Research

Formulation, development & evaluation of mucoadhesive buccal film containing mono-ammonium glycyrrhizinate for oral ulcers.

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ABSTRACT:

The present study focused on the formulation, development, and evaluation of a mucoadhesive buccal film containing mono-ammonium glycyrrhizinate (MAG) a bioactive compound extracted from Glycyrrhiza glabra (licorice root), for potential therapeutic application in the management of oral ulcers. To identify an optimal formulation, approximately 30 trial batches were initially prepared by varying concentrations of key polymers and further evaluated for different parameters. Based on the results, one batch was selected for further optimization using factorial design. The buccal film was prepared by the solvent casting method using mucoadhesive polymers Carbopol 934 and HPMC K100, where HPMC K100 was added for its film forming property and propylene glycol as a plasticizer. MAG, known for its anti-inflammatory, antimicrobial, and wound-healing properties, was incorporated as the active agent. Optimization of polymer and drug concentration was carried out using central composite design using design expert software, with batch F1 selected based on desirable properties such as thickness (0.15 mm), weight (66.96 mg), folding endurance (>300), drug content (99.8%), and ex-vivo muco-adhesion time (7.55 hrs.). In-vitro drug release showed sustained release with over 90% cumulative drug released in 6 hours, following first-order kinetics and diffusion-based mechanisms. Ex-vivo permeation and retention studies confirmed better mucosal retention (39.37%) and permeation of (37.18%) of MAG from the film, compared to the pure drug. The anti-inflammatory activity of MAG showed concentration-dependent inhibition (up to 70.55%) using the egg albumin denaturation assay. In-silico antifungal docking of MAG against Candida albicans protein (5TZ1) demonstrated strong binding affinity (-9 kcal/mol), supporting its potential antifungal activity. DSC analysis confirmed the amorphous dispersion of the drug within the polymer matrix, improving solubility. The film exhibited satisfactory tensile strength (54.4 mJ) and mucoadhesive strength (26 mJ). Stability studies revealed no significant changes in physicochemical parameters after one month. Overall, the optimized MAG buccal film showed potential as an effective, stable, and sustained-release formulation for localized treatment of oral ulcers.

KEYWORDS: Mono-ammonium glycyrrhizinate, in-silico antifungal activity, ex-vivo study, mucoadhesive film, oral ulcers.

INTRODUCTION :

Since the early 1980s, the concept of muco-adhesion has gained considerable interest in pharmaceutical technology. Adhesion can be defined as the bond produced by contact between a pressure sensitive adhesive and a surface.[1] Mucoadhesive drug delivery systems are getting more attention because they help medicines stay longer at the site of application, especially on mucosal surfaces like the mouth, nose, or vagina. These systems can improve how much of the drug is absorbed into the body and how well it works. Since the mucosal areas have a large surface and good blood supply, drugs can be absorbed quickly and more effectively.
Also, delivering drugs through the mucosa avoids the liver's first-pass metabolism and breakdown by stomach enzymes. This makes it a useful method for delivering sensitive or large-sized drugs like peptides and genetic materials (like oligonucleotides).[2]

Mucoadhesive drug delivery systems can be delivered by various routes:

1. Buccal delivery system
2. Oral delivery system
3. Vaginal delivery system
4. Rectal delivery system
5. Nasal delivery system
6. Ocular delivery system[3]

1. Mucoadhesive oral drug delivery system

Oral drug administration is the preferred and most common route for drug delivery. Several advantages of oral route-

1. It is patient-friendly.
2. Ease of self-medication.
3. Allows for a flexible and controlled dosing schedule in comparison to most other drug delivery systems.
4. Avoidance of first pass metabolism.
5. Increased bioavailability.
6. Increased residence time.
7. Enhanced therapeutic activity. [4]

Although the oral route is preferred for administration of drugs, it also presents major disadvantages such as first pass effect, gastrointestinal enzymatic degradation and delay between the time of administration and absorption, which is detrimental in the case of drugs with rapid onset requirements.
Within the oral mucosal cavity, delivery of drugs is broadly classified into two categories: a. Sublingual delivery, which is systemic delivery of drugs through the mucosal membranes lining the floor of the mouth and b. Buccal delivery, which is drug administration through mucosal membranes lining the cheeks (buccal mucosa).[5]

2. Oral ulcers

Ulcers are lesions on the surface of the skin or mucous membrane characterized by a superficial loss of tissue. There are many varieties of ulcers such as mouth ulcer, oesophagus ulcer, peptic ulcer and genital ulcer. Mouth ulcers/sores are common ailments that affect many people at some point in their life cycle. The sores appear on any of the soft tissues of the mouth including lips, cheeks, gums, tongue and floor and roof of the mouth as shown in Figure 1.[6] They appear to be round, white or grey, often swollen and red on the edges. If the mouth ulcers last for less three weeks, they are called “acute,” while if they last longer than 3 weeks, they are termed “chronic”. Mouth ulcers can be recurring in some circumstances.[7]

3. Oral cavity

The oral cavity (mouth) is divided into two main parts:

1. Oral vestibule – the space between your lips/cheeks and your teeth/jaw.
2. Oral cavity proper – the area inside your teeth, within the dental arches.

3.1 Anatomy & physiology of oral mucosa

The oral mucosa (lining of the mouth) has three main layers:

1. Stratified squamous epithelium – the outer layer.
2. Basement membrane – a thin layer under the epithelium.
3. Connective tissue – made up of the lamina propria and submucosa.

3.2 Permeability

The buccal mucosa (inside of the cheek) lets substances through 4 to 4000 times more than the skin, but less than the intestines.

In the mouth, permeability from highest to lowest is:

1. Sublingual (under the tongue) – most permeable
2. Buccal (cheek)
3. Palatal (roof of the mouth) – least permeable
3. This difference in permeability is due to the structural characteristics of each part.

3.3 Composition of Mucus

Mucus is a gel-like transparent secretion that plays a vital protective role in the oral cavity. It is primarily composed of water (95–99%), along with water-insoluble glycoproteins (mucins) that make up (1–5%) of its content. In addition, it contains enzymes, proteins, electrolytes, and nucleic acids. Functionally, mucus acts as a viscoelastic hydrogel, forming a protective barrier between the epithelial cells and the external environment, aiding in lubrication, hydration, and defence. Mucus also plays a crucial role in the retention and bioavailability of mucoadhesive drug delivery systems. Its negatively charged mucins can interact with positively charged polymers, enhancing adhesion and drug residence time.[8]

3.4 Types of Mucosae

There are three types of oral mucosa (lining tissues) in the mouth

a) Masticatory Mucosa
b) Specialized Mucosa
c) Lining Mucosa

Figure 1. Oral ulcers

4. Muco-adhesion & mechanism of muco-adhesion:

4.1 Muco-adhesion:

The word Muco-adhesion means – muco - mucus layer and adhesion – binding or attachment. It is defined as an interfacial phenomenon, where two materials are held together by interfacial attraction. Here one of the materials is artificial substance such as mucoadhesive polymer and second material is mucus layer.

4.2 Mechanism of muco-adhesion:

There are mainly 2 stages in the process of muco-adhesion:

a) Contact stage: here the mucoadhesive material is in tight contact with the mucus layer and wetting occurs.

b) Consolidation stage: here different physical and chemical attractive forces help the mucoadhesive material to bind to the mucus membrane and cause long lasting adhesion. After these two stages, the process of muco-adhesion is completed. (Figure 2 & 3)

Essentially, there are two theories explaining the consolidation step:

1. **Diffusion theory:** The diffusion theory and the dehydration theory According to diffusion theory, the mucoadhesive molecules and the glycoproteins of the mucus mutually interact by means of interpenetration of their chains and the building of secondary bonds.
2. **Dehydration theory:** According to dehydration theory, materials that are able to readily jellify in an aqueous environment, when placed in contact with the mucus can cause its dehydration due to the difference of osmotic pressure. The difference in concentration

gradient draws the water into the formulation until the osmotic balance is reached. This process leads to the mixture of formulation and mucus and can thus increase contact time with the mucous membrane.

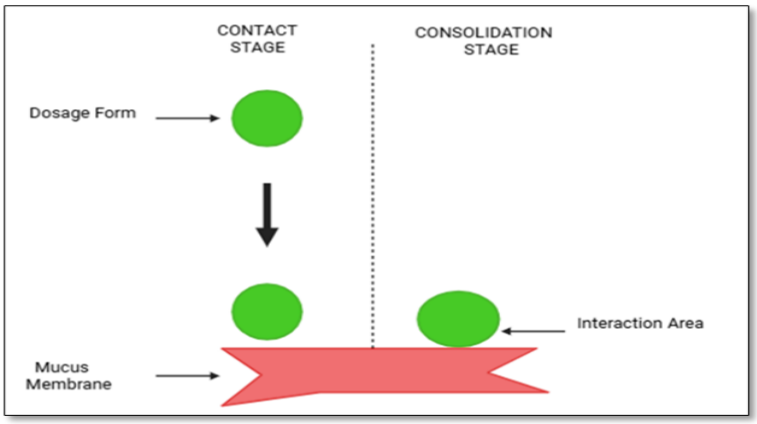


Figure 2. Mechanism of muco-adhesion

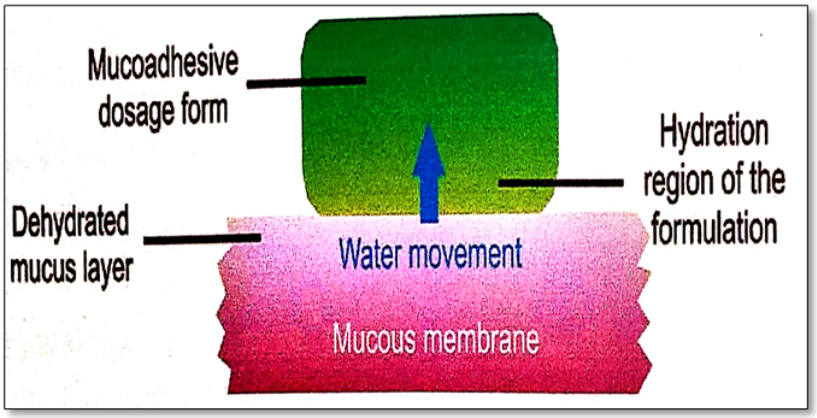


Figure 3. Dehydration theory

4.3 Advantages of oral mucoadhesive drug delivery systems:

1. Rapid onset of action.
2. Improved patient compliance.
3. Rapid absorption.
4. Prolonged residence time of dosage form.
5. Avoids first pass metabolism.

4.4 Disadvantages of mucoadhesive drug delivery systems

1. Potential for irritation.
2. Limited drug loading capacity.
3. In ocular formulation, the formulation might cause uneasiness and blur vision.[9]

5. Theories of Muco-adhesion:

Theories of muco-adhesion are classified into 2:(Figure 1.4)

1. Chemical theories
2. Physical theories

5.1 Chemical theories

5.1.1 Electronic theory

According to this theory, the mucoadhesive polymer and the mucus membrane has opposite electric charges and when they both come in contact with each other transfer of electron occurs causing formation of electric double layer at the interface.

This attractive forces within the double electric layer determines the mucoadhesive strength.[10]

5.1.2 Adsorption theory

According to this theory, the mucoadhesive polymer adheres or adsorbs on the mucus membrane by many secondary forces/ intermolecular forces such as- wander waal's forces, hydrogen bonds, electrostatic attractions, etc.[11]

5.2 Physical theories

5.2.1 Diffusion theory

This theory describes the interpenetration or diffusion of polymer and mucin chains to a depth resulting in formation of adhesive bond. The adhesive force increases with increase in penetration rate of polymer chains. The penetration rate depends on nature of mucoadhesive chains, contact time, diffusion coefficient etc.… (Figure 4.)

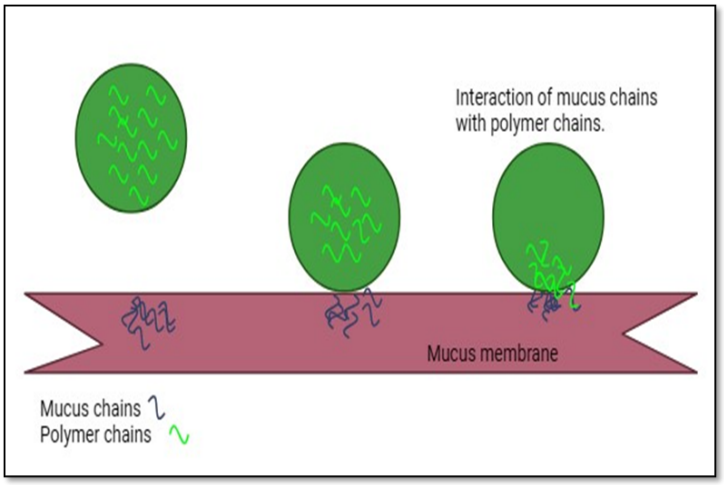


Figure 4. Diffusion theory

5.2.2 Wetting theory

This theory is generally applicable for liquids. It describes the ability of liquids to spread on the mucus surface for development of adhesion, and it states that lower the contact angle of liquid on the surface greater is the adhesion.[12] (Figure 5.)

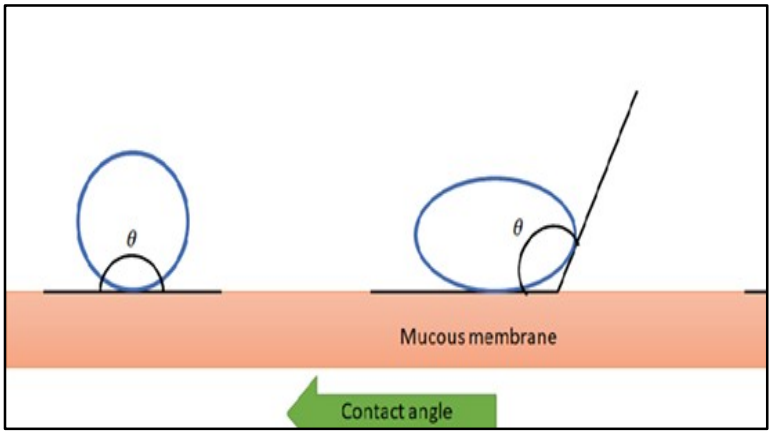


Figure 5. Wetting theory

5.2.3 Fracture theory

This theory describes the strength required to detach the two surfaces involved after adhesion. The maximum tensile strength by the ratio of the maximal detachment force Fm to the total surface area A0, which involves the adhesion interaction. (Figure 6.)

[Sm = Fm / A0] [13]

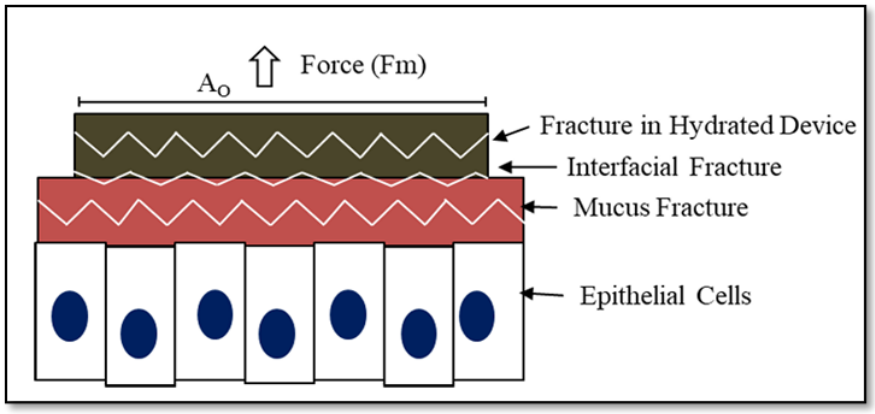


Figure 6. Fracture theory

5.2.4 Mechanical theory

This theory explains the formation of an interlocked structure when the liquid diffuses into the rough/irregular surface which results in adhesion. The rough surface provides greater surface area for interaction.[14] (Figure 7.)

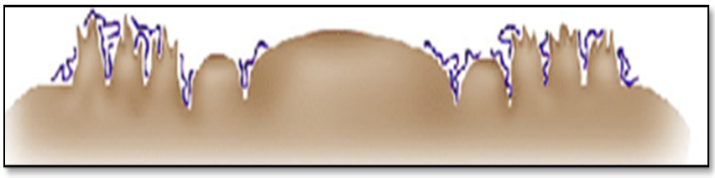


Figure 7. Mechanical theory

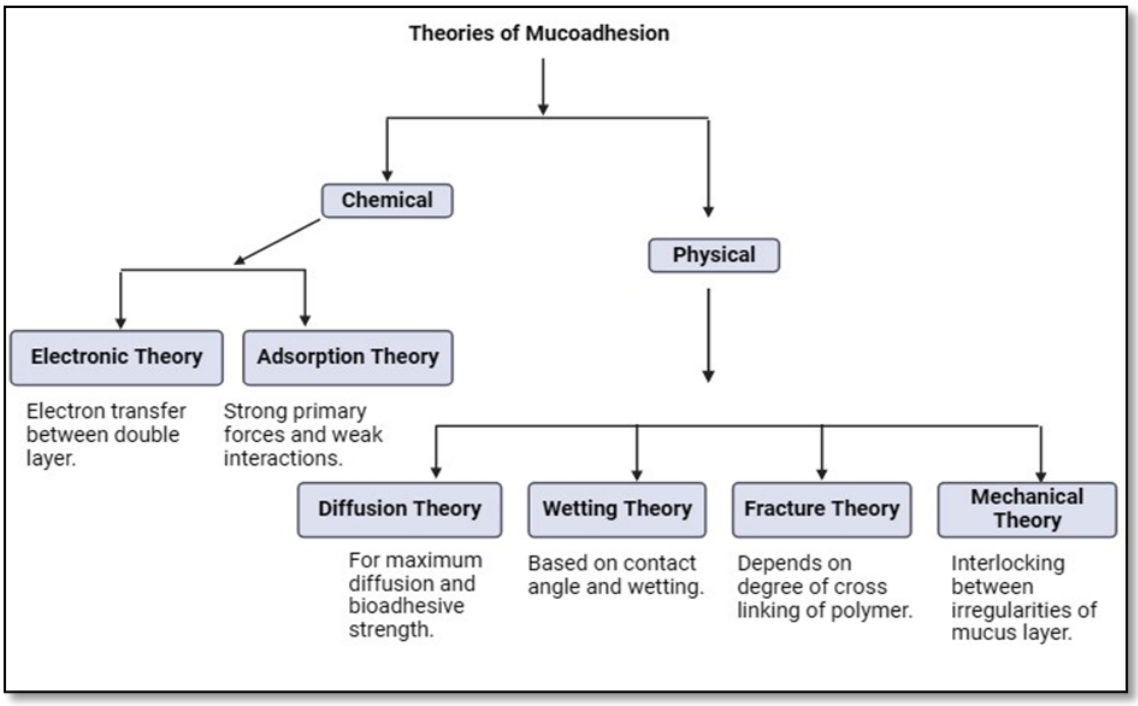


Figure 8. Theories of muco-adhesion

6. Mucoadhesive Films as Drug Delivery System

Films are a new type of medicine form that are becoming popular because they are easy to use, patient-friendly, and convenient. Recently, a special type called orally disintegrating films (or strips) has been developed. These films are stronger and less
likely to break than many of the mouth-dissolving tablets we already have, which often need special packaging. Mucoadhesive buccal films are another type of film.

Mucoadhesive films are retentive dose forms and release drug directly into a biological substrate. They have the same benefits and even more:

a) They are small and thin, so they are easier for patients to use compared to tablets.
b) They stick to the inside of the mouth (the cheek area), so the medicine can either:
i. Act locally (right where the film is placed, for example, to treat infections like oral thrush).
ii. Act systemically (enter the bloodstream through the cheek to affect the whole body).

Depending on how they are made, these films can:

a) Release medicine into the mouth
b) Release medicine through the cheek into the bloodstream, which bypasses the liver's first-pass metabolism. This can make the medicine work faster and more effectively.[15]

7. Methods to manufacture films

Several methods have been explored for making mucoadhesive buccal films, such as solvent casting, hot melt extrusion, inkjet printing, and 3D printing. Among these, solvent casting is the most commonly used because it is simple and low-cost.

Solvent casting

In this method, a casting solution is made by first dissolving water-soluble ingredients like polymers to create a thick, smooth liquid. Then, the drug (API) and other ingredients are mixed into it. This solution is spread out and dried to form a thin film. Finally, the film is cut into pieces that contain the right amount of the drug.

The process is simple, reproducible, and well-established, making it a convenient choice for formulation. However, it also has several limitations. The incorporation of small plasticizing molecules can alter the mechanical properties of the film, potentially affecting its performance. Achieving uniform drug distribution across the film can be challenging, and there is a risk of air bubble entrapment during preparation. Additionally, there is limited control over critical parameters such as film thickness and polymer concentration, which may impact the consistency and efficacy of the final product. (Figure 9.)

8. Types of mucoadhesive polymers

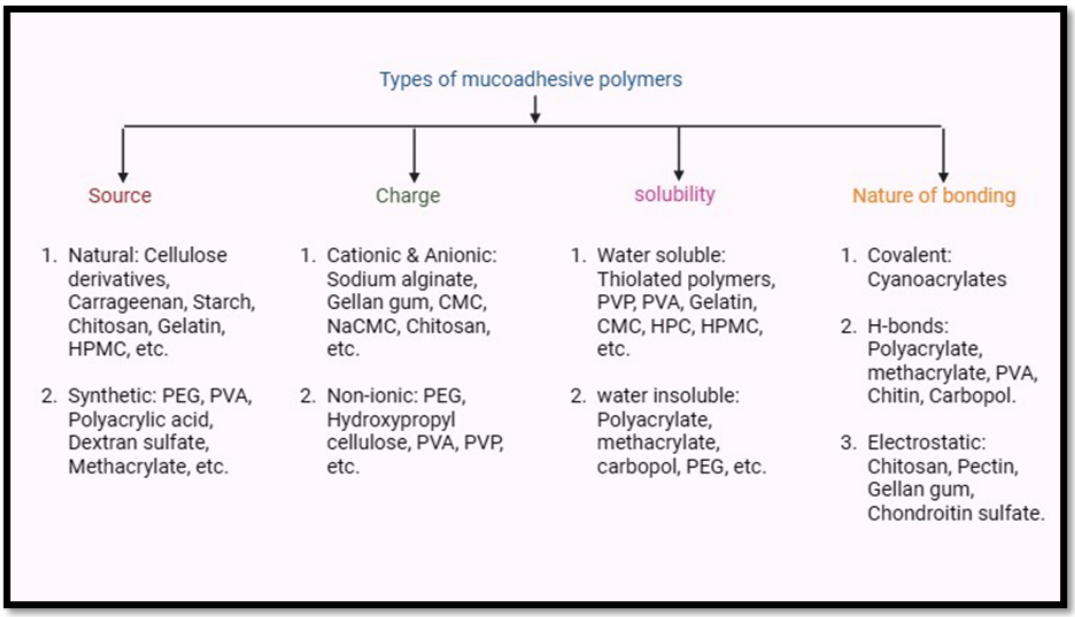


Figure 10. Types of mucoadhesive polymers

9. Mono-ammonium glycyrrhizinate in ulcer treatment

Glycyrrhiza Glabra also called as licorice, liquorice, sweet wood etc... is the most popular medicinal plant belonging to the Fabaceae family (also known as Leguminosae). It contains many chemical compounds such as flavonoids, triterpene saponin, etc…which are found to be useful for treating various oral and other diseases. Several active compounds are found in the roots of licorice including glycyrrhizin, glycyrrhizinc acid, glabrol, glycyrol etc. The glycyrrhizinic acid (GLA) or glycyrrhizin is the main active compound of liquorice and is a β-amyrin-type triterpenoid saponin and has various properties like anti-inflammatory, neuroprotective, antiviral etc…[16] Glycyrrhizin a triterpenoid glycoside is soluble in water, whereas the acid form of glycyrrhizic acid is not water soluble, but the ammonium salt of glycyrrhizic acid i.e. Mono-ammonium Glycyrrhizinate (MAG) is soluble in water and has many activities including anti-allergic, anti-cancer and many more.[17]

The ammonium salt of glycyrrhizic acid is characterized by an anti-inflammatory activity, anti-ulcer, anti-allergic, antidote, antioxidant, antiviral, anti-tumor, anticonvulsant activity.[18] MAG show anti-ulcer, anti-inflammatory, anti-bacterial, anti-viral, which play a major role in curing mouth ulcers, but the major reason for a selection of this drug is its sweet test which is mainly not seen in other drugs, so this property increases patient compliance.[19] During ulcers inflammation also occurs & GA shows anti-inflammatory effects by reducing the levels of IFN-γ, IL-12 and IL-17, and increasing the levels of IL-10, and modulating the balance between TNF-α and IL-10.[20]

10. Why only buccal film and not any other formulations

1. Local and Systemic Action

MAG can be used locally for treating oral inflammatory or viral conditions (e.g., oral ulcers, herpes simplex).It can also act systemically if absorbed through the mucosa, making buccal films versatile for both local and systemic therapies.

2. Patient Compliance and Convenience

Buccal films are easy to administer, making them particularly suitable for children and elderly patients who may have difficulty swallowing conventional tablets or capsules. They are also beneficial for patients requiring frequent dosing, as the film can dissolve slowly, helping to maintain consistent drug levels over time. Additionally, buccal films are discreet, portable, and convenient, which enhances patient compliance, especially in cases of chronic therapy.

3. Bypasses First-Pass Metabolism

Buccal delivery bypasses the hepatic first-pass metabolism, which is particularly advantageous for drugs like Mono-ammonium glycyrrhizinate (MAG) that have limited oral bioavailability due to extensive liver metabolism. By avoiding the gastrointestinal tract and liver, buccal administration allows more of the active drug to reach the systemic circulation in its intact form, thereby enhancing therapeutic efficacy.

4. Controlled and Sustained Release

Buccal films can be engineered for controlled or sustained drug release, offering prolonged therapeutic effects with reduced dosing frequency. This is beneficial for Mono-ammonium glycyrrhizinate (MAG), as it helps to maintain a steady drug concentration over an extended period, improving treatment outcomes and patient compliance.

5. Stability and Protection

Buccal films offer the advantage of protecting Mono-ammonium glycyrrhizinate (MAG) from degradation in the acidic environment of the stomach, which is a common issue with conventional oral formulations. By avoiding gastric exposure, the formulation enhances the stability of the drug, potentially improving its shelf-life and overall effectivenesss.

Therefore, this study was undertaken to develop and evaluate a buccal film for effective treatment of oral ulcers.

MATERIALS AND METHODS:

Materials

Mono-ammonium glycyrrhizinate was received as a gift sample from Sunpure extracts Pvt. Ltd, Ghaziabad India. Carbopol 934P, HPMC K100 were purchased from Loba chemie Pvt Ltd, Mumbai. All other chemicals were of analytical grade purchased from local suppliers.

Methods of preparation of mucoadhesive buccal film:

Buccal films of Monoammonium glycyrrhizinate were prepared by solvent casting method. HPMC k100 was weighed accurately and dissolved in a beaker containing 10ml distilled water. The beaker was then kept aside for five minutes for swelling of the polymer. Then, the solution was stirred continuously using magnetic stirrer

until it got dissolved. Simultaneously Carbopol 934 & monoammonium glycyrrhizinate was weighed accurately and dissolved in 10ml ethanol in another beaker, stirred continuously on magnetic stirrer until it got dissolved and kept aside. Then, both solutions were mixed together and to this solution propylene glycol was added while continuously stirring on magnetic stirrer. Then, the solution was stirred until the bubbles were removed. The mixed solution was then poured on to the petri plate. After drying overnight, the dried film was covered with aluminium foil and stored in desiccators until further use.[21]

PREFORMULATION STUDY

Organoleptic properties

1. Description
The sample of Mono-ammonium glycyrrhizinate was studied for organoleptic characters such as color, odor and appearance.

2. Melting point determination
Melting point was determined by open capillary method using melting point apparatus. The drug was placed in a sealed glass capillary and submerged in liquid paraffin within the melting point device. The temperature at which the drug melted was recorded. Precautions were taken to maintain the uniform heating of apparatus, in which the capillary containing drug was placed.

3. Fourier transform infrared spectroscopy (FT-IR)
Attenuated total reflectance (ATR) FT-IR was used for recording the peaks of functional group present. The sample of Mono-ammonium glycyrrhizinate was placed on the crystal of ATR-FTIR & the spectrum was recorded. The spectrum was scanned over the wave number range of 4000 to 400 cm^{-1}.

4. Compatibility study of Mono-ammonium glycyrrhizinate with polymers[22]
In this study, the drug-excipients physical mixtures were prepared on 1:1 ratio, weighed accurately and then they were gently mixed in a mortar. So, each method was applied on pure samples of drug and polymers individually and drug-polymer mixture separately. Then freshly prepared samples of pure drug, pure polymer and drug-polymer physical mixture were analyzed and evaluated as follows-

1. **Fourier Transform Infrared Analysis (FTIR) Studies:** FTIR is a procedure to identify the drug and it also to detect the compatibility of baclofen with used polymers. FTIR data of dried samples of pure MAG, polymers and physical mixtures of them were recorded in FTIR spectrophotometer using the potassium bromide disc method. Each sample was pulverized and intimately mixed with dried IR grade KBr powder at a weight ratio of 1:100, and then pressed using a hydrostatic press at a pressure of 10 tons for 5min at room temperature. The result disc was placed in the sample holder and the spectra were recorded over the wave number range 4000 to 400 cm−1 at a resolution
of 4 cm−1. The location of peak of pure MAG and pure polymer were analyzed and compared with spectra of their physical mixture.

5. Differential scanning calorimetry (DSC)
Differential scanning calorimetry (DSC) is thermal analytical technique in which the difference in the amount of heat required to increase the temperature of a sample and reference are measured as a function of temperature. Both the sample and reference are maintained at nearly the same temperature throughout the experiment. Generally, the temperature program for DSC analysis is designed such that the sample holder temperature increases linearly as a function of time, The reference sample should have a well-defined heat capacity over the range of temperature to be scanned. Differential scanning calorimetric of mono-ammonium
glycyrrhizinate (drug) & formulation was carried out using mettler toledo differential scanning calorimeter. Samples were placed in aluminium crucibles and DSC thermograms were recorded at the heating rate of 10^0C/min in the range of 0^{0C} to200°C. Nitrogen gas was purged at the heating rate of 50-80ml/min to maintain inert atmosphere.

6. Saturated solubility study of mono-ammonium glycyrrhizinate
The saturated solubility of the drug was determined in distilled water, ethanol & phosphate buffer pH 6.8. The 2 mL distilled water, ethanol & buffer pH 6.8 were taken in 5 mL glass vials. An excess amount of drug was added in each vial and closed with stopper. These glass vials were attached in an orbital shaker. The shaking was carried out for 48 hours with the speed of 50 rpm and in the entire study the temperature was maintained around 37 ± 0.5 °C. Then the resulting samples were filtered using Whatman filter paper with its pore size 0.22

μm. The filtrate was collected and after suitable dilutions with the same solvent the absorbance of the drug was analyzed with UV Visible Spectrophotometer (V-1800, Shimadzu, Japan) at the pre-scanned λ_{max} in particular solvent. Then the absorbance was converted into concentration using standard curve of drug in each concern solvent.[23]

Analytical method development

UV-visible spectrophotometric method for drug
a. Study of spectra and selection of analytical wavelength

Determination of absorption maxima value (λ max) was done using ultraviolet (UV)-visible spectrophotometer. Standard stock solution of Mono-ammonium glycyrrhizinate (1000μg/ml) was prepared in distilled water, ethanol & pH 6.8 phosphate buffer. For the selection of analytical wavelength, a solution of Mono-ammonium glycyrrhizinate of concentration 10 μg/ml was prepared by appropriate dilution of the standard stock solution with distilled water, ethanol & phosphate buffer pH 6.8 and scanned in the spectrum range from 200 to 400 nm. From the overlain spectrum of the drug, wavelength 258-260 nm was selected for analysis. The wavelength with maximum absorption was chosen for further analysis.

1. Calibration curve of mono-ammonium glycyrrhizinate in distilled water

The stock solution was freshly prepared by dissolving 10 mg of Mono-ammonium glycyrrhizinate in distilled water in a 10 ml volumetric flask and then making up the solution up to the mark using distilled water for obtaining the solution of strength 1000 μg/ml (stock I). From this primary stock, 1 ml of this solution was diluted to 10 ml with distilled water to obtain a solution of strength 100 μg/ml (stock II). From this secondary stock 1, 2, 3, 4, 5, 6, 7, 8 & 9ml were taken separately and made up to 10 ml with distilled water, to produce 10, 20, 30, 40, 50, 60, 70, 80 and 90 μg/ml, respectively. The absorbance was measured at 258 nm using a UV visible spectrophotometer. Similarly, standard graph of mono-ammonium glycyrrhizinate in distilled water was plotted.

2. Calibration curve of mono-ammonium glycyrrhizinate in ethanol

The stock solution was freshly prepared by dissolving 10 mg of Mono-ammonium glycyrrhizinate in ethanol in a 10 ml volumetric flask and then making up the solution up to the mark using ethanol for obtaining the solution of strength 1000 μg/ml (stock I). From this primary stock, 1 ml of this solution was diluted to 10 ml with ethanol to obtain a solution of strength 100 μg/ml (stock II). From this secondary stock 1, 2, 3, 4, 5, 6, 7, 8 & 9ml were taken separately and made up to 10 ml with ethanol, to produce 10, 20, 30, 40, 50, 60, 70, 80 and 90 μg/ml, respectively. The absorbance was measured at 258 nm using a UV visible spectrophotometer. Similarly, standard graph of mono-ammonium glycyrrhizinate in ethanol was plotted.

3. Calibration curve of drug in phosphate buffer (pH 6.8)

The stock solution was freshly prepared by dissolving 10 mg of Mono-ammonium glycyrrhizinate in phosphate buffer (pH 6.8) in a 10 ml volumetric flask and then making up the solution up to the mark using phosphate buffer (pH 6.8) for obtaining the solution of strength 1000 μg/ml (stock I). From this primary stock, 1 ml of this solution was diluted to 10 ml with phosphate buffer (pH 6.8) to obtain a solution of strength 100 μg/ml (stock II). From this secondary stock 1, 2, 3, 4, 5, 6, 7, 8 & 9ml were taken separately and made up to 10 ml with phosphate buffer (pH 6.8), to produce 10, 20, 30, 40, 50, 60, 70, 80 and 90 μg/ml, respectively. The absorbance was measured at 258 nm using a UV visible spectrophotometer. Similarly, standard graph of mono-ammonium glycyrrhizinate in phosphate buffer (pH 6.8) was plotted.

Optimization of mucoadhesive buccal film containing MAG

The selected trial batch was further used for the optimization process. The concentration of Carbopol 934 & HPMC K100 was chosen as independent factors as they affect the thickness, residence time & weight. Optimization is necessary to determine the ideal combination of these factors that will produce the buccal film with the desired characteristics for their intended use such as patient compliance, longer muco-adhesion time etc. By systematically varying the concentrations of Carbopol 934 & HPMC K100 & then evaluating the resulting buccal film properties, the optimal formulation can be identified to meet the specific requirements of the drug delivery.

Table 1. Optimization of buccal film containing MAG using 3^2 factorial design.

Independent Variable	Levels			Dependent Variable
	-1	0	+1	Thickness
X1= Conc. Of Carbopol 934 (mg)	180	200	220	Muco-adhesion time
X2= Conc. Of HPMC K100 (mg)	180	200	220	Drug content

Statistical parameters & experimental design

Table 2. Formulation design for optimization of MAG buccal film

Batch	Carbopol 934 (mg)	HPMC K100 (mg)	Propylene Glycol (ml)	Distilled water (ml)	Ethanol (ml)	MAG (mg)
F1	200	200	0.4	10	10	130
F2	200	220	0.4	10	10	130
F3	200	180	0.4	10	10	130
F4	220	180	0.4	10	10	130
F5	220	220	0.4	10	10	130
F6	180	180	0.4	10	10	130
F7	180	200	0.4	10	10	130
F8	180	220	0.4	10	10	130
F9	220	200	0.4	10	10	130

Evaluation of factorial batches of MAG buccal film

1. Physical appearance
The colour, transparency and smoothness of all factorial batches were evaluated.

2. Thickness (mm)
Films of (2×2 cm) were cut and thickness of films were measured using digital calliper with a least count of 0.01 mm at 3 different spots of the films and average was taken.

3. Weight variation
Weight variation was observed in films of 2×2 cm. 3-4 films were weighed individually and average was taken.

4. Folding endurance
Strip of prepared film (2 × 2cm) was folded repeatedly at the same place till it broke. The number of times the film could be folded at the place without breaking or cracking is equal to the value of folding endurance.[24]

5. Drug content
Films of 2X2cm were taken and dissolved in 100 ml of phosphate buffer of pH 6.8 in volumetric flask, 1-2ml of this solution was diluted with phosphate buffer (6.8 pH) to 10ml.The percentage drug content was determined by using UV Spectrophotometer by measuring the absorbance at 258nm. The percentage drug content was determined using the standard graph and the same procedure was repeated for three films.[25]

6. Ex-vivo muco-adhesion residence time
The Ex-vivo residence time for buccal film was measured using a locally modified dissolution apparatus USP. The medium was composed of 200mL of phosphate buffer pH6.8 maintained at 37±0:5 °C. A part of goat buccal mucosa of 3cm length was tied to the stirrer. After hydrating the film surface using phosphate buffer pH6.8, the film was set into contact with the mucosal membrane. The stirrer was secured vertically such that there is complete immersion of the film
into the buffer solution at the lowest point and was available out at the highest point. The time taken for the complete detachment of the film from the mucosal surface was recorded.[26]

7. In-vitro dissolution (drug release)

In vitro drug release study was carried out using, magnetic stirrer. The dissolution medium consisted of 50 ml of phosphate buffer pH 6.8. The release was performed at 37 ± 0.5 °C, at 50 rpm. One side of the buccal patch was attached to a glass disk with instant adhesive. The disc was put in the bottom of the beaker so that the patch remained on the upper side of the disk. Samples (1-5 ml) were withdrawn at pre-determined time intervals and replaced with fresh medium. The samples were then assayed spectrophotometrically at 258 nm.

7.1 Mechanism of release kinetics

The mechanism of release was determined by fitting the release data to the various kinetic equations such as zero order, first-order, Higuchi, Korsmeyer–Peppas. r2 values were determined by using DD Solver software.

8. Ex-vivo permeation study

The ex vivo buccal permeation of mono-ammonium glycyrrhizinate film was studied using fresh sheep buccal mucosa as a barrier. The buccal mucosa was collected from a freshly slaughtered sheep at a local slaughterhouse and used within 2 hours. The tissue was carefully cut and cleaned first with distilled water and then with phosphate buffer solution (pH 6.8). The test was done using a modified Franz diffusion cell, kept at a constant temperature of 37°C (±0.2°C). A small film (2 × 2 cm) of each test formulation was placed directly on the sheep cheek tissue. A Teflon bead was placed in the bottom compartment, which was filled with 15 mL of phosphate buffer at pH 6.8. This compartment was stirred with a magnetic stirrer to keep the liquid moving, and the temperature was maintained. Samples were taken at fixed time intervals, diluted as needed, and analyzed using a UV spectrophotometer at 258 nm. Each test was repeated three times (n = 3).[27]

9. Retention study

Retention experiment was performed in the same conditions of permeation experiment. The formulation was removed & buccal mucosa was extracted in order to quantify the MAG retained into the tissue. The extraction was performed with 50ml of PBS pH6.8 at 45°C for 1-2hr. Later 1-2ml of this solution was measured using UV-spectrophotometer and from the value of absorbance the % of drug retained in the tissue was calculated.[28]

10. In Vitro Anti-inflammatory Activity of Mono-ammonium glycyrrhizinate using egg albumin denaturation method

10.1 Preparation of reaction mixture

Non steroid (diclofenac sodium) was used as reference drug. About 10mg of diclofenac sodium drug powder was measured using a digital analytical balance and was added to 10 ml of ethanol, respectively. The solution was mixed well using a sonicator. Serial dilutions of 1000, 500, 200,100 & 10 µg/ml was prepared of mono-ammonium glycyrrhizinate and reference drug (diclofenac sodium). All samples contained 5.0 ml of total volume.

Reaction mixtures were prepared using 2.8 ml of phosphate-buffered saline (pH 6.8) and 0.2 ml of egg albumin solution. Then 2 ml of mono-ammonium glycyrrhizinate solution from each different concentration was mixed gently with reaction mixtures. A similar procedure was used for reference drug (diclofenac sodium) and they were used as positive controls for this study. The reaction mixtures were then incubated at 37±2°C for 30min & later heated in water bath at 70±2°C. After cooling, the absorbance was measured using calorimeter at 600nm. The % inhibition of protein denaturation was determined using the following equation.[29]

$$\% \text{ inhibition} = \frac{abs\ of\ control - abs\ of\ test}{abs\ of\ control} \times 100$$

11. Determination of In-silico Anti-fungal activity

In-silico antifungal activity of MAG (Active Compound-Glycyrrhizin) was performed against fungal protein of candida albicans (5TZ1).

Binding affinity

1. Protein preparation (5TZ1):

The crystal structure of sterol 14 alpha demethylase (CYP51) from Candida albicans in complex with the tetrazole-based antifungal drug candidate VT1161 (VT1) (PDB Id: 5TZ1) was used from the protein data bank

(PDB). Protein was prepared by removing water molecules, ligands, heteroatoms, and unnecessary chains. Hydrogen atoms were added, and energy minimization was performed using Discovery Studio or PyMOL to reduce steric hindrance.

2. Ligand preparation:
Some selected compounds were obtained from PubChem or drawn manually. The structures were energy minimized to achieve stable conformations. Ligands were converted to PDBQT format via Open Babel. Lipinski's rule of five was applied to assess drug-likeness.

3. Molecular docking (PyRx):
Molecular docking was performed using PyRx (Auto Dock Vina). The prepared protein and ligands were imported, and a grid box was defined around the active site of 5TZ1 (identified via literature and Discovery Studio). Docking was executed to obtain binding affinity values (kcal/mol). The best binding poses were selected based on lowest binding energy and RMSD values.

4. Docking analysis (Discovery studio & PyMOL):
Discovery studio was used to visualize key molecular interactions such as hydrogen bonding, hydrophobic, and electrostatic interactions. 2D and 3D interaction diagrams were generated. PyMOL was used to superimpose complexes, visualize binding conformations, and measure distances between ligands and active site residues.[30]

5.8 Accelerated stability studies
This stability study was done according to the ICH guidelines (Q1A(R2)), which required testing at 40℃ ± 2℃/75% RH ± 5% RH for a minimum of 6 months, the selected formula stored in the oven for 6 months, and all the evaluation parameters were measured & the drug content of Monoammonium glycyrrhizinate was measured using UV absorbance at λ max (258nm).[31]

RESULT:

Pre-formulation Studies

1. Organoleptic properties
The observed organoleptic properties of mono-ammonium glycyrrhizinate are mentioned in Table 3. and Figure 12.

Table 3. Organoleptic properties of mono-ammonium glycyrrhizinate.

Color	white
Texture	Amorphous powder
Odor	sweet

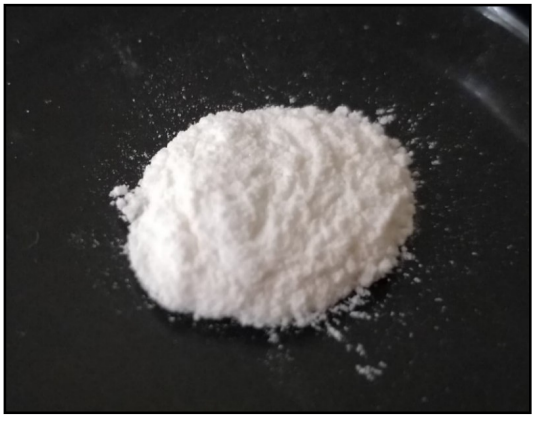


Figure 12. Monoammonium glycyrrhizinate powder

2. Melting Point Determination

The melting point of mono-ammonium glycyrrhizinate was found to be in the range of **196-209℃**, which matches with the standard reported melting point i.e. 190-217℃.

3. Fourier transform infrared (FTIR) spectroscopy study of Mono-ammonium glycyrrhizinate

FTIR analysis of mono-ammonium glycyrrhizinate (MAG) showed characteristic peaks confirming the presence of key functional groups, given in Table 4 & Figure 13. A strong peak at 1711 cm^{-1} indicated the C=O stretching of a carboxylic or ester group. Broad peaks in the range 3393–3298 cm^{-1} corresponded to O–H and N–H stretching, suggesting hydroxyl and amine groups. Aromatic C–H stretching appeared near 3109 cm^{-1}, while C–H (alkyl) stretching was observed around 2950–2842 cm^{-1}. Peaks between 1587–1419 cm^{-1} confirmed aromatic C=C bonds, and C–N / C–O stretching was evident between 1323–1047 cm^{-1}. Peaks above 3600 cm^{-1} suggested the presence of free hydroxyl groups, confirming the structure and purity of MAG.

Table 4. Interpretation of FTIR spectrum of Mono-ammonium glycyrrhizinate.

Functional group	Standard frequency (cm^{-1})	Observed frequency (cm^{-1})
Sharp, strong free O–H	3400 - 3900	3646
O–H / N–H	3200-3500	3298
Aromatic C–H	3030- 3100	3109
Aliphatic C–H	2850- 2960	2842
Carboxyl (C=O)	1680- 1750	1711
Aromatic C=C	1580-1600	1587
C–N (Ester or amine/ether stretching)	1240- 1340	1323, 1207 1047
Aromatic C–H bending	650-900	965 – 656

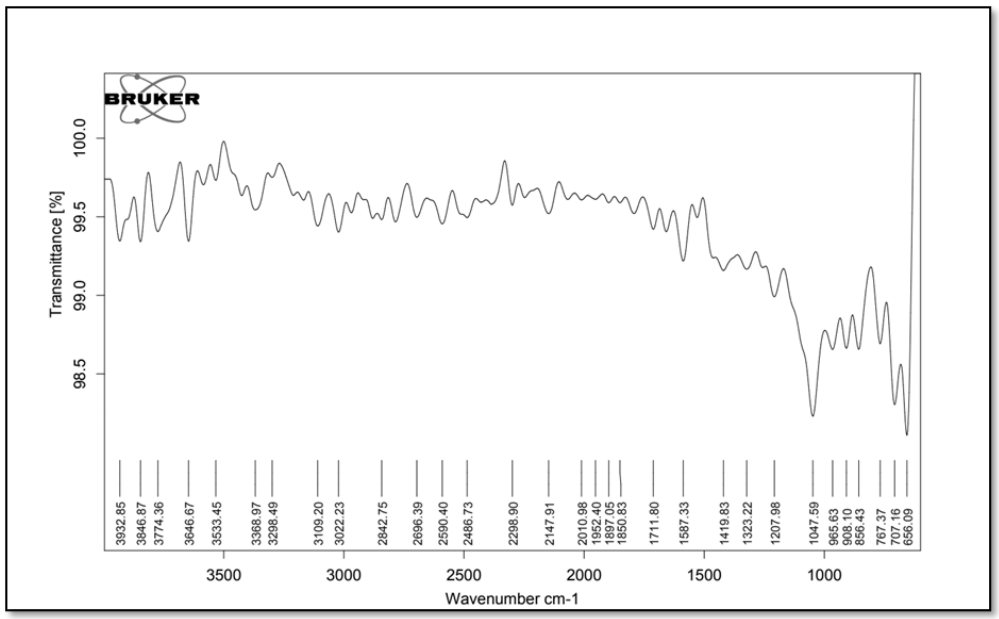


Figure 13. FTIR of pure MAG

4. Compatibility study of drug with excipients

The compatibility of monoammonium glycyrrhizinate (MAG) with various excipients (Carbopol 934, HPMC K100, and Propylene Glycol) was assessed using Fourier transform infrared (FTIR) spectroscopy.

The characteristic peaks of MAG were observed at ~3400 cm^{-1} (O–H/N–H stretching), ~2930 cm^{-1} (C–H stretching), ~1700 cm^{-1} (C=O stretching), and ~1000–1200 cm^{-1} (C–O stretching), as seen in Figure 14.

These peaks were preserved in the spectra of all formulations and physical mixtures, with only minor broadening or shifts in the O–H region, indicating possible hydrogen bonding or physical interactions. No new peaks or disappearance of functional groups were observed, suggesting no chemical incompatibility. Therefore, FTIR analysis confirmed the compatibility of MAG with all selected excipients, and the formulation is stable in terms of chemical structure, supporting their use in the formulation.

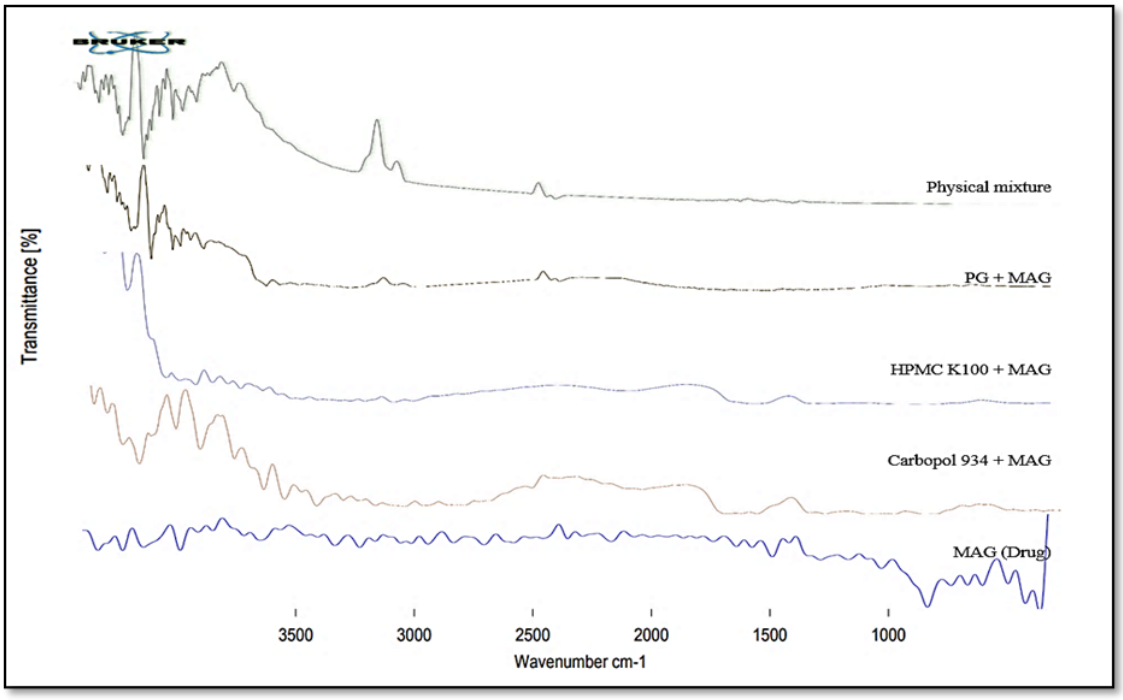


Figure 14. FTIR analysis showing compatibility of MAG with excipients

5. DSC

The DSC thermogram of monoammonium glycyrrhizinate (MAG) was obtained using a Mettler DSC system. The thermogram exhibited two significant thermal events as shown in Figure 15:

1. **First Endothermic Transition:**
   Peak Temperature: 86.05 °C
   This broad endothermic event is likely attributed to the loss of water of crystallization or a crystalline phase transition. It is not associated with the actual melting point of the compound.
2. **Second Sharp Endothermic Peak:**
   Observed at approximately 210–230 °C, this peak corresponds to the melting point of monoammonium glycyrrhizinate. This sharp endothermic event indicates the melting
   and/or initial decomposition of the compound, consistent with literature values for
   MAG. The total enthalpy change (ΔH) associated with these transitions,
   calculated as –131.57 J/g, supports the presence of a significant thermal event in this range.

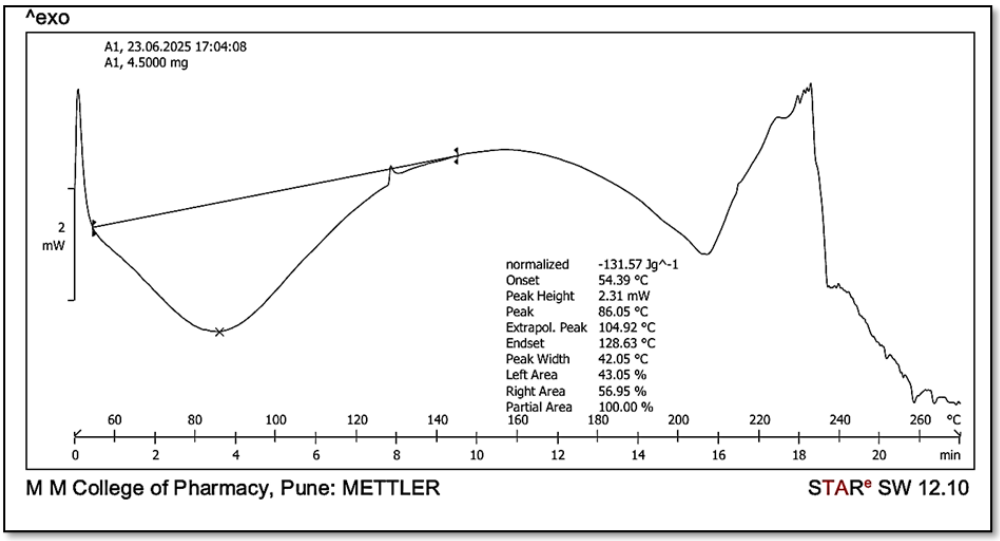


Figure 15. DSC of pure MAG

6. Saturated solubility study of Mono-ammonium glycyrrhizinate

Table 5 & Figure 16 shows the solubility of Mono-ammonium glycyrrhizinate in water, ethanol & phosphate buffer (pH6.8). Mono-ammonium glycyrrhizinate is found to be slightly soluble in water & ethanol, whereas it was found to be sparingly soluble in phosphate buffer (pH 6.8).

Table 5. Saturated solubility of MAG in various solvents.

Sr. No.	Solvent	Solubility (mg/ml)	Inference
1	Water	4.49±0.58	Slightly soluble
2	Ethanol	4.40±0.52	Slightly soluble
3	Phosphate buffer pH 6.8	29.98±0.1	Sparingly soluble

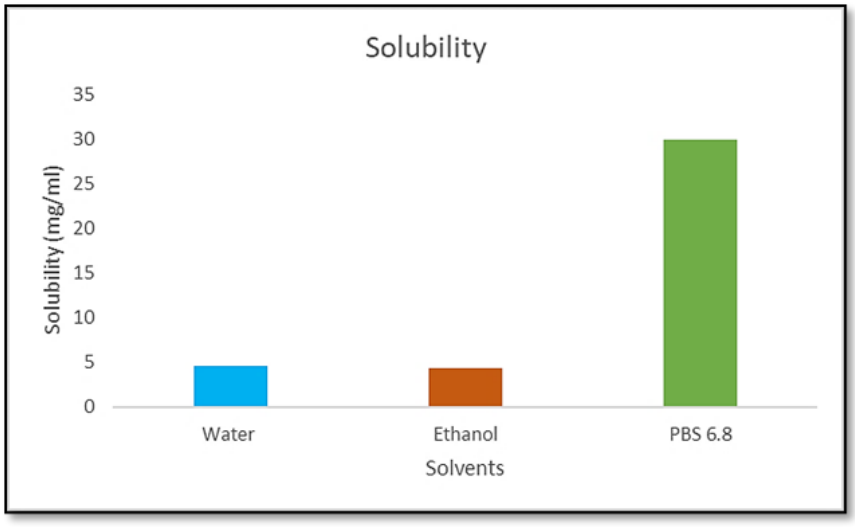


Figure 16. Solubility study of MAG

7. Analytical method development

UV-visible spectrophotometric method for drug

7.1 Determination of λ max

The maximum absorption wavelength (λ max) of mono-ammonium glycyrrhizinate was found to be 258 nm. This wavelength was selected as the working wavelength for further studies.
involving MAG. Figure 17. shows the maximum absorption wavelength of MAG.

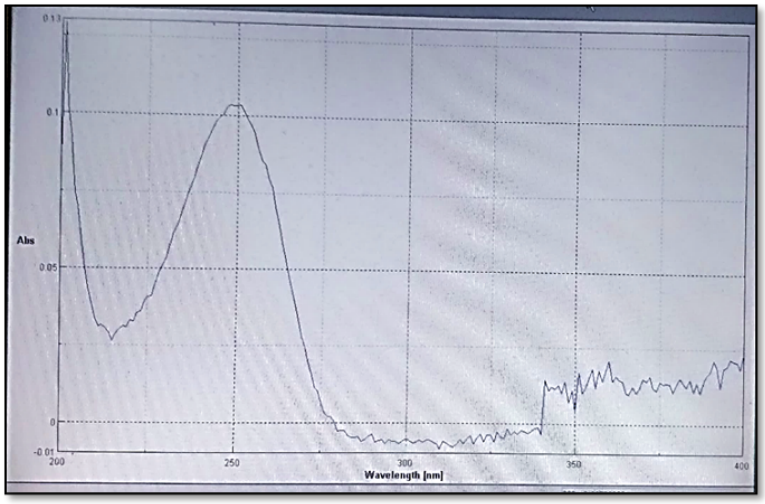


Figure 17. Determination of λ max of MAG by UV-spectrophotometer

7.2 Calibration curve of mono-ammonium glycyrrhizinate in distilled water

The developed calibration curve of mono-ammonium glycyrrhizinate in distilled water is shown in Figure 18. The absorbance values of mono-ammonium glycyrrhizinate in the concentration range of 10-100µg/ml is presented in Table 6. The calibration curve was found to be linear in the concentration range of 10-100µg/ml at the wavelength of 258nm. The coefficient of regression (R^2) of the calibration curve was found to be 0.9934, indicating a strong linear relationship between the concentration of mono-ammonium glycyrrhizinate and its absorbance. This suggest that the calibration curve obeys Beer-Lambert's law, which is a fundamental principle in spectrophotometric analysis.

Table 6. Calibration curve of MAG in Distilled water.

Concentration (µg/ml)	Absorbance
10	0.1191±0.02
20	0.1811± 0.047
30	0.3097±0.035
40	0.4184±0.095
50	0.5073±0.003
60	0.5682±0.011
70	0.6426±0.02
80	0.7184±0.023
90	0.8003±0.021
100	0.9009±0.05

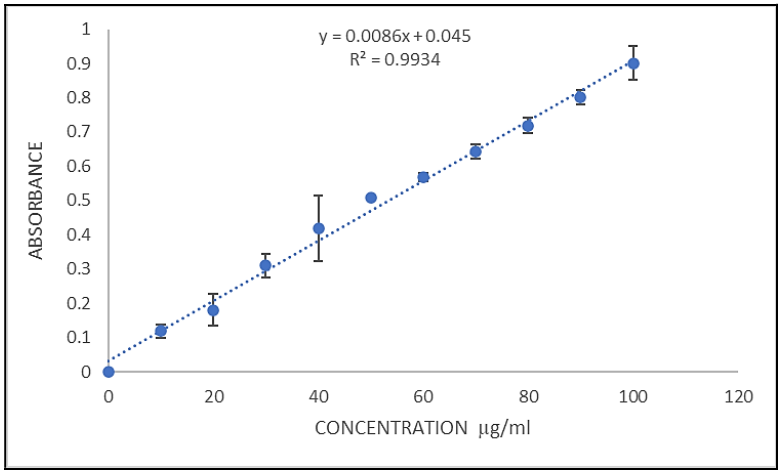


Figure 18. Calibration curve of MAG in Distilled water

7.3 Calibration curve of MAG in Ethanol

The developed calibration curve of mono-ammonium glycyrrhizinate in ethanol is shown in Figure 19. The absorbance values of mono-ammonium glycyrrhizinate in the concentration range of 10-100µg/ml is presented in Table 7. The calibration curve was found to be linear in the concentration range of 10-100µg/ml at the wavelength of 258nm. The coefficient of regression (R^2) of the calibration curve was found to be 0.9975, indicating a strong linear relationship between the concentration of mono-ammonium glycyrrhizinate and its absorbance. This suggest that the calibration curve obeys Beer-Lambert's law, which is a fundamental principle in spectrophotometric analysis.

Table 7. Calibration curve of MAG in Ethanol.

Concentration (µg/ml)	Absorbance
10	0.1469±0.02
20	0.2358±0.03
30	0.3339±0.015
40	0.4256±0.04
50	0.5506±0.09
60	0.6712±0.08
70	0.7645±0.05
80	0.8389±0.02
90	0.9338±0.05
100	1.0714±0.067

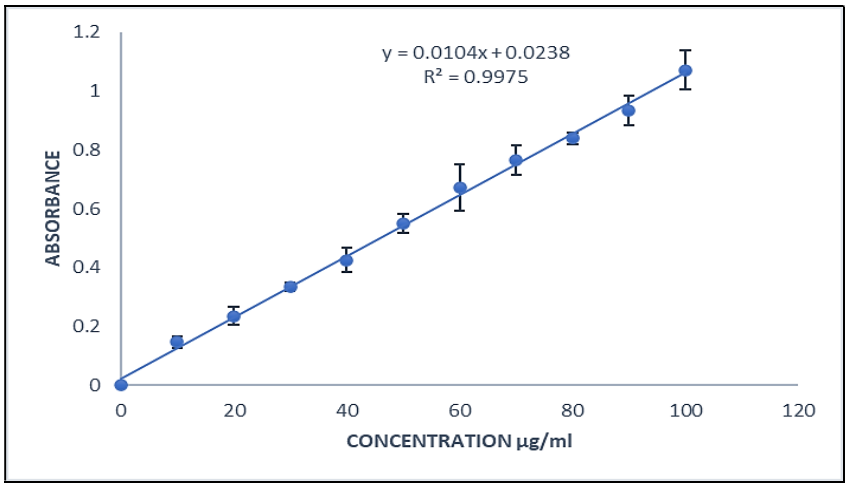


Figure 19. Calibration curve of MAG in ethanol

7.4 Calibration curve of MAG in phosphate buffer (pH 6.8)

The developed calibration curve of mono-ammonium glycyrrhizinate in phosphate buffer (pH 6.8) is shown in Figure 20. The absorbance values of mono-ammonium glycyrrhizinate in the concentration range of 10-100µg/ml is presented in table 8. The calibration curve was found to be linear in the concentration range of 10-100µg/ml at the wavelength of 258nm. The coefficient of regression (R^2) of the calibration curve was found to be 0.9941, indicating a strong linear relationship between the concentration of mono-ammonium glycyrrhizinate and its absorbance. This suggest that the calibration curve obeys Beer-Lambert's law, which is a fundamental principle in spectrophotometric analysis.

Table 8. Calibration curve of MAG in Phosphate buffer pH(6.8)

Concentration (µg/ml)	Absorbance
10	0.20928±0.02
20	0.29966±0.023
30	0.39861±0.02
40	0.57805±0.04
50	0.72207±0.031
60	0.89691±0.021
70	0.98565±0.043
80	1.10854±0.052
100	1.23282±0.02

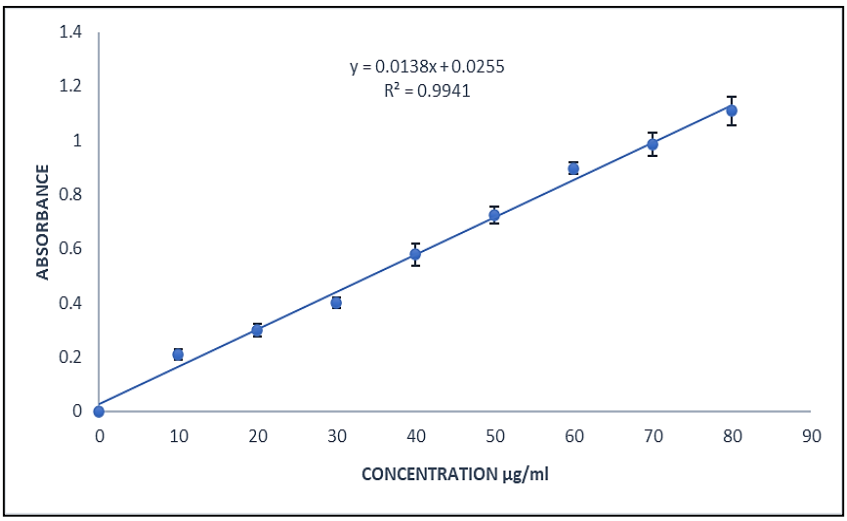


Figure 20. Calibration curve of MAG in Phosphate buffer 6.8

8. Optimization of mucoadhesive buccal film containing MAG

In the context of optimization techniques, the concentration of Carbopol 934 & HPMC K100 was selected as independent variables, & their impact on thickness, muco-adhesion time was evaluated. Out of the 4 trial batches, batch T3 was found to be most promising based on preliminary evaluation. This batch was then selected as the basis for further optimization. A 3^2 factorial design was selected as it allows for the study of the effect on response parameters by varying both variables simultaneously while minimizing the number of experimental runs. The results of the optimization for batches F1-F9 are presented in Table 9. and Figures 21. & 22.

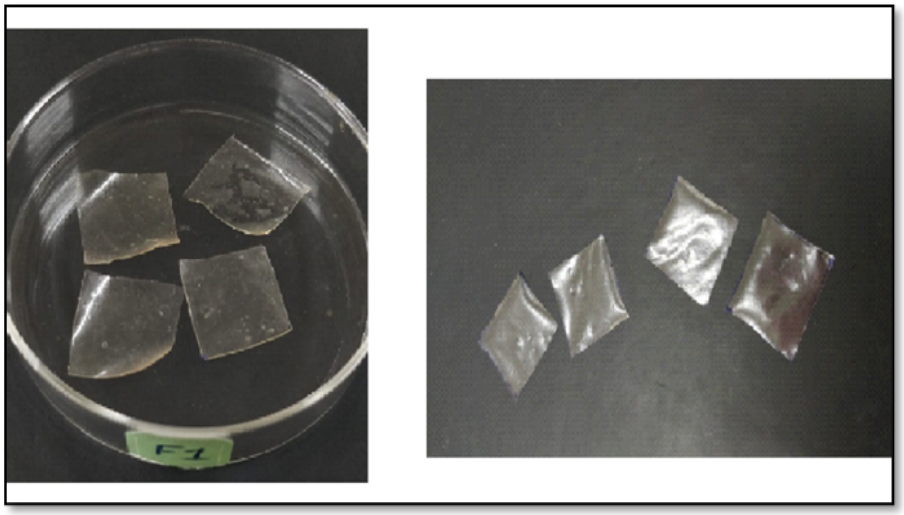


Figure 21. 2X2 cm² of optimized film

Table 9. Results of factorial batches of mucoadhesive buccal film

Batch	Physical appearance	Thickness (mm)	Weight variation (mg)	Folding endurance	Drug content (%)	Muco-adhesion time (hrs.)
F1	**Uniform transparent & smooth**	**0.15±0.0094**	**66.96±6.91**	**>300**	**99.8±0.005**	**>7.55**
F2	Uniform, transparent & smooth	0.11±0.0094	43.33±0.38	>300	90±0.002	>7.80
F3	Uniform, transparent & smooth	0.14±0.0047	69.23±10.64	>300	98±0.004	>6.85
F4	Uniform, transparent & smooth	0.14±0.0047	62.13±5.29	>300	98±0.0014	>7.36
F5	Uniform, transparent & smooth	0.17±0.012	70.33±7.54	>300	95±0.0012	>7.89
F6	Uniform, transparent & smooth	0.08±0.0047	42.1±1.24	>300	110±0.0011	>7.10
F7	Uniform, transparent & smooth	0.09±0.0047	45.43±3.83	>300	105±0.0013	>7.23
F8	Uniform, transparent & smooth	0.10±0.0047	60±0.53	>300	93±0.0022	>6.83
F9	Uniform, transparent & smooth	0.16±0.047	59.66±6.74	>300	94±0.0036	>7.80

9. Evaluation of factorial batches of buccal film

9.1 Physical appearance

All the films of trial batches were visually inspected for uniformity, transparency and smoothness. The results are mentioned in Table 9.. All the batches from F1-F9 were uniform, smooth & transparent as shown in the Figure 21 & 22.

9.2 Thickness (mm)

The thickness of films (2 × 2cm) was measured using digital caliper with a least count of 0.01 mm at 3 different spots of the films and average was taken. The results are mentioned in table 9 & shown in the Figure 22. The thickness of the buccal films varied from 0.17±0.012 to 0.08±0.0047mm. In optimized formula the thickness of film F1 was found to be **0.15±0.0094.** The thickness of film increases with increase in HPMC K100 concentration.

9.3 Weight variation

Weight variation of mucoadhesive buccal films 2×2 cm was observed. The results are mentioned in Table 9. The weight of buccal film varied from 42.1±1.24 to 70.33±7.54mg. The weight variation in optimized film F1 was found to be **66.96±6.91.**

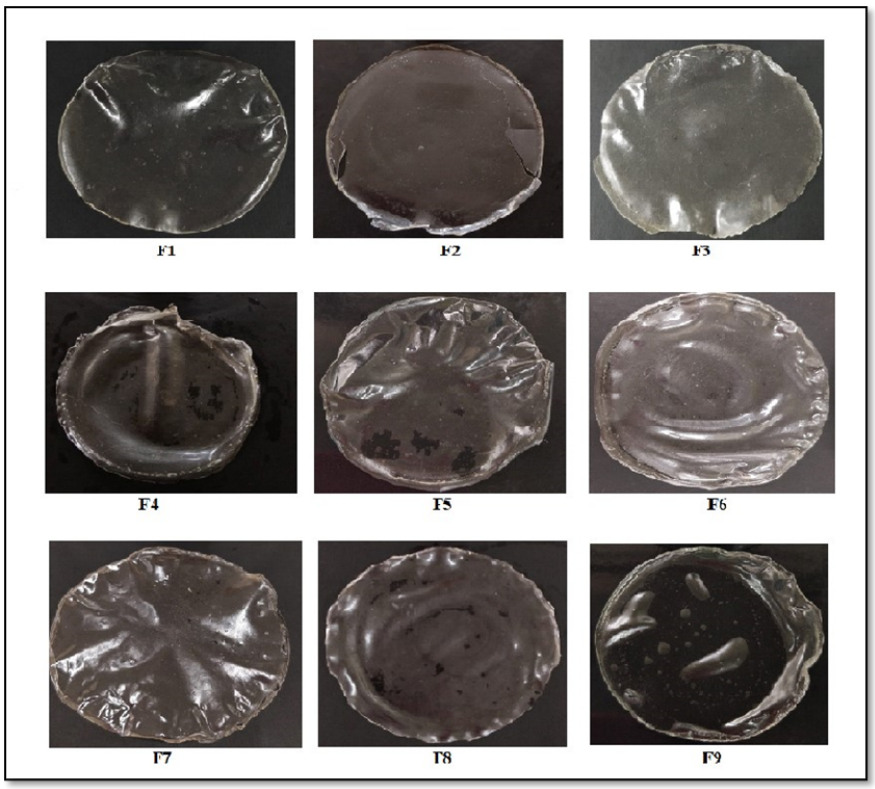


Figure 22. All mucoadhesive buccal films (F1-F9)

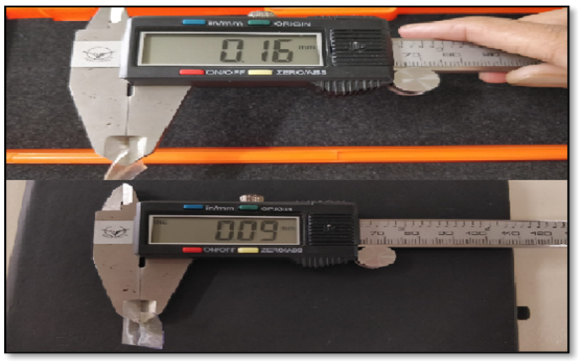


Figure 23. Film thickness using vernier caliper

9.4 Folding endurance

Folding endurance of mucoadhesive films (2 × 2cm) was measured and all of them were **>300**.The results are mentioned in Table 9. The folding endurance of film increases with increase in HPMC K100 concentration.

9.5 Drug content

The results obtained for the drug content of MAG mucoadhesive buccal film are shown in Table 9. Good uniformity of Monoammonium glycyrrhizinate was observed in all buccal films. The drug content was found to be within the standard range given in BP i.e. 85-115%. According to that the drug content of buccal films was found between 110±0.0011 to 90±0.002. The drug content of the optimized buccal film F1 was found to be **99.8±0.005%.**

9.6 Ex-vivo muco-adhesion residence time

The Ex-vivo muco-adhesion residence time of all buccal films was recorded using dissolution USP Type II apparatus, as shown in Figure 23. The results are mentioned in Table 9. The residence time of buccal films was observed between 6hrs to >7hrs. The residence time of optimized buccal film F1 was found to be **>7hrs**.

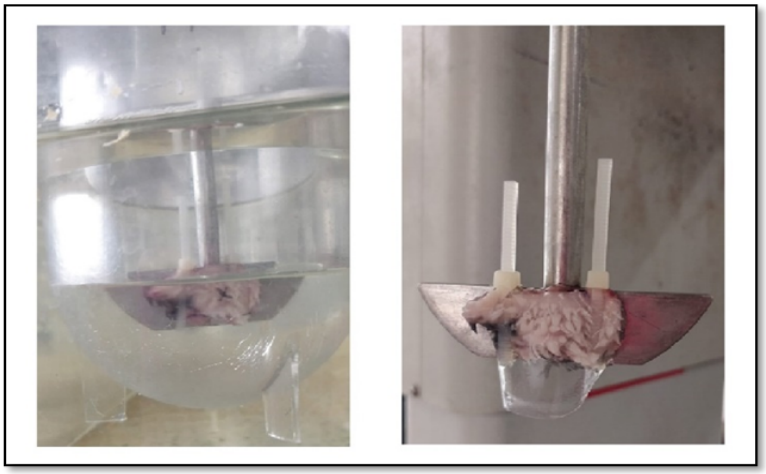


Figure 23. Ex- vivo muco-adhesion residence time

9.7 In-vitro drug release (dialysis method)

The release of pure drug (powder) was performed using the dialysis bag method & the release of MAG buccal film was also performed in-vitro. The result is given in the Table 10 & Figure 24. The result confirms that: the pure drug releases rapidly, while the buccal film offers a sustained release, which is desirable for localized treatment of oral ulcers, minimizing frequent dosing and enhancing therapeutic effect.

Table 10. In-vitro drug release study

Time	%CDR (Pure drug)	%CDR (MAG film)
5	0.0381±4.0	0.357±5.11
15	11.0691±5.32	9.2596±4.23
30	25.4845±4.43	23.2424±3.25
60	44.0095±5.81	40.512±4.14
120	57.2549±3.62	52.5794±3.86
180	69.3255±2.75	67.9925±3.41
240	80.2685±3.20	77.3347±5.60
300	88.7892±5.61	82.2601±4.42
360	95.6587±4.57	91.0738±3.28

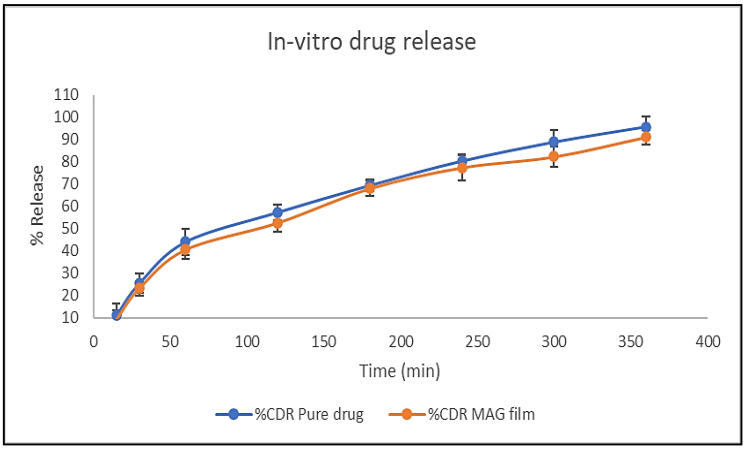


Figure 24. In-vitro drug release of pure drug and MAG film

9.7.1 Mechanism of release kinetics

Mechanism of release kinetics of MAG buccal film for different models- zero order, first order, Higuchi and Korsmeyer peppas was evaluated and the results of R^2 values are given the Table 11.

Table 11. Mechanism of release kinetics

Formulation	Mechanism of release kinetics			
	Zero order kinetics	1st order kinetics	Higuchi model	Korsmeyer-peppas
MAG buccal film	0.8194	**0.9858**	0.9723	0.9783

Among all the models given in the table 11., the first order kinetic model showed the highest correlation coefficient **(R^2= 0.9858)**, indicating that the drug release from the buccal film follows first order kinetics, where the rate of drug release is concentration dependent. The high R^2 values for Higuchi model **(0.9723)** & Korsmeyer- Peppas model **(0.9783)** also suggest that diffusion plays a significant role in the release mechanism. So, MAG buccal film followed first order kinetics with a strong contribution from diffusion-controlled release. This indicates concentration dependent release mechanism that is likely governed by a combination of erosion & diffusion, making formulation suitable for sustained & controlled drug delivery in the oral cavity.

9.8 Ex-vivo buccal permeation study (Franz diffusion method)

The ex vivo buccal permeation of Monoammonium glycyrrhizinate drug powder & film (F1) was performed using Franz diffusion apparatus & was recorded & calculated. The drug powder showed about 54.10% cumulative permeation at 7hrs. The optimized mucoadhesive buccal film showed 5.60% permeation after half hour & about 37.18% cumulative permeation at 7hr. The result is given in Table 12. & Figures 25 & 26.

Table 12. Ex-vivo % cumulative permeation of pure drug and optimized film (F1)

Time	%CP (Pure drug)	%CP (MAG Film)
0.5	8.90±4.51	5.60±2.52
1	14.50±5.18	6.80±3.65
2	22.30±3.56	12.38±2.81
3	29.20±5.02	13.99±4.52
4	36.80±3.2	15.52±3.25
5	43.50±2.31	21.06±4.21
6	49.20±4.12	27.94±2.92
7	54.10±3.53	37.18±4.11

Figure 25. Ex-vivo Franz diffusion apparatus

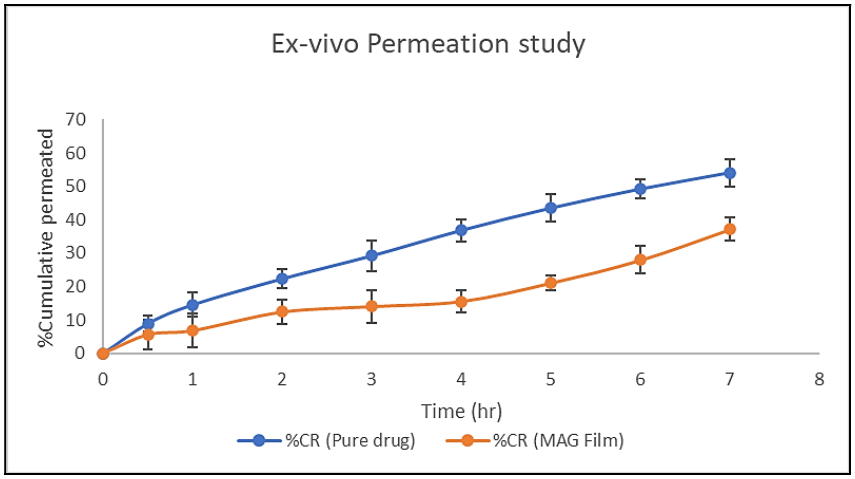


Figure 26. Ex-vivo % cumulative permeation of pure drug and optimized MAG film

9.9 Drug Retention study in mucosa

The retention study of Drug powder (MAG) & optimized film (F1) was performed. Almost **22.90%** drug was retained in tissue during permeation study MAG alone & **39.37%** of the drug was retained in the tissue during the permeation study of optimized film (F1).

So, from this, ex-vivo permeation study indicates that approximately **37.18%** of the drug permeated through the buccal mucosa over a period of 6 hours, while **39.37%** of the drug was retained within the buccal tissue.

The study revealed that MAG alone showed lower retention, likely due to the absence of mucoadhesive polymers. In contrast, when incorporated into a buccal film, the formulation exhibited prolonged mucosal retention, suggesting effective mucoadhesive interaction and enhanced localized drug delivery.

This suggests that the formulation provides both effective drug penetration and significant tissue retention. The high retention in the mucosa indicates that a substantial amount of drug remains localized at the site of action, which can contribute to sustained therapeutic effects.

10. Factorial models & response surface analysis

The results of the experimental design were statistically analyzed using Design Expert software, which provided valuable information & reaffirmed the utility of statistical design for conducting experiments. The relationship between the dependent & independent variables was interpreted by constructing contour plots (2D) & 3-dimensional (3D) response surface plots. These graphical representations helped to visualize & understand the interactions between the variables & their impact on the response parameters.

10.1 Thickness (Carbopol 934 and HPMC K100)

The counter plot & 3-D surface plot in Figure 27 shows the effect of Carbopol 934 & HPMC K100 concentrations on the thickness of the mucoadhesive buccal film. The color gradient represents different levels

of thickness, with red/orange areas indicating higher thickness and blue/green areas showing lower values/ thickness.
As these concentrations increases the thickness of the buccal film also increases. When both the concentrations are high, the thickness of film increases, but the solution becomes thick which is a limitation while pouring the solution onto a petri plate.
Similarly, the surface plot demonstrates that increasing the concentrations of Carbopol 934 & HPMC K100 leads to increase in the thickness of the buccal film.

ANOVA for linear model: Response 1: Thickness

Table 13. ANOVA for thickness

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	0.0067	2	0.0034	12.12	0.0078	significant
A-Carbopol 934	0.0067	1	0.0067	24.00	0.0027	
B-HPMC K100	0.0001	1	0.0001	0.2400	0.6416	
Residual	0.0017	6	0.0003			
Cor Total	0.0084	8				

The **Model F-value** of 12.12 implies the model is significant. In this case A is a significant model term.

Fit Statistics

Table 14. Fit statistics for thickness

Std. Dev.	**0.0167**		R^2	**0.8016**
Mean	0.1267		**Adjusted R^2**	0.7354
C.V. %	13.16		**Predicted R^2**	0.5936
			Adeq Precision	7.6210

The **Predicted R^2** of 0.5936 is in reasonable agreement with the **Adjusted R^2** of 0.7354; i.e. the difference is less than 0.2.

Coded equation

Thickness = +0.1267+0.0333A+0.0033B

It shows the effect of A & B on the thickness Y1 of the film.

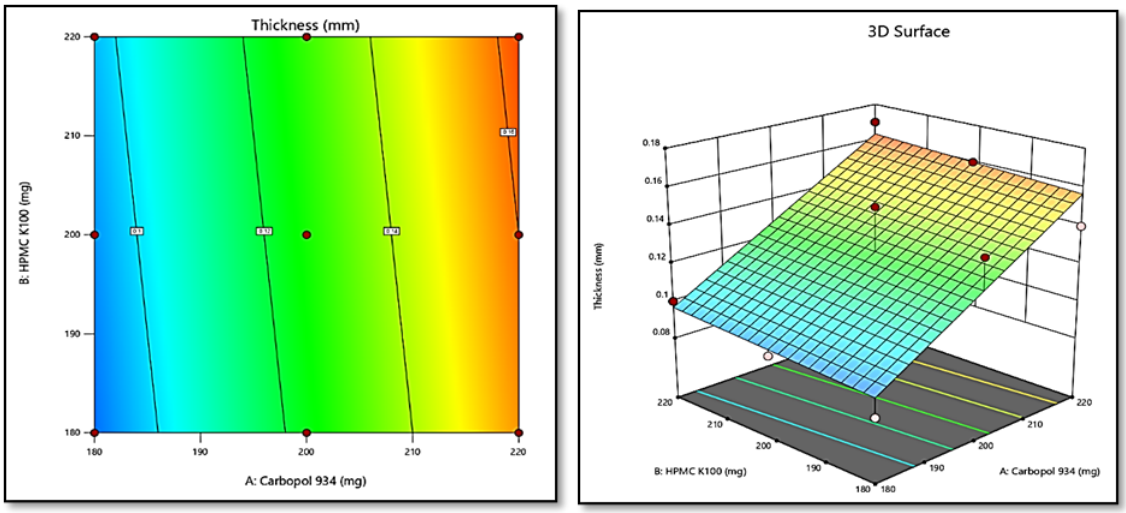


Figure 27. Counter plot & 3D surface plot for effect of Carbopol 934 & HPMC K100 concentration on the thickness of the buccal film.

6.6.2 Muco-adhesion time (Carbopol 934 & HPMC K100)

The contour plot & 3D surface plot in Figure 28, shows the effect of two formulation variables on the muco-adhesion time of the buccal film. The color gradient represents different levels of muco-adhesion time, with red/orange areas indicating higher adhesion time and blue/green areas showing lower values. As seen, increasing both concentrations (Carbopol 934 & HPMC K100), generally improves muco-adhesion time up to an optimal level. Beyond that, excess polymer may reduce adhesion due to overhydration or thickness.

Similarly, the surface plot demonstrates that increasing the concentrations of Carbopol 934 & HPMC K1 00 leads to increase in the muco-adhesion time.

ANOVA for linear model
Response 2: Muco-adhesion time

Table 15. ANOVA for Muco-adhesion time

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	0.8394	2	0.4197	5.17	0.0495	significant
A-Carbopol 934	0.5954	1	0.5954	7.33	0.0352	
B-HPMC K100	0.2440	1	0.2440	3.01	0.1337	
Residual	0.4871	6	0.0812			
Cor Total	1.33	8				

The **Model F-value** of 5.17 implies the model is significant.
P-values less than 0.0500 indicate model terms are significant. In this case A is a significant model term.
Fit Statistics

Table 16. Fit statistics for muco-adhesion time

Std. Dev.	**0.2849**		R^2	**0.6328**
Mean	6.38		**Adjusted R^2**	0.5104
C.V. %	3.86		**Predicted R^2**	0.0473
			Adeq Precision	6.2814

The **Predicted R^2** of 0.0473 is not as close to the **Adjusted R^2** of 0.5104 as one might normally expect; i.e. the difference is more than 0.2.
Coded Equation
Muco-adhesion time = +7.38+0.3150A+0.2017B
It shows the effect of A & B on the Muco-adhesion time Y2 of the film.

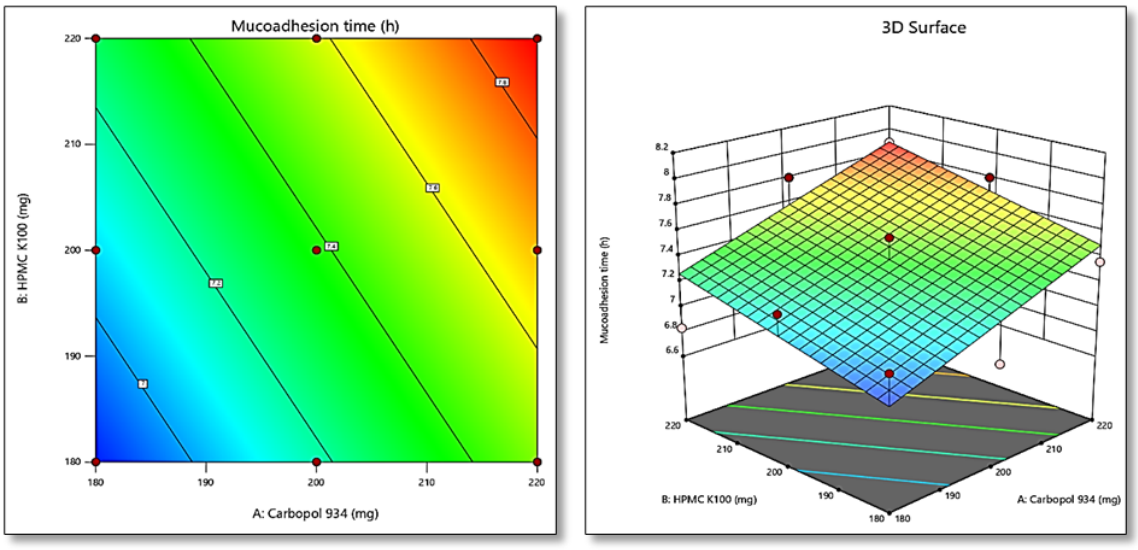


Figure 28. Counter plot & 3D surface plot for effect of Carbopol 934 & HPMC K100 concentration on the muco-adhesion time of the buccal film.

6.6.3 Drug content (Carbopol 934 & HPMC K100)

The contour plot & 3D surface plot Figure 29, illustrates the effect of Carbopol 934 and HPMC K100 concentrations on the drug content of the buccal film, higher drug content (up to 110%) is observed at lower levels of both polymers, indicated by the red zone.
As the concentrations of Carbopol 934 and HPMC K100 increase, the drug content decreases gradually toward 93%, as shown by the blue region. This decline may be due to higher viscosity or denser film matrix at increased polymer levels, which can hinder uniform drug distribution. Thus, optimal drug content is achieved at

lower polymer concentrations. Similarly, the surface plot demonstrates that increasing the concentrations of Carbopol 934 & HPMC K100 leads to decrease in the drug content of the buccal film.

ANOVA for linear model
Response 3: Drug content

Table 17. ANOVA for drug content

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	245.33	3	81.78	17.36	0.0045	significant
A-Carbopol 934	104.17	1	104.17	22.11	0.0053	
B-HPMC K100	60.17	1	60.17	12.77	0.0160	
AB	81.00	1	81.00	17.19	0.0089	
Residual	23.56	5	4.71			
Cor Total	268.89	8				

The **Model F-value** of 17.36 implies the model is significant. In this case A, B, AB are significant model terms.
Coded Equation
Drug equation = +98.11- 4.17A -3.17B+4.50AB
It shows the effect of A & B on the Drug content Y3 of the film.

Fit statistics

Table 18. ANOVA for thickness

Std. Dev.	**2.17**		R^2	**0.9124**
Mean	98.11		**Adjusted R^2**	0.8598
C.V. %	2.21		**Predicted R^2**	0.6834
			Adeq Precision	11.9788

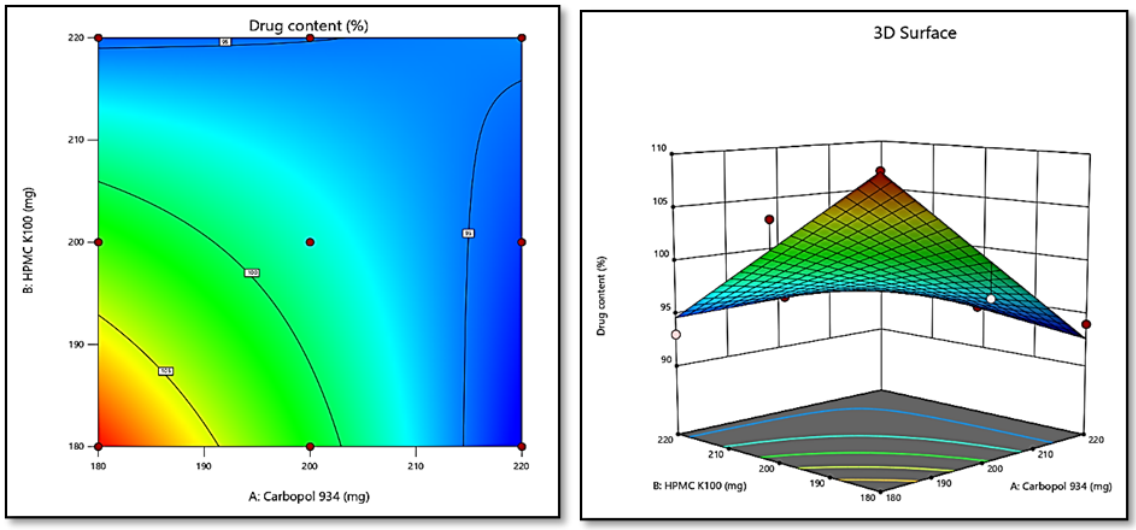


Figure 29. Counter plot & 3D surface plot for effect of Carbopol 934 & HPMC K100 concentration on the drug content of the buccal film

6.7 In vitro anti-inflammatory activity of mono-ammonium glycyrrhizinate using egg albumin denaturation method

The result of invitro anti-inflammatory activity of monoammonium glycyrrhizinate using egg albumin denaturation assay method is given in the table 19 & figure 30.
The results indicate a concentration dependent increase in anti-inflammatory activity for both MAG & std (diclofenac sodium). MAG exhibited moderate to good inhibition of protein denaturation, with a maximum inhibition of **70.55%** at 1000 µg/ml, while the std drug showed a stronger effect with **95.24%** inhibition at the same concentration.
So, MAG demonstrated notable anti-inflammatory activity in-vitro through its ability to inhibit protein denaturation. Although its activity was lower than the standard drug but it can show promising results.

Table 19. In-vitro anti-inflammatory activity of MAG

Sr No.	Concentrations (µg/ml) drug	Rate of Inhibition (%)	
		Diclofenac sodium	MAG
1	10	20.208	7.534
2	100	55.302	36.986
3	200	72.419	48.630
4	500	84.063	58.219
5	1000	95.240	70.547

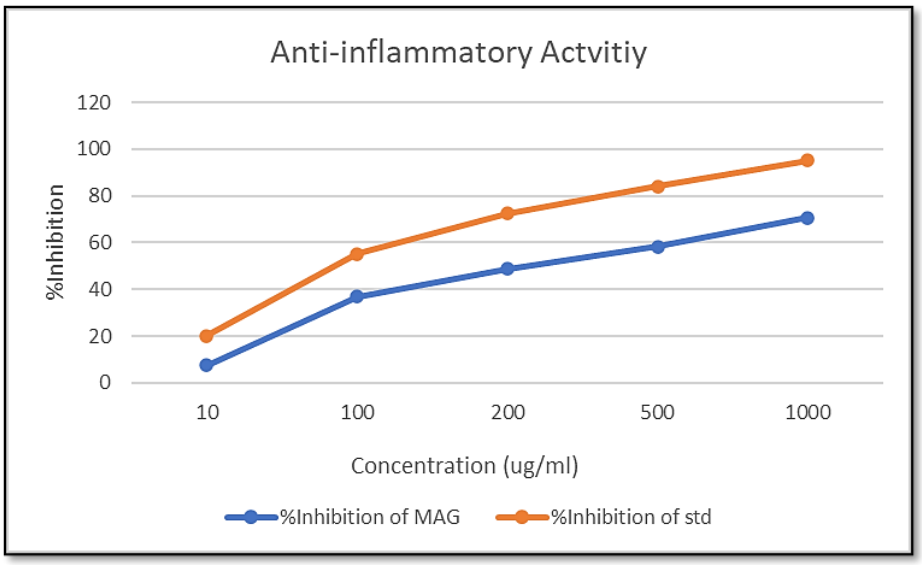


Figure 30. Anti-inflammatory assay of MAG

6.8 In-silico Anti-fungal activity of mono-ammonium glycyrrhizinate

The selected ligand exhibited a binding energy of **−9.0 kcal/mol** with the 5TZ1 protein, indicating strong affinity.

Docking analysis revealed three key hydrogen bond interactions-

1. UNK1:H → TYR505:O – Conventional H-bond (angle- 101.10°)
2. UNK1:H → SER507:O – Conventional H-bond (angle- 126.32°)
3. SER506: CB → UNK1:O – Carbon H-bond contributing to complex stability.

The 2D and 3D interaction diagrams confirm favourable orientation and binding of the ligand at the active site of 5TZ1, supporting its potential bioactivity. Figure 31 & 32.

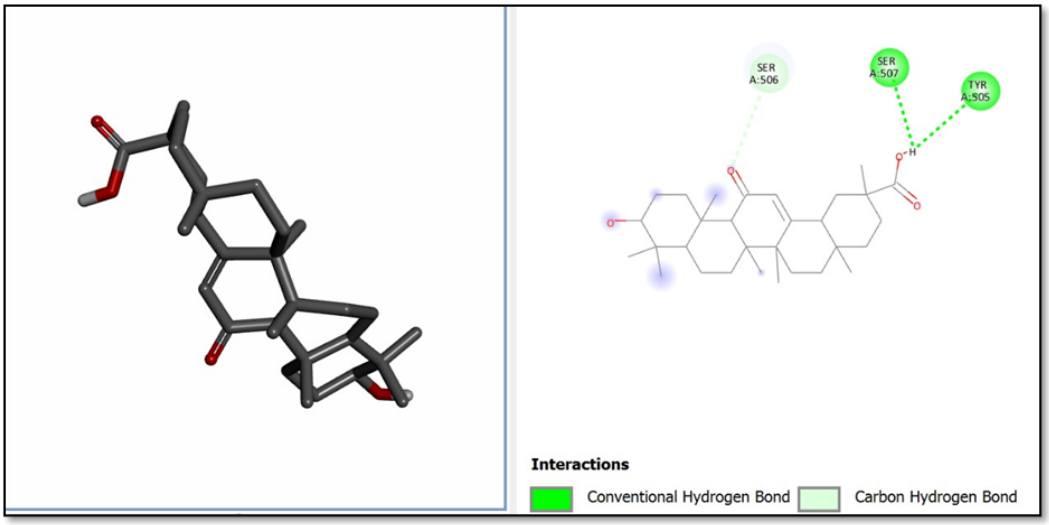


Figure 31. Glycyrrhizin (MAG) and 2D interaction diagram

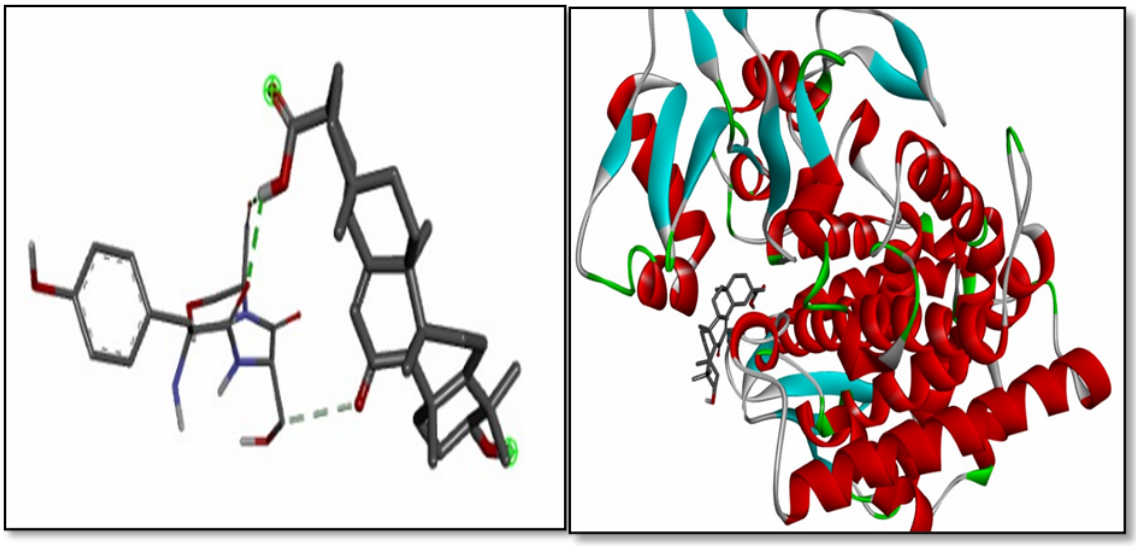


Figure 32. 3D interaction of 5TZ1 protein & Glycyrrhizin (MAG)

6.9 DSC of MAG mucoadhesive film.

The DSC thermogram of the drug-incorporated buccal film was recorded to assess the thermal behavior and possible interaction of the drug with the polymer matrix. Figure 33.

1. A single broad endothermic peak was observed at 72.83 °C, with a transition range between 51.87 °C and 102.87 °C.
2. The absence of a sharp endothermic peak around **210–230 °C** (characteristic of pure monoammonium glycyrrhizinate) suggests that the drug is no longer present in crystalline form within the film.
3. This shift and broadening of the peak indicate a possible **amorphization or molecular dispersion** of the drug within the polymer matrix.

The DSC thermogram of the drug-loaded film shows the absence of the drug's characteristic melting peak, confirming that the drug is molecularly dispersed or converted to an amorphous form within the film. This suggests successful incorporation and interaction of the drug with the polymer, which may enhance its solubility and bioavailability.

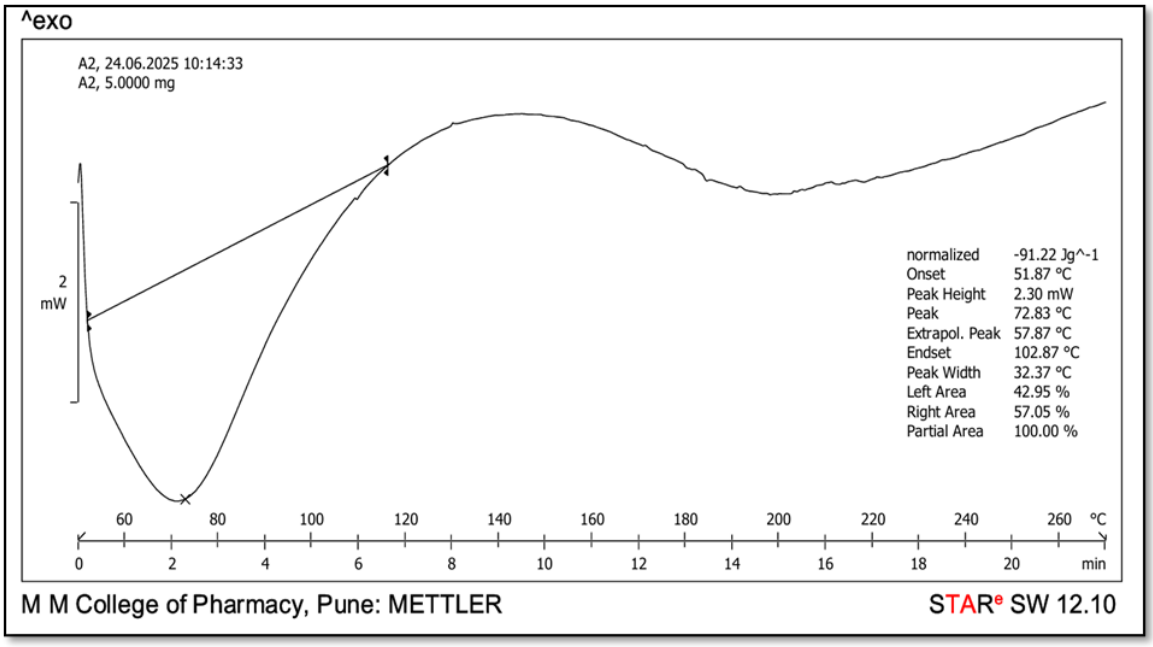


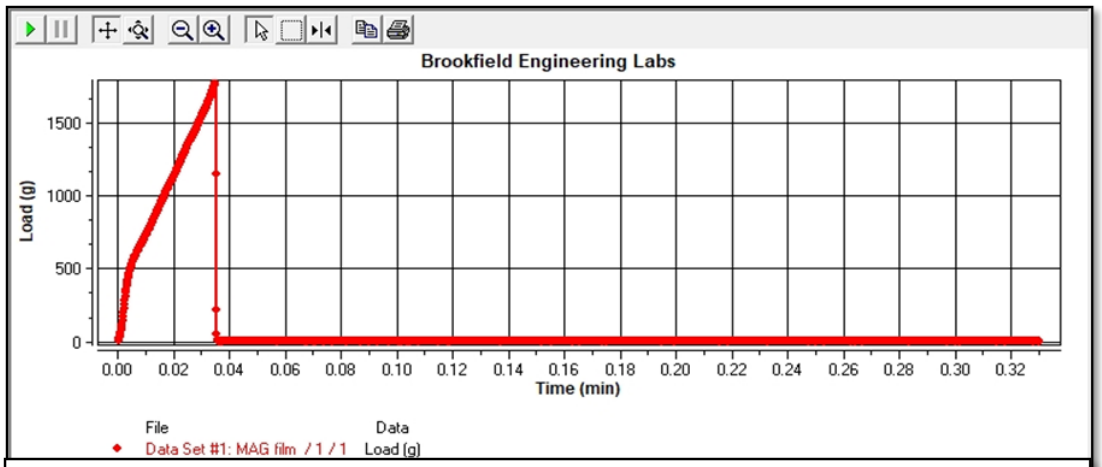
Figure 33. DSC of MAG film

6.10 Texture analysis of MAG mucoadhesive film.

Tensile strength: The tensile strength of the prepared buccal film (MAG film) was evaluated using a Brookfield Texture Analyzer under tension mode. The test was conducted with a target distance of 50 mm and a test speed of 2.5 mm/s. The film exhibited a peak load of 1777 g, indicating the maximum force the film could withstand before breaking. The deformation at peak load was 5.24 mm, reflecting the film's elongation capacity. The mean load during the test was 7 g, while the work done (energy absorbed until break) was 54.4 mJ, indicating good mechanical integrity (Figure 35). These results suggest that the film possesses acceptable tensile strength and flexibility suitable for buccal application.

Muco-adhesion strength: The mucoadhesion strength of the MAG film was evaluated using a Brookfield Texture Analyzer in tension mode with a test speed of 2.5 mm/s and a target distance of 50 mm. The results showed a peak load of 108 g, indicating the maximum force required to detach the film from the mucosal surface. The deformation at peak load was 1.73 mm, and the total work of adhesion was recorded as 26.0 mJ, reflecting the adhesive performance of the film. The final load was 43 g, supporting sustained adhesion (Figure 36). These findings suggest that the film exhibits satisfactory mucoadhesive strength for buccal application, ensuring prolonged retention at the site of action.

Figure 34. Texture analyzer



TexturePro CT V1.3 Build 15 **Brookfield Engineering Labs, Inc.**

DATA REPORT

Sample Description

Product Name:	MAG film	**Notes:**	
Batch Name:	1		
Sample:	1		
Dimensions:			
Shape:	Block		
Length:	0.00 mm		
Width:	0.00 mm		
Depth:	0.00 mm		

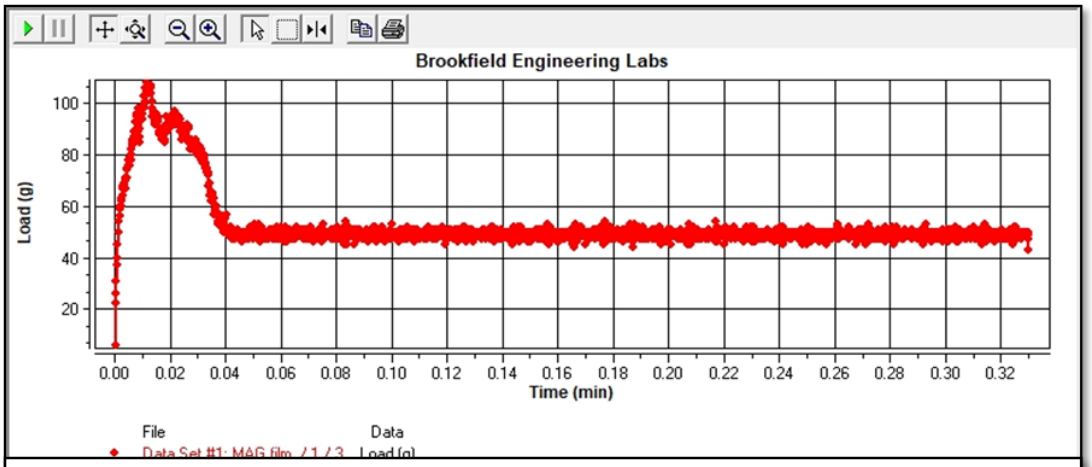
Test Method

Test Date:	19-06-2025	**Test Time:**	13:53:33
Test Type:	Tension	**Recovery Time:**	0.00 min
Target:	50.0 mm	**Same Trigger:**	False
Hold Time:	0.00 min	**Pretest Speed:**	0.5 mm/s
Trigger Load:	1 g	**Data Rate:**	100 points/sec
Test Speed:	2.5 mm/s	**Probe:**	TA3/100
Return Speed:	1 mm/s	**Fixture:**	TA-RT-KI
# of Cycles:	1	**Load Cell:**	10000g

Results

Mean Load:	7 g
	between 0.00 and 0.00 min
Peak Load:	1777 g
Deformation At Peak Load:	5.24 mm
Work:	54.4 mJ
Final Load:	-1 g

Figure 35. Tensile strength of MAG film



TexturePro CT V1.3 Build 15 — **Brookfield Engineering Labs, Inc.**

DATA REPORT

Sample Description

Product Name:	MAG film	**Notes:**	
Batch Name:	1		
Sample:	3		
Dimensions:			
Shape:	Block		
Length:	0.00 mm		
Width:	0.00 mm		
Depth:	0.00 mm		

Test Method

Test Date:	19-06-2025	**Test Time:**	14:28:09
Test Type:	Tension	**Recovery Time:**	0.00 min
Target:	50.0 mm	**Same Trigger:**	False
Hold Time:	0.00 min	**Pretest Speed:**	0.5 mm/s
Trigger Load:	1 g	**Data Rate:**	100 points/sec
Test Speed:	2.5 mm/s	**Probe:**	TA3/100
Return Speed:	1 mm/s	**Fixture:**	TA-RT-KI
# of Cycles:	1	**Load Cell:**	10000g

Results

Mean Load:	6 g
	between 0.00 and 0.00 min
Peak Load:	108 g
Deformation At Peak Load:	1.73 mm
Work:	26.0 mJ
Final Load:	43 g

Figure 36. Muco-adhesion strength of film

6.11 Stability studies

1 month stability study was performed & the results were evaluated for the parameters.

Table 20. Stability study

Month	Physical appearance	Thickness (mm)	Weight variation (mg)	Folding endurance	Drug content (%)	Muco-adhesion time (hrs.)
0 month	Uniform transparent & smooth	0.15±0.0094	66.96±6.91	>300	99.8±0.005	7.55
1 month	Uniform, transparent & smooth	0.13±0.001	53.28±2.14	>300	98.7±0.002	7.46

After 1-month storage, the MAG buccal film remained stable with no major changes in physical appearance, drug content, or mucoadhesive strength. Minor reductions in thickness, weight, and drug content were within acceptable limits.

The stability study results comply with ICH guidelines, indicating that the optimized buccal film formulation of MAG is stable under short-term storage conditions. It retains its physical integrity, drug content, and mucoadhesive properties, making it suitable for further development and long-term studies.

CONCLUSION:

The formulation, development, and evaluation of a mucoadhesive buccal film containing Mono-ammonium glycyrrhizinate (MAG) for oral ulcers involved creating a drug delivery system to enhance healing and provide symptomatic relief in oral mucosal ulcerations. A thorough literature review was conducted, during which a significant research gap was identified—specifically, the lack of a buccal film formulation of MAG for the treatment of oral ulcers. This gap provided the basis and rationale for undertaking the present study. The buccal film was formulated using solvent casting method by incorporating muco-adhesive polymers - Carbopol 934 & HPMC K100 to provide the adhesion to the buccal mucosa. Additionally, propylene glycol was added as a plasticizer to enhance the film-forming properties and flexibility of the final product. Mono-ammonium glycyrrhizinate, known for its anti-inflammatory, antifungal, and wound healing properties, was incorporated as the active agent to treat oral ulcers effectively.

The development process involved optimizing the concentration of polymers and MAG to achieve desirable mechanical properties -folding endurance, thickness, weight uniformity,drug content, and sustained drug release profile. The excipient concentration was optimized through blank trial batches (T1-T4) & the concentration of drug was optimized based on the percentage present in a marketed formulation to ensure efficacy and safety. Out of the 4 trial batches, the batch with optimum results was selected for further optimization using factorial design. To this optimized trial batch, color was added to enhance the visibility of the buccal film. However, this approach was not successful, as the color did not distribute uniformly throughout the film, resulting in inconsistent appearance. So, no colour was added.

The optimized trial batch was selected for further optimization using a factorial design- Central Composite Design (CCD). The optimization was based on independent variables – concentrations of Carbopol934 & HPMC K100, while dependent variables included - thickness, muco-adhesion time & drug content. The evaluation parameters included -Physicochemical properties: thickness, weight uniformity, folding endurance, drug content & muco-adhesion time. Mucoadhesive strength was also checked to ensure if the film adheres well to the mucosal surface without discomfort. In vitro drug release and permeation studies: to confirm controlled and sustained release of MAG over several hours. All the batches were successfully prepared & evaluated. The batch with optimum thickness, greater muco-adhesion time & appropriate drug content was selected i.e. F1. Optimized film (F1) had thickness- 0.15mm, weight- 66.96mg, folding endurance- >300, drug content- 99.8% & muco-adhesion time- 7.55hr. The central composite design revealed that an increase in the concentration of polymers led to a significant increase in both the thickness and mucoadhesion time of the buccal film, while simultaneously causing a decrease in the drug content.

The in-vitro drug release study showed a gradual and sustained release from the MAG buccal film compared to the pure drug. The film demonstrated a slower and more sustained release profile, reaching over 91.07% CDR by 6 hours. This indicates improved drug availability and prolonged retention from the buccal film formulation. The release kinetics of MAG buccal film followed first-order kinetics ($R^2 = 0.9858$), indicating concentration-

dependent drug release. High R^2 values for Higuchi and Korsmeyer-Peppas models also confirmed a significant diffusion-controlled mechanism.

Ex-vivo permeation study showed sustained drug permeation from the film 37.18% at 7 hrs compared to the pure drug 54.10%, supporting its controlled release potential. The drug retention study showed that the optimized MAG buccal film retained 39.37% of the drug in buccal tissue, compared to 22.90 % with pure drug. This highlights the film's superior mucoadhesive properties, enabling prolonged retention and localized delivery. Such retention supports sustained therapeutic action at the site of application.

The in-vitro anti-inflammatory study using egg albumin denaturation assay showed that MAG exhibits concentration-dependent inhibition, reaching 70.55% at 1000 µg/ml. While its activity was lower than diclofenac sodium 95.24%, MAG still demonstrated significant anti-inflammatory potential, indicating its promise as a natural anti-inflammatory agent.

In-silico antifungal study was conducted to prove the antifungal activity of the drug against fungal protein of candida albicans. 5TZ1 was the protein selected for docking & the binding energy was found to be -9kacl/mol. The 2D and 3D interaction diagrams confirm favorable orientation and binding of the ligand at the active site of 5TZ1, supporting its potential bioactivity.

DSC, tensile strength & muco-adhesion strength was also evaluated of the optimized film. DSC analysis confirmed that MAG (drug) is successfully incorporated in the buccal film in amorphous form, which indicated a strong interaction between the drug and the polymer, potentially leading to improved solubility and bioavailability.

The tensile strength of MAG buccal film was found to be 54.4mJ, which indicated excellent elasticity & mechanical integrity. The muco-adhesion strength was 26mJ, which confirmed good adhesive property essential for ensuring prolonged residence time at the buccal mucosa, allowing sustained drug release. So, the MAG buccal film exhibited satisfactory mechanical strength and mucoadhesive property.

Stability study was performed to assess how the quality of a drug product varies with time under the influence of environmental factors. After 1-month storage, the MAG buccal film remained stable with no major changes in physical appearance, drug content, or mucoadhesive strength. Minor reductions in thickness, weight, and drug content were within acceptable limits.

So, the developed Mono-ammonium glycyrrhizinate buccal film shows promising potential for the treatment of oral ulcers by delivering effective anti-inflammatory & anti-fungal activity. Its sustained drug release, strong mucoadhesive properties, a proven bioactivity through in-vitro & in-silico studies confirm its potential as a localized, effective therapeutics system for treatment & relief of oral ulcer.

CONFLICT OF INTEREST:

The authors have no conflicts of interest regarding this investigation**.**

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