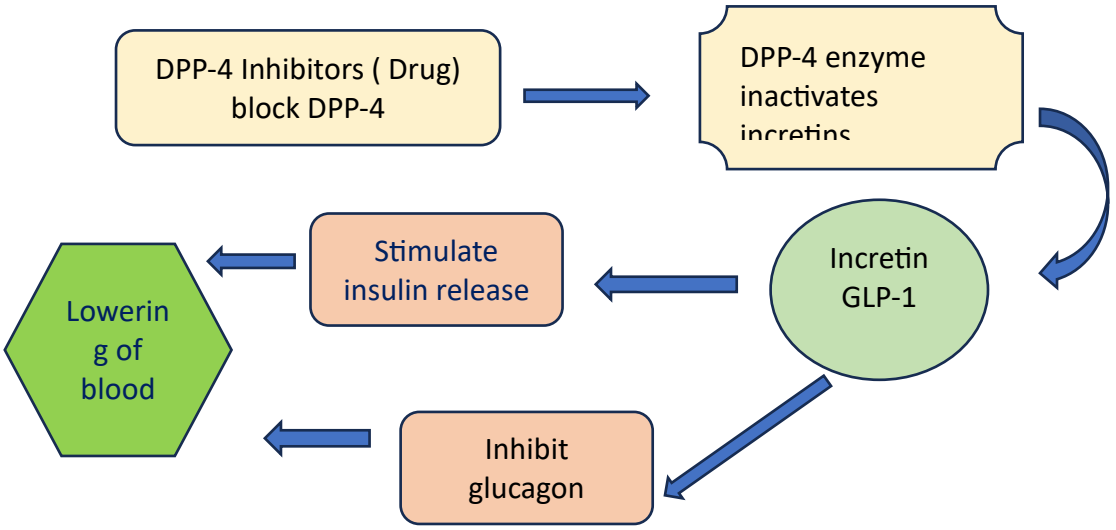


Fig3: Mechanism of action of Teneligliptin hydrobromide (DPP-4 inhibitor) (https://tmedweb.tulane.edu/pharmwiki/lib/exe/detail.php/dpp4.png?id=dpp-4_inhibitors)

Glucagon-like peptide-1 (GLP-1) is an incretin hormone that results in glucose-dependent insulin secretion, suppression of glucagon secretion, a delay in gastric emptying, and a decrease in caloric intake likely secondary to centrally mediated signaling. It arises from posttranslational processing of proglucagon primarily in intestinal L cells and is secreted in two major forms: GLP-1(7,36) and GLP-1(7,37)(12). The majority of known biological actions of GLP-1 depend on the presence of the two N-terminal amino acids; these are removed by the enzyme, dipeptidyl peptidase-4 (DPP-4), whose substrates are polypeptides with an alanine or a proline at the second position from the N-terminal side. Hence, the intact (7,36) and (7,37) peptides are often referred to as "active" GLP-1, whereas the truncated (9,36) and (9,37) peptides are known as "inactive" GLP-1. The activity, affinity, and wide distribution of DPP-4 results in GLP-1 having a half-life of approximately 1 min in the circulation(13).

The other class of pharmacotherapeutic agents that use the incretin system are DPP-4 inhibitors, which inhibit the principal enzyme responsible for the degradation of endogenous GLP-1. By decreasing clearance of GLP-1, concentrations of active GLP-1 are increased, resulting in a lowering of fasting and postprandial glucose concentrations(14).

In practice, DPP-4 inhibitors increase concentrations of both active incretin hormones, GLP-1 and glucose-dependent insulinotropic polypeptide (secreted by the enteroendocrine L and K cells, respectively, which are substrates for DPP-4). This results in improved β-cell responsiveness to prevailing glucose concentrations and suppression of glucagon secretion. Another often-overlooked subtlety of DPP-4 inhibitors is the fact that they likely decrease incretin hormone secretion through negative feedback inhibition by active hormone on enteroendocrine cells(6).



Physicochemical characterization of Teneligliptin hydrobromide:

United States Food and Drug Administration approved TNG which is a highly selective dipeptidyl peptidase-4 inhibitor for the treatment of type-II diabetes mellitus through the oral route.

Teneligliptin hydrobromide chemically is {(2S,4S)-4-[4-(3-methyl-1-phenyl-1H-pyrazol-5-yl)piperazin-1yl]pyrrolidin-2-yl}(1,3-thiazolidin-3-yl)methanonehemipentahydrobromide hydrate that exhibits an exclusive structure that is characterized by five consecutive rings(Fig2)(15). It occurs as a white to off-white powder. It is freely soluble in water, sparingly soluble in methanol, very slightly soluble in ethanol and insoluble in acetonitrile, having a molecular formula of $C_{22}H_{30}N_6OS.2.5\ HBr{\cdot}H_2O$ and a molecular mass of 628.86 g mol^{-1}.

Pharmacokinetics of Teneligliptin:

Reported evidence suggests that with teneligliptin 20 mg therapy, Tmax was 1 hour and t 1/2 was 18.9 hours. Maximum (89.7%) inhibition in plasma DPP-4 activity was noted within 2 hours and maintained >60% at 24 hours. Compared to placebo, active plasma GLP-1 concentration was higher throughout the day and even at 24 hours after administration of teneligliptin 20 mg. Metabolism of teneligliptin was majorly mediated through CYP3A4, a cytochrome P450 isozyme, and flavin-containing monooxygenases (FMO1 and FMO3). A weak inhibitory activity of teneligliptin on CYP2D6, CYP3A4, and FMO was noted, while there was no inhibitory activity on CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C8/9, CYP2C19, and CYP2E1. There was no induction of expression of CYP1A2 or CYP3A4.5 Reported evidence suggests that teneligliptin is metabolized and eliminated via both renal and hepatic routes. Approximately 34% of teneligliptin is excreted unchanged via the renal route, while 66% is metabolized and eliminated via the hepatic and renal routes(16).

Table 3: Approval status of Teneligliptin

Phase of development	Indication	Country
Marketed	T2DM	Japan
Marketed	T2DM	Argentina
Marketed	T2DM	India

Efficacy of Teneligliptin in renal impairment patient:

Because Teneligliptin can be used in T2DM patients with renal impairment, including those on hemodialysis, without the need for dose adjustment, the efficacy and safety of Teneligliptin in T2DM patients with CKD requiring hemodialysis has been assessed in several small observational studies. Teneligliptin is well tolerated by subjects with renal impairment or ESRD (end stage renal disease)(17). Dialysis was not expected to affect the efficacy or safety of teneligliptin. Dose adjustment may not be needed when teneligliptin is administered to subjects with mild, moderate, or severe renal impairment or ESRD(18).

Tolerability of Teneligliptin:

Teneligliptin was well tolerated in various clinical trials. Hypo glycemia can occur when other antidiabetic drugs are co administered. Intestinal obstruction may occur with teneligliptin and must be administered cautiously in patients with a history of intestinal obstruction or surgery(16). Reported evidence suggests that no QT prolongations were noted with teneligliptin 40 mg daily dose. Nevertheless, mild and transient QTc prolongation can be seen at a supra clinical dose of 160 mg/day given for a prolonged period(19).

Drug - Drug interaction:

Teneligliptin is metabolized by CYP3A4 and is a weak substrate of P-glycoprotein. Exposure to teneligliptin, when administered in combination with ketoconazole, is less than twice the exposure to teneligliptin alone, which suggests that drugs and foods that inhibit CYP3A4 are unlikely to markedly increase exposure to teneligliptin(20). No clinically relevant drug–drug interactions were observed when teneligliptin was co-administered with metformin, canagliflozin, glimepiride, or pioglitazone in healthy volunteers; therefore, no dose adjustment of teneligliptin is required when it is co-administered with these drugs. Furthermore, teneligliptin did not affect the pharmacokinetic properties of metformin, canagliflozin, glimepiride, or pioglitazone(21).

1.3. LIPOSOME

Liposomes are self-assembled bilayer (uni-lamellar) or multilayer (multi-lamellar) vesicles made up of Phospholipid and cholesterol, which have an aqueous core and a lipophilic surface (Liu et al., 2022). The aqueous core can entrap a Hydrophilic drug, whereas the lipophilic surface can entrap hydrophobic drugs. The size of the liposome ranges from 30nm to micrometer, and the phospholipid bilayer is 4-5nm thick. A liposome is being widely used for the delivery system of drugs, proteins, nucleic acids, and imaging agents, and the administration routes can be parenteral, pulmonary, oral, transdermal, ophthalmic & nasal route(22). There are several reasons for selecting liposomes as a drug delivery system. It gives outstanding stability to the encapsulated drug from physiological degradation, extends the half-life of the drug, controls the release of drug molecules, and provides sustained release & liposomes are excellent biocompatible and safe. Liposomes can selectively deliver the drug to the target site through passive or active targeting, thus decreasing the systemic side effects, elevating the maximum tolerated dose, and improving the therapeutic benefits(23).

STRUCTURE AND MAIN COMPONENTS OF LIPOSOMES

Depending on compartment structure and lamellarity Liposomes can be classified as:

a) Unilamellar vesicles (ULVs)
b) Oligolamellar vesicles (OLVs)
c) Multilamellar vesicles (MLVs)
d) Multivesicular liposome (MVLs).

OLVs & MLVs contain 2-5 and >5 concentric lipid bilayers, respectively. Based on particle size, ULVs can is further divided into small ULVs (30-100nm), large ULVs (>100nm), and giant ULVs (>1000nm). Most of the current commercial products are SUVs (Ex- Doxil) because of the long circulation time and the ability to passively target the disease site. The mechanism of internal lamella formation is explained as a thermodynamic response of the lipid bilayer to decrease the surface area-to-volume ratio of the liposomes caused by water egress in response to an external osmotic challenge. The abundant lamella provides a large space for the encapsulation of lipophilic compounds(24).

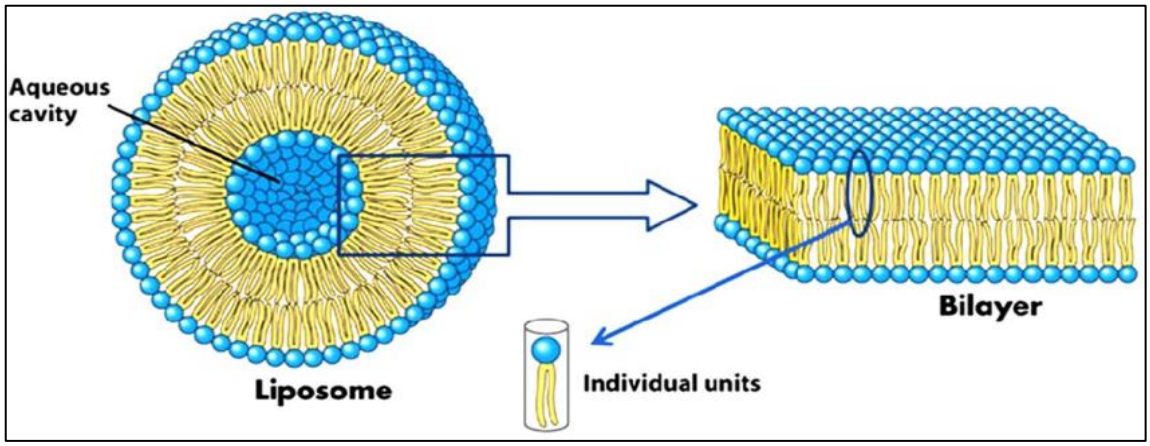


Fig 5: 3D structure of liposome (www.researchgate.net/figure/Cartoon-diagram-of-liposome-the-individual-units-are-the-lipid-molecules_fig2_301219384/download)

COMPONENETS OF LIPOSOME USED IN THIS STUDY

Cholesterol: Cholesterol is one of the main components of liposomes and can be used in almost all liposomes. The addition of cholesterol can promote the packing of lipid chains and bilayer formation, modulate the fluidity and rigidity of membrane, and further affects drug release, stability of liposome, and kinetics of exocytosis(25). The effects of cholesterol on bilayer properties are concentration-dependent. Low (2.5 mol %) and high (>30 mol %) concentrations of cholesterol showed little effect on the properties of the lipid bilayer(22).

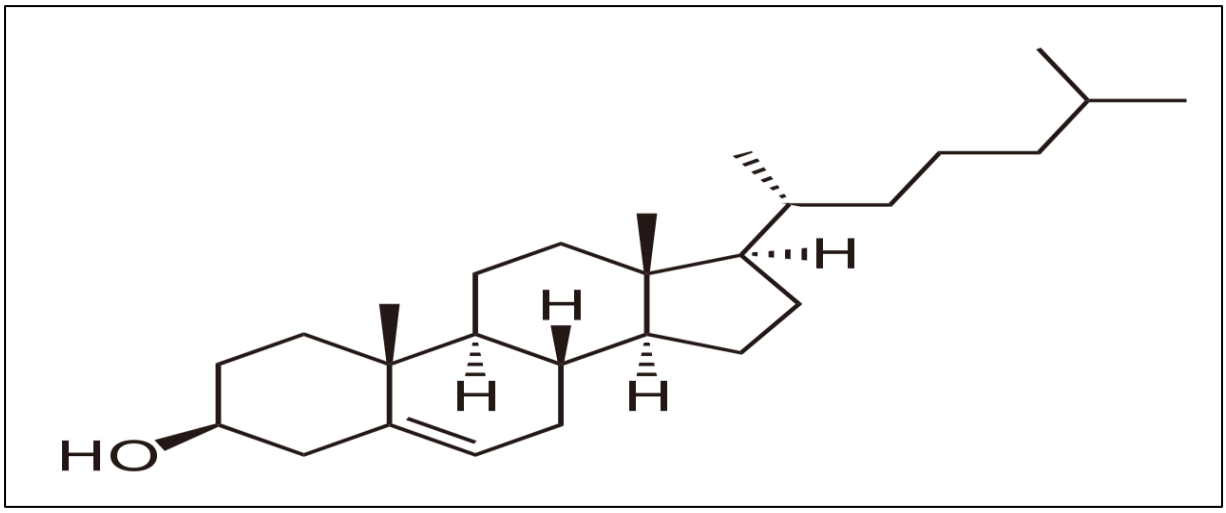


Fig 4: Structure of cholesterol
(https://en.wikipedia.org/wiki/Cholesterol#/media/File:Cholesterol.svg)

Soya lecithin- Lecithin generally refers to various triglycerides, Phospholipids, and glycolipids. Soya Lecithin, a phospholipid is a complex mixture containing 65-75% phospholipids together with triglycerides and smaller amounts of other substances. The major phospholipids include phosphatidylcholine, phosphatidylethanolamine, and inositol-containing phosphatides (Scholfield et al., 1981). However, the term lecithin, as applied in the field of biochemistry, specifically refers to pure phosphatidylcholine, which is a phospholipid that is derived from the phosphate fraction extracted from vegetables such as soybeans, sunflower, and rice beans. Normal phospholipids can be extracted from animal sources, including egg yolk, marine sources, and milk. For liposome formulation, lecithin sources other than soybean carry certain drawbacks. Therefore, lecithin from these sources is less stable when compared to soybean lecithin and other plant-derived lecithin. It has the MW-758.1 with a total polar surface area (TPSA) is 111 Å². Due to its less polar surface area, it is lipophilic in nature, and thus, the lipophilic drug will be encapsulated into the lipid bilayer encapsulate into the lipid bilayer(26).

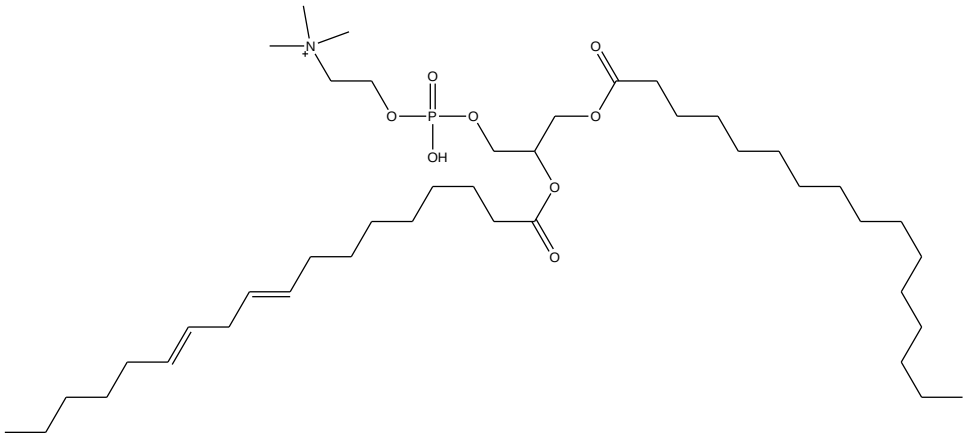


Fig 6: Chemical structure of soya lecithin

Cholesterol and soya lecithin both are biocompatible and biodegradable in nature. None of them have any serious toxic effects when given as a drug delivery system. There is inadequate evidence of human carcinogenicity of cholesterol [Cholesterol | C27H46O - PubChem (nih.gov)]. There are no such reported serious interactions in-between cholesterol, soya lecithin, and drug (Teneligliptin hydrobromide), which can inhibit the release of drug from the formulation.

ADVANTAGES OF LIPOSOME

- Liposomes increased the efficacy and therapeutic index of the drug (Akbarzadeh et al., 2013)
- Liposome increased stability via encapsulation
- Liposomes are non-toxic, flexible, biocompatible, and completely biodegradable for systemic and non-systemic administration
- Liposomes reduce the toxicity of the encapsulated agent
- Liposomes reduce the exposure to toxic chemicals in the human body
- It has the flexibility to couple with site-specific ligands to achieve active

DISADVANTAGES OF LIPOSOME

- Solubility of the drug sometimes decreases when incorporated into the liposome
- A liposome has a shorter half-life
- The tendency of phospholipids to undergo oxidation is higher
- There may be leakage or fusion of encapsulated drug
- The production cost of liposomes is high

METHODS TO PREPARE LIPOSOME

All the methods of preparing liposomes involve 4 basic stages:

1) Drying down lipids from organic solvent
2) Dispersing lipid in aqueous media
3) Purifying the resultant liposome
4) Analyzing the final product

METHOD OF LIPOSOME PREPARATION AND DRUG LOADING-

1) Passive loading technique
2) Active loading technique

The passive loading technique involves three different methods

- Mechanical dispersion method
- Solvent dispersion method
- Detergent removal method

The mechanical Dispersion method can be classified as

- Sonication
- French pressure cell: extrusion
- Freeze thawed liposomes
- Lipid film hydration by hand shaking, non-hand shaking, or freeze-drying
- Micro-emulsification
- Membrane extrusion
- Dried reconstituted vesicles

The solvent dispersion method can be classified as

- Ethanol injection
- Ether injection
- Reverse phase evaporation

The detergent removal method can be classified as

- Dialysis
- Detergent removal of mixed micelles
- Gel-permeation chromatography
- Dilution

EVALUATION OF LIPOSOMES

The Liposomal formulation is characterized to ensure their predictable in vitro and in-vivo performances. The liposome characterization procedures can be classified into three stages: physical, chemical, and biological parameters. Physical characterization parameters include size, shape, surface, and drug release profile. Chemical characterization includes studies in establishing the purity and potency of various lipophilic constituents, and biological characterization parameters are helpful in establishing the safety and suitability of formulation for therapeutic application.

Some of the parameters are:-

- **Vesicle shape and lamellarity:** This can be assessed by electron microscopic Techniques. Lamellarity of vesicles is determined by Freeze Fracture Electron Microscopy and P31 Nuclear magnetic Resonance Analysis
- **Vesicle size and size distribution:** They are liposome's most important characterization parameters. The size difference of liposome particles can affect the drug release. Drugs loaded on smaller size liposomes will be exposed to the particle surface leading to fast drug release. Smaller particles have a tendency to aggregate during storage and transportation of liposome dispersion. Liposome particle size and morphology can be measured by Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM), and Atomic Force Microscopy (AFM).

  The several tools used for determining particle size are as follows:

  - **Dynamic light scattering (DLS):** DLS is widely used to determine the particle size of Brownian liposomes in colloidal suspension in the nanometre and submicron range. The size distribution pattern of the vesicles can be obtained from the polydispersity index.
  - **Scanning electron microscopy (SEM):** SEM is a surface imaging method in which the incident electron beam scans across the sample surface and interacts with the sample to generate signals reflecting the topographical detail of the specimen surface. For SEM characterization, liposomes should be first converted into a dry powder which is then mounted on a sample holder and then it is coated with a conductive metal such as gold or platinum using a sputter coater. The sample is then scanned using a focussed beam of electrons. The incident electron causes the emission of elastic scattering of electrons referred to as backscattered electrons, in elastic scattering of electrons named low energy secondary electrons and characteristic X-Ray light called

cathodoluminescence from the atoms on the sample surface(27). Among these emissions, the detection of secondary electrons is the most common mode in SEM. The size, size distribution and shape of nanomaterials are directly acquired from SEM, but the process of drying and constraining samples may lead to shrinkage of the specimen and alter the characteristics of the nanomaterial(28). Also, many biomolecules that are nonconductive specimens, when scanned by an electron beam, tend to acquire charge and insufficiently deflect the electron beam leading to imaging defects. During sample preparation, coating an ultrathin layer of electrically conductive material onto the biomolecules is often required (29).

- **Transmission electron microscopy (TEM):** TEM works on the same principles of SEM, but in TEM, a focussed monochromatic beam of electrons is transmitted through a very thin foil of sample. In order to make the liposome withstand the instrument vacuum and facilitate handling, they are fixed by either using a negative staining material such as phosphor tungstic acid and derivatives or by plastic embedding. The surface characteristics of the sample are obtained when a beam of electron gets transmitted through an ultra-thin sample. Both SEM and TEM reveal the size and shape heterogeneity of nanomaterials as well as the degree of aggregation and dispersion, but TEM has the advantage over SEM in providing better spatial resolution and capability for additional analytical assessments (28).

- **Surface charge of liposome (Zeta potential):** Determination of the zeta potential of liposomes is very much important as it determines the interaction of liposomes with the biological environment. The colloidal stability of liposome is analysed through the measurement of zeta potential, and this potential provides an indirect measure of the surface charge. The measurement of zeta potential values provides an estimate of the storage stability of liposome, and high zeta potential values, either positive or negative, is preferred in order to maintain optimum stability and prevent aggregation of particles. Zeta potential is the overall charge a lipid vesicle acquires in a particular medium. It is a measure of the magnitude of attraction or repulsion between particles in general and lipid vesicles in particular.
- **Vesicle shape:** In addition to size and surface properties, the shape of liposomes also plays an important role in drug delivery, degradation, transport, targeting, and internalization(30). It has been observed that the efficiency of drug delivery carriers through macrophages is also dependent on carrier shape. Various electron microscopic

techniques are used to assess the shape and lamellarity of liposomes. The surface morphology of Liposomes can be assessed using field emission scanning electron microscopy (FESEM), which give clear, high-resolution pictures of the surface along with the overall nature of the formulation regarding the presence/absence of any aggregates or lump in the sample.

- **Lamellarity:** The average number of bilayers present in liposomes, i.e., lamellarity, can be determined by freeze-fracture electron microscopy or cryo-transmission electron microscopy (cryo-TEM) and by P-NMR. In a later technique, the signals are recorded before and after the addition of a broadening agent such as manganese ions with the outer leaflet of the outermost bilayers. Nowadays, freeze-fracture electron microscopy has become a very popular method for studying structural details of aqueous lipid dispersions (31).
- **Drug loading:** A high loading capacity is the measure of a successful nanoliposomal system because it reduces the amount of matrix material for administration. Drug loading and entrapment efficiency depend on the solid-state drug solubility in the polymer, which in turn is related to the polymer composition, the molecular weight, drug-polymer interaction, and the presence of the end functional group (32).
- **Entrapped volume:** It can be defined as the aqueous volume per unit quantity of the lipid. This entrapped volume is a crucial parameter that governs the morphology of liposomes. It is determined by measuring the quantity of water by replacing the external medium with spectrophotometrically inert fluid and then measuring the water signal by NMR.
- **Entrapment (percent capture):** It is essential to measure the quantity of material entrapped inside liposomes before the study of the behavior of this entrapped material in physical and biological systems since the effects observed experimentally will usually be dose-related. In general, two methods may be used, i.e., mini-column centrifugation and protamine aggregation
- **Drug release:** A number of methods are applied to determine the in-vitro release of drugs from the liposome. They are
  - Reverse dialysis bag technique
  - Dialysis bag diffusion technique and Shaking incubator technique

CHAPTER- 2

LITERATURE REVIEW

LITERATURE REVIEW:

Surendra Kumar Sharma et al. (2016)- In addition to diet and exercise, the recently created oral dipeptidyl peptidase 4 inhibitor teneligliptin is suggested for the therapy of type 2 diabetes mellitus (T2DM) in adults. Teneligliptin has lately been reasonably priced in Japan (Teneria®), Argentina (Teneglucon®), and India (Tenepure; Teneza). This is a step in the right direction for managing T2DM in underdeveloped nations, where the price of medicine is a barrier to access to healthcare and an out-of-pocket expense. Teneligliptin's effectiveness and safety in the treatment of type 2 diabetes are assessed in this review. Teneligliptin has been thoroughly examined in short-term (12 weeks) and long-term (52 weeks) studies in T2DM as a monotherapy with diet and exercise as well as in combination with metformin, glimepiride, pioglitazone, and insulin. Within 12 weeks of medication, these studies have shown a 0.8%–0.9% decrease in HbA1c. Teneligliptin has been shown to consistently enhance glycaemic control in two 52-week investigations. The safety profile of teneligliptin is comparable to that of other dipeptidyl peptidase 4 inhibitors, and it has been shown to be generally well tolerated(33).

Malviya V.R et al. (2020)- The goal of the current study was to create an oral dispersible film containing the drug teneligliptin hydrobromide using the polymer Pullulan and to test it using various criteria. The drug-excipient tests were conducted using Fourier transmission infrared spectroscopy (FT-IR) to identify any types of incompatibilities. Pullulan was used as the polymer in the solvent casting process to create the oral dispersible films. As a plasticizer, propylene glycol was employed. The produced films were assessed for characteristics such as surface pH, mechanical properties, drug content homogeneity, physical appearance, thickness, folding endurance, in vitro disintegration, taste evaluation, in vitro dissolution test, and stability study. When compared to other formulations, the T6 formulation was determined to be more optimised, stable, and adequate in terms of its evaluation parameters. The highest percentage drug release was determined to be 95.90% in 30 minutes, with the folding endurance measuring 282 1.59, disintegration time 05 0.57, thickness 0.064 0.001, tensile strength 5.89, and the percent elongation 26.08. The surface pH was found to be 6.4 and the drug content to be 99.90. The product was determined to be stable for 90 days during the formulation's stability trials. The developed teneligliptin film demonstrates to be a suitable candidate for safe and efficient

oral dispersible drug delivery. The oral dispersible film is easy to administer and very effective for the patients (34).

Miyako Kishimoto (2013): Dipeptidyl peptidase-4 (DPP-4) inhibitors are anti-diabetics that have lately come into the spotlight and have promising outcomes in improving glycaemic control with a low risk of hypoglycaemia and weight gain. Teneligliptin, a DPP-4 inhibitor, characterized by five consecutive rings, which produce a potent and longlasting effects. Teneligliptin is now used in cases when glycaemic control has not improved enough after diet control, exercise, or a combination of diet control, exercise, and medications from the sulfonylurea or thiazolidine class. Teneligliptin is given orally to adults once day at a dosage of 20 mg, with a daily maximum dose of 40 mg. No dose adjustment is required in patients with renal impairment because the metabolites of this medication are removed via renal and hepatic elimination. Teneligliptin's safety profile is comparable to those of other DPP-4 inhibitors that are currently on the market. Teneligliptin should only be given with caution to people who are predisposed to QT prolongation. One study found that teneligliptin taken before breakfast had postprandial blood glucose-lowering effects that persisted throughout the day and that the effects seen after dinner were comparable to those seen after breakfast or lunch. Thus, although clinical data for this new drug are limited, this drug shows promise in stabilizing glycaemic fluctuations throughout the day and consequently suppressing the progression of diabetic complications. However, continued evaluation in long-term studies and clinical trials is required to evaluate the efficacy and safety of the drug as well as to identify additional indications for its clinical use (35).

Junko Hanato et al. (2009)- Glucagon-like peptide-1 (GLP-1), an incretin hormone, is known to be a potent therapeutic candidate for the treatment of diabetes; however, due to its rapid enzymatic breakdown by dipeptidyl-peptidase IV, its clinical applicability has been severely limited. To protect GLP-1 from enzymatic degradation and improve pharmacological effects, liposomal formulations of GLP-1 were prepared using three types of lyophilized empty liposomes such as anionic, neutral and cationic liposomes. Electron microscopy and dynamic light scattering tests showed that GLP-1-loaded liposomes had a homogeneous size distribution with a mean diameter of 130–210 nm, and that inclusion of GLP-1 had no effect on the dispersibility or morphology of any individual liposome. Anionic liposomal formulation, out

of all liposomal formulations evaluated, has the highest GLP-1 encapsulation effectiveness (about 80%). In intraperitoneal glucose tolerance testing in rats, marked improvement of hypoglycaemic effects were observed in anionic liposomal formulation of GLP-1 (100 nmol/kg) with 1.7-fold higher increase of insulin secretion, as compared to GLP-1 solution. In pharmacokinetic studies, intravenous administration of anionic liposomal formulation of GLP-1 (100 nmol/kg) resulted in 3.6-fold higher elevation of serum GLP-1 level as compared to GLP-1 injection. Upon these findings, anionic liposomal formulation of GLP-1 would provide the improved pharmacokinetics and insulinotropic action, possibly leading to efficacious anti-diabetic medication (36).

Chun Y. Wong et al. (2018): Diabetes affects a large number of people, particularly in developed countries. To regulate the BSL, this chronic metabolic disease necessitates daily subcutaneous insulin injections. However, insulin injection has a low patient compliance rate and might result in unpleasant side effects such as pain, allergic response, discomfort, and nausea. As a result, non-invasive, safe, patient-compliant, and convenient medication delivery systems are preferable. As a result, non-invasive, safe, patient-compliant, and convenient medication delivery systems are preferable. This review demonstrates the oral delivery of insulin by liposomes. In general, conventional liposomes are not stable in physiological condition, but novel insulin-loaded liposomes have demonstrated better stability in vitro and hypoglycaemic effects in vivo. However, formulation design must be optimized by selecting appropriate preparation parameters and combining multiple absorption-enhancing strategies. Different strategies, such as surface coating, cell-specific targeting ligands, bile salts, and freeze drying, can be combined to improve the overall performance of liposomal formulations. Further studies are needed to examine the immunological response, long-term safety profile, biodistribution, and pharmacokinetic profile of liposomes for the development of successful oral insulin-loaded liposomal formulations (37).

Raz & M. Hanefeld et al. (2006): The aim of this study was to assess the efficacy and safety of sitagliptin (MK-0431) as monotherapy in patients with type 2 diabetes mellitus and inadequate glycaemic control (HbA1c Q7% and e10%) on exercise and diet. A total of 521 patients aged 27–76 years with a mean baseline HbA1c of 8.1% were randomised in a 1:2:2 ratio to treatment with placebo, sitagliptin 100 mg once daily, or sitagliptin 200 mg once daily,

for 18 weeks. The efficacy analysis was based on an all-patients-treated population using an analysis of covariance, excluding data obtained after glycaemic rescue. After 18 weeks, HbA1c was significantly reduced with sitagliptin 100 mg and 200 mg compared with placebo (placebo-subtracted HbA1c reduction: j0.60% and j0.48%, respectively). Sitagliptin also significantly decreased fasting plasma glucose relative to placebo. Patients with higher baseline HbA1c (Q9%) experienced greater placebo-subtracted HbA1c reductions. Homeostasis model assessment beta cell function index and fasting proinsulin: insulin ratio, markers of insulin secretion and beta cell function, were significantly improved with sitagliptin. The incidence of hypoglycaemia and gastrointestinal adverse experiences was not significantly different between sitagliptin and placebo. Sitagliptin had a neutral effect on body weight. Stagliptin significantly improved glycaemic control and was well tolerated in patients with type 2 diabetes mellitus who had inadequate glycaemic control on exercise and diet (38).

M. Kusunoki et al. (2014): Dipeptidyl peptidase-4 (DPP-4) inhibitors have been reported to improve the glycemic control and blood hemoglobin A1c (HbA1c) concentrations. However, there are few reports as yet suggesting that DPP-4 inhibitors may also improve insulin resistance and the serum lipid profile in the clinical setting. This study was aimed at investigating the effect of 14-week treatment with teneligliptin (20mg/day) on the homeostasis model assessment ratio (HOMA-R), an indicator of insulin resistance, and serum lipid profile in 9 patients with type 2 diabetes. The treatment produced a significant decrease of the blood glucose and HbA1c concentration (blood glucose: p=0.008; HbA1c: p=0.038), and also improved HOMA-R (p=0.039). Furthermore, the patients showed elevation of the serum HDL-cholesterol level (p=0.032), and a tendency towards reduction of the serum triglyceride level. The results indicate that teneligliptin acts not only to improve the blood glucose control, but also to improve the insulin resistance and serum lipid profile in Japanese type 2 diabetes patients (39).

Prabhat Agrawal et al. (2018): Present study is designed with an aim to determine the effectiveness of cost-effective DPP-4 inhibitor, Teneligliptin, over the other agent of the same class. The study was carried out in Postgraduate Department of Medicine, S.N. Medical College, Agra and 112 patients were selected as subjects with a selected inclusion criterion. Independent student's t-test was applied to compare the means. Mean standard deviation was

calculated for quantitative data. All p values were two-tailed and values p. Results show a 17.9% reduction in HbA1c with vildagliptin as compared to teneligliptin which shows 18.9% reduction in DPP4i naïve patients at the end of three months.11 Similar results were observed in our study where difference in HbA1c reduction between them is insignificant. In another study it was reported that gliptins (DPP-4 inhibitor) have slight lesser HbA1c lowering potential when compared to active glucose lowering agent like metformin. Though the former was reported of having better gastrointestinal tolerability. The study presents Teneligliptin, a DPP-4 inhibitor as an effective, antihyperglycemic second line agent with the use of other conventional antidiabetic agents. Along with it, Teneligliptin also offers low-cost treatment at an average reduced daily price of INR 39, when compared to other DPP-4 inhibitors (40).

Gemma Pujadas et al. (2016): Teneligliptin, a dipeptidyl peptidase-4 (DPP-4) inhibitor, has antioxidant properties and can reduce reactive oxygen species (ROS) levels in human endothelial cells exposed to high glucose. It also induces the expression of antioxidant genes through the Nrf2 pathway. Teneligliptin improves proliferation rates in endothelial cells exposed to high glucose by regulating cell-cycle inhibitors and reducing proapoptotic genes. It also promotes the expression of the anti-apoptotic gene BCL2. Teneligliptin ameliorates endoplasmic reticulum (ER) stress induced by high glucose, reducing the expression of ER stress markers. Teneligliptin has pleiotropic effects and may have a protective role in cardiovascular disease (CVD) due to its high tissue distribution and radical scavenging properties. It has been shown to improve endothelial function and reduce oxidative stress markers in animal models and patients with type 2 diabetes (41).

CHAPTER- 3

AIM OF THE RESEARCH WORK

AIM OF RESEARCH WORK-

The Disease Diabetes is very common throughout the world, and there are some established formulations for treating this disease. Still, none of them focus on the sustained release of the drug from the formulation to get lower drug toxicity and more extended drug action. So we tried to achieve a sustained release formulation by incorporating drug into liposomes.

The main aim of the research works are-

- Preparation of standard curve of Teneligliptin hydrobromide
- Development of liposomal formulation of Teneligliptin hydrobromide
- Characterization of physicochemical properties-
    - Particle size- average particle size of the liposomal formulation is determined by DLS (Dynamic Light Scattering)
    - Zeta potential- to determine the stability of the particle zeta potential is determined by zeta sizer.
    - FTIR study to determine the chemical interaction between the drug and excipient or between excipients
    - Drug loading and entrapment efficiency to check how much amount of the drug incorporated into the liposome
    - SEM to determine the surface morphology of the formulation
    - Invitro drug release study to determine the sustained action of the formulation

CHAPTER- 4

MATERIALS USED & METHADOLOGY

MATERIALS USED:

- **CHEMICALS**: Teneligliptin hydrobromide**,** Cholesterol, Soya-L-α-lecithin (SLE), Butylated Hydroxy Toluene (BHT), chloroform, Ethanol, Potassium dihydrogen phosphate, Disodium hydrogen phosphate, Sodium chloride are required for development of the formulation.

Table 4: List of the chemicals required for preparation of liposome with their source

NAME	SOURCE
Teneligliptin hydrobromide	Merck Lifescience Pvt. Ltd. (Mumbai, India)
Cholesterol	HiMedia Laboratories Pvt. Ltd. (Mumbai, India)
Soya-L-α-lecithin (SLE)	HiMedia Laboratories Pvt. Ltd. (Mumbai, India)
Butylated Hydroxy Toluene (BHT),	Qualigens Fine Chemicals (Mumbai, India)
Chloroform	Merck Lifescience Pvt. Ltd. (Mumbai, India)
Potassium dihydrogen phosphate	Merck Specialities Pvt. Ltd. (Mumbai, India)
Disodium Hydrogen Phosphate	E Merck Ltd. (Mumbai, India)
Sodium chloride	Merck Lifescience Pvt. Ltd. (Mumbai, India)
HPLC water	Merck Lifescience Pvt. Ltd. (Mumbai, India)
Ethanol	Changshu Hongsheng Fine Chemicals Co. Ltd., (Changshu city)
Hydrochloride acid	Merck Lifescience Pvt. Ltd. (Mumbai, India)
Sodium chloride	Merck Lifescience Pvt. Ltd. (Mumbai, India)

Table 5: List of the equipment used for the preparation of liposomal formulation and evaluation with their source

NAME	SOURCE
Rotary vacuum evaporator	Rotavap Superfit model-PBU-6, Mumbai, India
Low temperature circulating bath	Instrumentation India, Kolkata, India
Vacuum Desiccators	Tarson, Kolkata, India
Bath type sonicator	Tarson-O-Sonic, Mumbai, India
Advanced microprocessor UV-Vis single-beam spectrophotometer (Model Intech-295) Software-UV Professional V1.39.0	Model Intech-295, Gentaur GmbH, Aachen, Germany
Lyophilizer	Instrumentation India, Kolkata, India
Digital balance	Sartorius, Goetingen, Germany
High speed Ultra cold Centrifuge (Model Z 32 HK)	Hermle Labortechnik GmbH, Wehingen, Germany
Fourier-Transform Infrared Spectroscopy (FTIR) (ECO-ATR, Model ALPHA, Software-OPUS 7.5)	Department of Chemistry, PG Science building, Jadavpur University, Kolkata, India
Magnetic stirrer	Remi Equipments, Mumbai, India
pH meter	Eutech Instruments, Mumbai, India
Vortex mixture (Model CM100)	Remi Equipments, Mumbai, India
Distillation Plant	Sicco, Kolkata, India
All glass apparatus	Borosil, Mumbai, India
Pipette & Micro Tips, Centrifuge Tube	Tarsons Products Pvt. Ltd., Kolkata, India
Scanning electron microscopy	Center for Research in Nanoscience and Nanotechnology, University of Calcutta, Kolkata, India

DLS (Dynamic light scattering)	Department of Central Scientific Service, Indian Association for the Cultivation of Science, kolkata
Zetasizer	Department of Central Scientific Service, Indian Association for the Cultivation of Science, kolkata

CHAPTER- 5

METHODOLOGY

METHODOLOGY-

5.1. Preparation of calibration curve of Teneligliptin hydrobromide in PBS P^H 7.4 and Ethanol

5.1.1. Preparation of buffer solution-

Phosphate buffer saline pH 7.4 was prepared according to I.P protocol. 2.38 g of disodium hydrogen phosphate (Na_2HPO_4), 0.19 g of potassium dihydrogen phosphate (KH_2PO_4) and 8g of sodium chloride (NaCl) were dissolved in 1000 ml distilled water. pH was adjusted to 7.4 using pre-calibrated pH meter (Eutech. Instruments), 0.1N Sodium Hydroxide (NaOH) solution and 0.1N Hydrochloric acid (HCl) solution.

5.1.2. Preparation of stock solution-

2 mg of drug was dissolved in 2 ml of ethanol in a Eppendorf tube and 2 mg of drug was dissolved in 2 ml of PBS pH 7.4 in a Eppendorf tube using vortex mixer. The concentration of the both stock solution was 1000 µg/ml.

5.1.3. Preparation of standard solution-

0.10 ml of stock solution was taken in Eppendorf tube and diluted with PBS pH7.4 and ethanol respectively to make the 100µg/ml solution.

0.10, 0.16, 0.32 and 0.64 ml of the secondary stock solution (100µg/ml) were taken in Eppendorf separately and diluted with PBS pH7.4 and Ethanol respectively to make the concentration 10µg/ml, 16µg/ml, 32µg/ml and 64µg/ml.

For both PBS pH7.4 and Ethanol 0.1, 0.2, 0.4 and 0.8 ml of 10µg/ml solution were taken in Eppendorf separately and diluted with the respective solvent to prepare 1 ml solution of 1µg/ml, 2µg/ml, 4µg/ml and 8µg/ml.

5.1.4. Determination of absorption maximum of Teneligliptin hydrobromide-

For the determination of absorption maxima, about 1mg drug was dissolved in 10 ml of PBS (pH 7.4) using vortex mixer. The solution was then scanned under UV-VIS single-beam spectrophotometer (Model Intech-295, Gentaur GmbH, Aachen, Germany) using PBS pH7.4 as blank. A single characteristic peak at 242.5nm in PBS pH7.4 was obtained, which was close to the published lambda max of Teneligliptin (Teneligliptin-Wikipedia).

Same procedure was performed using Ethanol as a solvent and a single characteristic peak at 245.5 nm in Ethanol was obtained, which was close to published lambda max of Teneligliptin.

5.1.5. Preparation of the standard curve-

To prepare the standard curve of quercetin in all the cases, first, the blank solution was taken into UV-VIS single-beam spectrophotometer at lambda max-242.5nm, and the absorbance of the blank was set to zero. Various concentrations of drug solution in PBS pH7.4 (1,2,4, 6,8,16,32,64, µg/ml) were taken and their absorbances were measured in UV-VIS single beam spectrophotometer at lambda max-242.5nm from lower concentration to upper concentration up to 64 µg/ml. The same procedure was followed in the case of the drug in Ethanol with their respective standard solutions. All the absorbances for their respective solvent systems were noted down against their concentration. Values were then taken into MS Excel; a graph was prepared using concentration at the X axis and absorbance at the Y axis.

5.2. Preparation of nanosized liposomal formulation

Out of several methods of liposome preparation, in my work, I have used the conventional lipid layer hydration method (also called a thin-film hydration method) to develop nanosized liposomes. Both Blank liposomes and drug-loaded liposomes were prepared.

- **PREPARATION OF THIN FILM**- 150 mg soya lecithin, 75 mg cholesterol and pinch of BHT are dissolved in 10 ml chloroform in 250 ml round bottomed flask. For blank same amount of soya lecithin, cholesterol, BHT and chloroform were taken. The thin film was created using a Rotary vacuum evaporator at RPM-45, the Temperature of the Water bath 50°C, and the temperature of the circulator 4°C, and evaporation was continued under vacuum until the complete solvent got evaporated. The round bottom flask along with the thin film was then kept in a desiccator overnight for complete evaporation of any residual solvent.
- **HYDRATION-** Hydration of the thin film was done on day 2 using 70ml PBS pH7.4 using the rotary water bath for 45 min at RPM 45 and the temperature of the water bath 50°C. As drug is soluble in PBS, 10 mg drug is added in this step. For blank liposomal formulation no drug is added in this step. During hydration, lipid layer creates vesicles in the buffer system encapsulating the drug, and the vesicles created are mostly multi-lamellar.

- **SONICATION-** To create unilamellar vesicles, the content in the round bottom flask was then sonicated in bath sonicator for 45 min (3 cycles, 1 cycle for 15 min). The liposome dispersion was then kept in the dark place for 3 hours and then kept in the refrigerator overnight.
- **CENTRIFUGATION-** Centrifugation was done at day in High-speed Ultra cold Centrifuge (Model Z 32 HK) 3 to separate the liposome vesicles from the unentrapped drug and the lecithin and cholesterol which did not take part in a liposome preparation. To centrifuge, the first liposome dispersion was divided into two separate centrifuge tubes (50ml) in a manner that the weight of both the tubes remained equal. First cycle was carried out at 16000 RPM, 4°C for 30min, the supernatant was separated, the pellet was dispersed in fresh double distilled water, and it was again centrifuged at 16000 RPM, 4°C for 10 min (Washing phase). Again supernatant was discarded, pellet was dispersed in 3 ml of double-distilled water, kept in petri-dish, sealed using parafilm and kept in a refrigerator at -20°C.
- **LYOPHILIZATION-** The next day, Lyophilisation was done next day using Lyophilizer for 14 h. where dried liposomes were obtained. They were kept in Eppendorf tubes, sealed using parafilm, and stored at -20°C.

5.3. Evaluation

5.3.1. DRUG LOADING AND ENTRAPMENT EFFICIENCY STUDY OF LIPOSOMAL FORMULATION

2mg of blank and drug encapsulated freeze-dried liposome was taken in different Eppendorf tubes. 2ml PBS pH7.4 was added in each Eppendorf tube. They were then vortexed for 3 min. Then they were sonicated at a bath sonicator for 30 min (2 cycles, 15 min each) and then centrifuged for 15min at 4°C, 16000 RPM. During this process, the liposome vesicles will be disrupted, and the entrapped drug will come out and dissolve in the solvent (PBS). During centrifugation, the undissolved lipid and cholesterol will be precipitated as a plate. The supernatant containing the drug (for drug-loaded liposome) and without the drug (for blank liposome) were taken in fresh Eppendorf tubes. The absorbance of the supernatant of blank liposome and of drug-loaded liposome was measured at 242.5nm against the blank solvent (Ethanol).

The absorbance of drug = Absorbance of supernatant of blank liposome - Absorbance of supernatant of drug-loaded liposome

The percentage of drug loading and drug loading efficiency was calculated using the following formula:

- Theoretical drug loading (%) = [Amount of drug taken to prepare Liposome/ (Amount of SLE+CHL+BHT+Drug) x 100
- Practical drug loading (%) = (Amount of drug in Liposome/Amount of Liposome obtained) x 100
- Drug loading efficiency (%) = (Practical drug loading /Theoretical drug loading) x 100

5.3.2. Drug excipient-interaction study using FTIR spectroscopy

The pure drug Teneligliptin hydrobromide, cholesterol, soya lecithin, BHT, physical mixture of drug with cholesterol, soya lecithin, BHT and lyophilized formulation with and without drug were mixed separately, with infrared (IR) grade potassium bromide (KBr) in the ratio 1:100. Corresponding pellets were prepared by applying 5.5 metric ton pressure with a hydraulic press. The pellets were scanned in an inert atmosphere over a wave number range of 4000-400 cm^{-1} in a FTIR instrument at the Department of chemistry, PG Science building, Jadavpur University, Kolkata, India.

5.3.3. Study of surface morphology of nanoliposome by Scanning Electron Microscopy (SEM)

The external morphology of Teneligliptin nanoliposome was analysed by Scanning Electron Microscopy (SEM) at the Centre for Research in Nanoscience and Nanotechnology (CRNN), University of Calcutta, Kolkata, India. The freeze-dried particles were spread onto metal stubs and platinum coating was done by using ion-sputtering device. The coated particles were then vacuum dried and examined under Scanning Electron Microscope.

5.3.4. Particle size distribution study

The size distribution of reconstituted lyophilized liposomes was determined by Dynamic Light Scattering (DLS – ZETASIZER, Nano ZS 90, Malvern Instrument Ltd. UK) and analysed by DTS software at Department of Central Scientific Service, Indian Association for the Cultivation of Science, Kolkata.

The principle of the particle size determination by DLS is the measurement of the rate of fluctuation of the intensity of scattered light due to Brownian motion of particles. Determination of the intensity fluctuation yields the value of Brownian motion of the particles due to thermally induced collisions between the particles which are converted into particle-size by using DTS software. The mean particle diameter (Z average) was calculated by the software from the measured particle size distribution. The freshly prepared lyophilized formulation were suspended in double distilled water and poured into the cuvette and analysed by the instrument.

5.3.5. Polydispersity index (PDI)

Polydispersity index is a number which is a measure of size distribution of particles in a given sample. This value may be equal to one or greater or less than one. Generally for monodispersed sample the value is 0.05 and the values greater than 0.7 indicate that the sample has very broad size distribution and is probably not suitable for dynamic light scattering technique (Dynamic Light Scattering Common Terms Defined © 2011 Malvern Instruments Limited). Polydispersity index of the different formulations was measured by the instrument Zeta sizer nano ZS (ZETASIZER, Nano ZS 90, Malvern Instrument Ltd. UK).

5.3.6. Zeta potential measurement

Zeta potential is a measure of the magnitude of the electrostatic or charge repulsion or attraction between particles in a liquid suspension. This parameter ensures the dispersion stability of the nanoliposome formulation. Zeta potential of the different formulations were determined by the instrument Zeta sizer nano ZS (ZETASIZER, Nano ZS 90, Malvern Instrument Ltd. UK).

5.3.7. Invitro drug release study from the nanoliposome formulation through dialysis membrane

In a 250 ml conical flask, 50 ml of phosphate buffer saline (pH 7.4) was taken. The weighted amount (5mg) of lyophilized formulation was reconstituted in 1ml of PBS. Then it was taken into dialysis bag (Himedia dialysis membrane-60, Mumbai, India). Two ends of the dialysis bag were tightly bound with cotton threads. The dialysis bag was hanged inside the conical flask with the help of stand so that portion of dialysis bag with the formulation could dip into the buffer solution. The flask was kept on magnetic stirrer. Stirring was maintained with the help of magnetic bead at room temperature. Sampling was done by withdrawing 1 ml from released media with the help of micropipette and 1 ml of fresh buffer was added at definite

time intervals. Samples were analysed in a spectrophotometer at the wavelength of 242.5 nm. With the help of standard curve the drug concentration was measured.

CHAPTER- 6

RESULTS

RESULTS

6.1. UV Absorption spectra of Teneligliptin Hydrobromide

The method for the determination of absorption maximum (λ_{max}) was discussed in the previous section. After scanning the drug in single beam UV-Visible spectrophotometer from 800 to 190 nm, the λ_{max} was found at 242.5nm.

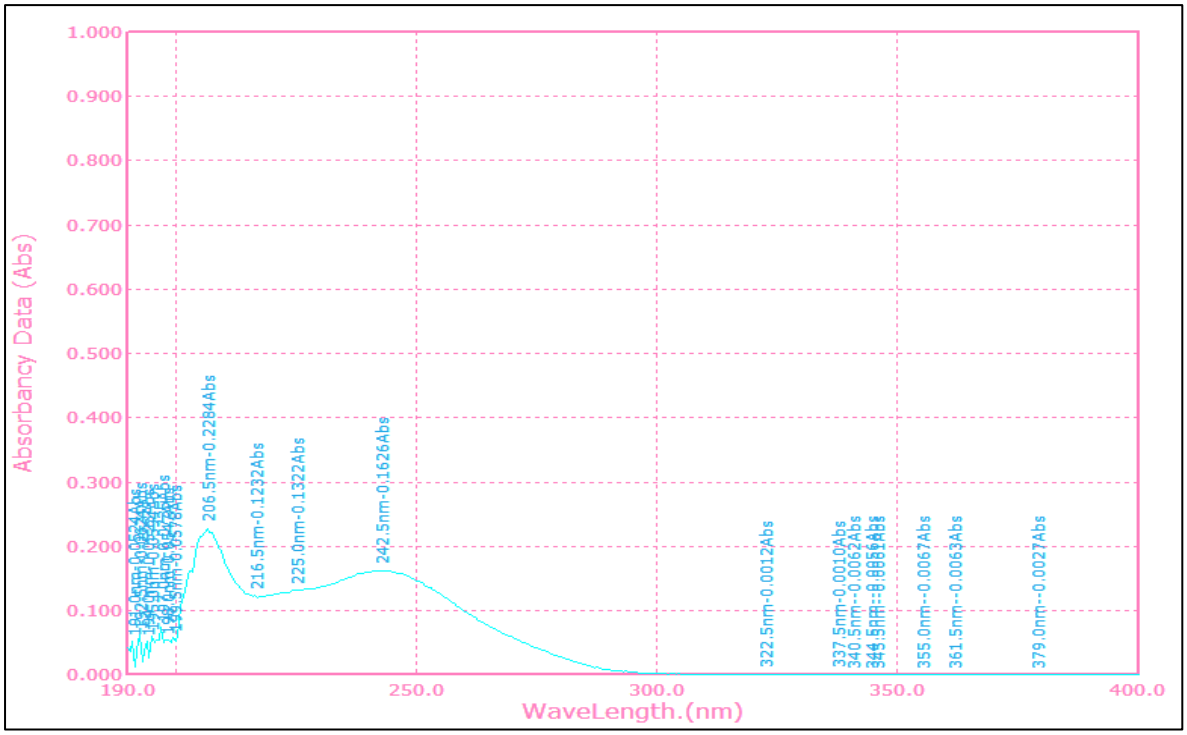


Fig 7: Lamda max of Tenegliptine hydrobromide in PBS (pH 7.4)

The calibration curve of Teneligliptin Hydrobromide

Two different calibration curves were prepared – one in PBS pH 7.4 & one in Ethanol for studying liposome entrapment efficiency and in vitro-drug permeation study. Each reading was made in triplicate, and the average value was taken. The dilutions prepared for the standard curves were as follows

PBS pH 7.4 – 1, 2, 4, 6, 8, 16, 32 µg/ml

Ethanol – 1, 2, 4, 6, 8, 16, 32 µg/ml

The standard curves are depicted below with their respective absorbance value against concentrations.

Table 6: The mean absorbance of Teneligliptin Hydrobromide against various concentrations in PBS pH 7.4 with standard deviations

CONCENTRATION (µg/ml)	MEAN ABSORBANCE (N=3)
0	0
1	0.011± 0.005
2	0.029± 0.002
4	0.064± 0.003
6	0.102± 0.003
8	0.138± 0.007
10	0.180± 0.007
16	0.290± 0.004
32	0.594±0.002

Here mean absorbance is represented as mean absorbance ± SD (n=3)

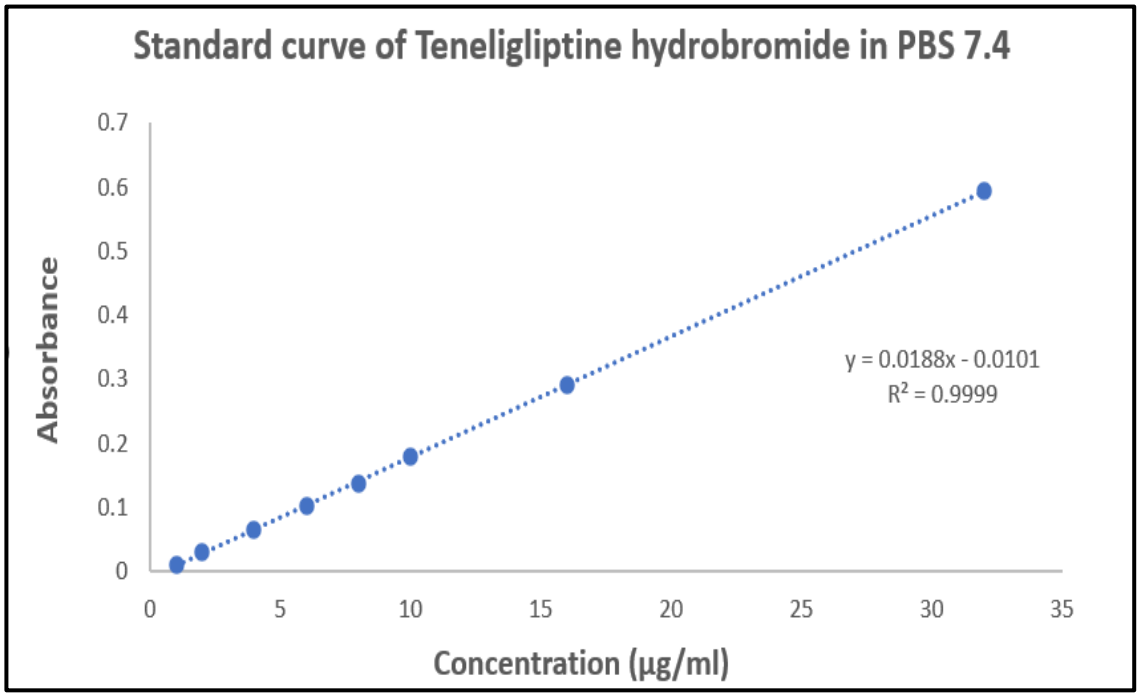


Fig 8: Standard curve of Teneligliptin hydrobromide in PBS 7.4

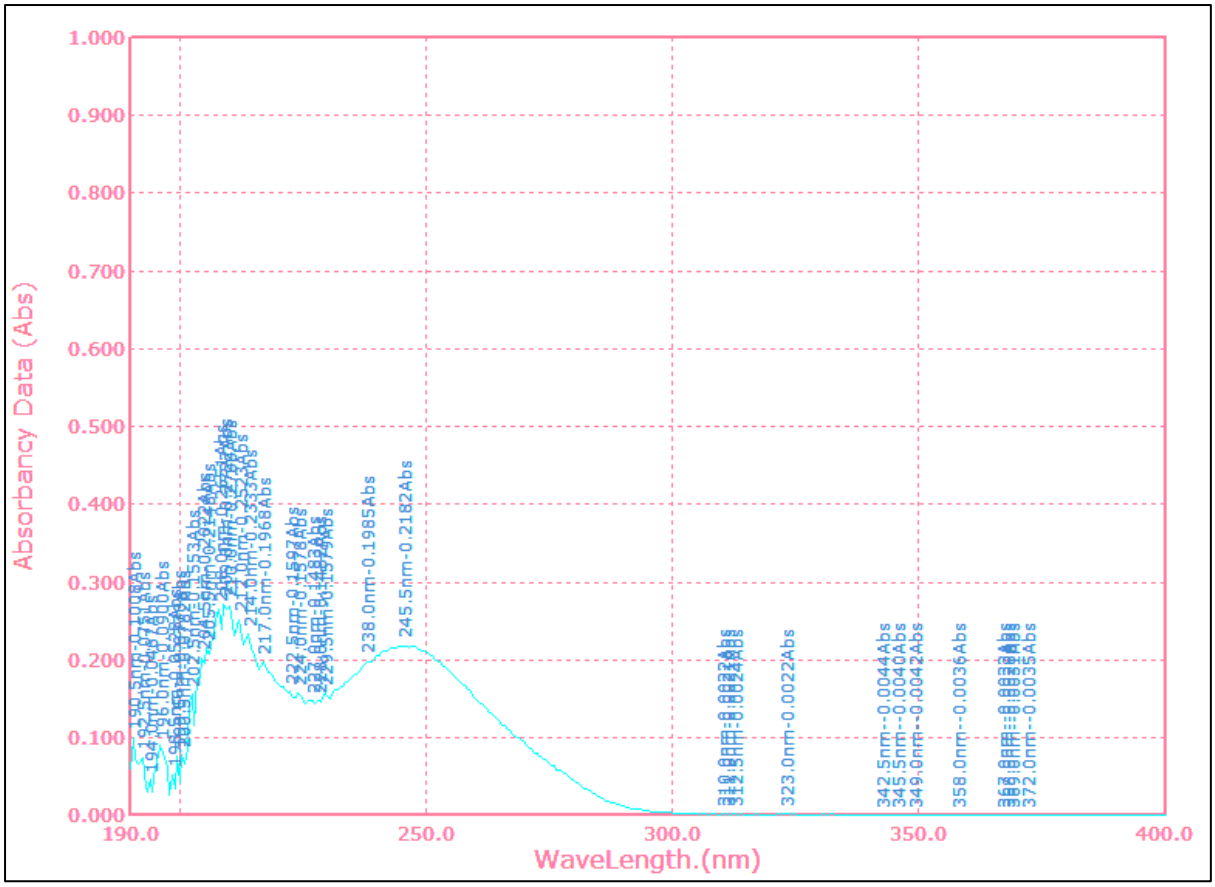


Fig 9: Lambda max of Teneligliptin hydrobromide in ethanol

Table 7: Mean absorbance of Teneligliptin hydrobromide against various concentration in Ethanol with Standard deviation

Concentration	Absorbance
0	0
1	0.014
2	0.041
4	0.089
6	0.135
8	0.179
16	0.356
32	0.682

Mean absorbance is represented as mean absorbance ± standard deviation (n=3)

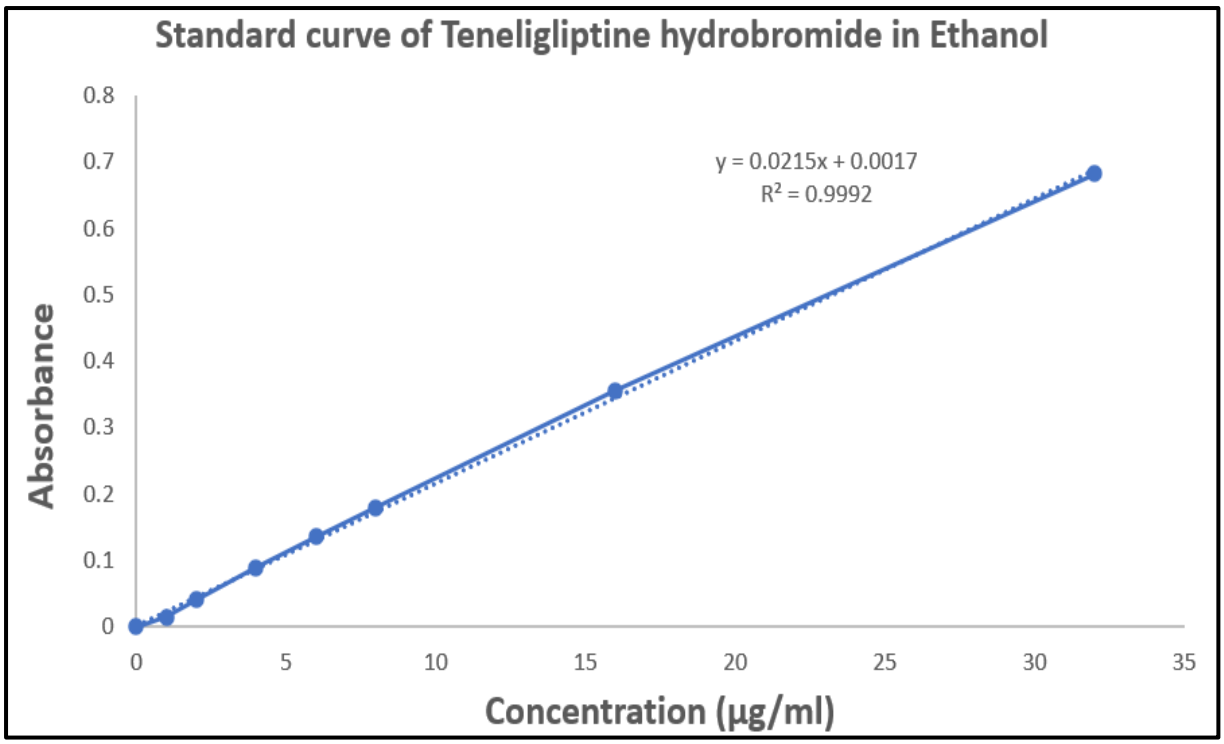


Fig 10: Standard curve of Teneligliptin hydrobromide in Ethanol

6.2. STUDY OF DRUG-EXCIPIENT INTERACTION THROUGH FOURIER TRANSFORM INFRARED (FTIR) SPECTROSCOPY

In the present study we evaluated the drug excipient interaction by FTIR spectroscopy. FTIR spectra asses the drug excipient interaction at the level of functional groups by determining their vibrational patterns. Here the spectra of drug, the individual excipients (Cholesterol & Soya Lecithin), blank liposomes (without drug) and drug loaded liposome have been depicted in the pictures from the **Fig 11** to **Fig 15.**

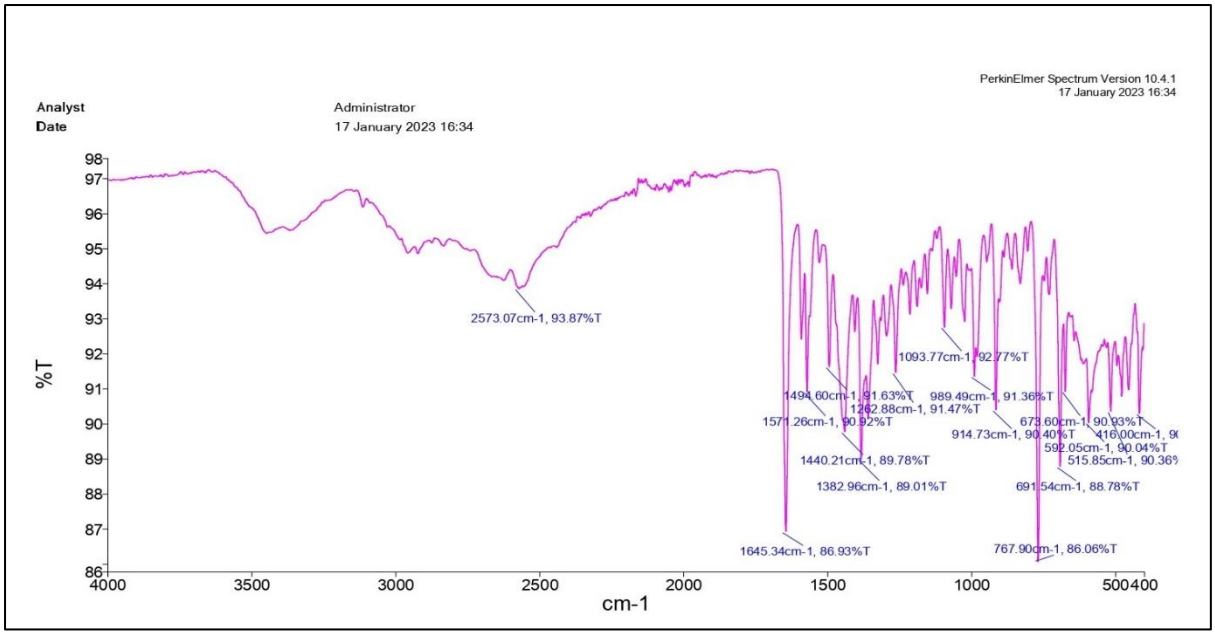


Fig 11: FTIR spectra of Teneligliptin hydrobromide

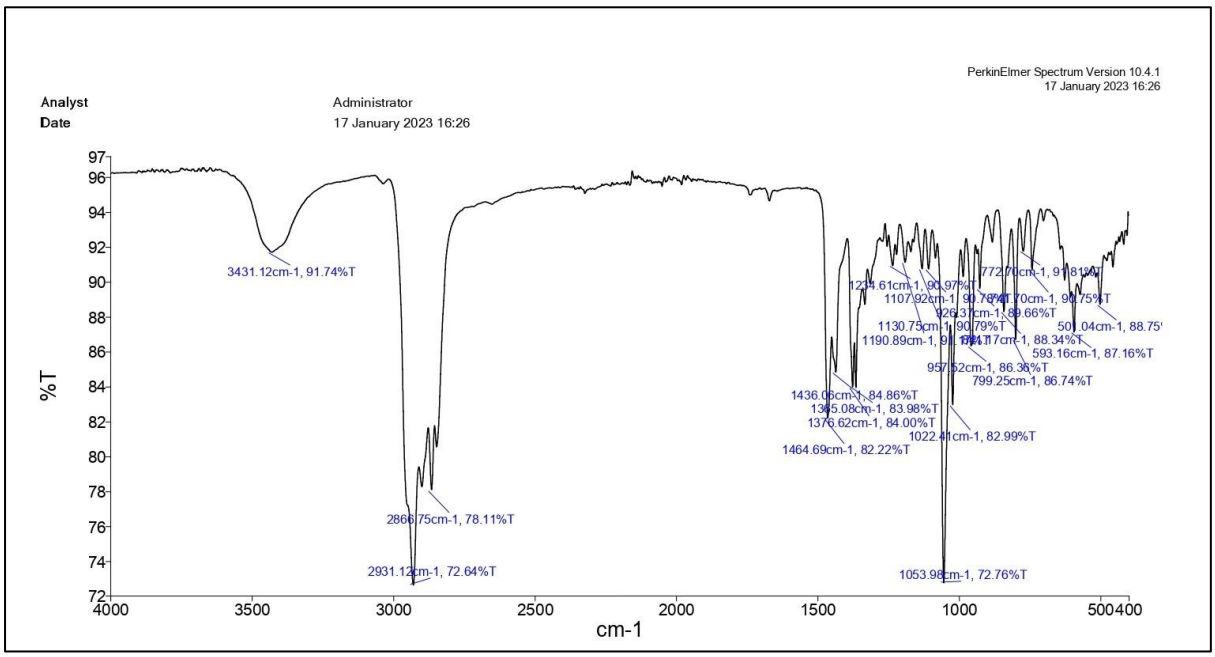


Fig 12: FTIR spectra of cholesterol

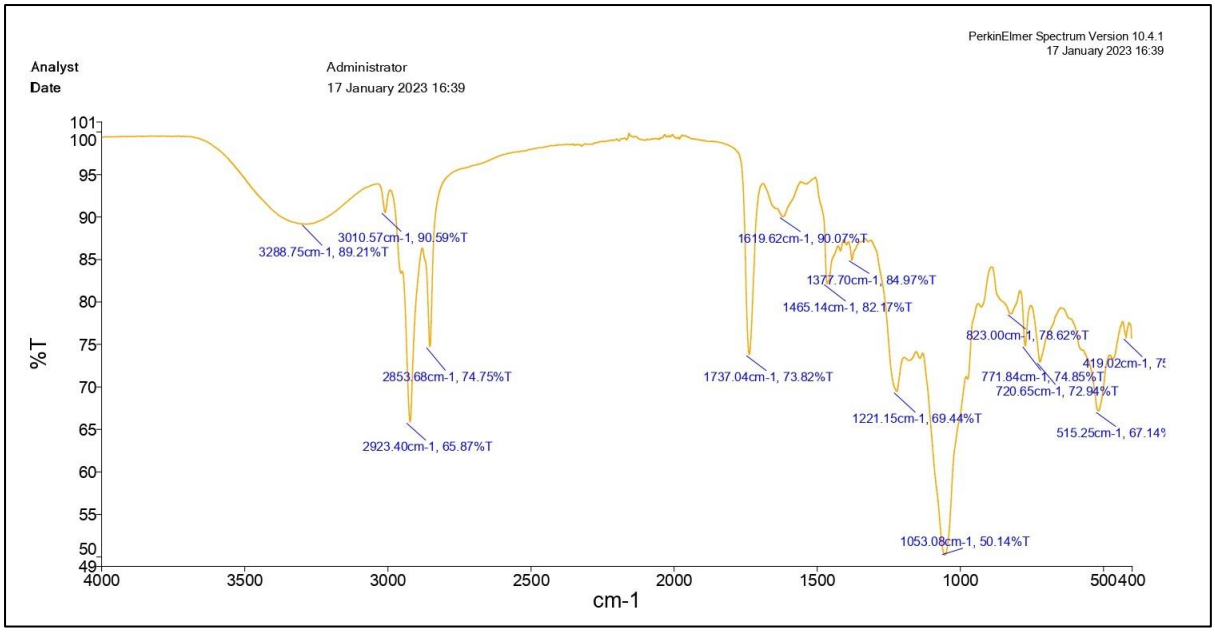


Fig 13: FTIR Spectra of Soya lecithin

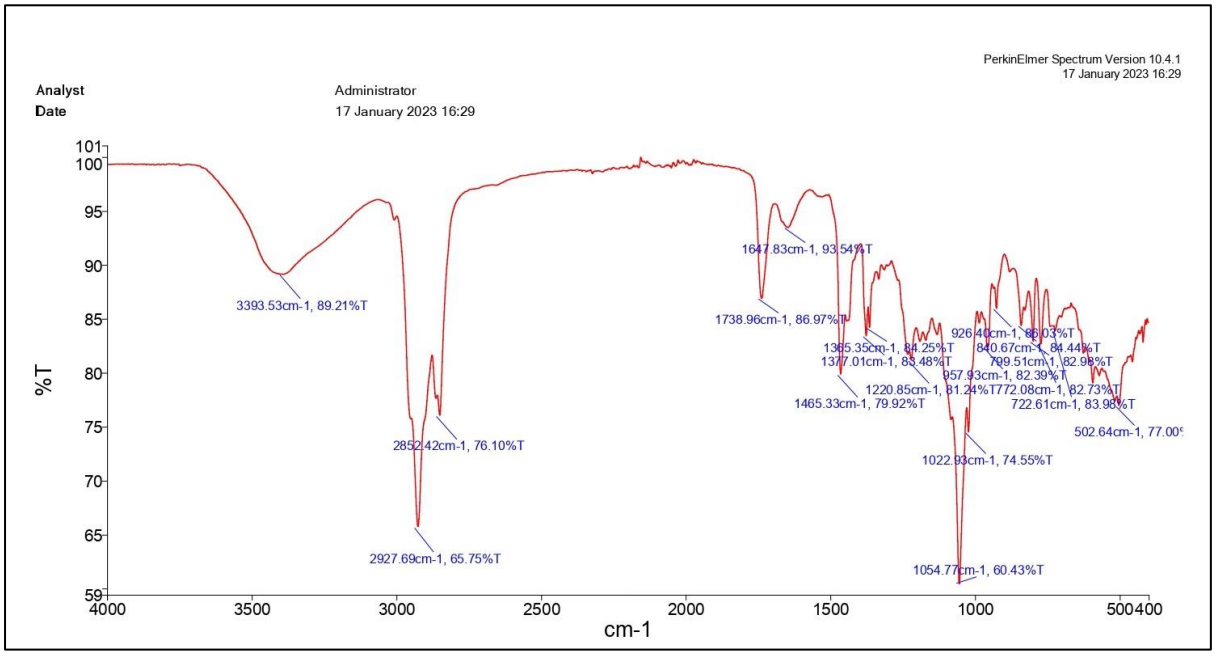


Fig 14: FTIR Spectra of Blank liposome (without drug)

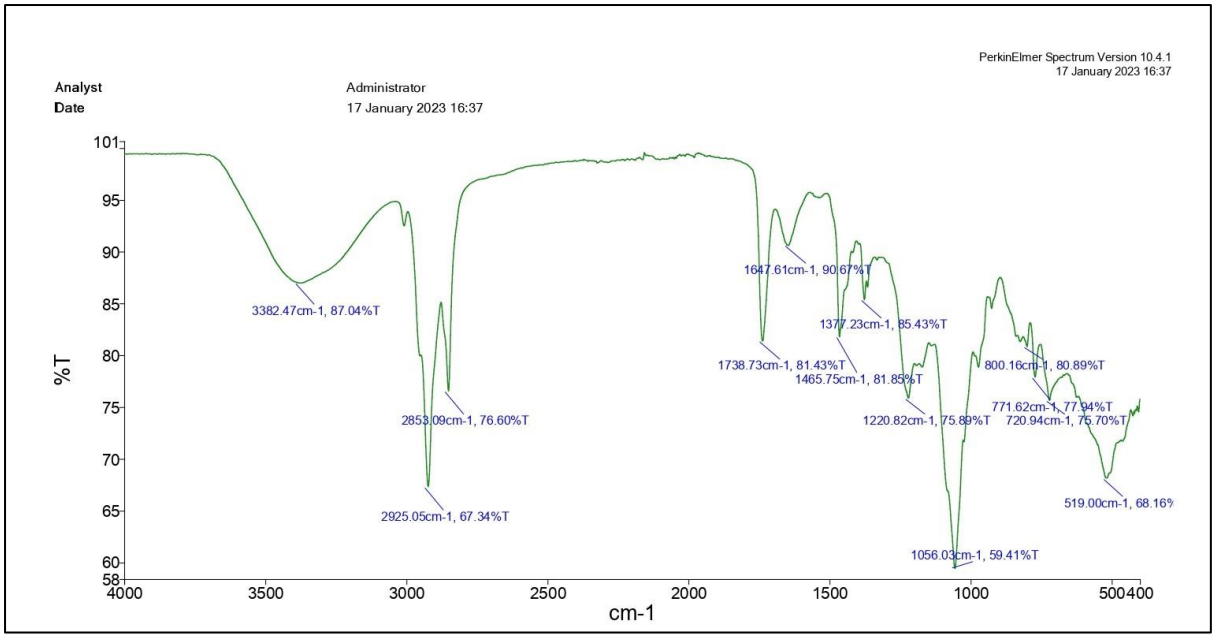


Fig 15: FTIR Spectra of drug loaded liposome

6.3. DRUG LOADING AND ENTRAPMENT EFFICIENCY OF LIPOSOME

The composition of experimental formulation along with respective drug loadings and entrapment efficiencies were given in the following table

Table 8: Composition, practical drug loading (%), entrapment efficiency (%) of the experimental liposome formulation

Formulation	Amount of drug taken (mg)	Amount of CHL taken (mg)	Amount of SLE taken (mg)	Theoretical drug loading (%)	Practical drug loading (%)	Entrapment efficiency
Liposome	10	75	150	4.23	2.52 ± 0.05	59.47 ± 0.11

6.4. SURFACE MORPHOLOGY STUDY OF LYOPHILISED LIPOSOMES BY SCANNING ELECTRON MICROSCOPY:

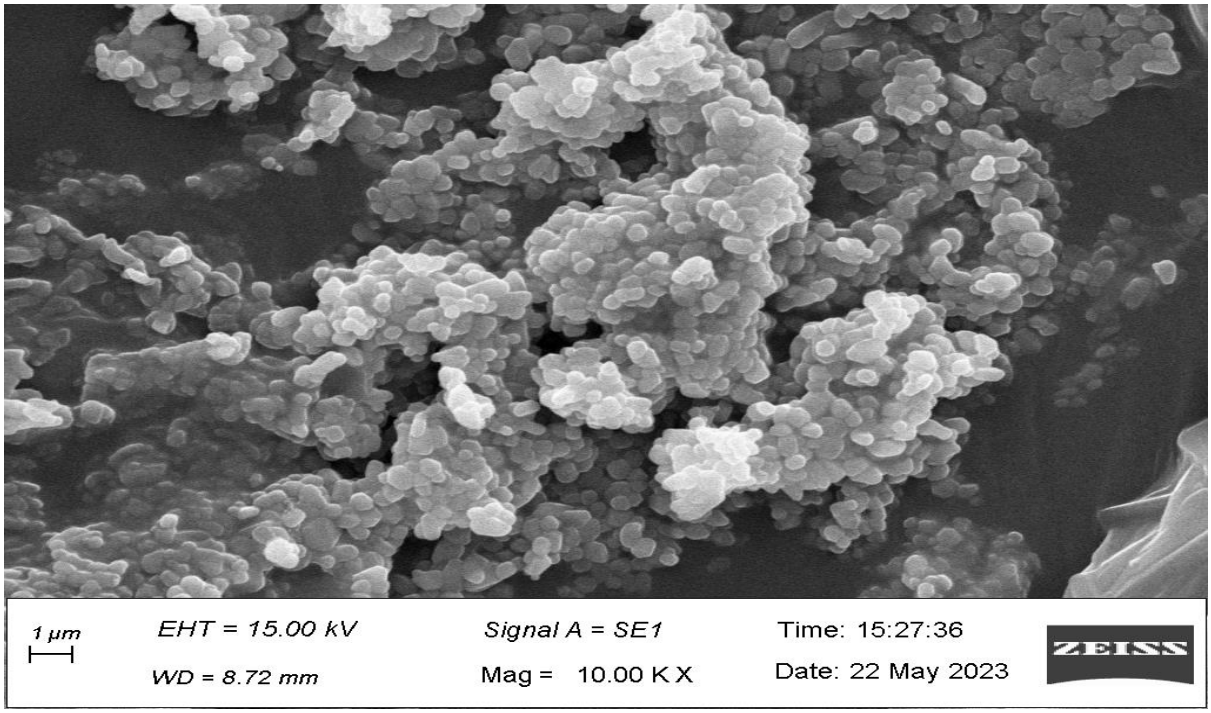


Fig 16: SEM image of Teneligliptin hydrobromide-loaded liposome at 10000x magnification

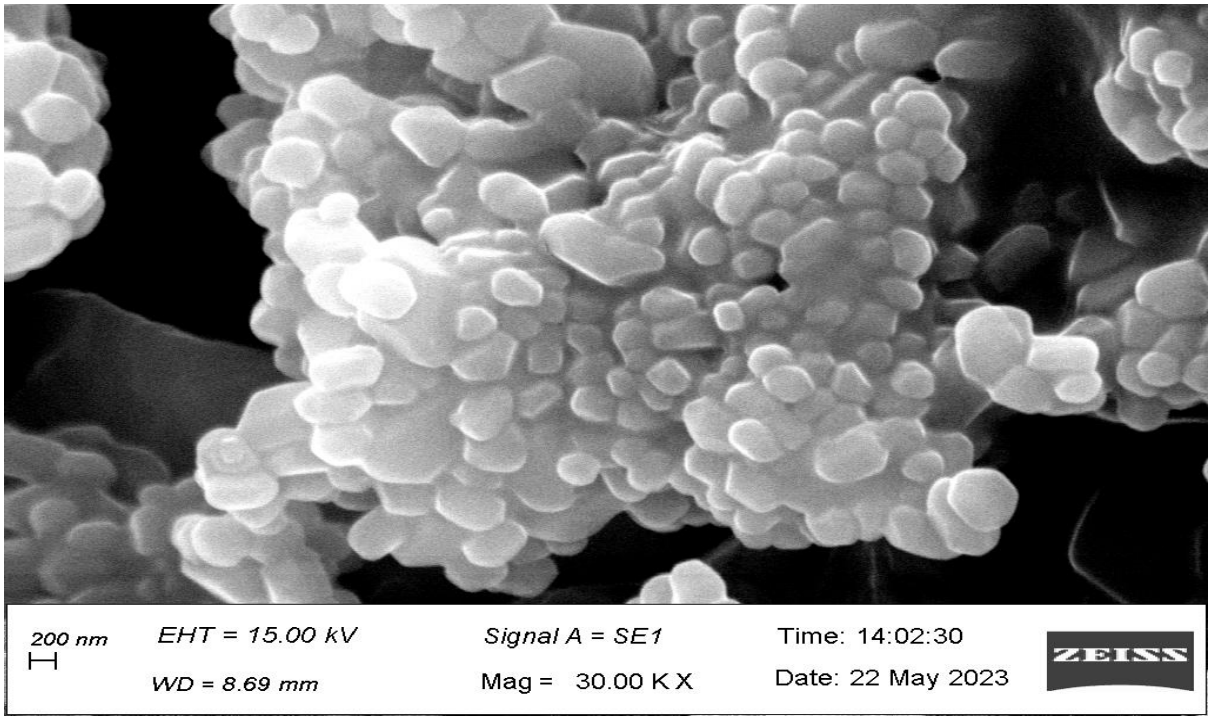


Fig 17: SEM image of Teneligliptin hydrobromide-loaded liposome at 30000x magnification

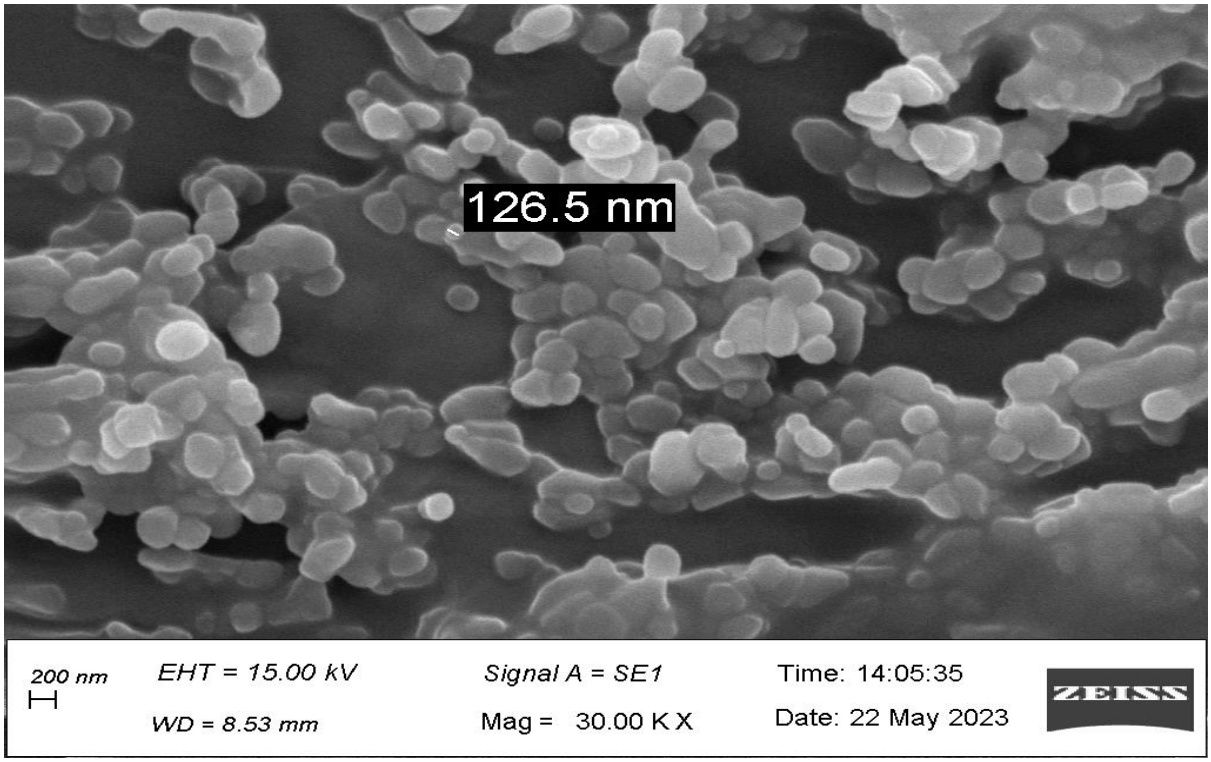


Fig 18: SEM image of Teneligliptin hydrobromide-loaded liposome at 30000x magnification with diameter

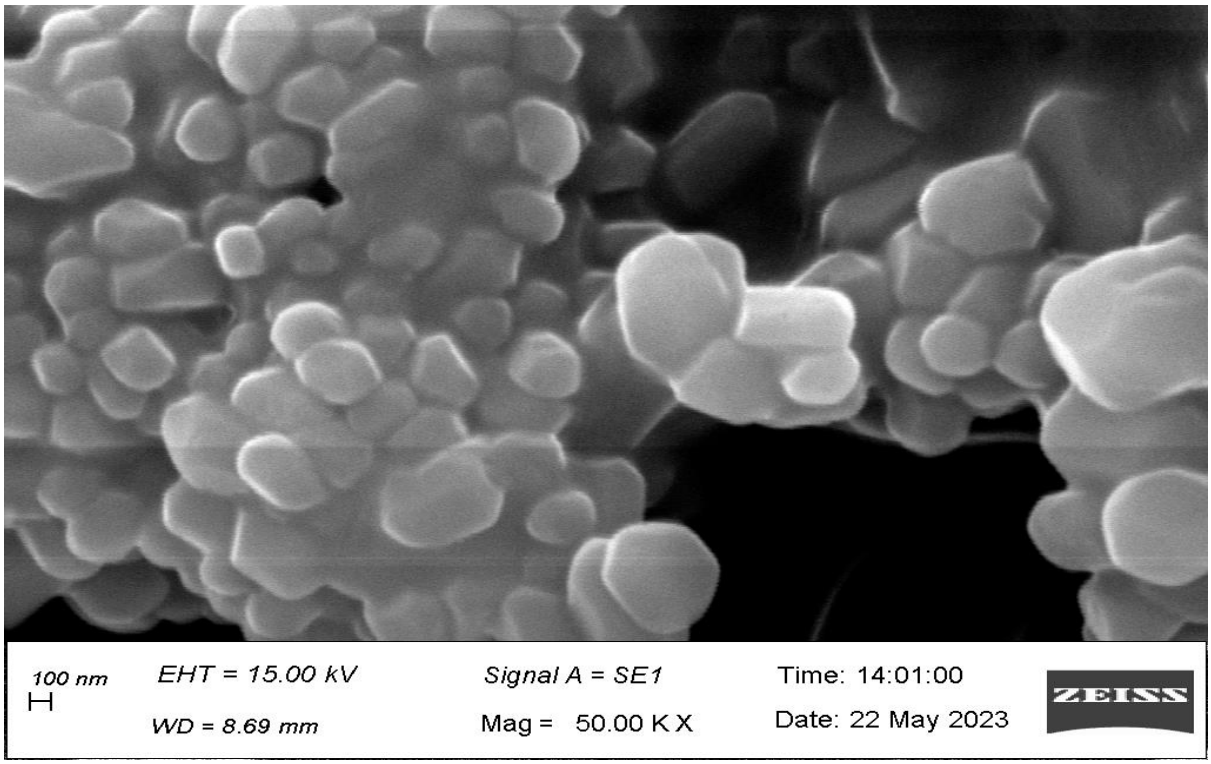


Fig 19: SEM image of Teneligliptin hydrobromide-loaded liposome at 50000x magnification

6.5. PARTICLE SIZE DISTRIBUTION STUDY

- **Particle size distribution study of TC-1**

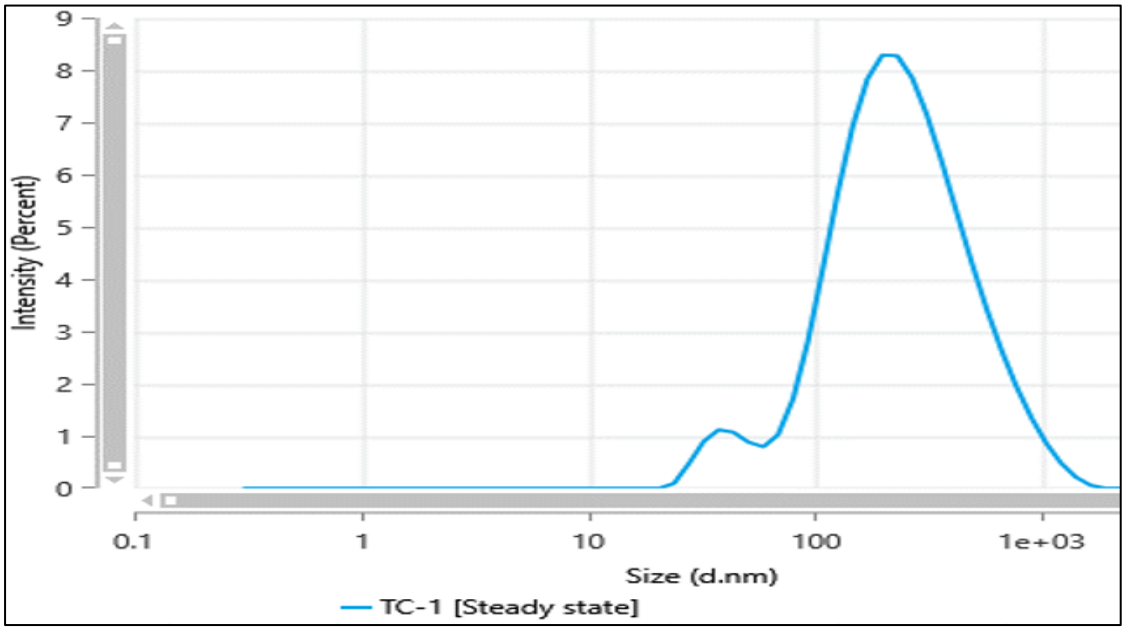


Fig 20: Shows the particle size distribution pattern of lyophilized formulation **TC-1.**

- **Particle size distribution study of TC-2**

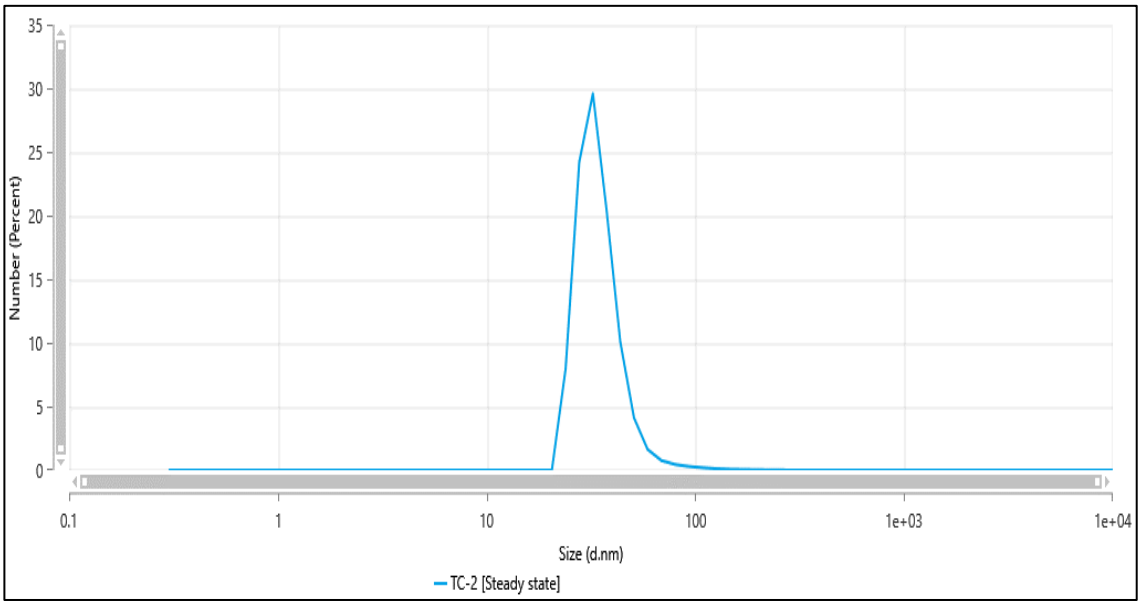


Fig 21: Shows the particle size distribution pattern of lyophilized formulation TC-2

Z- AVERAGE VALUE OF THE EXPERIMENTAL FORMULATIONS

Table 9: shows the Z- average value of different lyophilized formulation

Formulation code	Z- average (d.nm)
TC-1	206.7
TC-2	153.8

POLYDISPERSITY INDEX OF THE EXPERIMENTAL FORMULATIONS

Table10 : shows the polydispersity index of different formulations

Formulation code	Polydispersity index (PI)
TC-1	0.4219
TC-2	0.3581

6.6. ZETA POTENTIAL STUDY OF FORMULATION TC-1

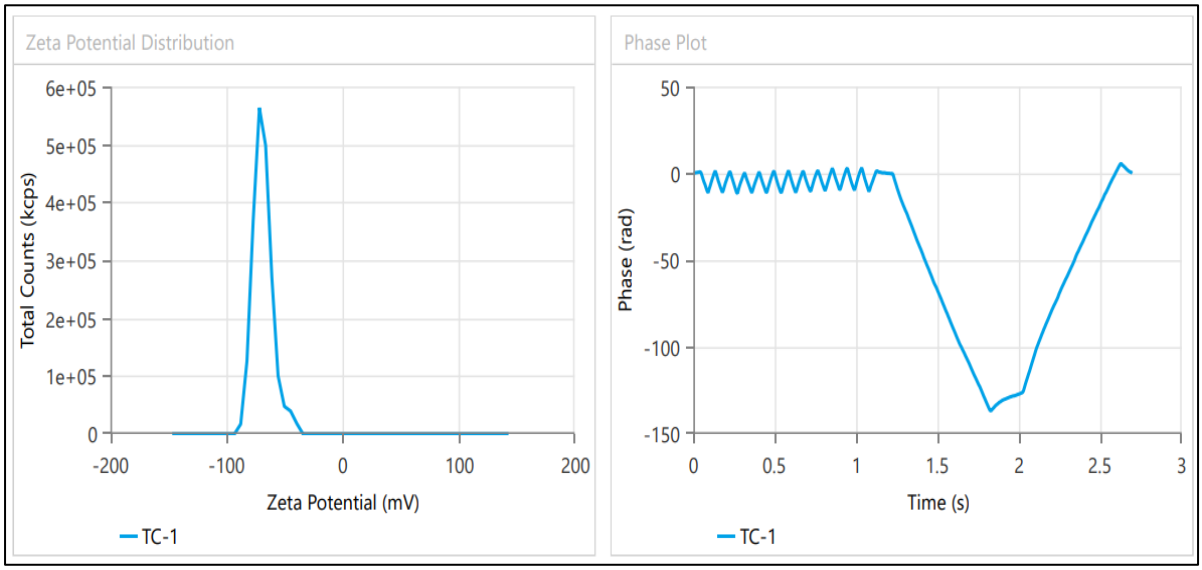


Fig 22: shows the zeta potential of formulation TC-1 and the value was -68.58 mV

ZETA POTENTIAL STUDY OF FORMULATION TC-2

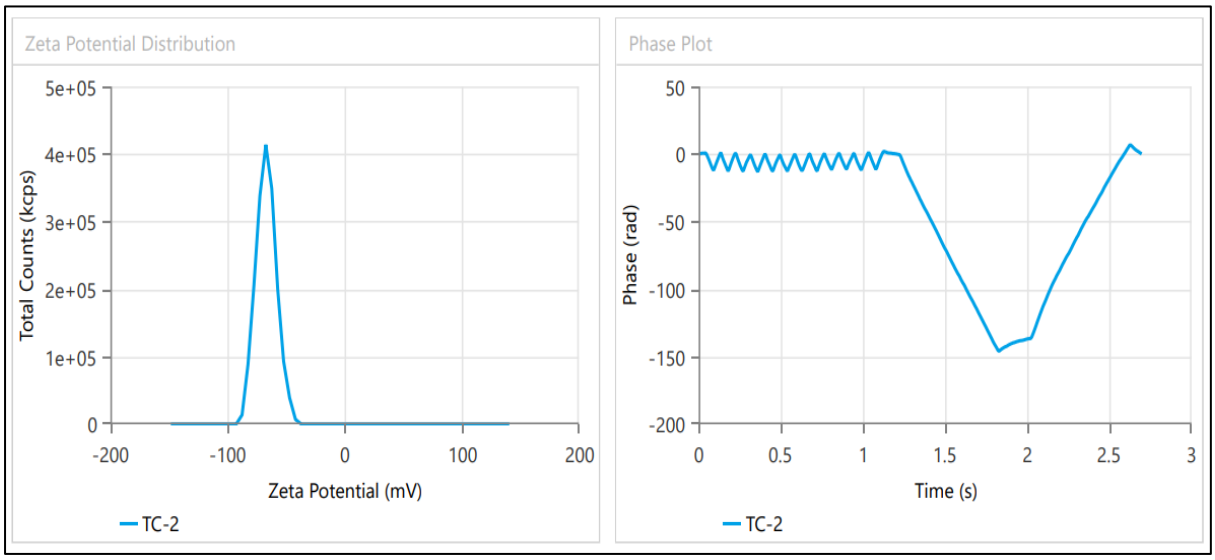


Fig 23: shows the zeta potential of formulation TC-2 and the value was -66.85 mV

6.7. DRUG RELEASE KINETIC STUDY

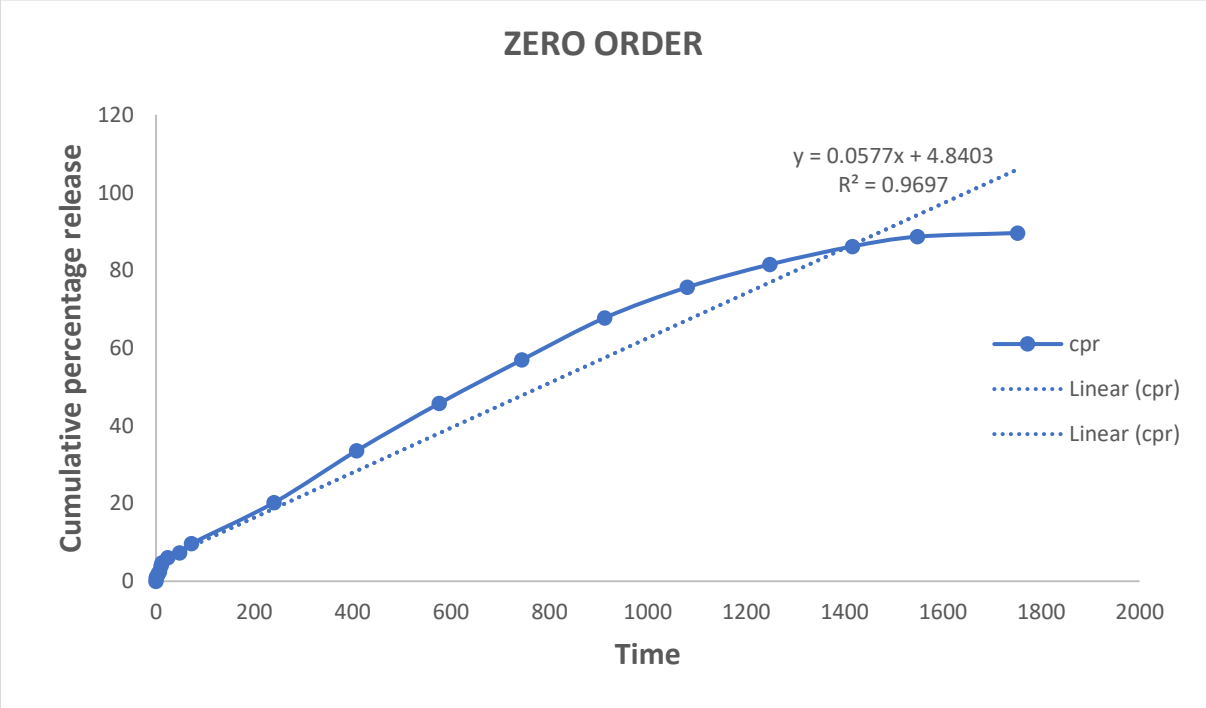


Fig 24: cumulative percentage drug release in PBS pH 7.4

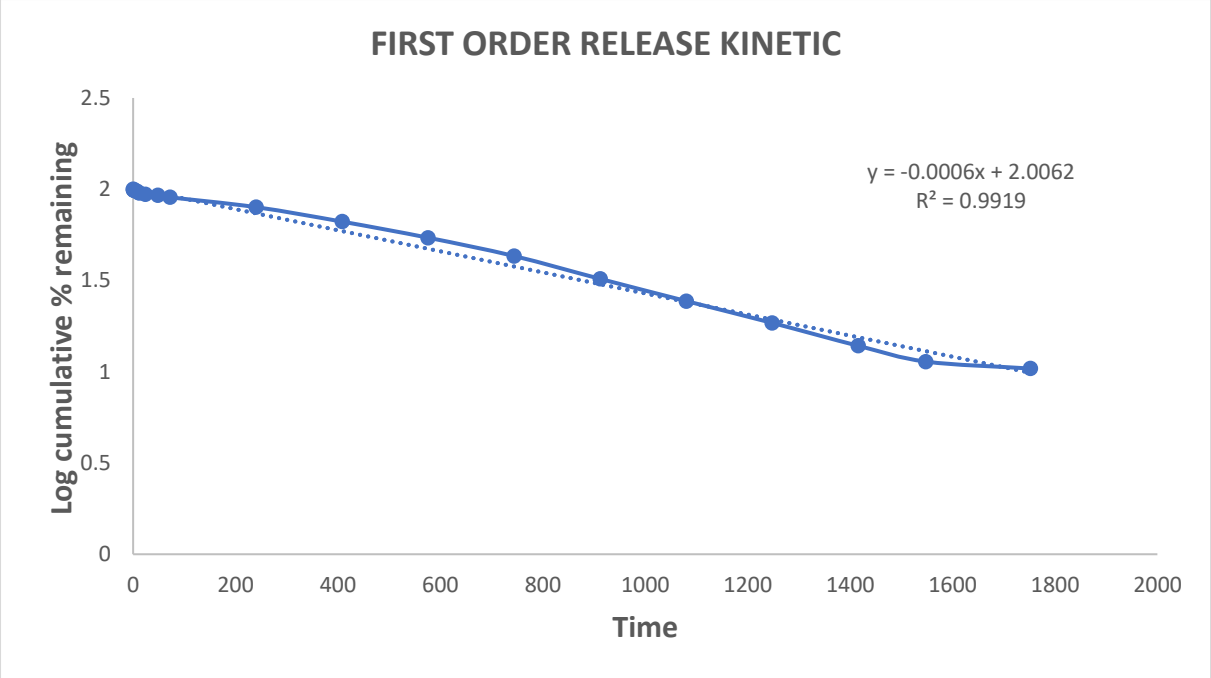


Fig 25: First order drug release kinetic model

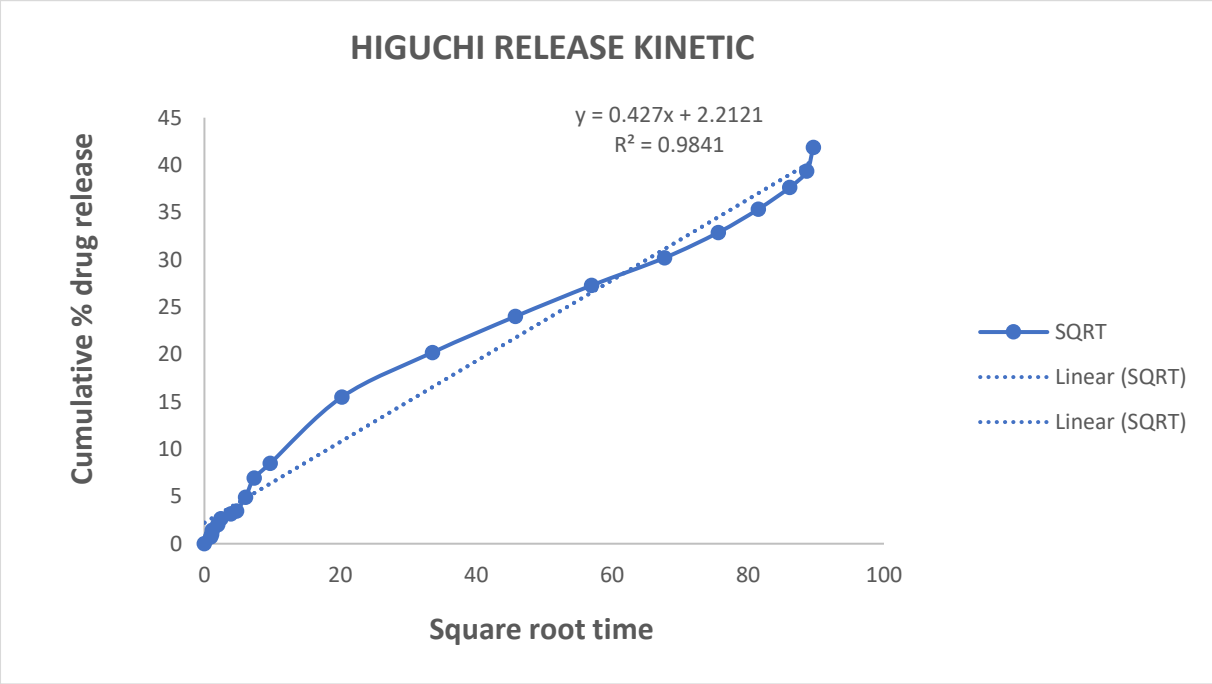


Fig 26: Higuchi drug release kinetic model

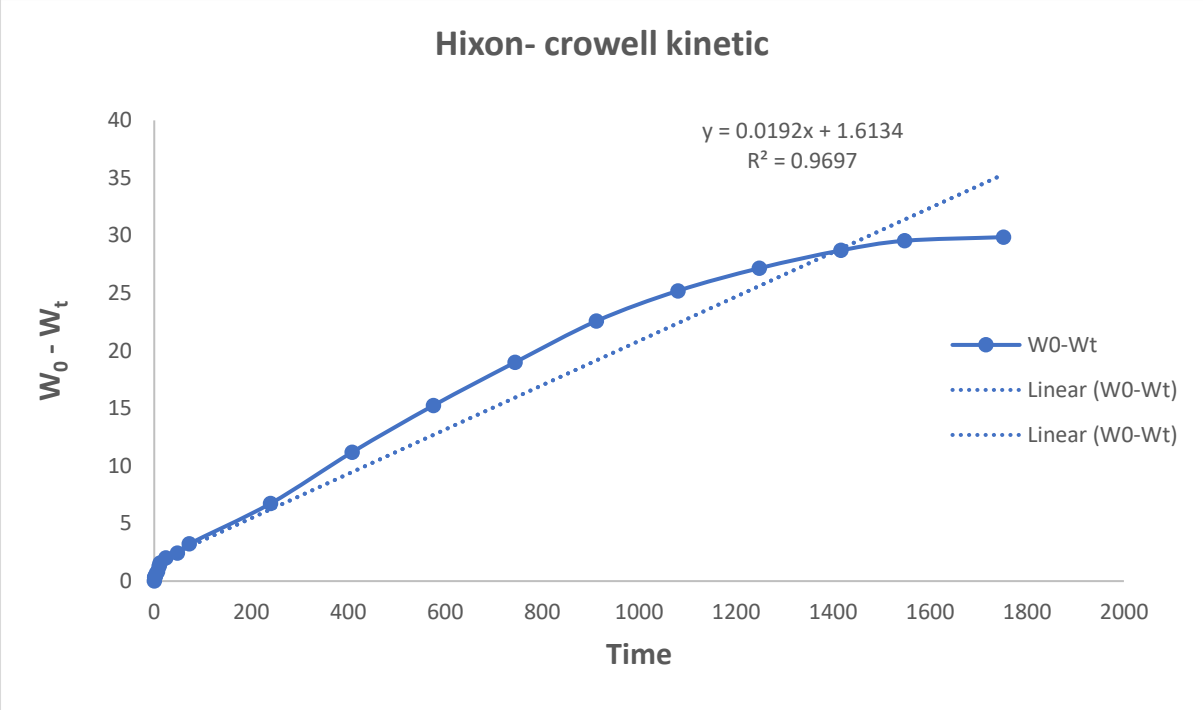


Fig 27: Hixon Crowell drug release kinetic model

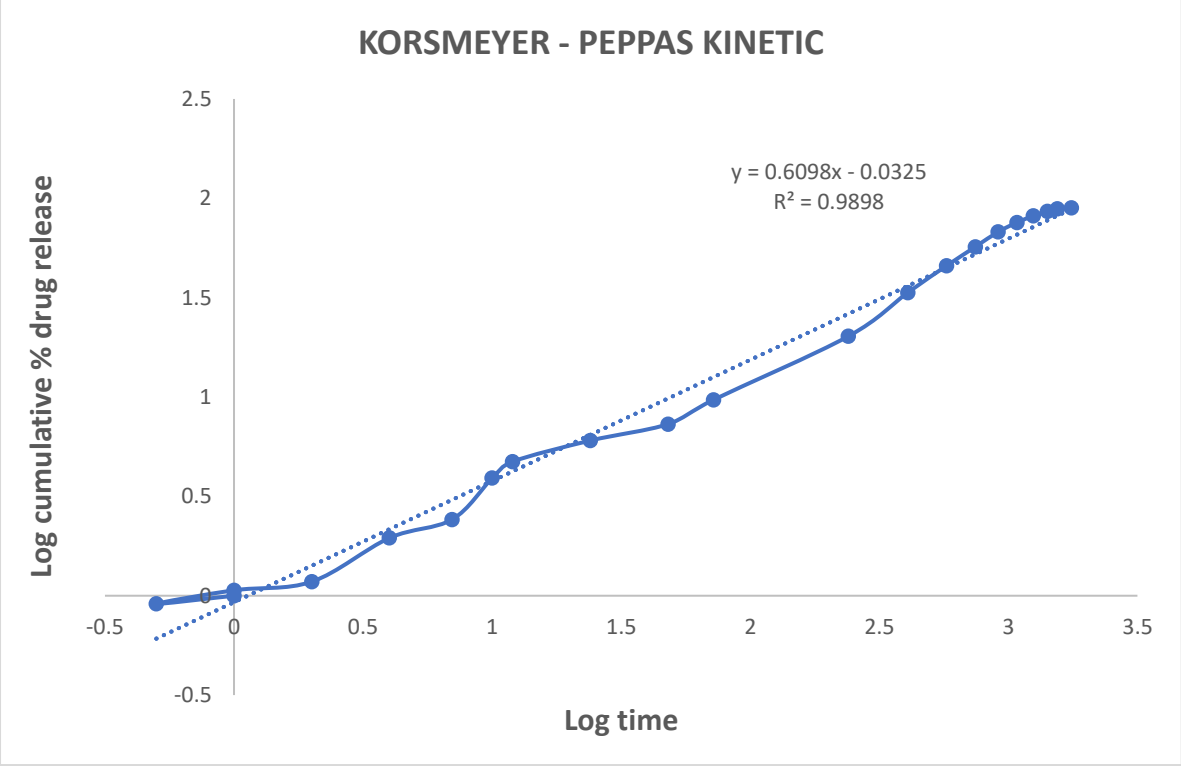


Fig 28: Korsmeyer-Peppas drug release kinetic model

CHAPTER-7

DISCUSSION

DISCUSSIONS

7.1. The UV absorption spectrum of Teneligliptin hydrobromide in different solvent systems

UV scanning of Teneligliptin hydrobromide in PBS pH7.4 & Ethanol yielded lambda max values at 242.5nm and 245nm, which was very close to the reported peak of Teneligliptin hydrobromide at 244.80nm [https://www.ncbi.nlm.nih.gov]. This confirms the authenticity and purity of the product being used and yielded the reference wavelength for spectrophotometric calculation in further studies.

7.2. The calibration curve off Teneligliptin hydrovromide in PBS pH7.4 & Ethanol

Two different calibration curves were prepared – One in PBS 7.4 & one in Ethanol to study in-vitro drug release and liposome entrapment efficiency. Each reading was made in triplicate, and the average values were taken, and standard deviations were measured. The correlation coefficient (R^2) values were 0.999 (PBS pH7.4) and 0.9992 (Ethanol). The values indicate the accuracy of the calibration curves used for further analysis. The low standard deviation in the graphs signified an accurate way of serial dilution to prepare stock and standard solutions.

7.3. Drug – excipients interaction study

Drug-excipient interactions were investigated using FTIR spectroscopy. The drug – excipient interaction is an important pre-formulation study which is a critical factor considered during liposome formulation. The stability of the drug in the formulation, drug release pattern from it and other physicochemical properties such as surface charge, shape, size etc related to the formulation depends on drug – excipients interaction. FTIR spectra assess the drug – excipient interaction at the level of functional groups by determining their vibrational patterns. Here the spectra of drug, the individual excipients (cholesterol and soya lecithin), liposome without drug and liposome with drug have been depicted in the result section.

The principle peaks of Teneligliptin observed at wave numbers 3445 cm^{-1} for N–H stretching of pyrrolidine ring, 3100-3000 cm^{-1} for C-H stretching vibration of aromatic ring, 2573 cm^{-1} for C–H stretching of pyrrolidine ring, 1645 cm^{-1} for C=O stretching vibration in the amide moiety, 1571 cm^{-1} for N –H bending of pyrrolidine ring, 1440 cm^{-1} for C=C stretching of aromatic ring and 691 cm^{-1} for C–S stretching of thiazolidine ring.

The liposome without drug showed the peaks of liposome at wave numbers 2927 cm^{-1} (Lecithin), 1377 cm^{-1} (Cholesterol), 1054 cm^{-1} (Lecithin) & 1025 cm^{-1} (Cholesterol). In case of drug loaded liposome shows no drug peak, indicating complete drug encapsulation within liposomal vesicle. Furthermore minor shifts in some of the peaks in formulation corresponding to cholesterol and soya lecithin could be due to physical interaction between the functional groups of the excipients and drug, most likely due to formation of Van der Waal's force of attraction or dipole – dipole interaction or weak hydrogen bond formation. These physical interaction might help in the formation of formulation.

7.4. Drug loading and entrapment efficiency

The theoretical drug loading was 4.23(%), whereas the practical drug loading was found to be 2.52%. The entrapment efficiency was found to be 59.47%. The drug, Teneligliptin hydrobromide is hydrophilic in nature, and thus it will be entrapped into the aqueous core in liposome.

7.4. Study of morphology by SEM

Size, shape and external morphology of the nanoliposome formulations were assessed by scanning electron microscopy (SEM). From the result it was found that lyophilised nanoliposomes had smooth surface and were in nano size range. The surface of the lipid vesicles had no leakage and the liposomes were uniformly distributed.

7.6. Vesicle size and size distribution

Particle size and size distribution study of the liposome formulation was carried out by dynamic light spectroscopy (DLS). The principle of the particle size determination by DLS is the measurement of rate of fluctuation of the intensity of scattered light due to Brownian motion of particles. Determination of dose intensity fluctuation yields the value of Brownian motion of particle due to thermally induced collision between the particles which are converted into particle-size by using DTS software. From the result it was observed that the particle size of the different formulation was within nano range and there was no predominant variation in the particle size distribution. The least variation in size distribution was observed for TC-1 (PI-0.4219) among the nanosized liposomes. This suggests that TC-1 had the narrowest range of size distribution among the experimental formulations.

7.7. In vitro drug release study

In-vitro drug release study data were collected over 168 hours, and the cumulative release percentage of drug from the formulation was plotted against time to determine the drug release pattern in various media. From the figures, it was seen that almost 89% of drug content were released from the formulation in PBS media. The graph clearly showed the sustained release of the drug from the formulation.

The drug release pattern was also correlated with the established models of release kinetics, namely first order, Higuchi, Hixson-Crowell, and Korsmeyer-Peppas. Considering the R^2 values (0.9919, 0.9841, 0.9697, 0.9898) in all the above-mentioned kinetics models in PBS media, it has been found that the drug release pattern from the formulation followed first-order kinetics model in PBS media, indicating that release of drug from the formulation decreases as the concentration of drug decreases in the formulation.

CHAPTER-8

CONCLUSION

CONCLUSION

In the present work, Teneligliptin hydrobromide loaded nanoliposomes were developed and their physico-chemical characterization was carried out invitro. From the result of liposomal size analysis, it was clear that all nanoliposome formulations were in nano size range with narrow size distribution range. Surface morphology study showed that the surface of the nanoliposome was smooth and liposomes were spherical in shape. The nanoliposomes are uniformly distributed. Higher value of zeta potential indicated the higher stability of the formulation. The optimized formulation showed a substantial amount of drug loading and a sustained release profile, as shown in the release curve, which will reduce the drug's side effects, decrease the dosing amount, and increase the dosing interval. However, animal studies of the formulation need to be done to check the appropriate therapeutic activity.

After fulfilling the in-vivo investigation, if we get a desirable result, this will open a new era of treatment over the existing medications for the diabetes.

CHAPTER- 9

REFERENCE

REFERENCE

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