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Metoclopramide Nanostructured Lipid Carrier Loaded In-Situ Gel via Nasal Route: Preparation, Characterization, and In vitro Evaluation

MASTER THESIS

MERYEM AYDİN

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This thesis has been deemed by the jury in accordance with the relevant articles of Yeditepe University Graduate Education and Examinations Regulation and has been approved by Administrative Board of Institute with decision dated ..... and numbered .....

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DECLARATION

I proclaim that this work constitutes my own work and that, it contains no material previously published or written by another, and that it has not previously been relied upon for the award of any other degree, diploma or other qualification except as appropriately acknowledged in the text.

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MERYEM AYDIN

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LIST OF SYMBOLS AND ABBREVIATIONS

API-LIP	Active Pharmaceutical Ingredient Loaded Liposome
Ca^{2+}	Calcium
CaCl2	Calcium Chloride
GG	Gellan Gum
EE	Encapsulation Efficiency
K^{+}	Potassium
KCL	Potassium Chloride
LISG	Liposomal In-situ Gel
LL	Loaded Liposome
LET	Liposomal Encapsulation Technology
MTC	Metoclopramide
Na^{+}	Sodium
NaCl	Sodium Chloride
NS	Nano System
SEM	Scanning Electron Microscope
SNF	Simulated Nasal Fluid
Sol-gel	Solution to gel
SPC	Soybean Phosphatidylcholine
UL	Unloaded Liposome

ABSTRACT

Aydin, M. (2022). Metoclopramide Nanostructured Lipid Carrier Loaded In-Situ Gel via Nasal Route: Preparation, Characterization, and In vitro Evaluation. Yeditepe University, Institute of Health Science, Department of Drug and Cosmetic Production Technology, MSc thesis, Istanbul.

This study is done to overcome the limitations of MTC occurred when taken by different routes of administration in order to improve its bioavailability and to increase patient compliance, also in an attempt to prolong their nasal cavity residence time when it is taken intranasally, and for the achievement of a prolong drug permanence into the site of administration. An innovator composite formulates, by associating the liposome (soybean and cholesterol) and in situ gel (gellan gum) delivery systems, has been created. Thin film hydration method, also called bangham method was used to prepare MTC loaded liposomes. The optimal liposomal formulation was determined using D-optimal mixture experimental design. All of the prepared formulations, there values of polydispersity index were lower than 0.6, which indicates monomodal size distributions. Also, the particle size of the MTC loaded liposomes were small, the formulation's mean particle was smaller than 150 nm. The encapsulation efficiency of MTC in liposomal formulation was between 71% and 94%. Furthermore, in this work, zeta potential values were negative for all our API-Lip formulations. In the present study, MTC loaded liposome were prepared and incorporated with gellan gum solution. Furthermore, MTC loaded in situ gel preparation and characterization studies were performed, in addition to liposomal morphology, determination of mechanical properties, rheological behavior, ex vivo and in vitro release study. Based on observed data the MTC loaded liposome formulation incorporated with gellan gum was showing significant results. Cumulative drug release from the MTC loaded liposome incorporated with gellan gum formulations after 5 h was found to be more than 80%. This study might point to the potential of an intranasal innovative composite formulation taken together, liposomes included the nasal in situ gel in terms ease of administration, drug dosing accuracy, good capability for reduction of the nasal mucociliary transport time with prolongation of residence time at the site of absorption, the

avoidance of antiemetic drug’s bitter taste and improved drug bioavailability. Therefore, it could be taken into consideration in pharmaceutical industries in the future.

Keywords: In-situ gel, Liposomes, MTC loded liposomes, Mucoadhesion, Residence time.

ÖZET

Aydın, M. (2022). Nazal Yol Yoluyla In Situ Jel Yüklenen Metoklopramid Nanoyapılı Lipid Taşıyıcı: Hazırlama, Karakterizasyon ve In vitro Değerlendirme. Yeditepe Üniversitesi, Sağlık Bilimleri Enstitüsü, İlaç ve Kozmetik Üretim Teknolojisi Anabilim Dalı, Yüksek Lisans tezi, İstanbul.

Bu çalışma, MTC'nin biyoyararlanımını iyileştirmek ve hasta uyumunu artırmak için farklı uygulama yollarıyla alındığında ortaya çıkan sınırlamaların üstesinden gelmek, ayrıca intranazal olarak alındığında burun boşluğunda kalma sürelerini uzatmak ve uygulama bölgesine uzun süreli bir ilaç kalıcılığının sağlanması amacıyla yapılmıştır. Lipozom (soya fasulyesi ve kolesterol) ve in situ jel (gellan zamkı) taşıma sistemlerini ilişkilendirerek yenilikçi bir kompozit formül oluşturulmuştur. MTC yüklü lipozomları hazırlamak için bangham yöntemi olarak da adlandırılan ince film hidrasyon yöntemi kullanıldı. Optimal lipozomal formülasyon, D-optimal karışım deney tasarımı kullanılarak belirlendi. Hazırlanan tüm formülasyonlarda, polidispersite indeksi değerlerinin 0.6'dan düşük olması monomodal büyüklük dağılımlarını gösterir. Ayrıca MTC yüklü lipozomların partikül boyutu küçüktü, formülasyonun ortalama partikülü 150 nm'den küçüktü. MTC'nin lipozomal formülasyonda kapsülleme etkinliği %71 ile %94 arasındaydı. Ayrıca, bu çalışmada, tüm MTC yüklü lipozom formülasyonlarımız için zeta potansiyel değerleri negatifti. Bu çalışmada, MTC yüklü lipozom hazırlandı ve jellan zamkı çözeltisi ile birleştirildi. Ayrıca lipozomal morfoloji, mekanik özelliklerin belirlenmesi, reolojik davranış, ex vivo ve in vitro salım çalışmasına ek olarak MTC yüklü in situ jel hazırlama ve karakterizasyon çalışmaları yapılmıştır. Gözlenen verilere dayanarak, jellan zamkı ile birleştirilen MTC yüklü lipozom formülasyonu, önemli sonuçlar gösteriyordu. 5 saat sonra jellan zamkı formülasyonları ile birleştirilen MTC yüklü lipozomdan kümülatif ilaç salımı, %80'den fazla bulundu. Bu çalışma, birlikte alındığında intranazal yenilikçi bir kompozit formülasyonun potansiyeline işaret edebilir, lipozomlar, uygulama kolaylığı, ilaç dozlama doğruluğu, nazal mukosiliyer taşıma ve emilim bölgesi, antiemetik ilacın acı tadından kaçınma ve gelişmiş ilaç biyoyararlanımı süresini azaltmak için iyi kapasite ve vücutta kalma süresinin uzaması açısından nazal in situ jeli içermiştir. Bu nedenle, gelecekte ilaç endüstrilerinde dikkate alınabilir.

Anahtar Kelimeler: İn situ jel, Lipozomlar, MTC yerleşimli lipozomlar, Mukoadezyon, Kalış süresi

1.INTRODUCTION

Over the past few decades, nanosized based drug delivering systems have been tremendously examined as a classic approach to bypass the low bioavailability of numerous pharmaceutical drugs. The delivery methods for nano particulates that use dendrimers, polymeric nanoparticles, solid lipid nanoparticles, micelles, noisome, and liposomes have been around for a while (1). Since they may be absorbed by cells more readily than bigger molecules, nanocarriers with improved biological and physicochemical qualities can be effectively utilized as delivery equipment for a variety of biologically active chemicals (2). Additionally, they increase the solubility of the medications, increase their bioavailability, lengthen the time of action, and enable targeted drug administration (3). Vesicular carriers offered additional advantages, like protection of active compounds from chemical and enzymatic degradation and the potential to lengthen the duration that medications are distributed in the bloodstream (4). Liposomes have received the most research attention among these carriers. Their allure is due to their constitution, which makes them biocompatible and biodegradable.

The aqueous core of a liposome is trapped by one or more lipid bilayers made of synthetic or natural lipids. Also, they contain naturally occurring phospholipids with minimal intrinsic toxicity and weak immunogenicity and biological inertness (5). Additionally, different lipophilic medications can be encapsulated in liposomes: extremely hydrophilic pharmaceuticals are wholly contained in the aqueous compartment, whereas strong lipophilic drugs are completely confined in the lipid bilayer (6). Through the choice of the bilayer's elements, the charge, fluidity, and rigidity of the bilayers are determined. Unsaturated long acyl chain phospholipids, such as di-palmitoyl-phosphatidyl-choline, generate an impermeable and rigid bilayer structure, while saturated short acyl chain phospholipids, such as soybean and egg, provide higher permeability and less bilayer stability (7,8). It has been proven that when hydrated in aqueous solutions, phospholipids spontaneously form closed structures. Depending on the type of medication, such vesicles with one or more phospholipid bilayer membranes can transport either aqueous or lipid medications. The lipid composition of the liposome is amphipathic which means it is both

hydrophilic and hydrophobic in aqueous media, so their self-assembling characteristics and thermodynamic phase properties, encourage the spherical bilayering of their hydrophobic components in an entropically focused manner. Lamellae are the name given to these layers (9). Spherical vesicles with particle sizes ranging from 30 nanometer to different micrometers are how liposomes are frequently described. The polar head groups of the lipid bilayers that surround the aqueous units that make up liposomes are orientated in the direction of the exterior and interior aqueous phases. The use of liposomes as a drug delivery system has increased both as an investigational and commercial system due to their decreased toxicity, biodegradability, biocompatibility, propensity to trap both hydrophilic and hydrophobic drugs, and ability to facilitate specific site drug delivery (10,11). To reduce drug toxicity and target specific areas, numerous studies have been conducted on liposomes (12,13).

Medical researchers use liposomal encapsulation technology, the modern delivering method, to deliver medications that act as curative promoters to specific bodily parts. The supply of necessary mixtures to the entire body was the goal of this kind of delivery system idea. LET is a process used to create liposomes, which are incredibly small foams that can contain a variety of compounds. These liposomes create a barrier around their contents that is impervious to the body's natural defense mechanisms against free radicals, stomach and mouth enzymes, bile salts, alkaline solutions, digestive juices, and intestinal flora (14).

The liposomes' contents are shielded from oxidation and decomposition. Until the liposome's contents are delivered to the precise target site of action, such as the gland, organ, or system where they will be used, this secure barrier, also known as the phospholipid shield, remains intact (15).

The favor of loading drug in liposomes, that can be applied in different dosage forms (ex. In-situ gels) are highlighted in Table 1.1:

Table 1.1: Benefits of loading drugs in liposomes

1. Solubility improvement of amphiphilic and lipophilic drugs
2. Passively attacking immune system cells, in particular mononuclear phagocytic system cells
3. Supplying a sustained release method for liposomes that are delivered locally or systemically.
4. Site prevention system
5. Site-specific targeting
6. Improving the transport of hydrophilic and charged molecules
7. Improved penetration into tissues

The intranasal drug delivery system has received too much attention in recent years since it is thought to be a potential drug delivery method for both systemic and local therapy (16). For the delivery of a wide variety of medications, the nasal cavity has reportedly become an appealing route with many targeting sites (17). Owing to the physiology and anatomy of the nasal passage, as an example it has large surface areas, porous endothelial membrane, highly vascularized epithelium, and the first pass metabolism avoidance, intranasal routes of administrations offer faster onset of pharmacological activity, minimal doses, lesser adverse effects, more rapid acquisition of therapeutic blood levels, and high amount of blood flow per cm^2 (18). Assessment of the benefits and drawbacks of intranasal drug delivery summarized in Table 1.2.

Table 1.2. The Benefits and Drawbacks of Intranasal Drug Delivery (19,20)

Benefits	Drawbacks
Preventing drug degradation brought on by first pass metabolism in the liver	The permeability of medications affected by conventional defense mechanisms such as ciliary beating and muco-ciliary clearance
Quick effect after rapid absorption	Limiting the amount of medication that can be administered into the nasal cavity to 25 – 200 µl
High bioavailability leads to the usage of lower drug doses	Adversely affected by pathological conditions
Easily accessible, non-invasive route	Nasal mucosal irritation

2.GENERAL INFORMATION

Furthermore, by relying on the nasal route's simple accessibility and ability to support self-medication, this method of administration has improved patient compliance. A thorough understanding of the nasal cavity is crucial before developing a nasal medicine delivery device. Despite its activity, the nose's primary function is olfaction since it humidifies, warms, and filters airborne particles (21). Therefore, the nose functions as a defense mechanism against external objects (22). The nasal cavity contains the olfactory, vestibular, and respiratory domains, which are three distinct functioning zones. Drug metabolism is possible in the olfactory epithelium. The vestibular area serves as a baffle system and filters airborne particles as a result. The location where medication absorption is best is in the respiratory mucosa (23). Figure 2.1 depicts the superior, middle, and inferior nasal turbinates, which are formed by the respiratory zone and project from the side walls of each nasal cavity half (24). Both serous cells and mucus cells, which produce a watery fluid and mucus gels, are present in the submucosal glands (25). The transcellular route, which means over the cells, and the paracellular route, which is known as between the cells, are the two primary routes by which medications are transported from the human nasal cavity throughout the nasal mucosa. Based on our formulation, lipophilic drugs follow trans-cellular route and indicate fast and effectual absorption comparable to oral or intravenous routes when they are given intranasally (26).

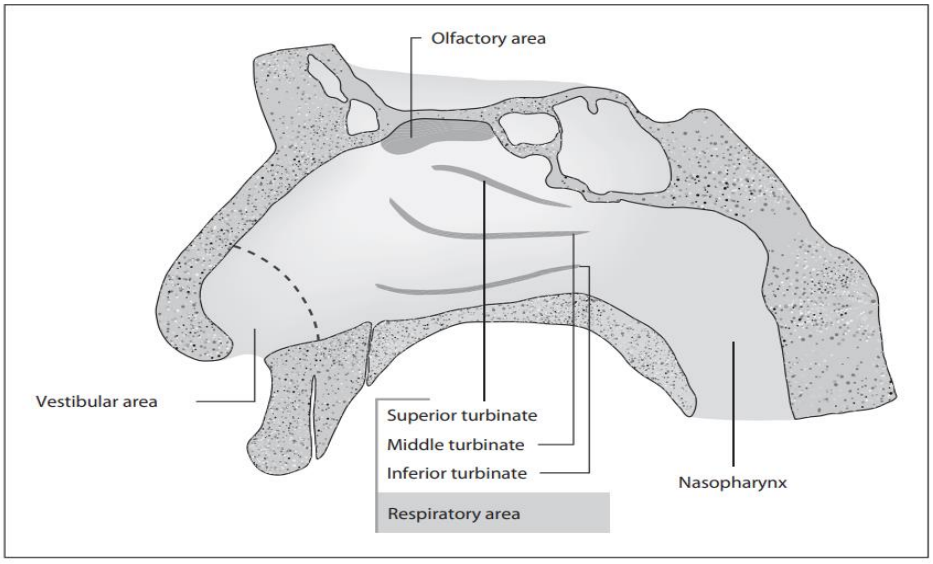


Figure 2.1. Nasal cavity's sagittal section (27)

The mucociliary clearance illustrates the physiological factor that is principally accountable for the reduction of residence time of the drug in the nasal medium, despite the fact that intranasal route of administration exhibits a variety of benefits according to its tolerability, efficacy, accessibility, and patient compliance. The term mucociliary clearance refers to the process of moving mucus and particulates that have been absorbed into the gastro-intestinal system. The cause of interaction between the moving mucus layer and the cilia beneath it, which move in a manner like a metachronal wave (27). Accordingly, the mucus layer is self-renewable every 15 to 20 minutes. This self-clearing process is in charge of the fast elimination of the medication from the nasal medium, which reduces the amount of time it takes for the medication to cure localized nasal ailments or enter the systemic bloodstream (28).

Coupling the drug to a carrier particle such as microspheres, liposomes, nanoparticles is done by an intelligent approach called carrier technology, by which it modulates the absorption and release characteristics of the drug. A novel way for a range of organic systems such as liposomes has been designed in order to efficiently deliver drugs to specific organs. During the few past decades, a substantial advance in medication delivery mechanisms have permitted efficient drug administration. Various drug targeting and drug delivery systems are lately under research and development in an effort to reduce

drug loss and degradation, to improve the drug's bioavailability and the fraction of the drug accumulated in the targeted zone, and for prevention of harmful side-effects (29).

Intranasal drug absorption is increased in addition to drug carriers by using mucoadhesive polymers to extend the dosage form's contact or residence time within the nasal mucosa. The ability of a polymer to adhere to a biological tissue for an extended period of time is what defines mucoadhesion (27,28). In an effort to prolong the time that drugs spend in the nasal cavity area after being administered as simple water solutions, a viscosity-enhancing approach has been developed: In recent times, it has seemed that intranasal in situ gelling formulations are a more effective substitute for nasal liquid formulations. When they come into touch with the nasal mucosa, the low viscosity fluid that is injected into the nasal cavity alters its structure to create a gel. This allows for a steady, continuous release of the medicine while also lengthening the contact period between the drug and the site of absorption in the nasal cavity. As a result, it is particularly helpful for medications that are regularly taken.

The term "mucoadhesive" refers to the method through which mucin interacts with a synthetic or natural polymer to indicate the drug delivery system's connection to the mucosal membrane.

The consecutive process that occurs in mucoadhesion contains:

- Primarily, the mucoadhesive system starts by water absorbance from mucus layer and followed by wetting and expansion.
- Secondarily, because the gradient of the medication's concentration across the epithelium will be improved as a result of the polymer's deep penetration of the mucous membrane and localization of the drug formulation in the nasal cavity.

During recent decades, In the scientific community, the creation of in-situ medication delivery systems has received far too much attention. The majority of these systems exhibit the oddity of being in a solution form prior to administration and then going through gelation once it reaches the body. For this reason, in situ gelling formulations have a longer residence duration, are simpler to administer, and provide sustainable release of drugs at the site of administration. Additionally, their administration frequency is reduced, and

patient comfort and compliance are improved (30). The fact that this system can be supplied through several routes in order to induce systemic or local action of the loaded drug is one of the reasons for the enormous success of these formulations (31-35). Additionally, they can be usefully employed as platforms for nano and micro drug delivery systems. Since it is liquid before nasal administration so it may easily be imparted gradually as a drop, an intranasal mucoadhesive in situ gel technology appears to be very desirable and allows correct medication dosing. Different conditions, such as temperature rises, pH shifts, and the presence of ions, can cause the solution to gel transition. Alginate, pectin, and gellan gum are examples of some of these well-known anionic polysaccharides that are ion sensitive polymers (36), which is a natural polysaccharide obtained from a bacteria Sphingomonas elodea, known as Pseudomonas elodea.

The molar ratios of b-D-glucose, b-D-glucuronic acid, and a-L-rhamnose residues make up the chemical structure below, which is made up of repeated units of a tetra-saccharide. Because it can form a strong, clear gel at a physiological ion concentration, it can provide an extended contact duration for the transfer of the medicine across the nasal membrane before the formulation is eliminated by the muco-ciliary clearance process. These qualities have sparked a lot of passion in gellan gum as a gelling agent, combined with the polymer's lack of toxicity, biodegradability, and biocompatibility (37).

Gellan gum

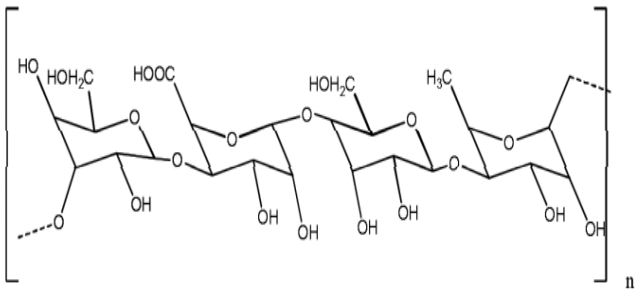


Figure 2.2. Gellan Gum Structure (38).

Gellan gum are cross-linked by monovalent such as sodium and divalent such as magnesium and calcium cations present in distinct physiological fluids including nasal fluids. The cross-linking mechanism produces a transition from solution to gel form, with

the development of powerful gel. Both the concentration and the type of cation are related to the viscosity of the cross-linked polymer and the rate of solution to gel transition (39). The formulation is cleared by the mucociliary clearance mechanism, the transition of a solution to gel and the creation of a strong gel at a physiological ion concentration can result in a longer contact time for drug transportation across the nasal membrane. Gellan gum has gained recognition due to the change of sol-gel at low ionic concentration, it is a prospective gelling agent for nasal drug administration. This is because the presence of calcium, sodium, and potassium ions in gellan gum causes great in situ gelation in the nasal cavity.

Since in situ gelling drug delivery methods have mucoadhesive qualities, which means they can interact with mucus, it is desirable to utilize them when applying formulations to mucosa in order to prolong the formulation's residence period (40).

Formulations with a dual purpose made of nano systems loaded into a muco-adhesive in situ gel with the ability to release their content after administration is the main manifestation of a new, creative a plan of action for extending the permanence drug into the site of administration and to enhance the diffusion of the drug across the mucus (41). The loaded NSs are better retained at the administration site thanks to this formulation's ability to make close contact with the mucus membrane; however, the loaded NSs do not necessarily need to bind to mucus components in order to cross the mucus barrier and reach the mucosal surface, which serves as their site of action or sit of absorption.

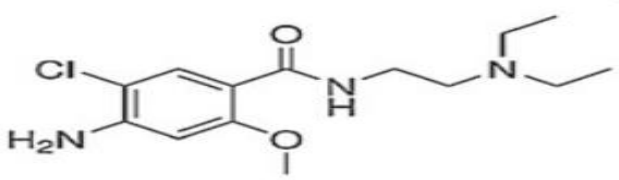
When treating local conditions that affect the nose and paranasal sinuses, such as infectious rhinitis or allergic, nasal epithelium lesions, sinusitis, and rhinosinusitis, nasal delivery via the nasal channel is the preferred method (42). Additionally, the nasal mucosa serves as a non-invasive alternate delivery method for medications with low systemic bioavailability. In fact, quick drug absorption has been made possible by the highly vascularized nasal epithelium that, following oral delivery, experience substantial first pass metabolism and/or stomach breakdown (43). Furthermore, it has been confirmed that the nasal administration route is advantageous for medications to go to the brain since it avoids the blood-brain barrier, this restricts several medicinal medicines' diffusional transport pathways following oral or parenteral administration. In the end, nose to brain delivery

ensures that medications are transported quickly and straight through the olfactory neuroepithelium from the nasal cavity to the central nervous system (44).

Additionally, the intranasal route of administration's faster absorption rate and quick commencement of action are crucial the supervision of emergency circumstances such severe nausea and vomiting. Particularly with antiemetics taken orally, parenteral administration is frequently hampered by vomiting before systemic absorption, which leads to low patient compliance (45). One of these antiemetics we have metoclopramide, a white crystalline powder which is freely soluble in alcohol, is a potent antiemetic with well-studied pharmacological and toxicological profile (46).

Antiemetic and anti-motion sickness medications have been found to quickly enter the systemic circulation when administered intranasally throughout the past few decades. In order to effectively treat acute nausea and vomiting, which are frequently brought on by cancer treatment or migraines, the extremely permeable and vascularized nasal mucosa ensures a rapid commencement of medication action (47).

Because nasal administration increases patient compliance and is pleasant and allows for self-medication, it encourages attention in the systemic drug delivery. Parenteral administration does not. Additionally, as vomiting and nausea are causes of gastric dysmotility and therefore of considerable variations in intestinal absorption of drugs after being administered orally (48), nasal delivery ensures a precise and consistent drug dosing. Metoclopramide represents substituted benzamide, antagonist of dopamine receptors, with direct effect on the chemoreceptor trigger zone and in particular the center of vomiting.



chemical structure of MTC

Figure 2.3. Chemical Structure of Metoclopramide (49).

By activating vagal afferents via the serotonin receptor known as 5HT3, radiotherapy and chemotherapy induce the release of serotonin in the small intestine, inducing vomiting reflux. Additionally, MTC prevents these reflexes from being induced (50). It stimulates the motility of the gastrointestinal tract without affecting the secretion of stomach, gallbladder, or pancreas and increases gastric peristalsis and gastric emptying. It also enhances duodenal peristalsis and tone of the cardia by reducing the tone of the pyloric sphincter (51). Contributing to these mechanisms, MTC is used in disorders associated with reduced gastrointestinal motility as gastroparesis, ileus, reflux, dyspepsia, nausea and vomiting due to migraine, motion sickness, chemotherapy, and other conditions. When taken orally, MTC is immediately absorbed from the gastrointestinal tract. However, conditions like vomiting or impaired gastric motility may reduce its absorption. As a result, MTC is distinguished by two circumstances, including a high variable bioavailability that ranges from thirty two percent to ninety eight percent due to its short half-life and quick first pass metabolism of 3 to 4 hours, necessitating three or four administrations a day (52).

The parenteral route has also several limitations, for instance poor patient compliance, heavy cost, safety consideration and non-feasibility of self-administration.

Antiemetic drug administration appears to be well-replaced by nasal delivery. The utility of the in-situ gel systems appears to be of a great formulation technique for extending the residence time of MTC in the nasal medium, however, given the limited permeability of MTC toward the nasal mucosal membrane and the mucociliary impact. Despite the fact

that the intranasal route has significant benefits in terms of patient compliance, tolerability, accessibility, and efficacy. The physiological element most responsible for reducing the length of time that drugs remain in the nasal environment is mucociliary clearance. This self-clearing mechanism is in charge of the medication's quick removal from the nasal cavity, which cuts down on the amount of time it takes for the medication to treat localized disorders in the nose or to enter the systemic bloodstream or the central nervous system (53). Intranasal in-situ gel formulation appears to be an efficient substitute for nasal liquid therapies for preventing the rapid drainage of medications when they are supplied like basic water solution and extending their residence duration in the nasal cavity. These formulations undoubtedly work best when delivered as low viscosity polymeric solutions because they ensure the best nasal deposition and transform into gels when they come into touch with the mucosa. Different chemical or physical stimuli, temperature, especially ionic strength, and pH can all affect the transition from solution to gel. The in vivo development of a polymeric network increases the amount of time the drug is in contact with the site of action and assures a prolonged release of the drug's active components (54).

As an alternative to in-situ gelling systems, double functioning formulations made of nano systems like liposomes loaded into a mucoadhesive in situ gel vehicle, having the ability to release NSs after administration, represent a recent novel plan to achieve an elongation for drug permanency into the administration site and to enhance the diffusion of the drug across the mucus. While the NSs do not bind to mucus components but should be able to cross the mucus barrier and reach the mucosal surface that represents their site of action, the mucoadhesive in situ gel can create direct contact with the mucus membrane to provide a greater retention of the loaded nano systems at the administration site.

The aim of the present work is to create a nasal dosage form formulation to overcome the limitations of MTC related mainly to the possibility of avoiding presystemic metabolism and emesis in the oral form, leading to variability in plasma levels, as well as the inconvenience in administration of other drug forms (rectal or injection). Moreover, to facilitate drug delivery by developing an innovative composite mixture, combining the liposome and in-situ gel delivery systems, suitable for intranasal administration, aiming to enhance absorption, muco-adhesion, increase stability and to ensure stable plasma

concentrations of our chosen active pharmaceutical ingredient. In this work, MTC intranasal formulations were developed as a potent antiemetic alternative therapy and to increase the extent of absorption through by passing of hepatic first pass metabolism.

3.MATERIALS AND METHODS

3.1 Materials

Metoclopramide (MTC) was purchased from ministry of defense drug company. Phytagel ® (gellan gum) and soybean phosphatidylcholine (SPC) were obtained from Sigma Aldrich, Munich, Germany. Cholesterol was purchased from Sigma Aldrich Chemie GmbH, Japan. Chloroform was purchased from Avantor Performance Materials, Poland S.A. Sodium chloride, potassium chloride, and calcium chloride from Sigma Aldrich Munich, Germany. Purified water used in HPLC and for preparation of the samples was produced with a Millipore Super Purity Water System (Sigma-Aldrich, Munich, Germany). Krebs buffer solution which is also obtained from Sigma-Aldrich, Munich, Germany.

3.2 Methods

3.2.1. Preparation of MTC Loaded Liposomes

Thin film hydration method, also called bangham method was used to prepare MTC loaded liposomes. MTC loaded liposomes were comprised of soybean phosphatidylcholine (SPC), Cholesterol, and chloroform. Using a bath sonicator, SPC and cholesterol were dispersed in chloroform. After that the MTC was added, and the mixture was mixed well. The temperature and rotational speed of the rotary evaporator are fixed between 40 °C and 50 °C. The organic phase was then expelled, and the lipophilic portion received the addition of an aqueous solution (hydrophilic phase) made of distilled water. This preparation is left for five minutes in bath sonication (55). After that, probe sonication was practiced for 5 minutes at a specified sonication frequency (70 kW) for five cycles (56). The resulting liposome solution was allowed to cool to room temperature (25 ± 0.5°C). Finally, the formulation is stored under a temperature of -4°C.

3.2.2. Characterization of MTC Loaded Liposomes

Polydispersity index, zeta potential and particle size have been checked.

Using dynamic light scattering particle size analyzer known as DLS, polydispersity index, zeta potential and particle size was illustrated by Malvern Instrument (Nano ZS

3600, USA) at room temperature (25 ± 0.5°C). The process is done by diluting 1 drop of the liposomal solution with 2 drops of purified water placed in the cuvette to be checked.

3.2.3. Determination of Encapsulation Efficiency

Liposomes separation from non-entrapped material is done by ultracentrifugation at 3500 rpm (Spectrafuge TM, USA) at 10 mins. After that, collection of the liposome supernatant and the determination of the amount of unentrapped MTC in supernatant was done by UV spectroscopy (Agilent 8457, USA) with 1mm cuvartz cuvette at room temperature (25 ± 0.5°C). A standard curve that plots known MTC concentrations (2.5–100 g) versus peak area at 273 nm was used to calculate the quantity of MTC in the supernatant. A standard curve was used to calculate the amounts of nonentrapped MTC from peak regions of the liposome supernatants under analysis (57).

An easy equation was used to determine encapsulation efficiency:

$$\textbf{Encapsulation Efficiency } \% = \frac{\text{Amount of MTC} - \text{Amount of free MTC}}{\text{Total amount of MTC used}} \; x \; 100 \qquad \text{Eq (1)}$$

Same quantity of MTC were utilized for determination of the encapsulation efficiency (58).

3.2.4. Experimental Design

Using D-optimal mixture experimental design, the ideal liposomal formulation was found. D-optimal design was used to assess the effects of four independent variables on four response variables, including zeta size, mean particle size, polydispersity index, and encapsulation efficiency. The independent variables were the amount of soybean (A), cholesterol (B), chloroform (C), and MTC (D). Previous studies were used to identify the parameter range for the current inquiry. In order to pick the best fitting model and improve the process. Table 3.1 displays the variables and their levels that were employed in the design (Result part).

The analysis of variance tables was performed, the effect and regression coefficients of the various linear models, as well as the correlations between the variables, were calculated. The experimental design resulted in 16 combinations. Based on comparisons of

many statistical characteristics offered by Design Expert® software Pro 13 program (Origin Lab Corporation, Northampton, MA, USA), including the coefficient of variation, the multiple correlation coefficient (R^2), and adjusted multiple correlation coefficient (adjusted R^2), the best-fitting mathematical model was chosen. The significance of all terms in the polynomial was judged statistically by computing the F value at the probability $p < 0.05$. Optimization of the fitted polynomials was done using numerical optimization and desirability function. By conducting experiments under the circumstances listed in Table 3.1, the ideal conditions were confirmed.

3.2.5. Liposomal Morphology

The properties of the liposomes were examined using scanning electron microscopy (SEM). To prevent electron charging, the liposomes that underwent lyophilization needed to be sputter-coated with gold using a Leica sputtering system (Leica EM ACE200, USA). Carbon tape was used to secure lyophilized liposome powders to a SEM stub. Before imaging, extra powder was removed by lightly hitting the stub. SEM pictures were captured using the instrument (Zeiss EVO 40, USA) at operating voltage 20 kV (65).

3.2.6. Gellan Gum Preparation

Gellan gum was prepared in four disparate concentrations (0.4%, 0.5%, 0.6%, and 0.7% w/v) in order to choose the optimal concentration. In the first beaker, 0.4% gellan gum in 20 mL distilled water was used, 0.08 g of gellan gum powder was dissolved in distilled water with continuous stirring using magnetic stirrer machine at 500 rpm for 30 minutes. In the second beaker, 0.5% gellan gum in 20 mL distilled water was used, 0.1 g of gellan gum powder was dissolved in distilled water with stirring continuously similar as before at 500 rpm for 30 minutes. In the third beaker, 0.6% gellan gum was dissolved in 20 mL distilled water in a weight 0.12 g. And finally, In the last beaker 0.7% gellan gum was dissolved in 20 mL distilled water in a weight 0.14 g with continuous stirring at 500 rpm for same period of time which is 30 minutes.

After that, simulated nasal fluid (SNF) which is an aqueous solution was prepared. In a 250 mL beaker, we added 0.59 mg/mL $CaCl_2$, 2.98 mg/mL KCl and 8.77 mg/mL of NaCl

in addition to 100 ml of distilled water (59). The formulation was placed on a magnetic stirrer with applied speed 500 rpm until a clear solution was obtained.

In four small glass plates, using a dropper same number of drops from the prepared SNF and gellan gum in three different prepared concentrations was added. From the observed result the best gellan gum formulation was the 0.6% w/v. Figure 3.4 in the result part will show the gel appearance for each concentration of gellan gum when it is mixed with SNF.

3.2.7. MTC L-ISG Preparation and Characterization

With continuous stirring at 500 rpm and 60 °C for an hour, the chosen concentration of gellan gum (0.6% w/v) was dissolved in distilled water. Generated gellan gum solution was cooled to room temperature (25 ± 0.5°C) in order to be incorporated with the below three formulations.

At the same time by using thin film hydration method two liposomal formulation was prepared using liposomal optimal formulation values given by D-optimal mixture experimental design, Soybean 1.97899%, Cholesterol 0.0810361%, Chloroform 97.84% and MTC 0.1%. One of these liposomal formulations is blank, it is unloaded with MTC, the second liposomal formulation is loaded with MTC.

The last one, 0.2 mL of ethanol was used to dissolve 10 mg of MTC in order to be incorporated also with gellan gum solution.

Using three small beakers, the first beaker containing 20 g of gellan gum solution, optimized MTC loaded liposomal formulation was added to it with uninterrupted stirring at 800 rpm for 30 minutes. The second beaker containing 20 g of gellan gum solution, optimized blank liposomal formulation was added to it with continuous stirring at 800 rpm for 30 minutes. Finally, the third beaker also containing 20 g of gellan gum solution, 10 mg of MTC dissolved in 0.2 mL of ethanol was added to it also with stirring it continuously at 800 rpm for 30 minutes. All the Formulations were kept in a refrigerator between 4 to 8 °C until usage (60).

On three glass plates, one drop of simulated nasal fluid was added in each glass plate and then one drop of the incorporated blank liposomal gellan gum mixture was added to glass plate number one, in the second glass plate one drop of the incorporated MTC loaded liposomal gellan gum mixture was also added, and in the last glass plate one drop of the incorporated MTC dissolved in ethanol mixed with gellan gum was added. The observed result was thick gel formulation as suspected.

3.2.8. Determination of pH

Calibrated pH meter was used to measure the pH of the composed in situ gel (Mettler Toledo, Switzerland). The measurement of the pH was repeated for three times and Calculations were made to determine the formulations' average pH values.

3.2.9. Determination of Mechanical Properties

Texture analysis of the prepared in-situ gel preparations were analyzed using a software-controlled penetrometer (TA-XT Plus, Texture Analyser Stable Micro Systems, UK) equipped with 2 kg load cell in texture profile analysis mode (the range was 0-100 N) (62). The sample holder was cylindrical probe with a diameter of 15 mm. Initial transfers of formulations were made to a petri dish of 3 cm diameter at room temperature (25 ± 0.5˚C). A delay of 15 seconds was allowed between the end of the first compression and the start of the second compression when an analytical probe was introduced twice into each composition at a set rate of 1 mm/s and a defined depth of 50 mm. Data collection and calculation was done using software belonging to the penetrometer (Exponent, version 6.1.7.0). Through the resultant force time curve the mechanical parameters, hardness indicating the force needed to reach a certain deformity, compressibility which is the work required to deform the product during the first pass of the probe, adhesiveness define the attractive forces between the surface of the sample and the surface, and cohesiveness of the in-situ gel formulations were determined (63). Experiments were carried out for three times.

3.2.10. Viscosity Method

A digital viscometer named Brookfield, equipped with spindle RV72 was performed to determine the viscosity study of the developed formulations. At two different

temperatures, the formulation's viscosity was observed, 25 ± 0.5°C and 34 ± 0.5°C, at 100 rpm shear rate (64).

3.2.11. Determination of Rheological Behavior

Kinexus rheometer (Malvern Instrument, USA) was used to study the rheological properties of the formulation. The rheometer is of the Couette type. The measuring system used was C18 concentric cylinders. The sample volume was about 2 mL. Formulation was added to the surface of the sample to prevent evaporation of solvent. Continuous shear analysis of each formulation was performed. In shear rate ramp mode and using parallel steel plate (CP4/40) (0.8 mm of the gap). Flow curves were measured ranging from 0,1 s^{-1} to 100 s^{-1}. The measurements are done at room temperature, 25.0°C and 34.0°C, the nasal temperature (64).

To ascertain the maximum strain amplitude for the gel, strain-controlled measurements were performed on each sample. The three-dimensional network of the gel is destroyed over a specific strain amplitude. As a result, measurements above this point do not capture the physical characteristics of a gel that are relevant for its resting state at the lower nasal temperature. The greatest strain was used for all subsequent rheological property measurements (65).

3.2.12. Fourier transform infrared spectroscopy (FTIR-ATR) analysis

In order to assess how formulation components interact with one another over time, the spectra of liposomal formulation were collected using a Fourier transform infrared (FTIR) spectrometer (NICOLET iS50, Thermo Scientific, USA). FTIR tests were performed on samples of (a) pure MTC, (b) gellan gum, (c) MTC loaded gellan gum, (d) gellan gum in a placebo formulation called a "blank liposome," and (e) MTC loaded liposome combined with gellan gum. The specimens were placed immediately over the equipment's crystal after the inserts were delicately cut with a scalpel blade. For each sample, several scans were performed, and the force applied to the specimen was changed to provide good transmittance findings. For each sample, this procedure was carried out three times.

3.2.13. Spreadability

Using a particular technique, the spreadability of the in-situ gel was assessed by adding 0.5 g of gellan gum inside a circle that was 1 cm in diameter and had been previously marked on a glass plate, over which a second glass plate was positioned (66). For five minutes, a weight of 1000 g was placed on the upper glass plate. 50 g of weight were added to the pan. According to Figure 3.1, the spreadability was calculated as the amount of time it takes for the upper glass slide to move over the lower plate. The increase in the diameter due to spreading of the gels was noted.

Spreadability (S) is calculated as follows:

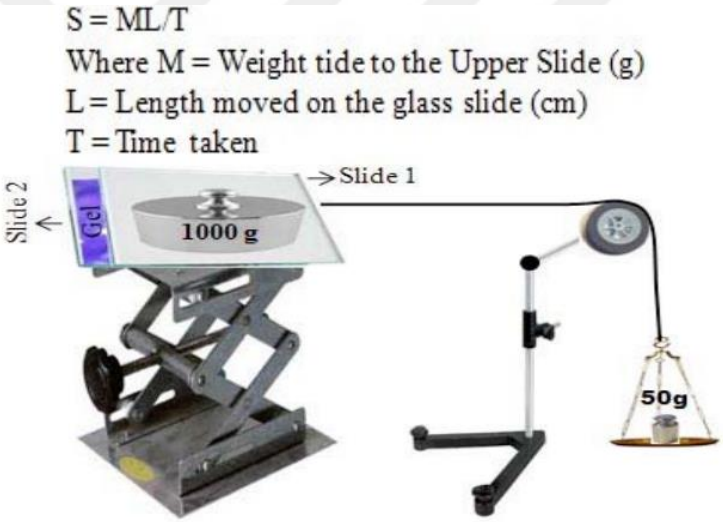


Figure 3.1. Assembly of Spreadability Measuring Device

3.2.14. Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) was employed to assess the formulation's thermal behavior. The thermal analysis of the blank in situ gel, blank liposome, drug loaded liposome, drug loaded in situ gel and the pure drug was performed using Differential Thermal Analyser (Setaram, DSC131, Fra3nce). The analysis was performed on approximately 8 to 10 mg samples placed in sealed aluminum pans. Nitrogen was used as the purge gas at a flow rate of 50 mL/min, and the thermogram was acquired at a scanning rate of 10 °C/min. Heat was used to scan each sample between 20 °C and 300 °C (67).

3.2.15. In-vitro Release Study

The release of the active ingredient from the formulation was examined using a Franz diffusion cell. An artificial membrane called cellulose acetate membrane (Spectra / Por Regenerated Cellulose, Molecular weight cut off 8-10 kDa) was employed for this. The experiment was conducted using Franz diffusion cells (Permegear, USA) with a diffusion area of 5.29 cm^2 and a volume of 20 mL. The receiving phase was Krebs buffer solution (pH: 6.6) (68, 69). The Franz cell's top was covered with closures after 2 g of formulations were placed inside an artificial membrane. 1 mL of sample was removed every 30 minutes to 6 hours by taking care to prevent air bubbles in the receiver compartment and replaced with fresh medium in the same amount (70). To determine the amount of drugs in the samples, HPLC analysis was used.

3.2.16. Ex-vivo Release Study

To accurately anticipate the actual drug release, natural membranes must be used. The sheep nasal mucosa was chosen for this study's experimental portion for the following reasons. Because of their big nares, anatomical similarities to the human nasal cavity in terms of nasal cavity surface area per kg body weight, accessibility of veins for cannulation, and their mild temperament in experimental settings, sheep offer a suitable model for testing nasal delivery of medications. Furthermore, it has been obtained those results from the sheep model closely match those from human data. The most important point is that the sheep mucosa must be freshly removed 10 minutes after the animal is sacrificed. In our study, we obtained post-slaughter sheep mucosa made in Acıbadem University Animal Experiments Center. There is main and important thing related with sheep mucosa, it should be used immediately as it is cut. Otherwise, the cells in the mucosa rapidly deteriorate and the transition study becomes meaningless. The cavity mucosa was carefully removed within 10 minutes of the animal's death, after which it was promptly submerged in KHS (about 900 mL), put on ice, and treated with O_2/CO_2 (95% /5%). Tissue was inserted in the nasal diffusion cell (Franz diffusion cell) with permeation area (cm squared). The cumulative of MTC permeated per unit area was plotted against time, and the slope of the linear portion of the plot was used as steady state flux (JSS). The permeability

coefficient (Kp) was calculated with Eq. (1), in which Cv is the total donor concentration of the formulation (71).

$$Kp = Jss/Cv \qquad \text{Eq (2)}$$

3.2.17. HPLC

The amount of MTC was measured using an HPLC system that included a gradient pump, an Agilent 1100 UV detector (Thermo Scientific, Germany), and a C18 column (Fisher Scientific Pittsburgh, PA) (5μm, 150 × 4.6 mm). At a temperature of 25 °C and a wavelength of 280 nm, the samples were examined. Acetonitrile and potassium phosphate buffer (pH 3.5) were combined in an 80:20 v/v ratio as the mobile phase. Flow rate was1.5 mL/min, and injection volume was 10 μL.

The validation of HPLC method was performed according to the international guidelines on analytical techniques for quality control of pharmaceuticals (ICH GUIDELINE) (72). Validation of the method was done to assess the linearity, limits of detection (LOD) and quantitation (LOQ), accuracy, precision, and specificity, as well as selectivity and stability, were all validated. Using a calibration curve made from standard solutions of MTC (5 mg/mL), the linearity between peak area and concentration was examined. The degree to which test findings generated by an analytical procedure are accurate in relation to the true value is known as recovery. As a test sample, the prepared standard solutions were injected six times at various levels.

3.2.18. Statistical Analysis

Statistical analysis was performed by one way analysis of variance (ANOVA) followed by Turkey's multiple comparison test with the software package prism v. 5.04 (GraphPad Software Inv., La Jolla, CA, USA). Results were expressed as mean values standard error of the mean (SEM) or as mean values standard deviation. A Student's t-test was used for statistical comparison/ analysis, and $P < 0.05$ was considered statistically significant.

4.RESULTS

4.1. Preparation and Characterization of MTC contained Liposomes

To determine the 4 various material compositions, D-optimal experimental design using Design-Expert® was used. Four independent variables' effects include: amounts of soybean, cholesterol, chloroform, and MTC on four response variables: zeta size, mean particle size, encapsulation efficiency and polydispersity index, and were evaluated as shown in table 4.1. The thin film hydration approach was used to create API-loaded liposomes (API-Lip), which were further characterized. Table 4.2 lists the fitting models, equations, and statistical parameters. To determine the significance level $P < 0.05$ was utilized. Design Expert® software produced equation-based three-dimensional response surface graphs. The optimal parameters for the formulations were then generated using the desirability approach (73,74).

The range polydispersity index values vary between 0.186 and 0.506, indicating monomodal size distributions (75). Also, the particle size of the MTC loaded liposomes were small, its mean particle size of all formulations was smaller than 128 nm ranging between 92.16 and 127.5. Encapsulation efficiency of MTC in liposomes was between 71% and 94%. Furthermore, in this work, zeta potential values were negative for all of our API-Lip formulations, as well-known the nanoparticles' surface charges may have an impact on the processes that lead to their transport from the nasal cavity. In reality, negatively charged nanoparticles are mostly transported by the olfactory route (76). Mucus can be more effectively penetrated by negatively charged nanoparticles that do not interact with it (77). Because they are tiny enough, negatively charged, and densely coated, nanoparticles seem ideally suited to penetrate mucous membrane.

Table 4.1. Optimal design with Component and Response Variables for the Preparation of MTC Loaded Liposomes

	Component 1	Component 2	Component 3	Component 4	Response 1	Response 2	Response 3	Response 4
Run	A: Soybean %	B: Cholesterol %	C: Chloroform %	D: metoclopramide %	zeta size	mean particle size (nm)	PDI	EE %
1	1.86	0.10	97.93	0.1	-47.0	127.5	0.190	86.03
2	1.90	0.09	97.90	0.1	-55.3	119.5	0.251	81.63
3	2.0	0.08	97.81	0.1	-38.6	114.4	0.186	90.36
4	1.99	0.10	97.80	0.1	-45.0	115.9	0.222	87.37
5	1.90	0.09	97.90	0.1	-55.3	119.5	0.251	81.63
6	1.89	0.08	97.92	0.1	-40.0	107.5	0.261	80.30
7	1.90	0.09	97.90	0.1	-55.3	119.5	0.251	81.63
8	1.90	0.09	97.90	0.1	-55.3	119.5	0.251	81.63
9	1.84	0.08	97.97	0.1	-40.7	101.2	0.302	73.45
10	1.81	0.10	97.98	0.1	-30.5	94.18	0.313	74.85
11	1.80	0.08	98.01	0.1	-35.6	112.4	0.506	71.83
12	1.80	0.08	98.01	0.1	-35.6	112.4	0.506	71.83
13	1.97	0.08	97.84	0.1	-41.0	92.16	0.328	93.71
14	1.94	0.10	97.85	0.1	-49.5	105.3	0.286	82.14
15	2.0	0.08	97.81	0.1	-38.60	114.4	0.186	90.36
16	1.93	0.08	97.88	0.1	-44.90	98.57	0.303	85.67

Table 4.2. Fitting Models, Equations, and Statistical Parameters of the Experimental Design

Response	Model	r^2	r^2 Adjusted	r^2 Predicted	Final Equation
Zeta size	Quadratic	0.9557	0.9335	0.8674	= +1.34343x10^5 A +7.64661x10^6 B +51.66782 C -81205.78985 A * B -1394.87178 A * C -76622.24486 B * C
Mean Particle Size (nm)	Cubic	0.9998	0.9995	0.8769	1/(mean particle size) = -43028.00352 A -5.14307x10^7 B +0.435772 C +7.58012x10^5 A * B +658.76614 A * C +7.73231 x10^5 B * C -5018.19548 A * B * C -2432.57808 A * B * (A-B) +2.28838 A * C * (A-C) +2586.71053 B * C * (B-C)
Polydispersity Index	Cubic	0.9996	0.9990	0.7646	1/(PDI) = +3.96465 x10^{7}A +3.60533Ex10^{10} B -360.25365 C -5.37382 x10^8 A * B -6.06968 x10^5 A * C -5.41924 x10^8 B * C +3.57648 x10^6 A * B * C +1.76484 x10^6 A * B * (A-B) -2108.08769 A * C * (A-C) -1.81212E+06 B * C * (B-C)
Encapsulation Efficiency (%)	Cubic	0.8204	0.7928	0.7028	1/(EE) = -22829.04963 A -1.10232 x10^7 B +0.218251 C +1.53119 x10^5 A * B +349.27612 A * C +1.65912 x10^5 B * C -982.94276 A * B * C -422.44433 A * B * (A-B) +1.21162 A * C * (A-C) +556.27412 B * C * (B-C)

The optimum conditions were verified by conducting experiments at the conditions determined. Also, for detection of optimal formulation expert design was done depending on the given values (shown in Table 4.1.).

The responses of the four variables conducted from MTC loaded liposome were determined under 16 different experimental conditions (Figure 4.1.).

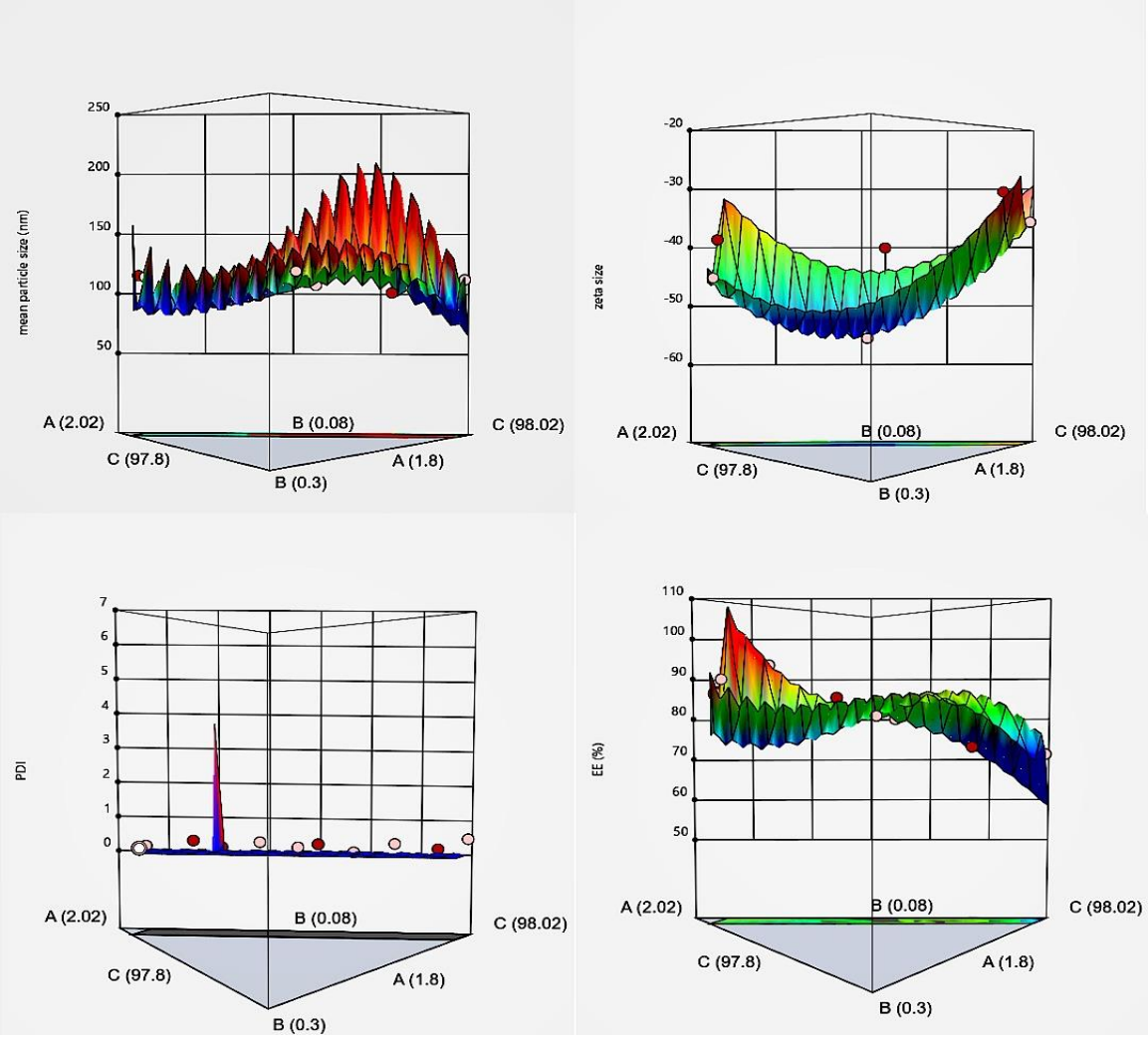


Figure 4.1. 3D Surface Plot for the Response of four variables (soybean, cholesterol, chloroform, MTC) conducted from Metoclopramide Loaded Liposome on mean particle size, zeta size, polydispersity index and encapsulation efficiency. 3D surface plots.

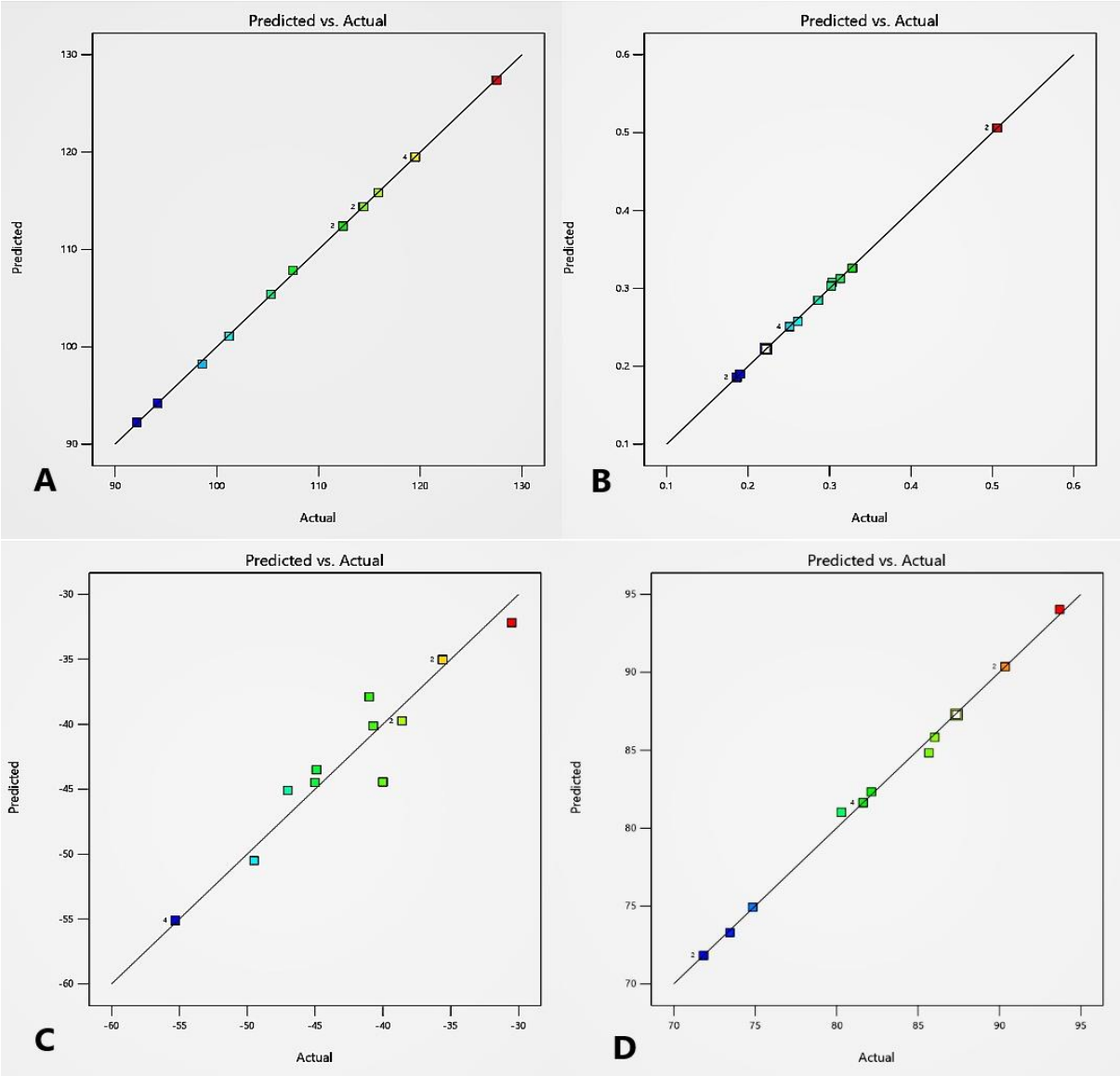


Figure 4.2. Influence of API-Lip component (soybean, cholesterol, chloroform, and MTC) amounts on mean particle size (A), polydispersity index (B), zeta size (C), and encapsulation efficiency (D). Predicted vs. Actual graphs.

4.2. Choosing an Optimum Formulation

The procedure was optimized for each of the three responses after creating the simplified model polynomial equations that link the dependent and independent variables. Based on the criteria of achieving the smallest value of particle size with significant encapsulation efficiency, the best formulation was chosen. Utilizing the extensive grid

search and feasibility search features offered by the Design Expert program, the final ideal experimental parameters were determined. Numerical optimization of wall material amounts using the desirability function has been performed. The optimization parameters are presented in Table 4.3.

Table 4.3. Numerical optimization of wall material amounts using desirability function.

Independent Variables		
	Amount level	**Predicted Optimal Amount**
Soybean	1.8-2	1.97
Cholesterol	0.08-1	0.08
Chloroform	9.78-98.02	97.84
Response Variables		
Encapsulation Efficiency	81.63	83.21
Mean Particle Size	119.50	107.3

Desirability 1.

The results showed that experimental values did not significantly differ from the predicted values ($p > 0.05$). The optimal composition of MTC loaded liposome has been determined as follows: 0.49 g of Soybean, 0.02 g of cholesterol, 25 mL of chloroform and 0.01 g of MTC.

Table 4.4. Given values of the optimal formulation

Optimal Formulation			
Soybean (%)	Cholesterol (%)	Chloroform (%)	Metoclopramide (%)
1.97899	0.0810361	97.84	0.1

4.3. Liposomal Morphology

The surface morphology of the liposomes was evaluated using SEM analysis. SEM picture was taken to obtain more information about the morphology of the prepared API-Lip (Figure 4.3). In Figure 4.3, it could be seen that the particles were almost spherical, and the SEM images taken are supportive of the nanosized measurement results.

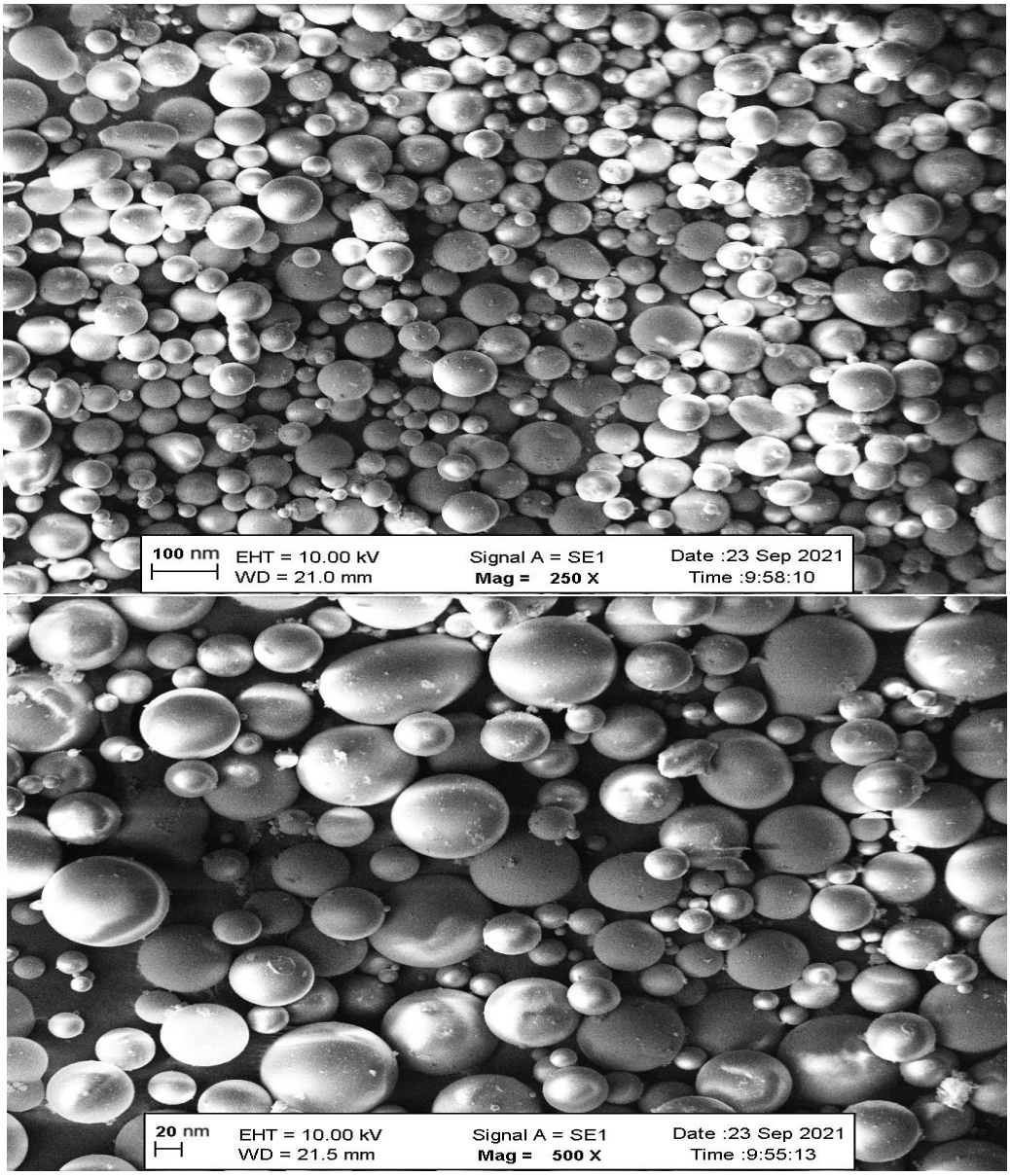


Figure 4.3. Surface morphology of MTC loaded liposomes imaged by scanning electron microscope at magnifications 250 X and 500 X.

4.4.Gellan Gum preparation

As mentioned in the method part 2.2.6., the choice of gellan gum concentration was mainly dependent on the thickness of the gel composed by mixing different concentrations of gellan gum (0.4, 0.5, 0.6, and 0.7%) with a simulated nasal fluid solution prepared from Nacl, KCl and $CaCl_2$. The best gel appearance was obtained from the concentration of 0.6% gellan gum. The figure below will show the gel appearance for each concentration of gellan gum**.**

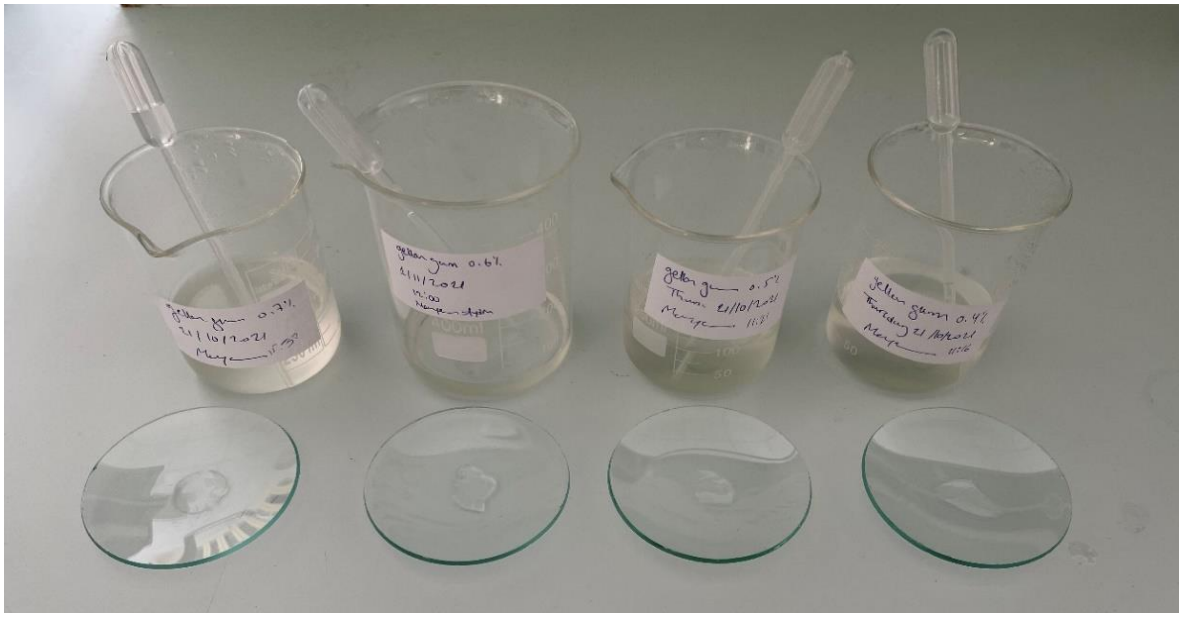


Figure 4.4. Gel Appearance at Different Concentrations of Gellan Gum

4.5. MTC L-ISG Preparation and Characterization

Liposomal and non-liposomal MTC loaded in situ gel in addition to blank liposomal formulations were prepared in the ratios indicated in Table 4.5. Three formulations are done, non-liposomal in situ gel formulations were added to the formulation by first preparing the gelling agent which is gellan gum and then dissolving and adding the specified amount of MTC in the appropriate amount of ethanol. For liposomal in situ gel formulations, firstly, the liposomes were prepared by using soybean and MTC at the specified ratios by using thin film hydration method after prepared liposomal formulation in-situ gel (gellan gum) were prepared. Liposomes were added to the gels, which were

mixed at 500 rpm at room temperature (25 ± 0.5°C) for 30 minutes, using the directly-corporation method.

Table 4.5. Ratios of Liposomal and non-liposomal metoclopramide loaded in situ gel in addition to blank liposomal formulations

Formulation code	Drug (MTC) (mg)	Gellan Gum (%)	Soybean (%)	Cholesterol (%)
MTC-G	10	0.6	-	-
L-G	10	0.6	1.97899	0.0810361
UL-G	-	0.6	1.97899	0.0810361

4.6. Storage Stability Studies of Liposomal Formulations

The MTC loaded liposomal stability after being combined with the in-situ gel's components was monitored for six months. The colloidal dispersions were stored at 4 °C in sealed containers. Aliquots were taken out and measured for mean size, polydispersity index, Zeta potential, and encapsulation efficiency at predetermined intervals. After six months, there was no statistically significant difference, and comparable information was found in other articles.

Table 4.6.: Stability of the Optimal formulation on Week 0,1,2,4, and 5

OPTIMAL FORMULATION's STABILITY				
Variables	**Zeta Size (mV)**	**Mean Particle Size (nm)**	**Polydispersity Index**	**Encapsulation Efficiency (%)**
WEEK 0	-48.3 ± 0.06	107.3± 0.17	0.201± 0.03	84.75± 0.81
WEEK 1	-49.9± 0.07	111.1± 0.28	0.181± 0.02	83.62± 0.12
WEEK 2	-51.6± 0.08	109.7± 0.23	0.179± 0.02	82.66± 0.13
WEEK 4	-47.3± 0.07	110.6± 0.28	0.212± 0.07	78.33± 0.07
WEEK 5	-48.2± 0.09	110.1± 0.23	0.193± 0.01	77.61± 0.14

Zeta size was varied between -47.3 ± 0.07 and -51.6± 0.08, the mean particle size was showing variation between 107.3± 0.17 and 111.1± 0.28, for the polydispersity index the values were varied between 0.179± 0.02 and 0.201± 0.03, the last response which is

encapsulation efficiency was varied between 77.61± 0.14 and 84.75± 0.81, which shows no significant difference in the resulted ratios.

4.7. Mechanical Properties

In general, nasal in situ gel compositions should have the appropriate mechanical properties for simple administration and high mucosal spreadability. To learn more about the gel structure and assess how well formulations resist to compressive loads and the ensuing relaxation, texture profile analyses (TPA) were carried out. Hardness, compressibility, adhesiveness, and cohesiveness were used to describe the mechanical characteristics of the formulations. Table 4.7. contains the outcomes that were obtained.

Table 4.7. Mechanical properties of the formulations. Data means ± SD, n = 6. Different letters in each column denote statistical difference at $p \leq 0.05$.

Formulation	Hardness (N)	Adhesiveness (N.mm)	Cohesiveness	Compressibility (N.mm)
GELLAN GUM	0.581 ± 0.033^{a}	1.003 ± 0.095^{d}	0.814 ± 0.021^{a}	2.286 ± 0.193^{a}
UL-IN SITU	$0.577 \pm 0.092^{a,b}$	2.074 ± 0.117^{c}	0.859 ± 0.036^{a}	2.283 ± 0.322^{a}
API-IN SITU	0.493 ± 0.031^{c}	3.106 ± 0.194^{b}	0.429 ± 0.018^{b}	2.016 ± 0.329^{a}
LIP-INSITU	0.481 ± 0.020^{c}	4.498 ± 0.429^{a}	0.403 ± 0.047^{b}	1.996 ± 0.174^{b}

The stress needed to dislodge the formulation from the container is explained by the formulation's hardness and compressibility. These features measure sample compression-induced deformation. To make it simple to remove the gel from the container, compressibility should be low (78). The development of intranasal formulations faces a considerable difficulty in achieving a variety of attributes, including improved spreadability, mucoadhesion, and appropriate viscosity, which support comfortable administration and patient compliance. By examining the physical gel structure, texture profile analysis (TPA) enables the evaluation of the mechanical characteristics of semi-solid formulations. Mechanical parameters (viscosity, density exponent, cohesiveness, and adhesiveness) were determined from the resulting force-time curve of TPA diagrams, and

the findings are shown in Table 4.7. In terms of the study of the textural profile, a higher adhesiveness of the liposomal in situ gel in compared to the UL and API in situ gels would indicate a higher viscosity. Adhesiveness, a mucoadhesion-related attribute, is used to describe the relative characteristics of each candidate formulation. It is defined as the effort needed to separate the probe from the sample in which its cohesive connections were broken (79). Higher adhesiveness values are advantageous because they may result in increased mucosal surface adhesion and longer retention times, both of which may improve clinical efficacy. Lower values for cohesiveness represent better spreadability.

Figure 4.5. Texture Profile Analysis Machine

4.8. Viscosity

The viscosity of the formulation ranged between 14.07±0.02 and 17.83±0.01 cP when it was incorporated with liposome. The viscosity was increased when the formulations were prepared with 0.6% gellan gum and adding the MTC or liposome. The highest viscosity range is achieved when MTC is loaded into liposomal formulation and incorporated with gellan gum.

Table 4.8. Viscosity Ranges from Different Formulations

Formulation	Viscosity (Cp)	Temperature (°C)
Gellan gum	14.07 ± 0.02	25
	17.01 ± 0.02	34
API Gellan gum	14.19 ± 0.05	25
	17.28 ± 0.01	34
UL-Lip-Gellangum	14.52 ± 0.02	25
	17.39 ± 0.03	34
L-Lip-Gellan gum	15.08 ± 0.03	25
	17.83 ± 0.01	34

4.9. Rheological Behavior

One of the most crucial factors for predicting the in vivo behavior of in situ gels is the assessment of their rheological properties. Application easiness and retention within the application area are particularly impacted by rheological characteristics. In addition to the other formulations, the MTC loaded formulation was primarily chosen and its rheological behavior assessed. In order to track changes in the gel structure, the rheological properties were assessed at both room temperature (25°C ± 0.5°C) and nasal temperature (34°C ± 0.5°C).

In an attempt to mimic the scenario in the nasal application we combined the formulation in a 1:1 ratio with simulated nasal fluid. This is the ideal study case since it represents the scenario in which no dilution happens after delivery and additional electrolytes are supplied to cause formation of a gel.

High elasticity and concentration independence were observed for strain sweep (0.5 %) measurements in-situ gel formulation created in simulated nasal fluid.

Measurement results at room temperature and nasal mucosa temperature show that the decrease in shear stress of liposomal formulations is more noticeable at (34° C ± 0.5°C).

It was observed that the rheological properties of all formulations were better at nasal temperature and the non-Newtonian flow properties were more pronounced.

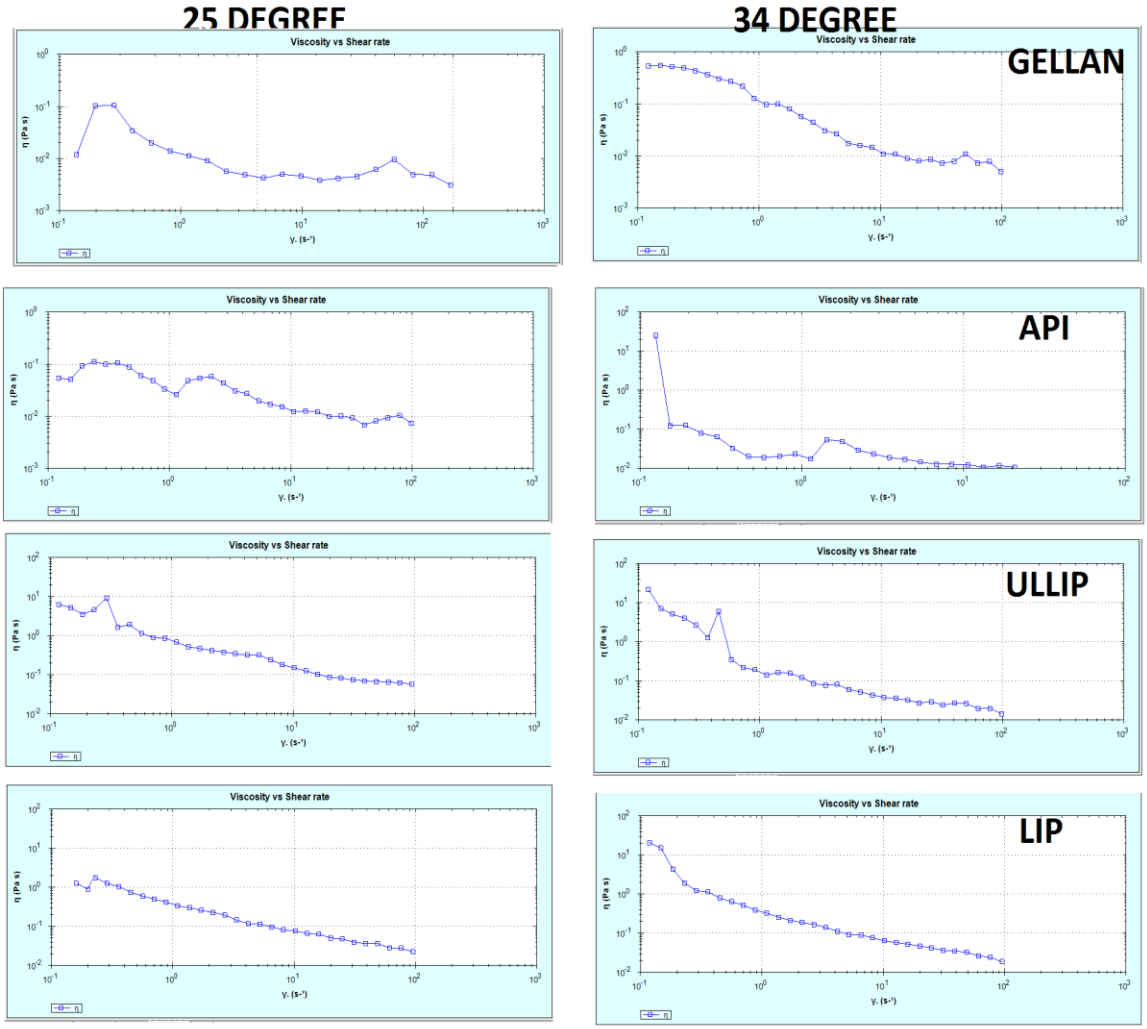


Figure 4.6. Evaluation of Rheological Properties for In-situ gels at both Body and Nasal Temperature

4.10. Fourier Transform Infrared Spectroscopy (FTIR-ATR) Analysis

Different solid-state forms of an organic chemical have been examined using FTIR spectroscopy to investigate changes in molecular conformations, crystal packing, and hydrogen bonding configurations.

In the FTIR spectrums of pure MTC represented as follows: 3394.39 cm^{-1} (OH stretching mode of hydrate), 3302.81 cm^{-1} (symmetric NH2 stretching vibration), 3192 and 2942.51 cm^{-1} (NH stretching mode of amide), 1661.41 cm-1 (OH bending mode of

hydrate), 1629.09 cm^{-1} (NH_2 scissoring and/or C=O stretching bands) and 1537.61 cm^{-1} (amide N–H band), respectively. It was found to be in agreement with the reference article (67). The major OH group signal at 3394 cm^{-1} and 3192 cm^{-1} is prominent which is belong to gellan gum. In addition, stretching vibrations belonging to the aromatic C-O group are seen in the spectrum of 2942.57 and 2635.90 cm^{-1}. The signal at 1628.54 cm^{-1} is due to the glycosidic link in gellan, which is also prominent. Again, these results appear to be in agreement with the reference (80).

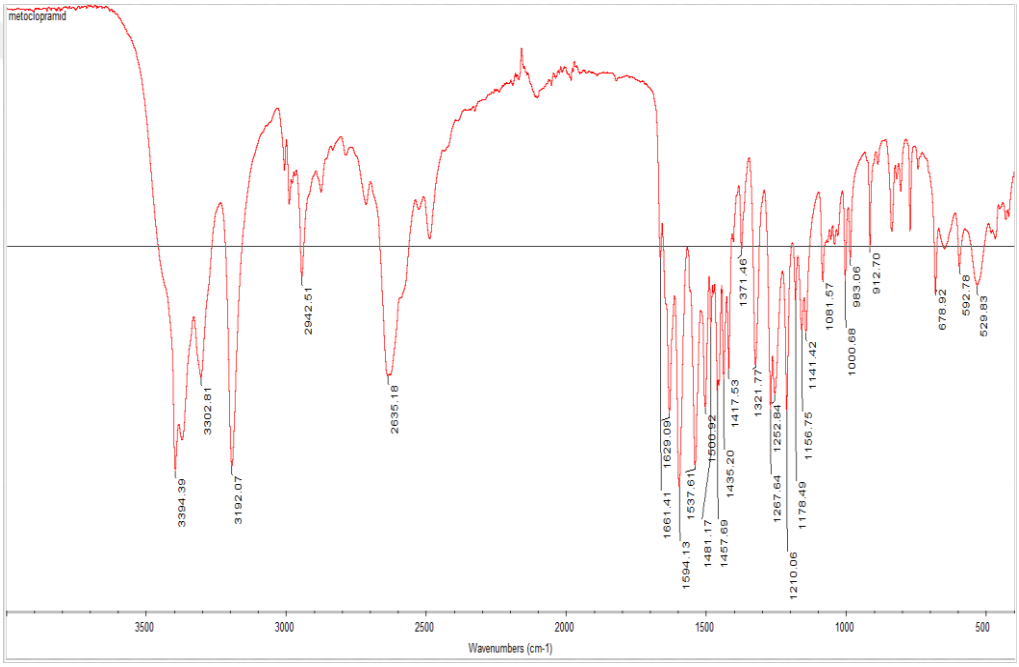


Figure 4.7. FTIR Spectrum of Pure Metoclopramide

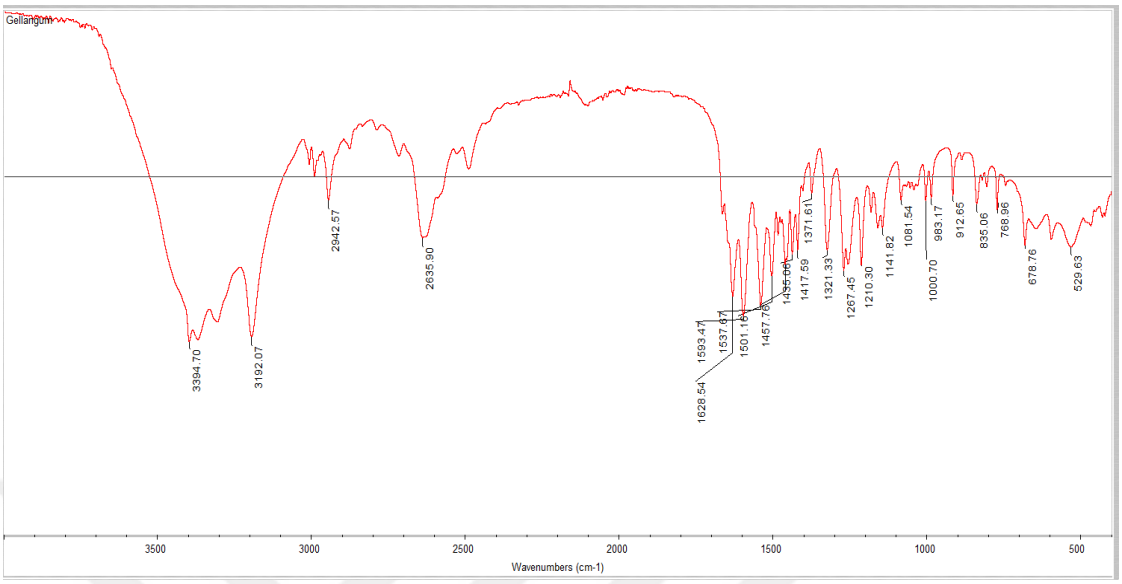


Figure 4.8. FTIR Spectrum of Gellan Gum

In the spectrum of MTC loaded gellan gum, it is seen that the stretching vibrations of the -OH group shift to 3327.61 cm^{-1} due to the -NH_2 group of MTC. It is seen that the stretching vibrations originating from the glycosidic bonds of Gellan gum and the C=0 vibrations of MTC also give the spectrum of 1636.19 cm^{-1} as a single peak. The spectrum resulting from the -OH stretching vibrations of gellan gum in the placebo formulation from the -CH_2 group of soy lecithin appears to signal as a single spectrum at 3331.82 cm^{-1}. In addition, the C=0 band of the cholesterol content in the liposome gives a signal at 1636.09 cm^{-1} (81). This proves the liposome found in gellan gum since there are no interactions between the polymers and other ingredients.

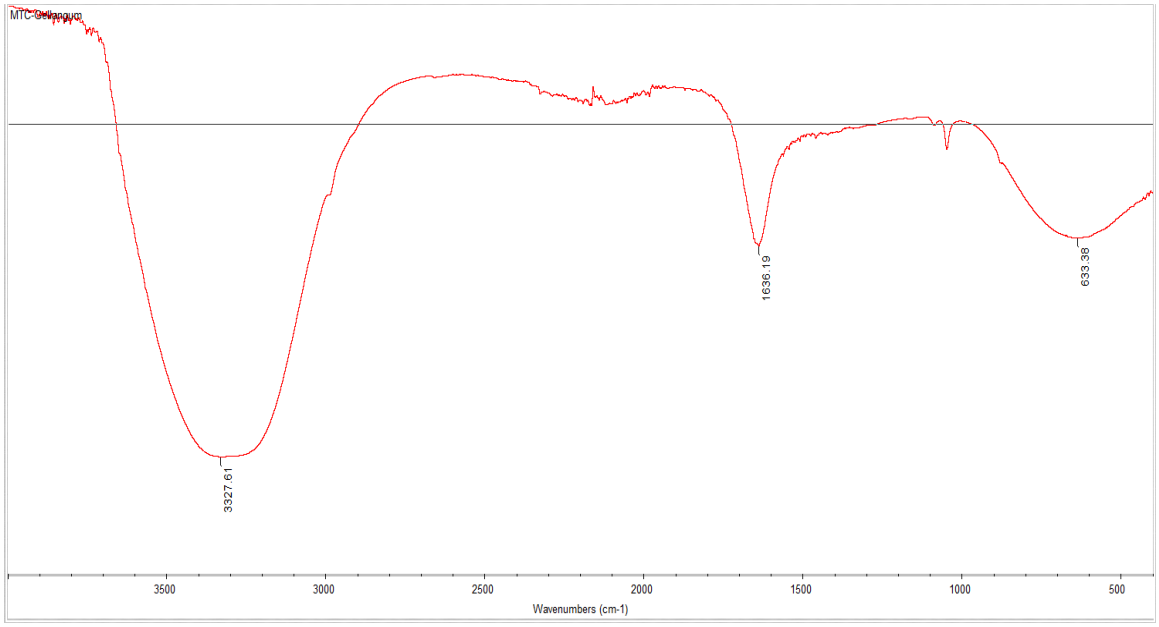


Figure 4.9. FTIR Spectrum of Metoclopramide incorporated with Gellan Gum

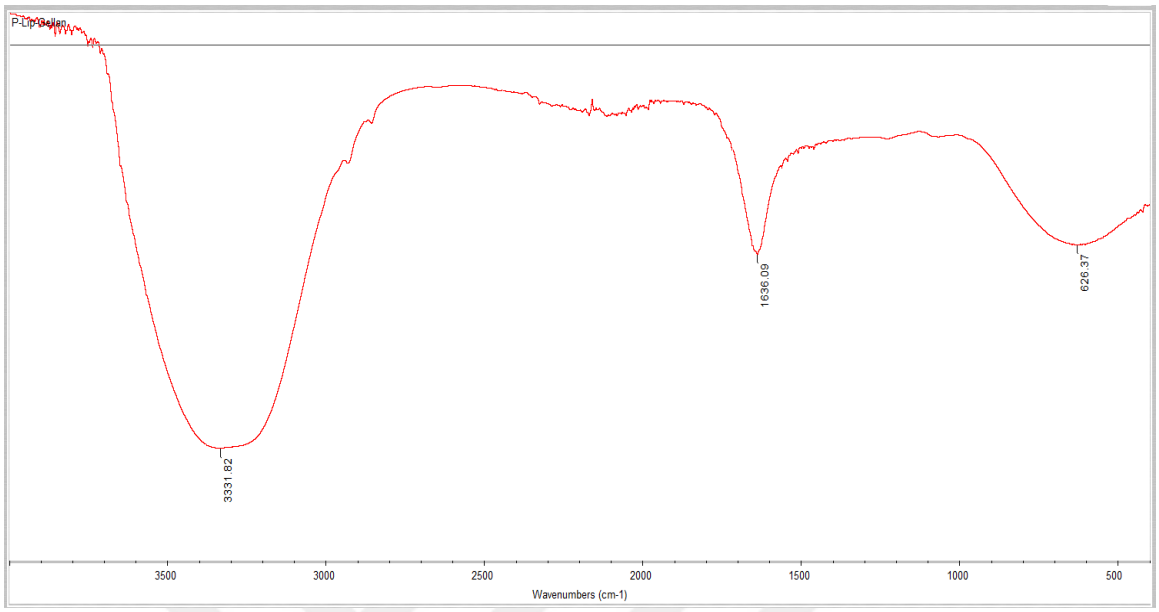


Figure 4.10. FTIR Spectrum of Blank Liposome incorporated with Gellan Gum

In the MTC loaded liposome formulation, the peaks of MTC were not observed much, which proves that most of the MTC is in the liposomal formulation. It is seen that vibrations of glycosidic bonds originating from gellan gum give spectrum at 1636.06 cm^{-1}, stretching vibrations of -PO_2 originating from soy lecithin at 637.09 cm^{-1}.

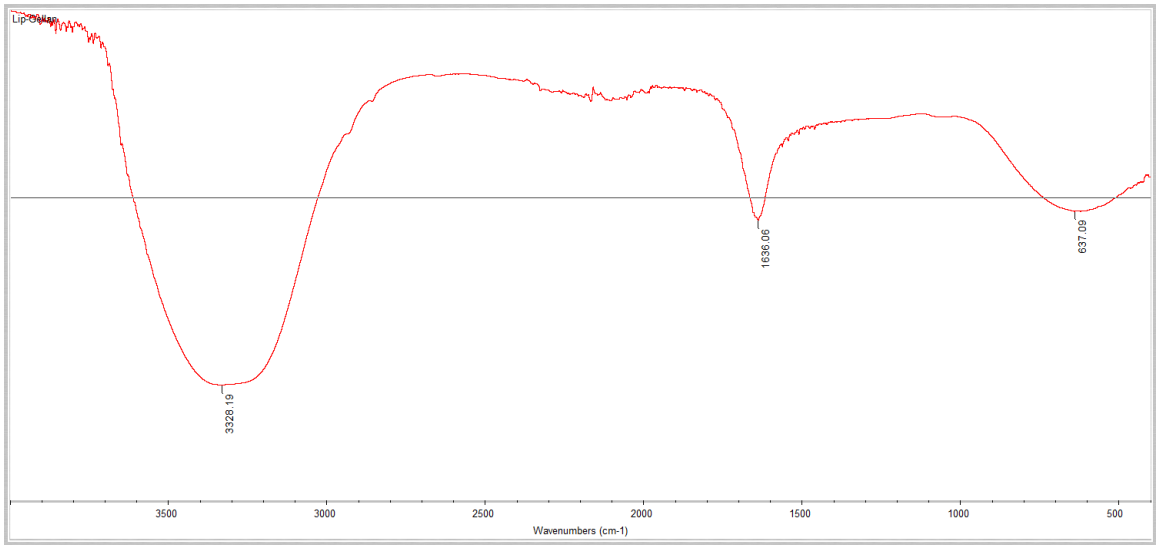


Figure 4.11. FTIR Spectrum of Metoclopramide Loaded Liposome incorporated with Gellan Gum

4.11. Spreadability (gcm/sec)

Table 4.9. Spreadability values for all the formulations done. Data means ± SD, n = 6. Different letters in each column denote statistical difference at $p \leq 0.05$.

Gellan gum	83.3 ± 1.3^{c}
Blank liposome + Gellan Gum	$93.3 \pm 2.4^{a,b}$
Loaded liposome + Gellan Gum	96.6 ± 3.4^{a}
MTC + Gellan Gum	90 ± 4.5^{b}

The spreadability results showed that MTC loaded liposome formulation show the most effective results for spreadability among the other formulations.

4.12. Differential Scanning Calorimetry

Figure 3.12. shows the DSC thermograms of MTC and formulations by using a heating rate of 3 °C/min from 25 to 200 °C. It is clearly indicated one endothermic peak at 112,5 was observed in the DSC curve of MTC (67,82). In addition to seeing a single endothermic peak in the thermogram of gellan gum, the onset temperature is observed at 94.5 °C, while the peak melting temperature is observed at 142.3 °C. It is thought that the reason for this is because the bonds between glyceryl and acetyl found outside the molecule begin to break (83,84). In the thermograms of Gellan gum – blank liposome and gellan gum – loaded liposome formulations, the onset temperature of the endothermic peaks is 93.8 °C and 94.1 °C, respectively; melting degrees were observed as 128.3 °C and 131.8 °C. The reason for the increase in the melting point in the GG-Blip formulation is thought to increase the cross-links of Gellan gum due to the need for more energy (85,86). The temperature increase in the GG-Lip formulation is attributed to the endothermic energy requirement due to both gellan gum and MTC content. In the GG-MTC formulation, the melting point of MTC was observed to be characterized by a single endothermic peak at 135.3°C due to gellan gum.

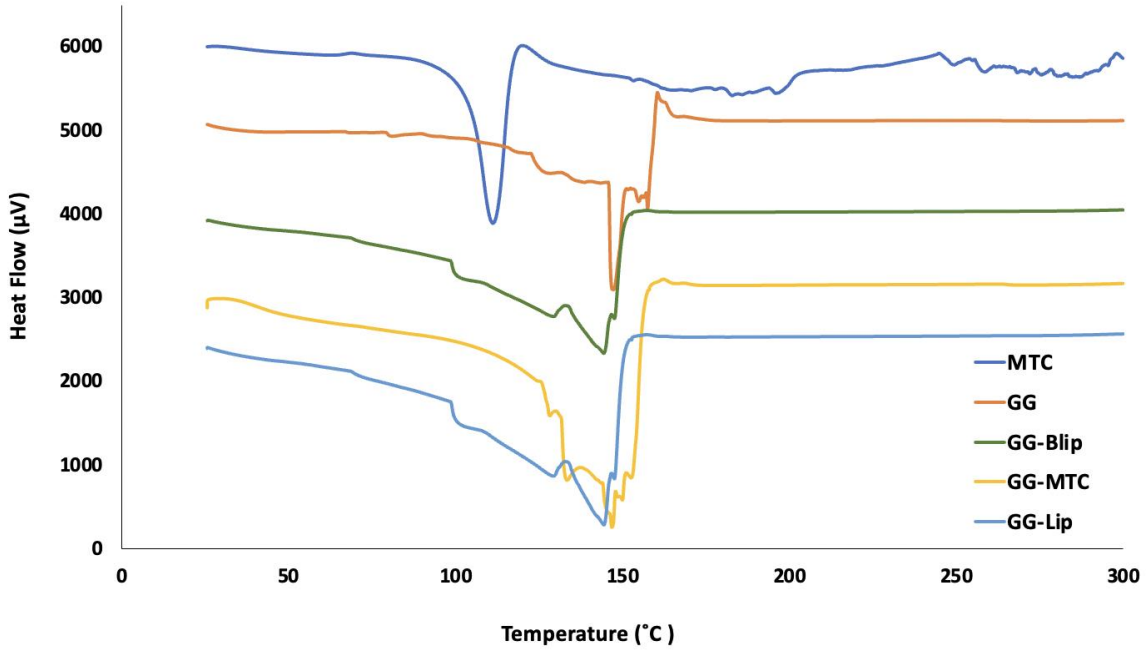


Figure 4.12. DSC Thermogram of MTC and other formulations

4.13. HPLC Study

The limit of detection and limit of quantification were 2.555 and 0.005 g/mL, respectively, and the technique was linear from 0.0117 to 3 mg/mL. Each sample was tested twice, and a calibration curve was created. Depending on the stability of the samples, at least three analytical runs were performed (the first for samples of 2, 4, and 8 hours, and then perhaps another three for samples of 24 hours, 48 hours, and 72 hours). Finally, data are presented for intra-, inter-day accuracy- and precision-estimation. The samples are intercalated with the control standards.

Samples	R.T (min)	Linearity range (mg/ml)	Linear equation	r^2
Analytical	4.3±0.1	0.011 - 3	y= 11175000X +91196	0.9998

*Average of three days; X: unknown concentration Representative chromatograms

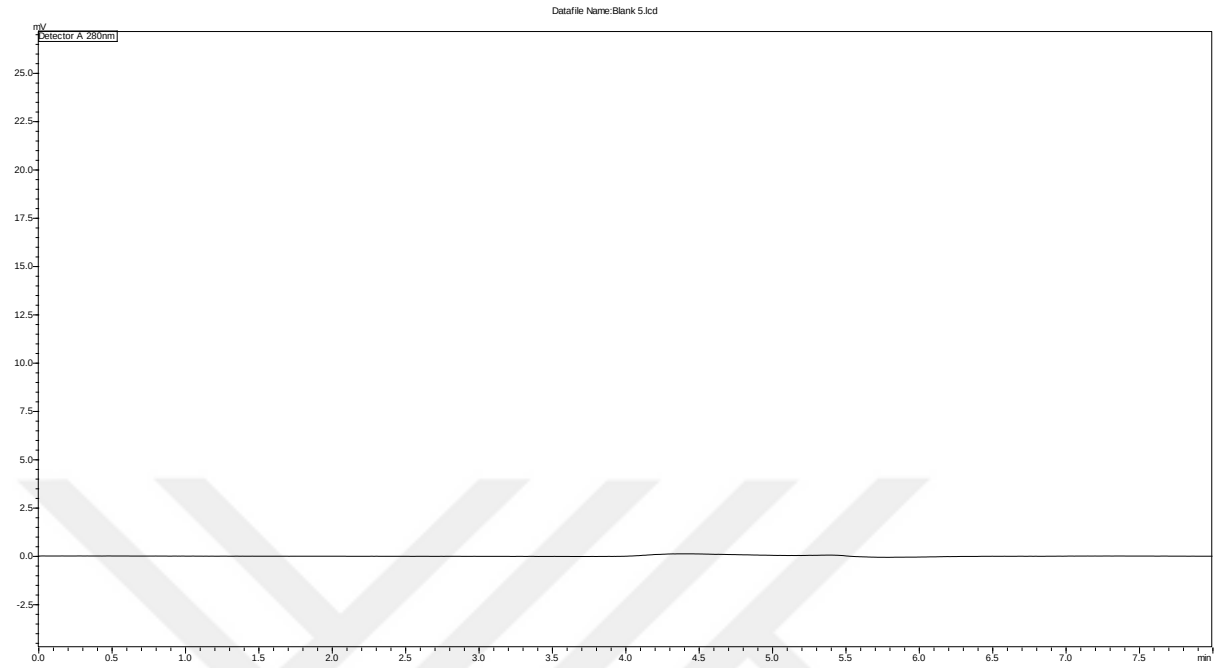


Figure 4.13: Chromatogram of blank mobile phase

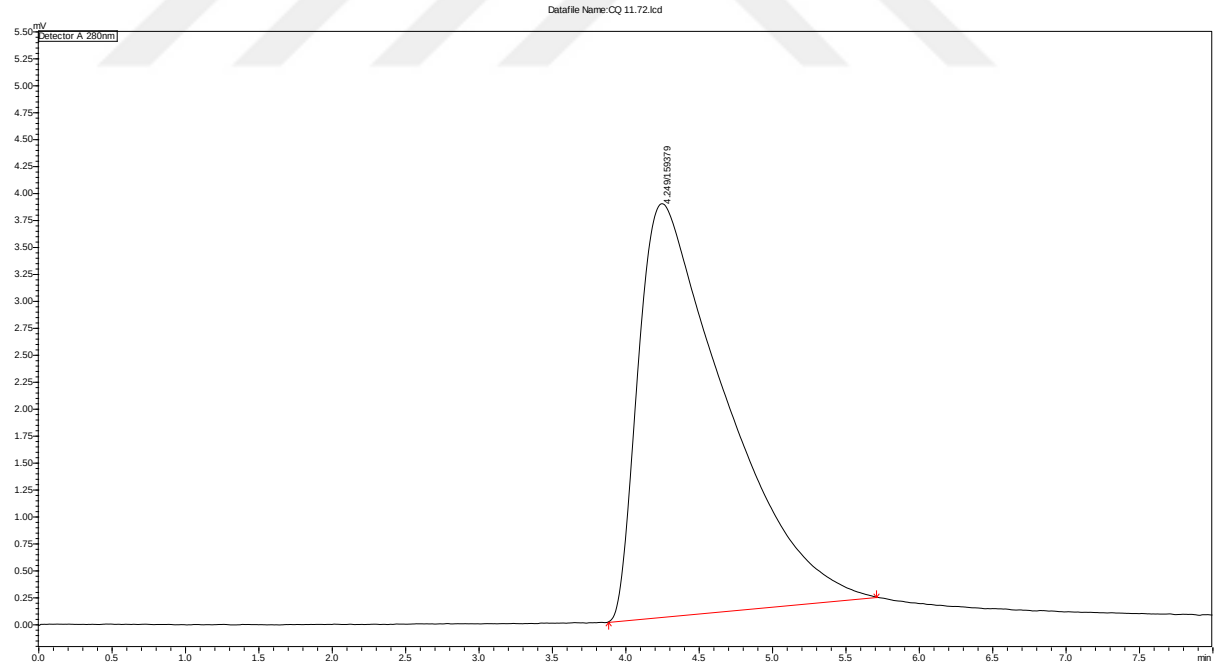


Figure 4.14: Chromatogram of 0.011 mg/ml metoclopramide HCl

The retention time of MTC was 4.3 ±0.1 min. Blank mobile phase did not show any interference with MTC peak. The method was specific for MTC and linear between 0.011-3 mg/mL.

4.14. In-vitro Release Study

By comparing MTC-GG, LL-GG, and UL-GG, in-vitro drug release behavior was investigated. Krebs buffer solution (pH: 6.6) was employed as the diffusion medium for the drug release tests of formulations, which were carried out utilizing the Franz diffusion cell and dialysis membrane. Figure 4.15 displays the release profiles for the formulations. Cumulative drug release from the MTC loaded liposome incorporated with gellan gum formulations after 5 h was found to be more than 80 %. The inflection point in the release profiles showed that gel was forming in the diffusion cell's donor compartment. A portion of the drug may be loaded into the gel matrix during gel formation; as a result, the drug release rate is slowed down by the polymer's cross-linking. Additional findings revealed that the highest amount of the drug released in the first hour mostly depended on the fact that in the Loaded liposome incorporated with gellan gum, the liposome was used as drug carriers, and during the course of the investigation, this pattern continued.

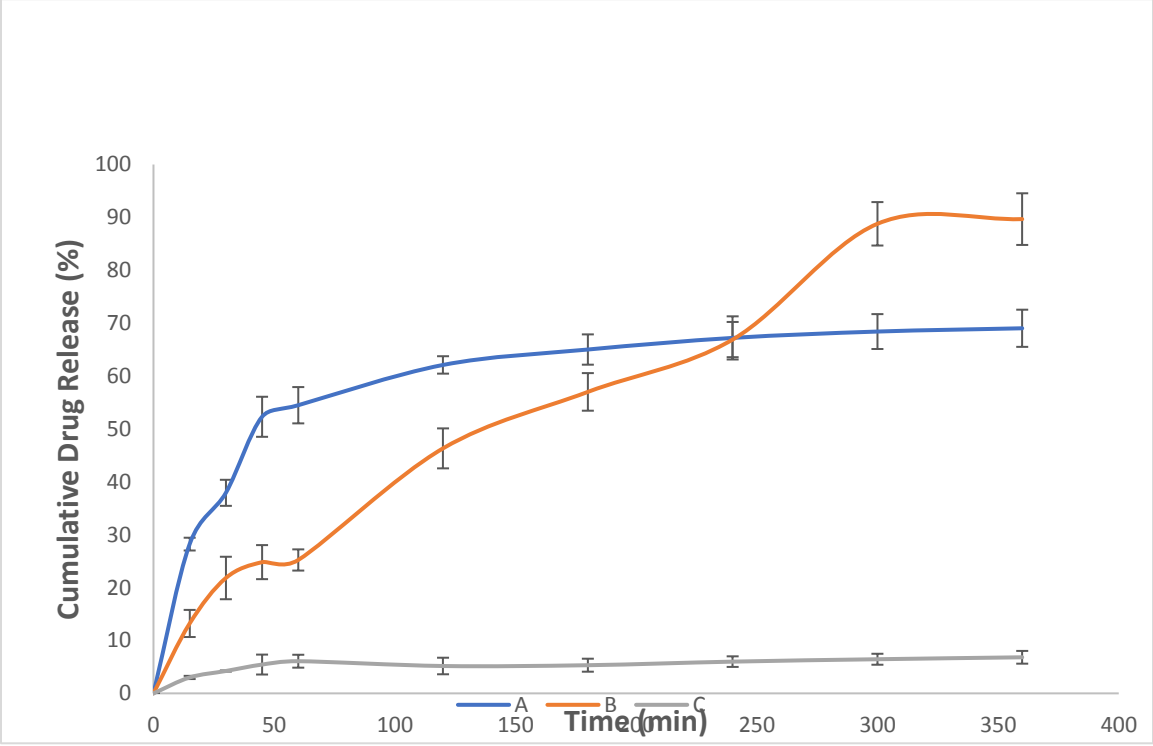


Figure 4.15. *In vitro* drug release studies of MTC loaded ISG formulation

A- Unloaded liposome with gellan gum

B- Loaded liposome with gellan gum

C- MTC with gellan gum

By comparing MTC-GG, LL-GG, and UL-GG, in-vitro drug release behavior was investigated. Krebs buffer solution (pH: 6.6) was employed as the diffusion medium for the drug release tests of formulations, which were carried out utilizing the Franz diffusion cell and dialysis membrane. Figure 4.15 displays the release profiles for the formulations. Cumulative drug release from the MTC loaded liposome incorporated with gellan gum formulations after 5 h was found to be more than 80 %. The inflection point in the release profiles showed that gel was forming in the diffusion cell's donor compartment. A portion of the drug may be loaded into the gel matrix during gel formation; as a result, the drug release rate is slowed down by the polymer's cross-linking. Additional findings revealed that the highest amount of the drug released in the first hour mostly depended on the fact that in the Loaded liposome incorporated with gellan gum, the liposome was used as drug carriers, and during the course of the investigation, this pattern continued.

4.15. Ex vivo Release Study

Ex vivo permeation tests on the sheep nasal mucosa were performed on formulations A and B. For formulation A, it was discovered that more than 80% of the drug had permeated after 5 hours. Additionally, the permeability coefficient (P) was determined.

In order to compare the liposomal MTC-loaded gel to the same gel formulation carrying the simple medication, ex-vivo penetration tests via excised nasal sheep mucosa were performed. The findings of these experiments are given in Table 4.10 below, along with the total quantity of MTC that was absorbed, apparent permeability coefficient, and drug permeation patterns. The new gel formulation was really able to enable a controlled and extended distribution of the medication for more than 5 h in both situations, as can be

shown by the observation of a virtually linear penetration profile of MTC.

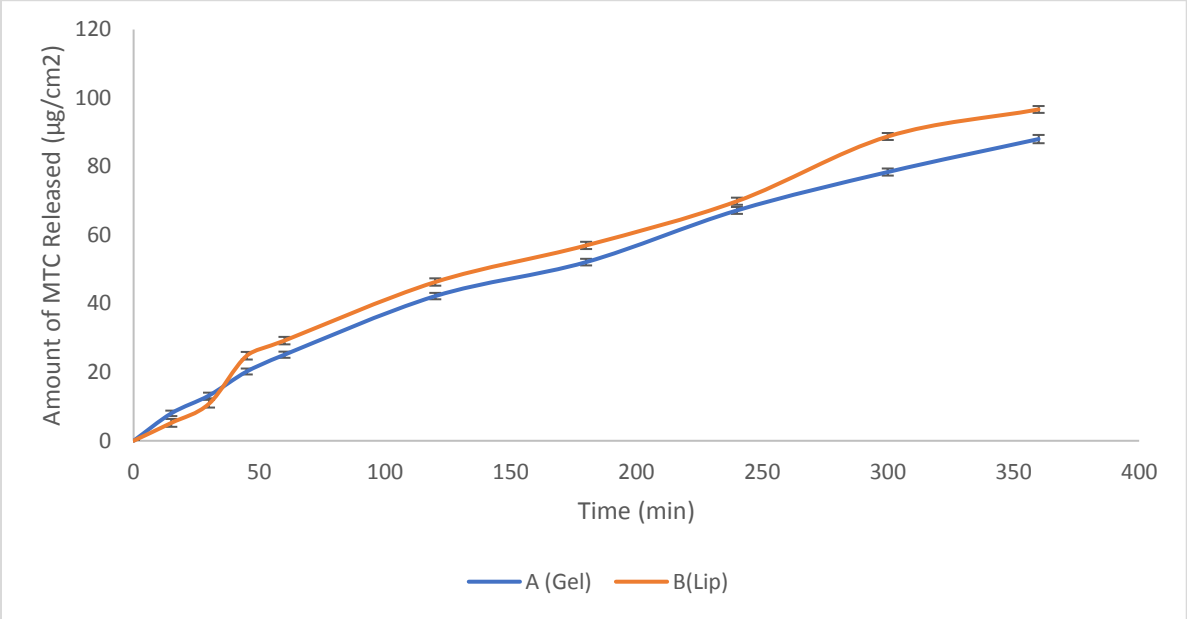


Figure 4.16. Ex-vivo Release Study showing Drug Permeation Profiles

Table 4.10. Results of Ex-vivo Release Study in terms of AUC

	Amount of MTC Release (%)	Jss (µg/cm2.h)	Pcoeff (cm/h)
A	77.29± 0.28[a]	32.28 ± 0.82	17.76 ± 0.39
B	57.11± 0.15[b]	30.19± 0.76	17.11 ± 0.26

5.DISCUSSION and CONCLUSION

This study is done to point on the potential of an intranasal innovative composite formulation based on liposomes included in-situ gels in terms ease of administration, accuracy of dosing, prolonged nasal residence, and improved drug bioavailability. Based on or work, The optimal liposomal formulation was determined using D-optimal mixture experimental design. All the prepared formulations, there values of polydispersity index were lower than 0.6, which indicates monomodal size distributions. Also, the particle size of the MTC loaded liposomes were small, the formulation's mean particle was smaller than 150 nm. The encapsulation efficiency of MTC in liposomal formulation was between 71% and 94%. Furthermore, in this work, zeta potential values were negative for all our API-Lip formulations. In the present study, MTC loaded liposome were prepared and incorporated with gellan gum solution. Furthermore, MTC loaded in situ gel preparation and characterization studies were performed, in addition to liposomal morphology, determination of mechanical properties, rheological behavior, ex vivo and in vitro release study. Based on observed data the MTC loaded liposome formulation incorporated with gellan gum was showing significant results. Cumulative drug release from the MTC loaded liposome incorporated with gellan gum formulations after 5 h was found to be more than 80%. This study might point to the potential of an intranasal innovative composite formulation taken together, liposomes included the nasal in situ gel in terms ease of administration, drug dosing accuracy, good capability for reduction of the nasal mucociliary transport time with prolongation of residence time at the site of absorption, the avoidance of antiemetic drug's bitter taste and improved drug bioavailability. Therefore, it could be taken into consideration in pharmaceutical industries in the future.

I'd also like to add that this thesis topic was orally presented in **"20th International Pharmaceutical Technology Symposium-IPTS 2022"** which was held online between February 21-23, 2022.

Letter of Acceptance for the oral presentation:

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Dear Meryem Aydin, February 1, 2022

Thank you for your abstract submission to **20th International Pharmaceutical Technology Symposium-IPTS 2022,** which will be held online between February 21-23, 2022.

We would like to inform you that your abstract entitled **"Preparation and Characterization of Metoclopramide Nanostructured Lipid Carrier Loaded In Situ Gel"** has been accepted by the scientific committee for oral presentation. You can find the details of your presentation below.

We look forward to your presentation at the symposium.

Best regards,

Prof. Erem Bilensoy
Co-Chair of IPTS 2022

Prof. Levent Öner
Chair of IPTS 2022

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