"STUDY ON DESIGN AND VALIDATION OF STABILITY INDICATING RP-HPLC & HPTLC METHODS FOR ESTIMATION OF ACTIVE PHARMACEUTICAL INGREDIENTS"

A Thesis

Submitted towards the Requirement for the Award of

Degree of

Doctor of Philosophy

In Pharmacy

Under the Faculty of Pharmacy

By

Stuti Pandey

Enrollment No.:- 161555817435

Under the Supervision of

Prof. (Dr.) Jitendra K Malik

Professor

P.K. UNIVERSITY

2024

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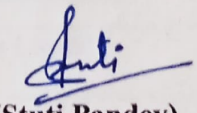

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LIST OF ABBREVIATION

LOQ	Limit of Quantitation
LOD	Limit of Detection
METH	Methanol
RP-HPLC	Reversed Phase High Performance Liquid
ICH	International Conference on Harmonization
SD	Standard Deviation
RSD	Relative Standard Deviation
USP	United States Pharmacopeia
UPLC	Ultra Performance Liquid Chromatography
API	Active Pharmaceutical Ingredient
HPLC	High Performance Liquid Chromatography
RPC	Reversed-phase Chromatography
NPC	Normal Phase Chromatography
RT	Retention time
HPTLC	High Performance Thin Layer Chromatography
Rf	Retardation Factor
MS-MS	Tandem Mass Spectrometry
FT-IR	Fourier-transform infrared spectroscopy
APL	Apremilast
mg	Milligram
µg	Microgram
pH	Potential Hydrogen
C18	Column- Octadecylsilane
SD	Standard Deviation
RSD	Relative Standard Deviation
NMT	Not more than
NLT	Not less than
DP	Degradation Product
ICH	International Council for Harmonization

Abs.	Absorbance
AR	Analytical grade
AUC	Area under curve
BP	British Pharmacopoeia
Temp	Temperature
IR	Infrared
IP	Indian Pharmacopoeia
nm	Nanometers
JP	Japanese Pharmacopoeia
LC	Liquid Chromatography
R^2	Correlation Coefficient
RP	Reverse Phase
RSD	Relative Standard Deviation
R.T.	Retention Time
SD	Standard Deviation
STD	Standard
USP	United States Pharmacopoeia
UV	Ultraviolet
WHO	World health organization

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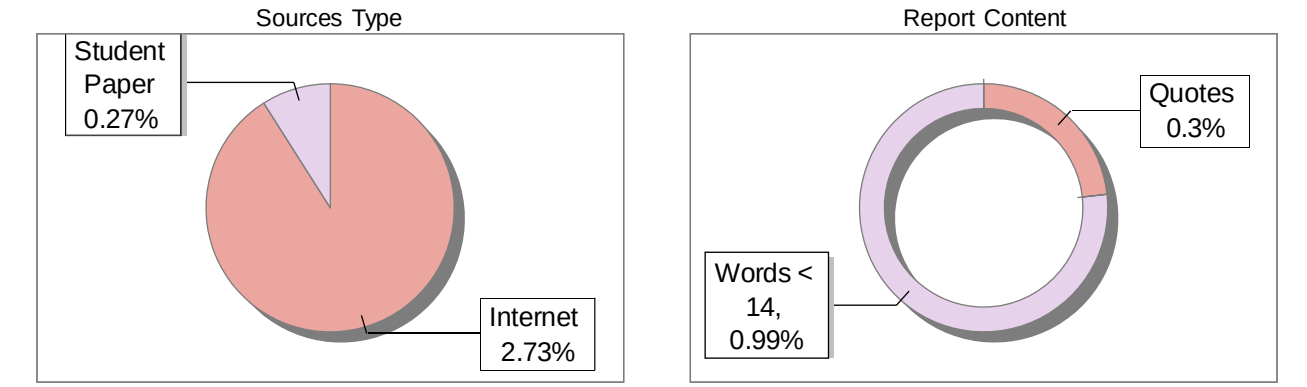

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ABSTRACT

Quality of drug product is very vital as it involves life. The newly developed pharmaceutical formulations need to be analyzed in order to establish the purity, potency and efficacy of drug product. A novel gel formulation containing glabridin and pellet formulation of apremilast were selected for present study.

Aim: Glabridin is a bioactive phytoconstituent present in licorice (Glycyrrhiza glabra Linn). The aim of the present investigation was to design an appropriate form of glabridin, using vesicular delivery system. To develop & validate HPLC method for estimating glabridin in transfersomal gel formulation.

The aim of the second part of the study is to develop and evaluate modified release apremilast pellets for the treatment of Rheumatoid arthritis (RA). This disease directly depends upon circadian rhythm of the body i.e., the maximum joint stiffness generally higher during the morning. The modified release apremilast pellets can be taken before bedtime and capable of releasing drug after predetermined lag time. To develop and validate new stability indicating RP-HPLC and HPTLC methods for apremilast.

Methods: The glabridin-loaded transfersomal gel was developed to treat a variety of hyperpigmentation disorders. Transfersomes were prepared by film hydration method and characterized by Entrapment efficiency, Infrared spectroscopy and Scanning electron microscopy. In-vitro diffusion studies of gel formulation were performed. The HPLC process has been developed and validated to study glabridin in transfersomal formulation. The HPLC separation was carried out on a Kromasil C8 column (250 mm X 4.6 mm, 5m) with acetonitrile & acetate buffer (70:30 v/v) as a mobile phase and a flow rate of 1 ml/min. Quantification was performed at a detection wavelength of 228 nm. In compliance with the International Conference on Harmonization standards, the procedure is validated for accuracy, precision, reproducibility, robustness, and identification, as well as quantification limits.

Apremilast pellets were prepared using extrusion-spheronization technique. The Apremilast pellets were prepared by using blend of microcrystalline cellulose (MCC), lactose, tamarind kernel powder and crospovidone. The process variables such as

Spheronizing speed, amount of Spheronizing aid (MCC) and binder (TKP) were optimized and reported. The obtained pellets were subjected for determination of percentage yield, hardness, physicochemical properties and particle size analysis. A reversed phase HPLC method has been developed and validated for estimation of apremilast, Neosphere C18 (250 mm X 4.6 mm, 5µm) column was used as stationary phase.

The optimal composition of the mobile phase, Acetonitrile: (30mM) potassium dihydrogen phosphate buffer pH-3 (Adjusted with o-phosphoric acid) (60: 40 v/v), and flow rate 1.0ml/min. HPTLC method has been developed and validated for estimation of apremilast in bulk and pellets using aluminium plate precoated with silica gel 60F254 as stationary phase. The optimal composition of the mobile phase selected for analysis was toluene: Ethyl acetate (4: 6 v/v). The method is validated for accuracy, precision, reproducibility, robustness, and identification, as well as quantification limits.

Result and Discussion: Prepared Transfersome were predominantly brownish and spherical in shape. The infrared spectra revealed the absence of drug excipients interaction. Scanning electron microscopy and deformability studies confirmed spherical and deformable nature. In-vitro release studies and stability studies of glabridin loaded transfersomal gel.

According to statistical analysis, the approach is precise, reproducible, selective, and effective for the study of glabridin. The proposed developed and validated high-performance liquid chromatography system for quantifying glabridin can control and standardize glabridine.

The apremilast pellets batch (F-6) prepared with MCC (12gm) and TKP (3gm) spheronized at 1000rpm for 90 sec showed good results in terms of yield, drug content, particle size.

The Proposed RP-HPLC and HPTLC method was found to be a more sensitive, precise, economic and less time consuming and cost effective.

Conclusion: Transfersome based novel glabridin delivery system has been developed successfully. The formulation showed better potential of delivery system as compared to conventional glabridin gel. In-vitro release studies & stability studies with HPLC analysis

shown appropriate results indicate that the glabridin loaded transfersomal gel would be efficacious and stable. The proposed RP-HPLC method for estimating Glabridin in transfersomal gel formulation is sensitive, accurate, precise, and reproducible.

A significant result obtained with the study indicates that modified release apremilast pellets prepared by extrusion spheronization technique can successfully be further explored and employed in treatment of Rheumatoid arthritis. The stability indicating HPTLC and HPLC methods were developed and validated for estimation of apremilast in pellet formulation, which found to be simple, accurate, and reproducible.

Keywords: glabridin, transferosomes, gel, HPLC, method validation, apremilast pellets, Extrusion, Spheronization, HPTLC

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CHAPTER-1

1. INTRODUCTION

1.1 General description

A goal of Analytical chemistry is to comprehend the chemical composition of all matter and to develop techniques to encode such compositions. Analytical chemistry is dedicated to developing new methods for determining the chemical compositions of natural and synthetic materials. This science's procedures are used to identify the compounds that may be present in a material and to calculate the precise concentrations of those substances. (Khopkar, 1998, Christen, 2003, Jeffery et al 1989, Skoog, 2004 & Sharma, 2005)

The discipline of analytical chemistry consists of:

a) Qualitative Analysis:

Qualitative analysis deals with the detection of elements, ions or compound present in the sample. (Beckett et al 2002; Chatwal, 2002)

b) Quantitative Analysis:

Quantitative analysis is concerned with determining the quantity of one or more constituents contained in a sample.

Analytical chemists aim to increase the accuracy of existing techniques in order to meet the growing need for improved chemical measurement. Clinical laboratory testing is based on analytical chemistry, in medicine. Analytical chemistry is frequently used to assess environmental quality by testing for potential pollutants. The quality of the end product, i.e., the drug product, is extremely important in the pharmaceutical sector because it includes human life. The pharmaceutical analyst plays a critical role in pharmaceutical quality control by performing stringent checks. To perform this important task and to cope up with the requirement of analysis, variety of analytical methods and techniques are available. (Dong, 2006; Sethi, 2001)

1.1.1 Analytical method--Selection:

The first step in selecting or developing a technique is to determine what will be tested and how accurately it will be measured. Unless a set of methods is available to assess product quality, validation programmes may have limited validity.

It is best to avoid having several sources of critical components (reagents, columns, and

TLC plates).

Before being transferred, it must be fully optimized to ensure that its properties are all met

Classification---analytical methods:

The Methods are classified under two broad categories as follows

1. Classical Methods:

A. Volumetric Methods: The volume or mass assessed in volumetric, also known as titrimetric, methods.

B. Gravimetric Methods: The mass of the analyte or a chemical formed from the analyte was determined using gravimetric techniques.

However, with the passage of time and the introduction of instrumental ways to replace them, the scope of their broad use is diminishing.

2. Instrumental Methods:

These methods rely on measuring physical qualities.

There are many techniques available for the analysis of analytes:

a) Spectroscopic Analysis

1. Ultraviolet and visible spectrophotometry,
2. Fluorescence and phosphorescence spectrophotometry,
3. Atomic spectrophotometry (emission & absorption),
4. Infra-red spectrophotometry,
5. Raman spectroscopy,
6. X-ray spectroscopy,
7. Radio chemical techniques including activation analysis,
8. NMR spectroscopy,
9. ESR spectroscopy.

b) Electrochemical Techniques

1. Potentiometry,
2. Voltammetry,
3. Voltametric techniques,
4. Stripping techniques,

5. Amperometric techniques,
6. Coulometry,
7. Electrogravimetry,
8. Conductance techniques.

c) Chromatographic Methods

1. Gas chromatography (GC),
2. High performance liquid chromatography (HPLC),
3. High-performance thin layer chromatography (HPTLC)

d) Miscellaneous Techniques

1. Thermal analysis,
2. Mass spectrometry,
3. Kinetic techniques.

e) Hyphenated Methods

1. GC-MS,
2. ICP-MS,
3. GC-IR,
4. MS-MS.

UV and HPLC are the most extensively utilised techniques for quantitative analysis of medicinal compounds among all the techniques described above. (Weston et al, 1997; Willard et al, 2001; Swadesh 2000)

1.2 Chromatography

1.2.1 Introduction to chromatography:

M. Tswett, a botanist, invented chromatography for the first time in 1906 in It is, however, used for preparative purposes to a limited extent. Simultaneously separation of mixture components and its quantitative estimation can be performed. Samples can be gaseous, liquid, or solid, and their complexity comprising a wide spectrum of chemical species. Furthermore, the analysis may be performed on highly expensive and complicated equipment on one hand, and a basic, affordable thin layer plate on the other. Chromatography defined as, It is a separation process that is achieved by distribution of

the components of a mixture between two phases, a stationary phase and a mobile phase. Those components held preferentially in the stationary phase are retained longer in the system than those that are distributed selectively in the mobile phase. As a consequence, solutes are eluted from the system as local concentrations in the mobile phase in the order of their increasing distribution coefficients with respect to the stationary phase‖.

In practice, the distribution system can be a column, such as a tube filled with particle matter, to which the stationary phase is bound or coated. To elute the sample, the mobile phase travels through the column under pressure. To elute the sample, the mobile phase (a liquid) is set to percolate up the plate. Just before the front of the stationary phase, the sample can be placed into the mobile phase stream. (Weston et al, 1997; Willard et al, 2001; Swadesh, 2000; Swarbrick, 1997)

1.2.2 Principle of Chromatographic Separation:

Chromatographic methods are dynamic processes in which the sample mixture is transported over or through a stationary phase medium by a mobile phase. Interaction happens when the sample comes into contact with the stationary phase. The differential affinity of each component with the stationary phase causes partitioning or separation of the components in the mixture.

A detector responds with a signal change when the separated component emerges or elutes, resulting in a chromatogram that is displayed against time.

1.2.3 Types of Chromatography:

The chromatographic methods can be categorized in two ways.

A) Physical Classification:

- **Column chromatography**: The stationary phase is contained within a small tube through which the mobile phase is passed under pressure.
- **Planar chromatography**: The stationary phase is supported on a flat plate or paper.

B) Fundamental Classification

The second and more fundamental categorization of chromatographic methods is based on the types of mobile and stationary phases used in the transfer of solutes between phases, as well as the types of equilibria involved.

1.3 High performance liquid chromatography: (*HighPerformanceLiquid Chromatography & Capillary Electrophoresis - 1st Edition*, n.d.; Publishers, n.d.) The fastest-growing analytical method for drug analysis is high-performance liquid chromatography (HPLC). Its ease of use, excellent specificity, and broad sensitivity range make it suitable for analyzing a wide variety of pharmaceuticals in both dosage forms and biological fluids.

Liquid chromatography (LC) is a kind of physical separation that takes place in a liquid medium. The mobile phase (a moving liquid) and the stationary phase (a stationary solid) are used to separate a sample into its constituent components (or analytes) (sorbents packed inside a column). The flowing liquid, for example, might be an organic solvent like hexane, whereas the stationary phase could be porous silica particles packed in a column. HPLC is a contemporary kind of LC in which the mobile phase is injected at high pressure through small-particle columns. The chromatographic process is shown in Fig. 1.1-a, in which a combination of analytes A and B migrate down a column packed with packing material and are separated into two different bands (stationary phase). The dynamic partitioning of analytes shown in Fig. 1.1-b. Component B's movement in the column is slowed because each B molecule has a higher affinity for the stationary phase than the A molecule. The concentration of each separated component band in the effluent is monitored by an in-line detector, which produces the "chromatogram" illustrated in Fig. 1.1-c.

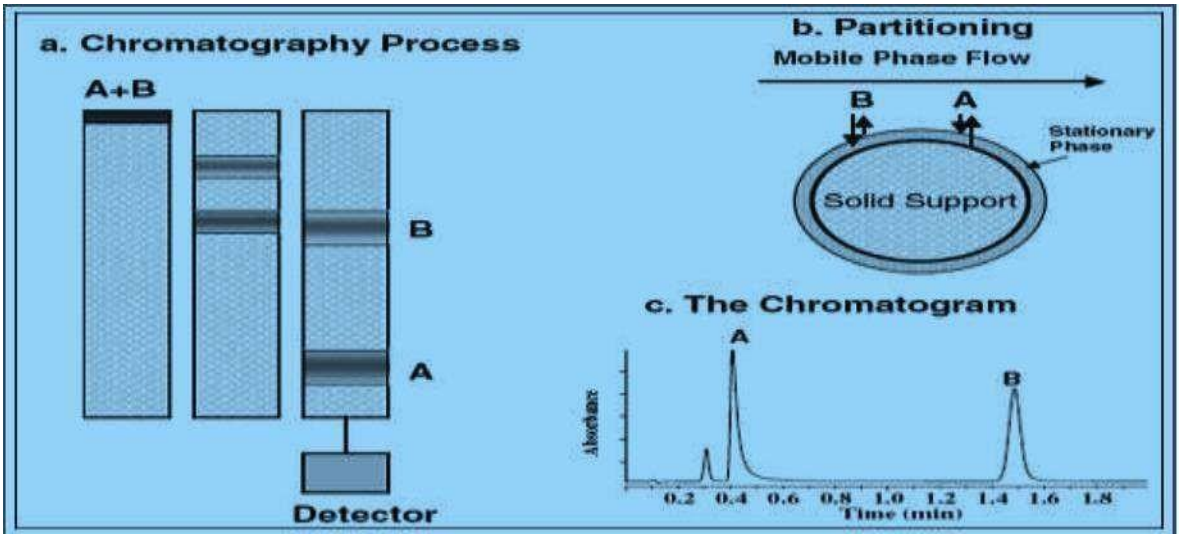

Figure 1.1: (a) migration of two bands (b) partitioning process of analyte molecules A and B. (c) signal from a UV detector - elution of components A and B.

1.3.1 Advantages and limitations:

HPLC is a multicomponent separation method capable of analyzing real-world materials and complicated mixtures. Few methods can equal 0.5 percent Relative Standard Deviation's flexibility and accuracy (RSD). For unattended analysis and report creation, HPLC uses advanced autosamplers and data systems.

Detection limits may now be extended to nanogram, picogram, and even femtogram levels thanks to a slew of extremely sensitive and specialized detectors. It can recover numerous labile components quantitatively in milligram to kilogram amounts as a preparative method. Most significantly, HPLC can handle 60% to 80% of all known chemicals, while GC can only handle approximately 15%. HPLC has several well-known drawbacks and limitations. First, no universal detector, flame ionization detector equivalent in GC, thus detection is difficult. Second, since the separation efficiency is much lower, complicated mixture analysis is more challenging. Finally, HPLC has a large number of operational parameters, making it more challenging for a beginner. Through instrumental and column advancements, these restrictions have been greatly reduced.

1.3.2 Modes of HPLC:

A) Normal-phase-chromatography(NPC)

NPC is a classic separation mode based on the adsorption/ desorption like (typically silica or alumina) Fig. 1.2 a. with the silanol groups, polar analytes move slowly across the column. A surface layer of water is thought to decrease the activity of the silanol groups, resulting in more symmetrical peaks. NPC is very effective for separating nonpolar molecules and isomers, as well as fractionating complicated samples by functional groups or cleaning up samples. One of the primary drawbacks of this mode is the ease with which sample components might contaminate the polar surfaces. Bonding polar functional groups to the silanol groups, such as amino- or cyano-moiety, helps to mitigate this issue.

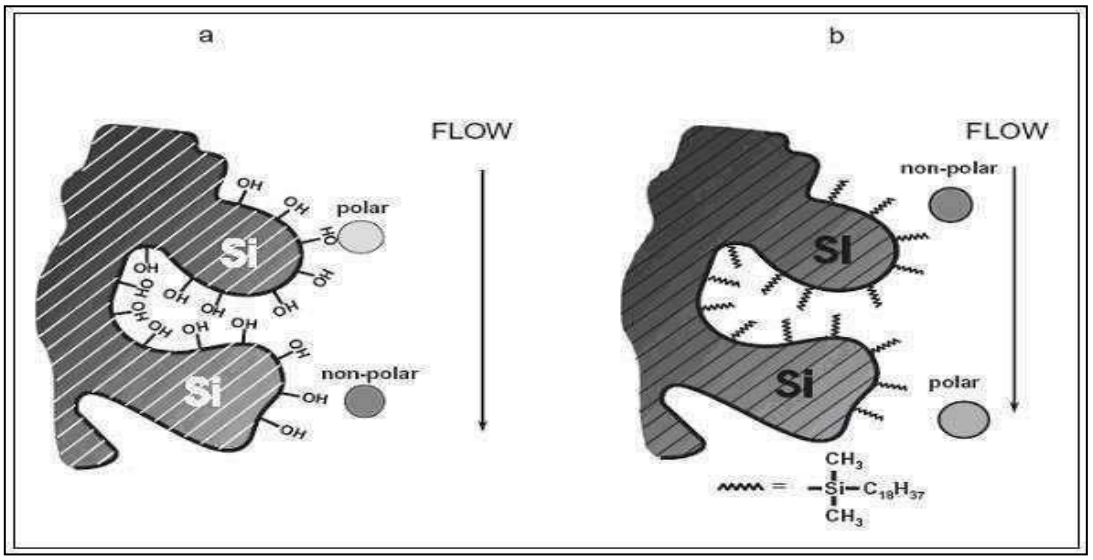

Fig 1.2: Schematic diagrams depicting separation modes of (a) normal-phase chromatography (NPC) and (b) reversed-phase chromatography (RPC).

B) Reversed-phase-chromatography (RPC)

Solid particles covered in nonpolar liquids were the first stationary phases. On silica support, they were rapidly replaced by more permanent bonding hydrophobic groups, whereas polar analytes elute first. This elution order of ―polar first and nonpolar last‖ is the reverse of that observed in NPC, and thus the term ―reversed-phase chromatography.‖ A polar mobile phase, such as a combination of methanol or acetonitrile with water, is commonly used in RPC." The mechanism for separation followed is basically solvophobic or hydrophobic interaction. RPC is the most widely used HPLC mode, accounting for more than 70% of all HPLC studies. It may be used to analyse polar (water-soluble), medium-polarity, and nonpolar analytes. Ion-suppression or ion-pairing techniques can be used to separate ionic analytes.

C) Ion-exchange-chromatography(IEC)

Cationic exchange (sulfonate) or anionic exchange (quaternary ammonium) groups linked to polymeric or silica materials are common stationary phases Fig. 1.3a.. Mobile phases mostly contains buffers, the examination of ions and biological components are common uses. Ion chromatography is a branch of IEC that involves utilising a high-

performance ion-exchange column and a specialised conductivity detector to analyse low quantities of cations or anions.

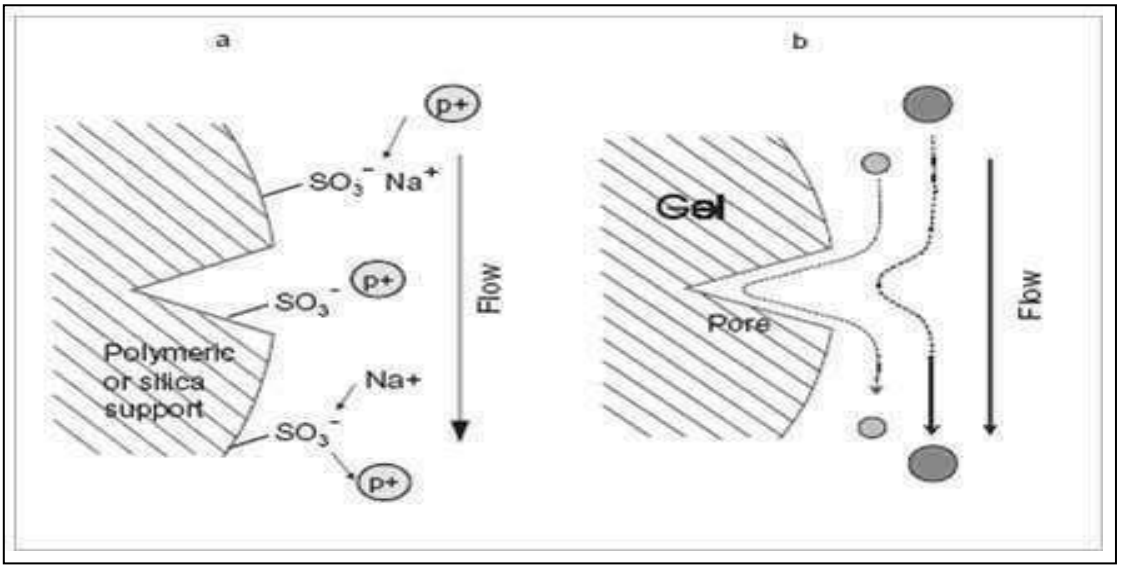

Fig 1.3: Schematic diagrams (a) ion-exchange chromatography (IEC (b) size-exclusion chromatography (SEC)

D) Size-exclusion-chromatography (SEC)

Size-exclusion Chromatography is a separation method that is purely dependent on the molecular size of the analyte. A big molecule is excluded from the pores and migrates fast down the column, as seen in Fig. 1.3b, but a tiny molecule may penetrate the pores and migrates more slowly. When used to determine the molecular weights of organic polymers, it is known as gel permeation chromatography (GPC), and when used to separate water-soluble biological materials, it is known as gel-filtration chromatography (GFC). The GPC column is filled with controlled-pore-size cross-linked polystyrene beads and rinsed with common mobile phases like toluene and tetrahydrofuran.

1.3.3 Instrumentation of HPLC:

The various components of HPLC are Mobile Phase Reservoir and solvent System treatment, Pumps (Displacement Pump, Reciprocating Pump, Pneumatic Pump), Sample Injectors, Pre columns, Liquid chromatographic column (Analytical Column), Detectors

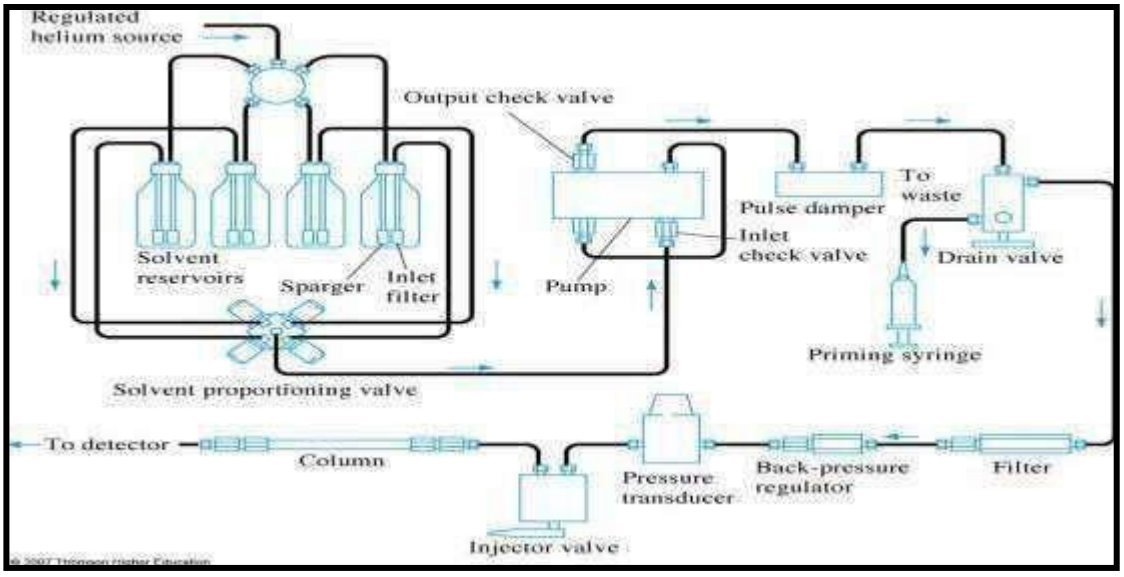

Fig 1.4: Schematic diagram of HPLC system

1. Mobile Phase Reservoir and Solvent System Treatment: Glass or stainless steel reservoirs are included in modern HPLC systems. It is frequently equipped with a degasser system to eliminate dissolved gases, most commonly O2 and N2, which cause interference by creating bubbles in the column and detection system.

Degasser may consist of:-

Vacuum pumping system, a distillation system, Devices for heating and stirring the solvent, Devices for sparging (in which the dissolved gases are swept out of the solution by fine bubbles of inert gas of low solubility).

Isocratic System: A separation that employs a single solvent of constant composition is called as isocratic system.

Gradient System: In this technique the proportion of the two or more solvent is varied in a programmed way.

2. Pump: Pumps are necessary to deliver a constant flow of mobile phase at pressures ranging from 1-550 bars. Pumps capable of producing pressure up to 8000 psig.

The various types of the pump used are: Constant displacement pumps or syringe pump,

Dual piston reciprocating pump, Pneumatic pump or constant pressure pump.

3. Injection System: two types, a. those in which the sample is injected directly in column and the sample is deposited in column inlet and subsequently swept into the column by the mobile phase.

4. Precolumn: The precolumn is primarily used to remove contaminants from the solvent and so protect the analytical column from contamination. These are the columns that have the same chemical packing as the analytical column. Because the particle size is big, the pressure drop across the pre-column is insignificant in comparison to the analytical column.

5. Liquid Chromatographic Column (Analytical Column): HPLC columns are composed of high-quality stainless steel that has been polished to a mirror shine on the inside. Standard analytical columns have an internal diameter of 4-5 mm and a length of 1000 cm. Shorter columns 3-6 cm in length containing a small particle size packing material (3-5m) produce similar or better efficiencies in terms of the number of theoretical plates (approximately 7000) than 20 cm columns containing 10 m irregular particles, and are used when short analysis times and high sample throughput are required. Micro bore columns with a diameter of 1-2 mm and a length of 10-25 cm have the advantages of a lower detection limit and lower solvent usage.

6. Detectors:

A detector is required to detect the presence and amount of sample in the efflulent sample component in the column. The output of the detector is an electrical signal that is proportional to the mobile phase and/or the characteristics of the solute. Bulk property detectors, such as the Refractive Index detector, monitor properties that are shared by both the mobile phase and the solute.

Alternatively, if the property is mostly owned by the solute, such as UV/visible light absorption in an electrochemical property, the detector is referred to as a solute property detector.

The detectors in the HPLC analysis of pharmaceutical substances are describe below.

a) Photometric detectors. (Single wavelength detector,Multi-wavelength

detector,Variable wavelength detector, programmable detectors,Diode array detectors)

b) Fluorescence detectors

c) Refractive index detectors

d) Electrochemical detectors.

Note: The detector should not be operated below the ultraviolet cut-off of the solvent and the solvent should be of spectral grade quality to ensure the absence of absorbing impurities.

2. Quantitative analysis can be carried out by one of the following methods*:*

a) The internal standard method:

Internal standards are used to achieve the best precision in quantitative chromatography because sample injection uncertainties are avoided. The ratio of analyte involves adding a carefully measured quantity of an internal standard to each standard and sample.

Selection of proper Internal Standard:-

- Capacity factor (k‟) different yet elute in reasonable time.
- Retention time of internal standard must not be too long.
- Should not interact with the mobile phase and the drug.
- Should have sufficient absorbance at the selected wavelength.
- Should be stable in the mobile phase.

b) External standard method:

There are separate injections of a fixed volume of sample and a fixed volume of reference solution. Peaks are integrated, and sample concentration is determined by direct comparison.

(Peak Area of Sample / Peak Area of Standard) x Conc. of standard = Sample Concentration.

This requires precision in sample injection volume.

c) Area normalization method:

The area normalisation method is another way , in this approach, and after accounting for variations in detector sensitivity to different chemical types, the concentration of the analyte is determined by the ratio of its area to the total area of all peaks.

Terms of HPLC:

A summary of the nomenclature is shown diagrammatically in following (Fig. 1.5)

The various terms

- The peak maximum
- The injection point
- The dead point
- The dead time (to)
- The dead volume (Vo)

Thus, Vo = Qto where Q is the flow rate in ml/min and to is dead time.

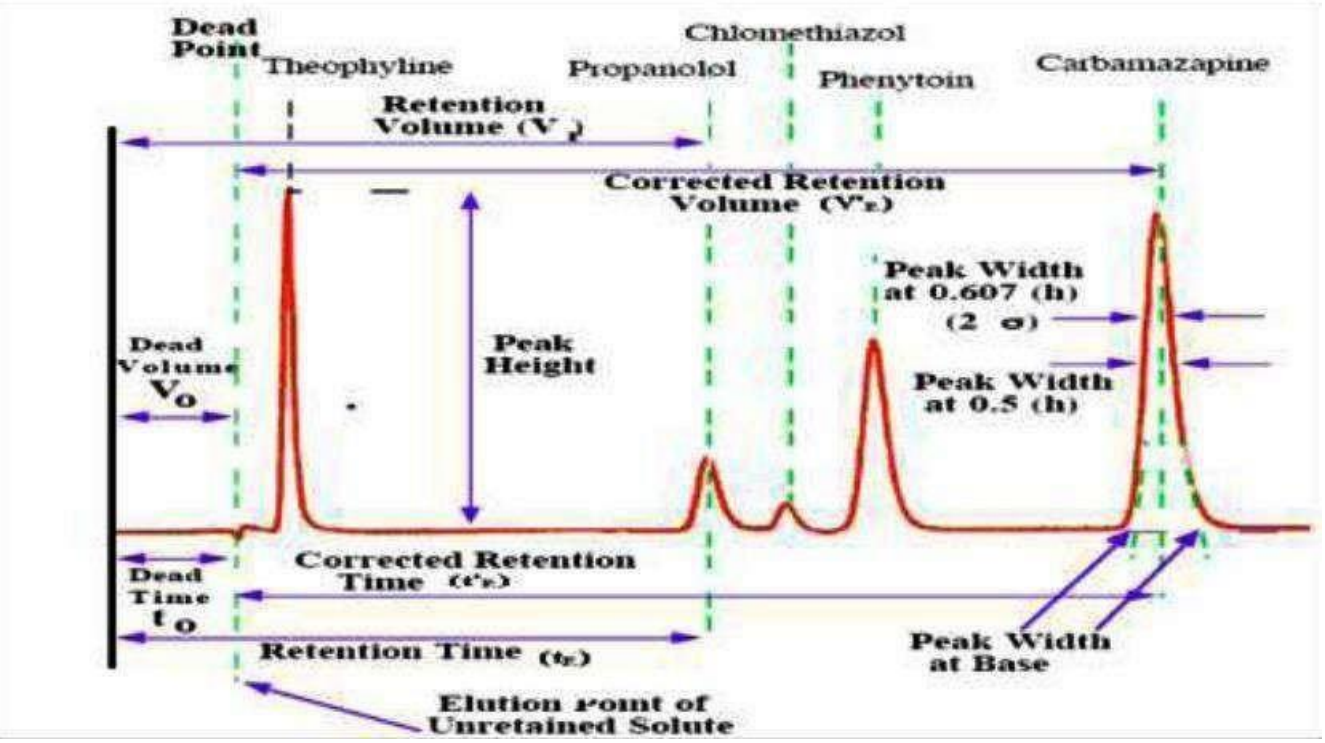

Fig. 1.5: The terms of a chromatogram

- The time spent between the injection site and the peak maximum is known as the retention time (tR). Every solute has a characteristic retention time.
- The volume of mobile phase travelled through the column between the injection point and the peak maximum is known as the retention volume (VR). VR = QtR, where

Q is the flow rate in millilitres per minute and tR is the retention period. Each solute will have its own unique retention volume.

 The time spent between the dead point and the peak maximum is called the corrected retention time (t'R).

 The volume of mobile phase travelled through the column between the dead point and the peak maximum is the corrected retention volume (V'R). It will also be the retention volume minus the dead volume. Thus, V'R = VR - Vo = Q (tR - to) where Q is the flow rate in ml/min.

 The distance between the peak maximum and the geometrically generated baseline underneath the peak is known as the peak height (h).

 The peak width (W) is the distance measured at 0.6065 of the peak height between each side of a peak (0.607h). When dealing with chromatography theory, the peak width measured at this height is comparable to two standard deviations (2s) of the Gaussian curve and hence has importance.

 The peak width at half height (W0.5) is the measurement of the distance between either side of a peak at half its height. The peak width measured at half height has no significance with respect to chromatography theory.

 The distance between the intersections of the tangents traced to the sides of the peak and the geometrically generated peak base is the peak width at the base (Wb). When dealing with chromatography theory, the peak width at the base is comparable to four standard deviations of the Gaussian curve and hence has importance.

- **Fundamental parameters of HPLC:**

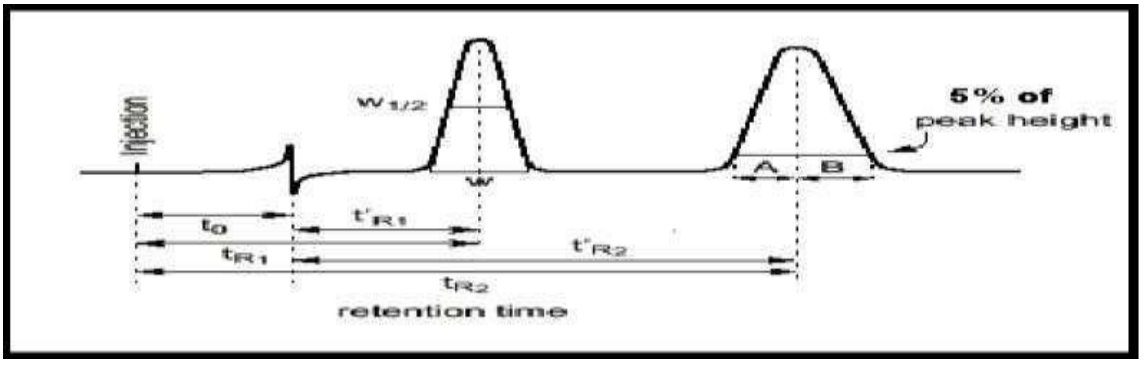

Fig. 1.6: Fundamental parameters of chromatography

1. Retention time:

It is the time taken to elute out the solute's peak maximum from the injection site, or the time required to elute out half of the solute from the injection site. The total retention time (tR1 or tR2) is the amount of time it takes for a sample component to move from the column inlet to the column end (detector).

2. Capacity factor (k'):

It is the measurement of a sample peak's location in a chromatogram. It is unique to a certain material. The stationary phase,the mobile phase,the temperature, the packing quality, and other factors all influence k. Its value should fall anywhere between 2 and 10.

$$k'_1 = \frac{t_{R1} - t_0}{t_0} \qquad k'_2 = \frac{t_{R2} - t_0}{t_0} \qquad (1)$$

3. Relative retention (α):

The ratio of two capacity factors, is the separation factor. The capacity of a chromatographic system to distinguish between two substances is described by relative retention. Impurities in the mobile phase (such as water content) have a significant impact on relative retention.

$$\alpha = \frac{k'_2}{k'_1} \qquad \text{---------- (2)}$$

4. Tailing factor (T):

The asymmetry factor of a peak is usually close to the tailing factor of the same peak, although the two numbers cannot be translated directly. The tailing factor is calculated as 5% of the highest peak height. The T value ideally be less than 2.

$$T = \frac{W_{0.05}}{2f} \quad \text{---------- (3)}$$

Where, **f**- *is Distance between maxima of two peaks*

$W_{0.05}$- *is width of peak at 5 % height.*

5. Resolution (Rs):

It's a metric for how well neighbouring bands in a chromatogram are separated; obviously overlapping bands have low Rs values. The breadth and retention duration of two neighbouring peaks are used to compute it. If Rs is less than 2.0, the calculation's accuracy is low.

$$Rs = \frac{2(t_2 - t_1)}{W_1 + W_2} \quad \text{---------- (4)}$$

Where, t_1 *and* t_2*are the retentiontime of first andsecond adjacent bands,*

W_1 *and* W_2 *are their baseline bandwidths.*

6. Number of theoretical plates (N):

It describes the quality of a column packing and mass transfer phenomena. Larger the N, the column is capable to separate complicated sample mixtures.

$$N = 16\,(t_{R1} / w)^2 \quad \text{or} \quad N = 5.54\,(t_{R1} / w_{1/2})^2 \quad \text{---------- (5)}$$

It can also be calculated by following formula.

$$\text{Plate number (N)} = \frac{3500 \times L\ (cm)}{dp\ (\mu m)} \quad \text{----------} (6)$$

Where, **L** *= Length of the column (cm)*

dp *= Diameter of the particle (μm)*

7. Height equivalent to theoretical plate (h):

The height equal to a theoretical plate h, HETP is the length required to achieve chromatographic equilibrium between the mobile and stationary phases. Because there will be a high number of theoretical plates, 'h' should be as minimal as feasible. The value of 'h' is a criteria for column quality; values are affected by particle size, flow velocity, mobile phase (viscosity), and, most importantly, packing quality.

$$h = \frac{L}{N} \quad \text{----------} (7)$$

where *L* is the column length and *N* is the plate number

8. Symmetry factor:

Symmetry is measured at 10 % of peak height. Ideally symmetry should be 1,

i.e. A = B. where B= Peak half width, A= Front half width

$$\text{Symmetry} = \frac{B}{A} \quad \text{----------} (8)$$

9. System suitability:

System suitability tests, according to the USP, are an important element of

chromatographic techniques. These tests are used to ensure that the system's resolution and repeatability are sufficient for the analysis. Plate count, tailing factor, resolution, and repeatability are determined and compared to the specification set for the methods. These characteristics are assessed during the system suitability "sample" study, which consists of a mix of major components and anticipated by-products.

Table 1.1: System suitability parameters and recommendations

Parameter	**Recommendations**
Capacity factor	The peak should be well –resolved from other peaks and the void volume, generally k′> 2.0
Repeatability	RSD ≤ 2 % for N ≥ 5 is desirable
Relative retention	Not essential as long as the resolution is stated.
Resolution (R_S)	R_S of > 2 between the peak of interest and the closest eluting potential inteferent
Tailing factor (T)	T of ≤ 2
Theoretical Plates	In general should be > 2000

1.4 High Performance Thin Layer Chromatography (HPTLC):

HPTLC (also known as Planar Chromatography) is a sophisticated separation method with a global reputation for flexibility, dependability, and cost effectiveness. It is one of the micro-analytical techniques, along with HPLC and GC, that play a significant role in research and regular laboratories. The simplest basic separation method accessible to the analyst today is HPTLC. It may be thought of as a time machine that speeds up your work and allows you to perform a lot of things at once, which is impossible to achieve with other analytical approaches. (Thorat, *et al* 2017; Attimarad, *et al* 2011; Nanda, *et al* 2010; Nanda, *et al* 2010; Nanda, *et al* 2010; Kothapalli, *et al* 2022; Kothapalli, *et al* 2015; Bhole, *et al* 2015; Bhole, *et al* 2017; Chitlange, *et al* 2017; Bhole, *et al* 2018)

HPTLC is the most simple separation technique available today, because of following advantages:

1. Extreme adaptability

2. Parallel separation of a large number of samples in a short amount of time

3. Exceptional clarity and visual assessment of all samples and sample components at the same time

4. Sample preparation is simplified because the stationary phase is only used once.

5. Because all fractions of the sample are kept on the plate, repeated assessments of the plate with varied parameters are possible.

6. A less amount of mobile phase and sample was required.

1.4.1 Schematic Procedure for HPTLC:

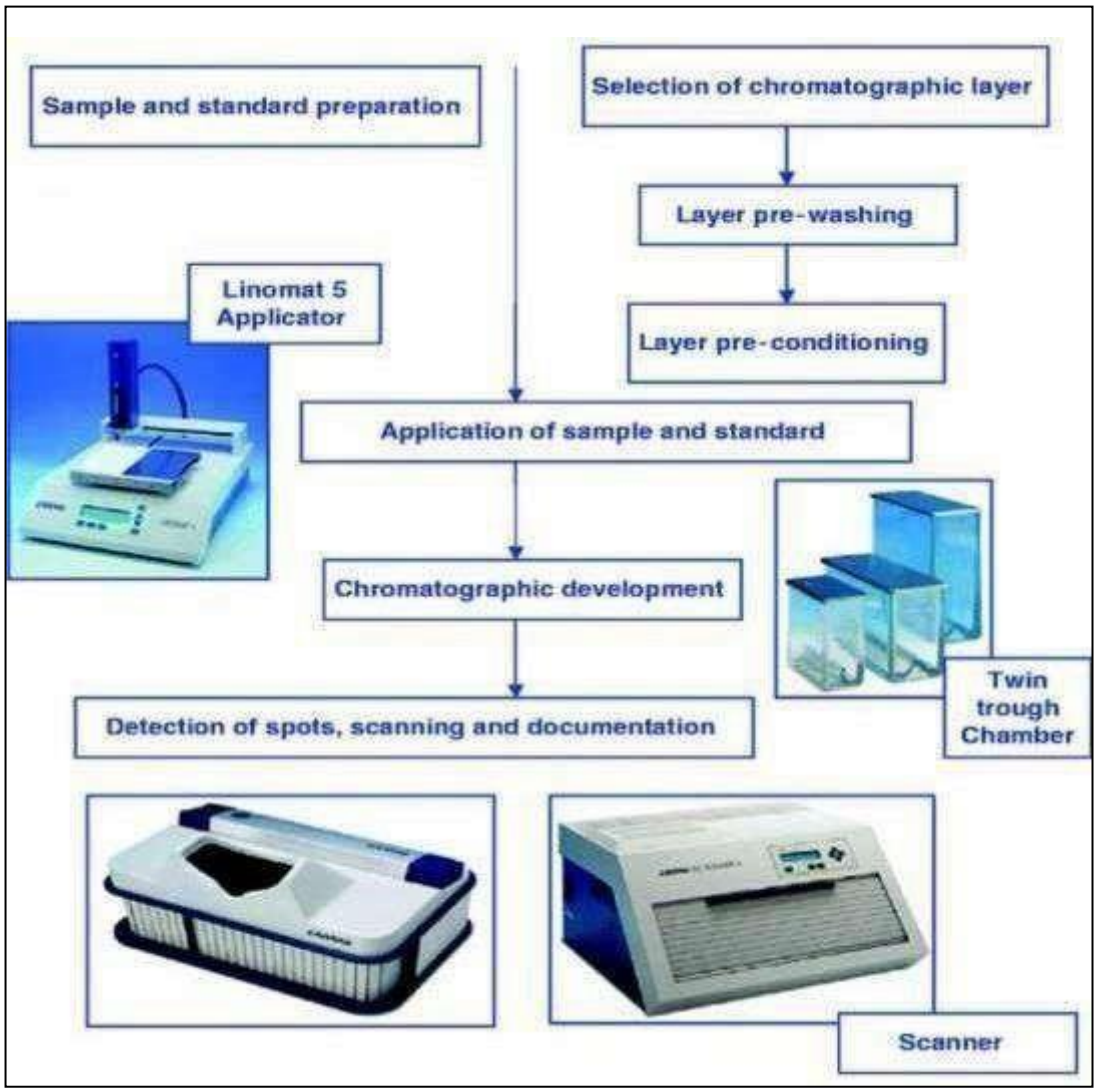

Fig. 1.7: Schematic Procedure for HPTLC

Each stage may need a unique strategy that must be thoroughly validated to identify potential sources of errors.

1.4.2 Various steps involved in HPTLC:

A) Sample preparation and derivatization

The sample preparation technique is to dissolve the sample with a sufficient concentration of analyte for direct application to the HPTLC plate, with full recovery of intact compounds of interest and minimal matrix. As a result, selecting an appropriate solvent for a specific analysis is critical. The solvent for dissolving the sample in normal phase chromatography utilising silica gel precoated plates should be non-polar and volatile as much as feasible. At the origin, polar liquids are likely to cause circular chromatography. Polar solvents are typically employed to dissolve the material in reverse phase chromatography. When a molecule does not respond to UV or has no fluorescence, derivatization processes are fundamentally necessary for identification.

Stationary and mobile phase

On various support materials, Silica gel 60 F254 with a pore size of 6 nm and fluorescent indicator is commonly employed as a sorbent layer (glass, aluminium, plastic). For quantitative analysis, precoated plates of 20 × 20 cm with an adsorbent thickness of 100 - 250 m are commonly employed. The selection of a suitable mobile phase is dependent on trial and error, with chemical characteristics of the solute and solvent, analyte solubility, adsorbent layer, and other factors being taken into account, as well as the analyst's personal expertise.

B) Activation of precoated plates

Plates that have been exposed to high humidity for an extended period of time are activated wiyh the help of oven maintained at 110°C-120°C for 30 minutes. Water that has been physically adsorbed on the surface of the sorbent is removed in this phase.

C) Sample application

It's the most important stage in getting high resolution for HPTLC quantitation. A typical application amount of 6–20 µl with a 6 mm land band size is suggested. Distances of 12–15 mm from the side and 8 mm from the bottom are commonly used.

The sample and standard are applied in a band, equal Rf values, and less spot broadening. Because of the uniform distribution and higher amount that may be applied, bands are favoured over spots. Most commonly used sample applicators are shown in figure given below.

Fig. 1.8: CAMAG sample applicators in HPTLC

D) Preconditioning (Chamber saturation)

Saturation of the chamber has a significant impact on the separation profile. The solvent evaporates from the plate mostly at the solvent front when the plate is placed into an unsaturated chamber during development. As a result, a higher amount of the solvent is required over a given distance, resulting in an increase in Rf values. Solvent vapours quickly become equally dispersed throughout the chamber if the tank is saturated (by lining it with filter paper). When a plate is put in a saturated chamber, it quickly becomes pre-loaded with solvent vapours, requiring less solvent to travel a given distance and resulting in lower Rf values. The amount of time it takes to reach saturation is determined by the type and content of the mobile phase, as well as the layer thickness. Saturation is not required for low polarity mobile phases; nevertheless, saturation is desired for highly polar mobile phases.

Chromatographic development

Ascending, descending, two-dimensional, horizontal, multiple over run (continuous),

gradient, radial (circular), anti-radial, multi-dimensional, forced flow planar chromatography are the most common types of chromatographic development.. In TLC development, twin trough chambers (Fig 1.9), Vario chambers (Fig 1.10), V- shaped chambers, Circular and Anti-circular U chambers are employed. A migration distance of 5 to 6 cm is sufficient for HPTLC plates. Plates are taken from the chamber after development and dried to eliminate any residues of mobile phase.

Common problems:

a) Tailing: This can happen if there are traces of contaminants present, or if there are several ionic species of the chemicals being chromatographed. By buffering the mobile phase system with an acidic (1-2 percent acetic acid) or basic (ammonia) solution, this can be minimised. It maintains the materials to be separated in non-ionic forms.

b) Diffusion: This is represented by chromatographic plate zones. This can be caused by non-uniformity, longitudinal diffusion between the mobile and stationary phases, or stationary phase non-equilibrium.

Fig. 1.9: Twin Trough chamber

Fig.1.10 : Vario chamber

E) Zone detection

When the development process is finished, the plate is taken out of the chamber and dried to eliminate any remaining mobile phase. Physical, chemical, and biological-physiological approaches can all be used to locate the zones. Visual inspection at 254 nm or 366 nm in a UV cabinet is used to identify HPTLC.

F) Photo and Video documentation

To assist the analysts and researchers in the traceability of analytical data, photo and video documentation are necessary.

G) Chromatogram evaluation by densitometry

The measurement of UV-Visible absorbance, fluorescence, or fluorescence quenching directly on a layer without scraping or elution is known as densitometry. To perform an HPTLC densitometric analysis, three or four standard and purified samples are placed on the same plate. After development and detection, the chromatogram is scanned (if necessary). The quantity of analyte in the sample indicated by scan area is extrapolated from the standard curve using a calibration curve consisting of scan area of standard versus amount of analyte.

1.4.3 Factors influencing-HPTLC separation and resolution of spots:

- **Type of stationary phase (sorbent)**

Commercially available sorbets for HPTLC include silica gels of various grades, aluminium oxide of various grades, florisil, and kieselguhr G.

- **Type of pre-coated plate**

The use of HPTLC pre-coated plates is required for quantitative analysis. Various manufacturers provide precoated plates with various support materials (glass, aluminium, plastic) and layers in various formats and thicknesses.

- **Mobile phase (solvent system)**

The chemical characteristics of analytes and the sorbent layer should be considered while selecting mobile phases. It is usually best to avoid using mobile phases with more than three or four components since it is difficult to achieve consistent ratios of various components.

- **Solvent purity**

Resolution, spot definition, and Rf repeatability have all been reported to be affected by the solvent grade employed in preparative mobile phases.

- **Size of developing chamber**

First, Stahl emphasised the relevance of the degree of saturation. In a standard rectangular chamber, this ratio is 1:20, while in a narrow chamber, it is 1:0.1 to 0.5.

Saturation of chamber (pre-equilibrium)

- Saturation of the chamber has a significant impact on the separation profile. As a result, a higher amount of the solvent is required over a given distance, resulting in an increase in Rf values.

- **Sample volume to be spotted**

Substance zones that are too big from the start result in poor separation since spots tend to get larger and more dispersed as they develop. This problem is exacerbated in the case of substances with high Rf values. As a result, it is suggested that the solution be applied in tiny increments with intermediate drying.

Size of initial spot (band width)

The resolution is improved by increasing the distance between separate spots and decreasing the sample's starting band width.

- **Solvent level in the chamber**

Solvent level should be lower than the position of the spots applied on the plate so that they would not be washed away while placing the plate into the chamber.

- **Temperature**

R_f value usually increases with rise in temperature.

1.5 Validation of Analytical Method

Validation is defined as "documented evidence which gives a high degree of confidence that a process, system, facility will consistently produce a product meeting its predetermined specifications and quality attributes. **(**ICH, Q2 (R1) Guideline 2005., ICH, Q2A Guideline 1994., ICH, Q2B Guideline (1996)., Nash, *et al* 2003.)

1.5.1 Method validation:

The process of showing that an analytical technique is suitable for its intended purpose is known as method validation. The United States Pharmacopoeia (USP), the International Conference on Harmonization (ICH) provide a framework for undertaking such validations for pharmaceutical procedures.

1.5.2. Parameters for method validation:

a) Accuracy

The closeness of agreement between the values, which is recognised either as a conventional true value or an acceptable reference value obtained, is expressed by the accuracy of an analytical method. The accuracy studies' findings are presented as a percentage recovery. (The findings must be followed within a 98% to 102 %.)

b) Precision

The degree of dispersion (closeness of agreement) between a set of measurements acquired from serial sampling of the same homogeneous sample under the required conditions is expressed by the precision of an analytical technique. It's usually stated as a % of the standard deviation. Precision may be considered at three levels:

i) Repeatability

The term "repeatability" refers to the precision of a measurement made under the same operating circumstances over a short period of time. Intra-assay precision is another name for it. (Repeatability studies must have a relative standard deviation of less than 2%.)

ii) Intermediate precision

iii) Intermediate precision refers to the precision of results obtained from different laboratories, different days, different analysts, different equipment, reagents, and so on. (Studies of intermediate accuracy must have a relative standard deviation of less than 2%.)

iv) Reproducibility

The term "reproducibility" refers to the consistency of results among laboratories.

c) Specificity

The capacity to assess the analyte definitively in the presence of components that may be present is known as specificity. Impurities, degradants, matrix, and other substances are examples of these.

d) Limit of Detection

The lowest quantity of analyte in a sample that can be detected but not necessarily quantified as an exact number is the detection limit of an individual analytical process. The limit of detection (LOD) is a concentration at a certain signal-to-noise ratio.

S/N= 2/1 or 3/1 - ----------(9)

*Where,***S=** *Signa,* **N=***Nois:*It may be calculated based on standard deviation (SD) of the response and slope of the curve(S).

LOD= 3.3(SD)/S ..... (10)

Where, **SD=** *Standard deviation,***S=** *Slope*

e) Limit of Quantitation

The lowest quantity of analyte in a sample that can be quantitatively measured with suitable precision and accuracy is the quantitation limit of a particular analytical method. The limit of quantitation (LOQ) is a concentration at a certain signal-to-noise ratio. The injected quantity in chromatography quantitation is the amount that produces in a peak with a height 10 times that of the baseline noise level.

S/N = 10/1 .... (11)

*Where,***S=** *Signal,***N=***Noise:*It may be calculated based on standard deviation (SD) of the response and slope of the curve(S).

LOD = 10 (SD)/ S - -------- (12)

Where, **SD=** *Standard deviation,***S=** *Slope*

f) Linearity

The capacity of an analytical technique to produce test findings which is directly proportional analyte concentration in the sample (within a defined range) is known as

linearity. (For linearity studies, the correlation coefficient must be more than 0.999.)

g) Range

The range of an analytical method is the range of analyte concentrations in a sample for which the analytical procedure has been proven to have a sufficient degree of precision, accuracy, and linearity.

h) Robustness

The robustness of an analytical methodology is a measure of its capacity to remain unaffected by small but deliberate changes in method parameters, and it reflects its reliability when used frequently.

i) Ruggedness The robustness/ruggedness of an analytical technique is a measure of its ability to stay unaffected by modest but purposeful changes in method parameters, and it indicates its dependability throughout regular use.

1.5.3 Forced Degradation (Stress Degradation Studies)

Objectives:

The goal of stability testing is to gather information that will allow for shelf-life proposals and storage recommendations.

The goal of stability studies is to figure out how a medical product's quality changes over time and under the impact of various environmental variables.

On the basis of the information gathered, storage settings are advised that will ensure that the medical product's quality, in terms of safety, effectiveness, and acceptability, is maintained during the projected shelf-life (i.e., during storage, distribution, dispensing and use). It also enables the justification of any active component overage used to ensure potency at the end of the shelf life. The Stability Indicating Method can be defined as ―Validated Quantitative analytical method that can detect the changes with time in the chemical, physical or microbiological properties of the drug substance and drug product, and that are specific so that the content of active ingredient, degradation can be accurately measured without interference.

Stress testing is intended to "determine the intrinsic stability of the molecule by establishing a degradation pathway in order to identify the likely degradation products

and to validate the stability indicating power of the analytical procedure used," according to the International Conference on Harmonization (ICH) guidelines.

Stress testing is required by the ICH guidelines „Stability testing of novel drug substances and products' (QIA) to elucidate the substance. It indicates that the breakdown products generated under various conditions should take into account the effects of temperature, humidity, oxidation, photolysis, and hydrolysis susceptibility throughout a wide pH range. The effect of temperature is advised to be studied in 10°C increments above the accelerated temperature test setting (e.g. 50°C, 60°C, etc.) and humidity at a level of 75 percent or more, according to the guideline. However, no information is given on the research of oxidation, photolysis, and hydrolysis at various pH levels. The ICH recommendations Q3B, headed "Impurities in New Drug Products," highlight the need of providing documented proof that analytical techniques are established and adequate for detecting and quantifying the degradation product. It also necessitates the validation of an analytical method to show that impurities specific to the novel therapeutic ingredient do not interact with or segregate from defined and unspecified degradation products in drug products. Stress testing is an important part of the medication development process. Predictive degradation information may be gathered early in the process by creating critical stress testing samples (i.e., partially degraded sample strained under various conditions), which can be of substantial value to a drug firm in terms of time and money.

Temperature, humidity, light, oxidative agents, and sensitivity over a wide range of pH values should all be considered during stress testing. It is also suggested that stability samples be analysed with the help of established stability indicating testing procedures. (Singh et al 2002;, ICH, Q1A (R2) Guideline, 2003; . ICH, Q1B, Guideline, 1996).

- ***Overview of regulatory guidance:***

• Stability-indicating technique development and validation

• Determination of drug substance and drug product degradation pathways

• Distinguishing between degradation products linked to drug compounds and those related to non-drug substances in formulations (e.g., excipients)

• Degradation product structure elucidation

• Determination of a medicinal substance molecule's inherent stability.

Degradation studies

a) Acid and Alkali Hydrolysis:

In 0.1- 2 N HCl / 0.1 N NaOH, the hydrolytic breakdown of a new medication in acidic and alkaline conditions may be investigated. Testing can be terminated at this step if reasonable deterioration is seen. However, if no degradation occurs under these conditions, a greater strength HCl / NaOH solution should be used over a longer period of time. If complete degradation is seen after exposing the medicines to the initial condition, the acid/alkali strength as well as the reaction temperature can be reduced.

b) Oxidative Degradation:

To test for oxidation, use hydrogen peroxide in a concentration range of 3 to 30%. When exposed to 3 percent hydrogen peroxide, several medicines undergo significant deterioration. In some situations, even under severe conditions, exposure to large concentrations of hydrogen peroxide does not result in substantial deterioration. The behaviour is consistent with expectations, as some medicines are oxdisable while others are not. Even in the presence of a high amount of oxidising agent, the latter are not anticipated to alter.

c) Photolytic Degradation:

UV light: Photolytic experiments should be conducted using a mix of cool white and UV fluorescent light. Exposure energy should be a minimum of 1.2 million lux hours fluorescent light, with the intensity raised by 5 times if decomposition is not detected. If no decomposition occurs, the medication can be classified as photo stable.

d) Heat degradation:

Stress testing for dry heat degradation can be carried out by heating the medication powder at a higher temperature in an oven. If there isn't enough deterioration in the initial experiments, the heating duration can be raised to 24 hours.

1.6 Formulations of Transferosomes formulation

1.6.1 Introduction

1.6.1 a The Human Skin &Pigmentation:

The skin is the biggest human organ and acts as a protective barrier, allowing water to enter while keeping dangerous substances out. The skin, along with the kidney, controls body temperature and maintains the body's water-electrolyte balance. It is also one of the five human sense organs. (Hadgraft, 2001) Minor physiological changes may alter pigmentation patterns in both temporary (such as during pregnancy) and permanent ways. (Costin & Hearing, 2007)

- **Skin Composition:**

The skin is the body's first line of defense against the environment, transmitting information about both health and personal identity. The skin plays important role in the body's physiological state by serving as a huge physical barrier against various factors(Wolff et al., 2008). Besides these functions, the skin acts as an immune network and offers a unique UV protection mechanism via its pigments (UV-R)(Brenner & Hearing, 2008).The three layers of the skin are the epidermis, dermis, and hypodermis, with the hypodermis. The layers and components that make up the system.

The epidermis is a stratified epithelium that is 5–100 m thick and lacks blood and nerve supply (although it may reach 600 m on the palms and soles) (Serup et al., 2015). It is made up of a variety of cell types, the most significant of which are keratinocytes and melanocytes, which account for 95% of the epidermis and are arranged into four layers. Merkel cells and melanocytes are at least two distinct kinds of neural crest-derived cells. In the stratum granulosum, non-dividing flattened polyhedral keratinocytes produce keratino hyalin granules. Langerhans' cells are the skin's antigen-presenting cells and are involved in many immunological responses, including allergic contact dermatitis. Keratinocytes differentiate further as they progress, cornified cells with abundant keratin but no cytoplasmic organelles result from the basal layer to the stratum corneum.. (Elias, 2005).

The dermis, connective tissue, and fibroblast-rich layer that houses the skin's neural, vascular, lymphatic, and secretory systems and is 2 to 4 mm thick, is connective tissue

and fibroblast-rich layer that houses the skin's neural, vascular, lymphatic, and secretory system. Fibroblasts, which are the most prevalent cell type, generate and destroy the extracellular matrix (ECM). This matrix is a highly structured structure composed of collagen, elastin, and reticular fibers. Additionally, the dermis contains immune system multifunctional cells such as macrophages and mast cells, the latter of which can elicit allergic reactions through the secretion of bioactive mediators such as histamine.

The following structures are found in the dermis:

Glands of excretion and secretion. Sebaceous glands generate sebum, which is high in triglycerides and cholesterol, and lubricates and waterproofs the skin. Hair shafts are often connected to them.

Hair follicles and nails (Cotsarelis et al., 1990; Ito et al., 2005). During wound healing, these stem cells are most visible.

Melanocytes are divided into three types:

Melanin synthesis is a complex process that takes place within membrane-bound organelles called melanosomes in highly specialized cells known as melanocytes. Melanosomes are delivered to neighboring keratinocytes through dendrites. The epidermal melanin unit is the anatomical link between keratinocytes and melanocytes, and each melanocyte is anticipated to be in contact with 40 keratinocytes. (Fitzpatrick & Breathnach, 1963) Many critical steps are required are described below.(Boissy & Nordlund, 1997)

A) Melanocyte precursor cells develop and migrate. The dermis, epidermis, and hair follicles are mainly affected, as are the uveal tract, stria vascular, as well as the brain's leptomeninges. Humans migrate their dermis during the tenth and twelve weeks of development, whereas their epidermis migrates two weeks later. Steel factor binds to the KITreceptor on melanocytes and melanoblasts, for example, and is also known as stem cell factor (SCF).

B) Melanoblast differentiation into melanocytes: Melanoblasts become melanocytes once they reach their final destinations, which are already established.

C) Melanocyte been detected in the fetal epidermis as early as 50 days after conception. Dermal melanocytes decline in number during pregnancy and almost disappear before

delivery.

D) Melanosome development and melanin production: Melanocytes generate melanosomes, which are highly structured after they have formed in situ.

Electron microscopy may detect in pregnancy (EM). Melanosomes are into four levels (I–IV) depending on their shape, quantity, quality, distribution during after melanin produced (Fig. 1.12) (Kushimoto et al., 2001; Seiji et al., 1963)

Nascent melanosomes are formed near the Golgistacks in the perinuclear area. TYR, on the other hand, has been found in Golgi vesicles and has been demonstrated to be trafficked to stage II melanosomes. (Berson et al., 2001; Kushimoto et al., 2001), which contain tyrosinase and have limited melanin deposition. Melanin production begins after this, and the pigment is evenly deposited on the interior fibrils, indicating that the melanosomes have reached stage III.

In highly pigmented melanocytes, their last developmental stage (IV) is identified. To manufacture various kinds of melanin, at least three enzymes are needed inside melanosomes. While tyrosinase is responsible for the key stages of melanogenesis (including the rate-limiting first phase , also involved in the modification of melanin into various kinds. (Basrur et al., 2003)

1.6.1b Hyperpigmentation:

Hyperpigmentation occurs when certain regions of the skin produce and deposit too much melanin, leading them to become darker than the surrounding skin. (Fig. 1.11) It is more prevalent in individuals who have a non-Caucasian or olive complexion. (Ebanks et al., 2009) Phototoxic response to scented goods may all cause hyperpigmentation. The endocrine system has a significant role in melanin synthesis. Chemical messengers or hormones are sent to the body via the endocrine system. A melanocyte-stimulating hormone is one of these hormones (MSH). In diseases like Nelson's Syndrome and Addison's illness, MSH promotes the synthesis of melanin in the skin. (Jablonski, 2004)

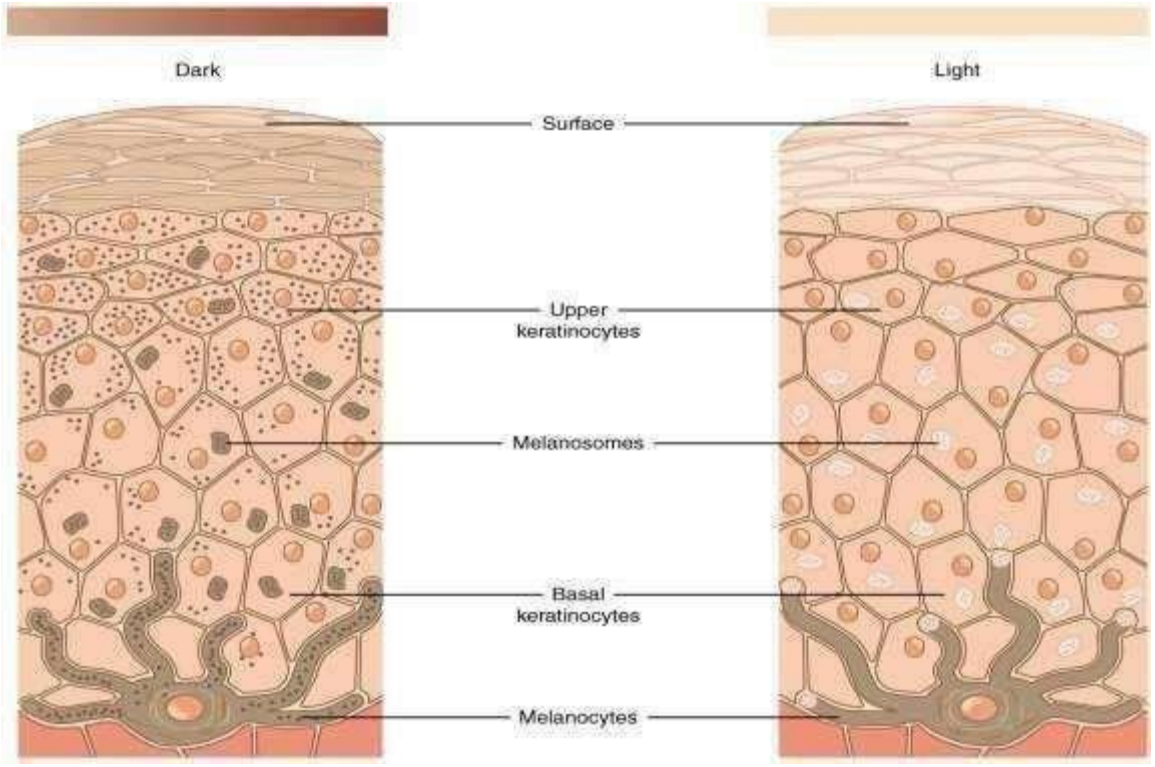

Figure 1.11: Difference between dark and light skin

1.6.1c Hyperpigmentation Induced…External Factors:

A) UV influence on human pigmentation:

The skin develops two defensive barriers in response to UV-R exposure: the stratum corneum thickening and the development of a melanin filter in epidermal cells.

The thickest stratum corneum, which makes them very resistant to UV damage.

- Keratinocytes, which respond to UV-R (bursts of mitoses) and increased synthesis of endothelin-1 (ET-1) and proopiomelanocortin (POMC), are thought to trigger this process, resulting in a new need for melanosomes. After UV irradiation, the mitotic rate of basal keratinocytes increases one day, peaks two days later, and then plateaus for one week. It then starts to erode, and after 1–2 months, assuming no further exposure, the skin returns to its original thickness. To fulfil the growing need, increased rates of melanosome transfer to keratinocytes after UV-R for melanosomes created by keratinocyte proliferation. (Brues, 1992)
- UV comprises only 5 to 10% of the radiant energy absorbed at Earth's surface from

the sun, with the rest split evenly between visible (40%) and infrared (40%) wavelengths UV-A, UV-B and UV-C (320–400 nm), (280–320 nm), (200–280 nm) respectively are the three forms of UV radiation; the latter, like most wavelengths 280 nm. 19–50% of solar UV-A is estimated to reach the depths of melanocytes, compared to only 9–14 percent of solar UV-B. As a result, whereas UV-A exposure stimulates melanin pigmentation, the resulting tan appears to be transient and less protective against UV-induced damage than tans produced by UV-B exposure. Even though the amount of UV-A reaching the Earth's surface is many orders of magnitude higher, UV-A has 1000 times fewer erythema-producing effects than UV-B (Wang et al., 2001). In cultured human melanocytes, UV-A has also been proven to cause cancer (Garland et al., 1993; Stern et al., 1997). Melanin acts as a natural sunscreen in the skin by neutralizing Reactive Oxygen Species (ROS) produced by a variety of sources, including UV-B (Lindelöf et al., 1999; Stern & PUVA Follow up Study, 2001). It was originally thought that the higher the concentration of melanin, the lesser the danger of UV-R-induced DNA damage.

- Despite the popular notion that dark skin types are UV resistant and hence unharmed by UV, this study discovered that even the darkest UV-resistant skin types suffered significant DNA damage at levels as low as one erythema dosage (MED).

B) Tanning caused by ultraviolet radiation (UV-R): Tannin is described as an increase in skin pigmentation over the baseline constitutive level that is physiologically activated by UV-R in humans. UV-induced skin darkening increases the number of melanocytes and speeds up melanin production and dendricity. Two stages of the tanning reaction have been identified: delayed tanning. It begins right after exposure, peaks in 1–2 hours, and then fades between 3 and 24 hours after exposure.(Gilchrest et al., 1996) Exposure to UV-B, as well as UV-A and visible light, produces delayed tanning, resulting in a long-lasting tan. It's a lengthy process in which the skin darkens 48–72 hours after exposure, peaks 3 weeks later, and takes another 8–10 months to return to its normal melanin level.(Nishizuka, 1986; Sies & Stahl, 2004) Delayed tanning is induced by qualitative and quantitative changes in melanocytes, which become larger, more dendric, and have a wider dispersion.

- At all embryonic stages, indicating an increase in TYR and melanosome production,

melanization, and number conveyed to keratinocytes. As a result, melanocyte populations and melanogenesis rise, causing tanning to be delayed. (Friedmann et al., 1990; Gordon & Gilchrest, 1989)

- The development of chloasma-like pigmentation of the cheeks, forehead, and nose has been linked to the use of oral contraceptives.(Goh & Dlova, 1999; Pathak & Stratton, 1968)A microscopic study of the epidermis revealed increased melanogenesis and bigger melanocytes. Skin darkening has been connected to antiepileptic medicines (mainly hydantoins) (Levantine & Almeyda, 1973). Females have higher melanin concentrations than men, with Caucasians being the most affected(Robins, 1972). Chloroquine has a strong affinity for melanin, causing skin darkening. Many studies have discovered melanin in the dermis of chloroquine-treated people (Levy, 1982). Skin hyperpigmentation is caused by the drug levodopa. Because DOPA (like levodopa) is normally converted to melanin inside melanosomes, DOPA therapy (like levodopa) may boost melanin formation, possibly by extracellular oxidation, but there is no good evidence in the literature to support this notion.
- Heavy metals, which can occur after long-term use of drugs containing arsenic, bismuth, gold, or silver, can also induce hyperpigmentation. (Molokhia & Portnoy, 1973)

1.6.1 d Internal Factors-Induced Hyperpigmentation:

A) Hormonal effects on skin pigmentation in humans:

- Melasma, chloasma, or the masks of pregnancy are all terms for hyperpigmentation that occurs during pregnancy. The cheekbones, upper lip, chin, and forehead are all affected. This syndrome is characterized by asymmetrical hypomelanosis.
- Although pregnancy is the most prevalent cause of melasma, additional variables also, hereditary effects, some cosmetics, endocrine or hepatic disorders, and certain antiepileptic medicines can also play a part in its development. UV-R is the most significant of the environmental sources (Barankin et al., 2002). Increased melanin deposition in the epidermis and dermis characterizes melasma hyperpigmentation(Grimes et al., 2005; Kang et al., 2002). The number of melanocytes did not increase, but produced eumelanin in particular (Grimes et al.,

2005). The number of melanosomes shown to be higher in lesional skin than in nonlesional skin. Melasma has been associated with elevated levels of estrogen, progesterone, and MSH (Smith et al., 1977; *Williams Textbook of Endocrinology - 12th Edition*, n.d.). Cellular proliferation reduces in melanocytes treated with estrogen in vitro (Ranson et al., 1988).

- Melasma patients' melanocytes may be more vulnerable to the stimulating effects of estrogens and maybe other non-steroid hormones by nature. A recent study reveals that estrogens have the same molecular mechanisms in the skin as they do in non-reproductive tissues (Verdier-Sévrain et al., 2006). 17-estradiol has been demonstrated to use both genomic and nongenomic signaling pathways in epidermal keratinocytes (Thornton, 2005).Although estrogens have such a large impact on skin, little is known about their cellular and molecular mechanisms of action, and their impact on pigmentation is unknown. Estrogens work by connecting with and activating certain Estrogen Receptors (ERs), that are produced by genes on separate chromosomes (Enmark et al., 1997; Thornton, 2005). They are 60% similar, bind 17-estradiol with about equivalent affinity, and have similar binding profiles for a variety of natural and synthetic ligands. (Couse et al., 1997; Kuiper et al., 1997). B) Skin hyperpigmentation as a result of the inflammatory response:
- Individuals with darker skin are more likely to develop post-inflammatory hyperpigmentation, in which the number of melanocytes is normal, but the amount of melanin is higher. synthesis at the cellular level(Tomita et al., 1987).

1.6.1 e Treatment Options for Hyperpigmentation:(Cayce et al., 2004)

Hyperpigmentation may be treated in several ways:

- Brightening chemicals work by blocking several steps of the melanin synthesis pathway, preventing pigmentation from forming in the first place.
- Exfoliating with AHAs, having chemical peels, using retinoids such as Tretinoin or Retinol, brightening chemicals such as Vitamin C, or a lightening agent such as Hydroquinone can all assist to remove melanin deposits.
- Ablative lasers that destroy layers of skin and IPL (intensity pulsed light) that shatters melanin pigment granules are two mechanical ways for eliminating melanin deposits.

Lasers are the most effective method for removing cutaneous pigment because they may enter the dermis.

- You can use injections to slow down the production of melanin (the key ingredient is glutathione). Table 1.2 lists the most often used lightening and brightening agents.

Table 1.2: Most Common used Lightening & Brightening agents:(Jimbow et al., 1974; Palumbo et al., 1991; Pathak et al., 1986)

Plant-Derived agents	**Chemical agents**	**Exfoliating agents**
Glabridin	Hydroquinone	Azelaic Acid
Pine Bark	Retinoids (e.g. Retinol, Tretinoin)	Lactic Acid
Pycnogenol	Vitamin C	Phytic Acid
Indian Gooseberry	Niacinamide	Glycolic Acid
Kojic Acid	Glucosamine	Salicylic Acid
Cucumber	Resorcinol	Trichloroacetic (TCA)
Arbutin	Peptides (e.g. Nonapeptide-1, Oligopeptide-68, Oliogpeptide-34.	Enzymes (e.g. papain, bromelain)

1.6.2 Introduction to Vesicular drug delivery systems:

A unique drug delivery system guides to target during the treatment time and distributes medications to that site at a pace and extent regulated by the body's needs (Bhalaria et al., 2009), whereas a drug delivery system delivers at site for a defined. The fundamental motivation for developing new delivery techniques is to either delay the pharmaceutical release or maintain a therapeutic concentration that is effective while causing fewer negative effects(Jadhav et al., n.d.). Several carrier systems for drug delivery across multiple channels have been created to enable regulated and targeted medicine administration (Biju et al., 2006). Among the strategies used in drug, distribution are patented technologies that vary medicine release profiles andpharmacokinetics to improve product safety and effectiveness. Patient compliance has improved as a result of this method(Sharma et al., 2009). In dermal and

transdermal distribution, the skin serves to achieve localized and systemic effects(Touitou, 2002). One of the most essential strategies for properly treating skin problems is to provide medications directly to the skin. As a result, a wide variety of medications have been utilized to treat various fungal infections on the skin(Katare et al., 2010). Novel dermal systems have distinct external and interior structures and compositions than traditional formulations (creams and ointments) and have been created as possible carriers to transport antifungal medications to the target site and improve epidermal permeability across the skin (Gupta et al., 2004). There have been numerous attempts to produce lipid-based vesicular carriers to improve topical pharmaceutical administration(Sinico & Fadda, 2009). According to several studies, these vesicles offer a promising future in terms of improving topical medicine administration and in-vivo drug release(Jerajani et al., 2000).

1.6.3 Transposable (Deformable Vesicle)

In 1991, Gregor Cevc created the term Transfersome (TFs) as well as the underlying concept. A Transfersome is a complex aggregation that is exceedingly flexible and stress-sensitive in its broadest sense. Transfersome successfully overcome transport barriers and, as a result, Drug carrier for targeted drug administration and therapeutic agent sustained-release(Cevc et al., 1996). Traditional liposomes are hundreds of times less elastic than these vesicular transfersomes, making them perfect for skin penetration. By pressing themselves along the stratum corneum's intracellular sealing lipid, transfersomes get through the barrier to skin penetration. This is achievable because of deformability, which allows for self-adapting entry in response to the mechanical stress of the environment. Transfersomes can penetrate stratum corneum via two separate intracellular lipid routes with varying bilayer properties(Cevc, 1991; Cevc & Blume, 1992).

1.6.4 Penetration Mechanism of Transfersomes:

When supplied under the right conditions, transfersomes can transfer 0.1 mg of lipid per hour and cm2 of undamaged skin. This value is substantially higher than the transdermal concentration gradients that normally drive it. This high flux rate is caused by natural "transdermal osmotic gradients," which are accessible over the skin and have a significantly more pronounced gradient. The skin penetration barrier provides an osmotic gradient that keeps a water activity difference between the live epidermis (75% water content) and the dead epidermis and prevents water loss through the skin and the

practically dry stratum corneum (near the skin surface) (15 percent water content). Even when transdermal water loss is excessive, ambient air serves as a perfect sink for the water molecule, resulting in a very stable gradient. As a result, most lipid bilayers can withstand dehydration by themselves. As a result, all lipid vesicles made from polar lipid vesicles migrate from relatively dry places to sites with a lot of water.

When a lipid solution (transfersomes) is applied to the skin surface, it is partially dried due to evaporation of water, and the lipid vesicles since this "osmotic gradient" and migrate along with it to avoid total drying. Because surfactant-based transfersomes have greater rheologic and hydration qualities than the surfactant responsible for their increased deformability, they can only do so if they are sufficiently flexible to pass through the skin's tiny pores. Liposomes and other less deformable vesicles are limited to the skin's surface, where they dry and fuse completely, resulting in poorer penetrating power than transfersomes. The flexibility of transfersomes is enhanced in this aspect, allowing them to completely exploit the transepidermal osmotic gradient (water concentration gradient).

At least one amphipathic (such as phosphatidylcholine) is present in the carrier aggregation, which self-assembles into a lipid bilayer in aqueous solvents and closes into a simple lipid vesicle. Lipid bilayer flexibility and permeability are significantly enhanced by adding at least one bilayer softening component (such as a biocompatible surfactant or an amphiphile medication) (Ramesh, 1997). If you're looking for a unique way to express yourself

The resultant Transfersome vesicle, which has been designed for flexibility and permeability, can simply and quickly change its form to the environment by changing the local concentration of each bilayer component to the bilayer's local stress (Fig:1.12).

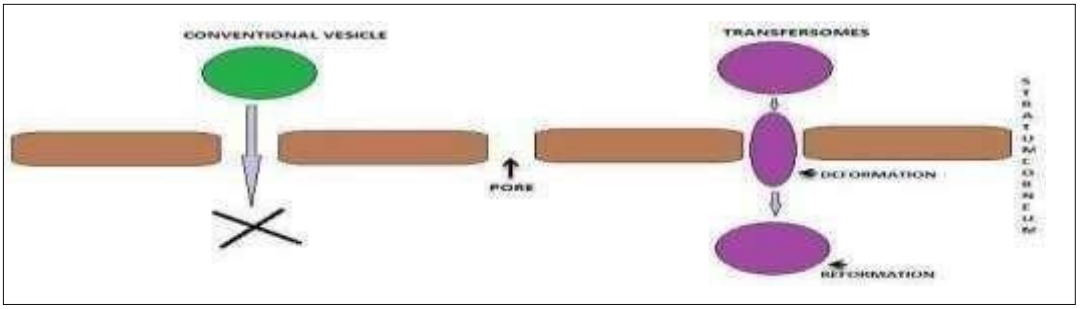

Figure 1.12: Mechanism of Transfersomes penetration.

Transfersomes are frequently formulated with phospholipids, surfactants, alcohol, color, buffering agent, and other ingredients. Table 1.3 lists the different elements needed in the creation of Transfersomes.

Transfersomes can be made using the same method as was used to generate liposomes. According to the report, two strategies are used to create deformable vesicles. Thin-film hydration is the first way, while a modified handshaking method is the second. To create uniform-sized vesicles and homogeneous dispersion, the traditional rotary evaporation sonication procedure is also commonly utilised. Any technique for preparing transfersomes has two phases. The hydrated thin film is then sonicated to the required size, and the sonicated vesicles are subsequently homogenised by extrusion through a polycarbonate membrane. The thin-film hydration approach is most commonly used for transfer preparation: hydration produces a thin film, which is then sonicated to the desired size. Extrusion across a polycarbonate membrane homogenises the sonicated vesicles. A volatile organic solvent is used to dissolve the phospholipid-surfactant mixture (chloroform-methanol).A rotary evaporator is used to evaporate the organic solvent above the lipid transition temperature. The final traces of solvent are vacuumed away overnight. Rotating at 50-100 rpm at a sufficient temperature hydrates the deposited lipid sheets with buffer. At normal temperature, the resultant vesicles expand for 2-3 hours.

Table 1.3: Materials for transfersomes

Class	**Example**	**Uses**
Phospholipids	Dipalmitoyl phosphatidylcholine, Distearoyl phosphatidylcholine, Soya Phosphatidylcholine	Vesicles forming component

Surfactants	Sod.Cholate, tween-80, Span-80, Sod.deoxycholate,	For providing flexibility
Alcohol	Ethanol, methanol	As a solvent
Buffering agents	Saline phosphate buffer, phosphate buffer	As a hydrating medium
Dyes	Rhodamine,Fluorescein, Nile red	For CSLM study

Need of Work

- An extensive literature search revealed that there is need of novel formulations and there are only a few methods available on RP-HPLC analysis of Apremilast and Glabridin in new Pharmaceutical formulation.
- Glabridin is available in the form of oral and topical (cream) dosage forms for the treatment of hyperpigmentation. Glabridin, as a member of flavonoids family, act by inhibiting tyrosinase enzyme, which regulates the production of melanin. Therefore, flavonoid glabridin has been introduced for the treatment of hyperpigmentation with fewer side effects and interaction with other drugs, non-carcinogenicity. IF administered orally, there are problems like very poor solubility in biological fluids that results into poor bioavailability after oral administration and Large amount of drug required to administer. If used in form of conventional creams, Poor permeability of some drugs through the skin, larger particle size drugs which are not easy to absorb through the skin and Enzyme in epidermis may denature the drugs. So, a need was felt to develop Transfersomal topical gel containing Glabridin in nano encapsulated form in a vesicular drug delivery system. This would be transfersome as nanocarrier. So, it is thought

worthwhile to develop and validate new stability indicating RP-HPLC method of glabridin.

- Apremilast drug is reported for Rheumatoid arthritis (RA) treatment. This disease directly depends upon circadian rhythm of the body i.e., the maximum joint stiffness generally higher during the morning. The modified release apremilast pellets can be taken before bedtime and capable of releasing drug after predetermined lag time. So, it‟s useful if available in the form of pellets. So, it is thought worthwhile to develop and validate new stability indicating RP-HPLC and HPTLC method for Apremilast.

Aim & Objective of Work

- To design and evaluate transfersomal gel for topical delivery of Glabridin.
- To formulate Glabridin loaded transfersome by film hydration technique followed by probe sonication and to evaluate the transfersomes.
- To design and validate an RP- HPLC method for determining Glabridin in transfersomal formulation that isstable.
- To formulate apremilast pellets and evaluate for various characteristics. Develop and verify a stability-indicating RP-HPLC and HPTLC method for estimating Apremilast in both bulk and pellet form.

Plan of Work

I. To design and evaluate transfersomal gel for topical delivery of Glabridin

- Literature survey
- Selection and procurement of drug and excipients
- Characterization of drug:

  Melting point, FTIR Spectroscopy, UV Spectroscopy, Diffraction scanning calorimetry (DSC)
- Formulation development:

  Formulation of Transfersome loaded glabridin by using film hydration method.
- Evaluation and characterization of transfersome by:

Appearance, Entrapment efficiency, Vesicle size analysis, Drug content, In-*vitro* release study

- Characterization of optimized formulation:

  Particle size analysis,Zeta potential, Scanning electron microscopy(SEM), Diffraction scanning calorimetry(DSC)

- Formulation of glabridin loaded transfersomal gel
- Characterization of gel:

  Appearance, pH, Drug content determination, Spreadability, Viscosity, *In-vitro* drug release

II. To develop and validate an RP-HPLC method for determining Glabridin in transfersomal formulations that is stable..

- Selection of suitable detection wavelength
- Optimization of mobile phase composition
- Optimization of chromatographic conditions
- To determine linearity range
- Studyof system suitability parameters
- To perform analysis of formulation by proposed method
- To validate the developed method as per ICH guidelines

III. To formulate apremilast pellets and evaluate for various characteristics

- Formulation of Apremilast pellets
- Evaluation of pellets

  Particle size analysis, physicochemical properties of Apremilast, Hardness, Drug Content, Pellets Shape, Differential scanning calorimeter,

IV. Develop and evaluate a stability-indicating RP-HPLC and HPTLC method for estimating Apremilast in both bulk and pellet form.

- Selection of suitable detection wavelength
- Optimization of mobile phase composition
- Optimization of chromatographic conditions
- To determine linearity range
- Studyof system suitability parameters

CHAPTER-2
REVIEW OF LITERATURE

Review of literature done to identify the status of research on novel formulations of Glabridin and Apremilast as well as the available analytical methods and solvent systems. Through literature review at national and international level revealed the need of development of novel formulation and development of analytical methods for Glabridin and Apremilast.

Literature review on Transfersomes:

- **(Opatha et al., 2023):** The benefits of transdermal administration methods over traditional oral and parenteral ones have sparked considerable attention in the last few years. They are self-administered, noninvasive delivery devices that help patients stay compliant while also releasing medicinal substances at a regulated rate. Transfersomes have a high degree of elasticity and may bend and compress as a whole vesicle through holes hundreds of times smaller than the vesicle itself. Here, the goal is to describe the concept of transferosomes as well as the mechanism of action. Other topics covered in this review include different preparation methods and characterization factors that affect the properties of Transfersomes, as well as their recent use in transdermal drug administration. Transfersomes are ultra-deformable carriers that are more effective than traditional vesicular systems in delivering a wide range of medication compounds through the epidermal barrier. To move transferosomes further into the skin, a strong osmotic gradient is required. The most important thing to remember about transferosomes is that they are custom-built vesicular systems that must be tuned for particular medicines to produce the most effective formulations and final pharmacological reactions.

- **(Balata et al., 2023):** The purpose of this study was to create a once-daily transferosome-loaded IvabradineHCl IVB transdermal film. The surfactant type, drug:phospholipid ratio, and surfactant:phospholipid ratio were used to construct transferosomes using a modified ethanol injection technique. Entrapment efficiency, particle size, zeta potential, in vitro drug release, optical and

transmission electron microscopy imaging, differential scanning calorimetry (DSC), and Fourier transform infrared spectroscopy were all used to characterize the transferosomes (FTIR). The best transferosomes and pure medication were then formed into a transdermal film and tested for content homogeneity, folding endurance, weight and thickness, moisture content, moisture absorption, tensile strength, and penetration through rabbit excised ear skin. The pharmacokinetic characteristics of the transfersomes integrated film were compared to those of the same drug dosage in an oral solution. From sodium lauryl sulfate-based transfersomes, a maximum of approximately 82.03 percent entrapment efficiency, size of 206.7 nm, the zeta potential of 88.3 Mv, and 66.7 percent drug release after 24 h were achieved at a drug: phospholipid and surfactant: phospholipid ratios of 1:20 and 75:25 w/w, respectively.

- **(Allaw et al., 2023):** To enhance the carrier capabilities of transfersomes and the effectiveness of mangiferin in the treatment of skin lesions, the moisturizing qualities of glycerol, the penetration increasing capacity of propylene glycol, and the bioadhesive properties of mucin were combined. Mangiferin was added to transfersomes and glycoltransfersomes that had already been mucin-modified. In vitro and in vivo, the physicochemical properties, as well as the effectiveness against oxidative stress and skin wounds, were evaluated. Glycoltransfersomes increased mangiferin accumulation in the epidermis and dermis, protected fibroblasts from oxidative stress, and increased their proliferation. Glycoltransfersomes' wound healing and anti-inflammatory properties were verified in vivo. The results revealed that glycoltransfersomes transport mangiferin to injured skin at optimum levels, enhancing its wound-healing capabilities and validating the efficacy of the formulation approach used. Indeed, the two water cosolvents employed in this study, glycerol and propylene glycol, work together with the elastic vesicles (transfersomes) to improve their well-known capacity to cross biological membranes. These new carriers increase the effectiveness of mangiferin in tissue regeneration, making them potential wound healing compositions.

- **(Vishwanathan et al., 2022):** . The goal of this study was to develop and validate an HPLC method for estimating glabridin from Glycyrrhizaglabra extract. The

HPLC separation was done on an 18e column with a gradient elution of 0.2 percent acetic acid in water acetonitrile, and the method was validated according to ICH guidelines.

- **(Reddy et al., 2023):** . It covered the current state of transfersomes as a carrier system, with a focus on their applications, as well as a review of transfersome manufacturing procedures.
- **(Choi et al., 2023):** The goal of this work was to evaluate a new vesicular system comprising ethosomes and transfersomes to a standard liposome to increase topical administration of ginsenoside Rh1 isolated from red ginseng. In comparison to ethosome and traditional liposome, ginsenoside Rh1-loaded vesicles were generated and assessed for various parameters, and transfersomes gave much greater skin permeation of ginsenoside Rh1. As a result, ginsenoside Rh1-loaded transfersomes may have topical therapeutic effects, according to the current study.
- **(Patel et al., 2020):** Fluconazole-loaded transfersomes were generated utilising various lipid and surfactant ratios, as well as various surfactants (span-80, tween-80, and SDC). This research implies that Fluconazole-loaded transfersomes could be employed as a Topical drug delivery system.
- **(Akhtar et al., 2020):** The recently created innovative carrier for increased topical drug delivery was summarised, with a focus on topical delivery via these vesicles, with various elements of all these carriers being highlighted.
- **(Gaur et al., 2023):** The goal of the study was to characteriseProtransfersome gel as a TDDS of ketoprofen for effective and long-term drug delivery. By developing 18 formulations with sodium deoxycholate and brij 35 as an edge activator, the various formulation parameters were adjusted. Formulations including cholesterol have a high entrapment capacity, whereas those containing isopropanol have a little advantage in terms of entrapment when compared to those containing ethanol.
- **(Ghanbarzadeh et al., 2023):** The goal of this work was to use ordinary liposomes, ethosomes, and transfersomes to improve the transdermal penetration of diclofenac sodium, a poorly water-soluble medication. The loaded medication quantity and

vesicle size of the produced formulations were measured.

- **(Lei et al., 2023):** The goal of this work was to create novel tacrolimustransfersomes (TFs) for treating atopic dermatitis in mice, and to compare the therapeutic efficacy of these TFs to commercial tacrolimus ointment and liposomes-gel. On mice with atopic dermatitis, topical use of TFs-gel had the best therapeutic effect. As a result, TFs demonstrated improved performance and an effective skin target for tacrolimus topical distribution.
- **(Kaur et al., 2022)**: This work presented a general description of the transfersome and its components, as well as a detailed discussion of the edge activator and transfersome elasticity, and explained how elasticity is formed by incorporating an edge activator into the lipid bilayer structure.
- **(Mohammed et al., 2022):** Ibuprofen was developed and studied as a transdermal medication delivery system using transfersome vesicles. It was studied by encapsulating the medication in several formulations made up of varying ratios.
- **(Prasanthi et al., 2022):** It looked at how effectively conventional, deformable, and ethosomal vesicles worked as drug delivery methods, as well as how they might work as permeation enhancers or transdermal drug carriers. Deformable liposomes and ethosomes can penetrate the stratum corneum, and using vesicles as a carrier system could lead to new transdermal and dermal medication delivery systems.
- **(Gavali et al., 2023):** It examined several transfersome production procedures, including their benefits and drawbacks, drug loading strategies and the factors that influence them, and drug release, with a focus on the mechanism of drug release.
- **(Patel et al., 2019):** The goal of this study was to see how well a transfersomes formulation could distribute Curcumin. Curcumin is a powerful anti-inflammatory plant that is frequently utilised. The formulations were optimised by adjusting several process variables such as lecithin effect, surfactant ratio, effect of various solvents, and surfactant effect. The transfersomes were made using a modified hand shaking method with surfactant. The researchers concluded that transfersomes made from PC:Span-80 in the ratio 85:15 are a potential way to

enhance Curcumin permeability over time.

- **(Pandey et al., 2019):** The paper gave an overview of transfersome and described the various methods of synthesis, characterisation, and prospective applications for drug molecule administration.

Literature review on Glabridin and HPLC analysis of glabridine:

- **(Yewale et al., 2022) :** The effects of several organic solvents (ethanol, dichloromethane, ethyl acetate, and acetone) on the extraction efficiency of glabridin and total flavonoids (TF) from Glycyrrhizaglabra roots were investigated in this research. The extract yield of Glycyrrhizaglabra roots ranged between 3% and 6%, with ethanol being the most efficient solvent followed by acetone, ethyl acetate, and dichloromethane. Dichloromethane produced the highest extraction yields of TF and glabridin, followed by ethyl acetate, acetone, and ethanol, suggesting that non-polar solvents aid in the optimum extraction of TF and glabridin. Additionally, results showed for the first time that the extraction efficiency of flavonoids is unaffected by the recovered solvents, save for ethanol, indicating that the solvent's moisture-absorbing capacity determines the extraction efficiency of such compounds. The glycyrrhizin concentration of all extract types was rather low (0.1 to 1%), except for the extract produced with water, which contained 10% glycyrrhizin, as anticipated given that glycyrrhizin is a polar molecule. Notably, researchers found that ethyl acetate separated just glabridin with no evidence of glycyrrhizin, which is a novel discovery.

- **(S. Kulkarni et al., 2023) :** The purpose of this article is to discuss the development and validation of an HPLC technique for the simultaneous determination of glabridin (GB), glycyrrhizic acid (GA), and total flavonoids (TF) in liquoriceper ICH recommendations. Additionally, this paper examined and discussed factors affecting the stability of GB, GA, and TF. Gradient chromatography was used to separate the samples using an Inertsil ODS 3V column (250mm4.6mm ID, 5m particle size) at 40°C with KH2PO4 buffer as

mobile phase A and pure acetonitrile as mobile phase B at a flow rate of 1 mL/min. The proposed technique was effectively tested for GB, GA, and TF in liquorice and showed good selectivity, linearity, sensitivity, precision, and accuracy. Additionally, this study presents data on the stability of GB, GA, and TF in liquorice extract when subjected to six distinct experimental conditions, including temperature, light, humidity, acid hydrolysis, base hydrolysis, and hydrogen peroxide oxidation. The proposed technique is straightforward, sensitive, and accurate, and has been verified following regulatory requirements and successfully used in stability studies of GB, GA, and TF.

- **(Alam et al., 2018) :** The review of the literature showed that no effort has been made to simultaneously determine glycyrrhizin and glabridin biomarkers in G. glabra roots, rhizomes, and/or herbal formulations. For this aim, a reversed-phase HPTLC technique was designed and validated with the benefits of being cost-effective, economical, and environmentally benign due to the avoidance of corrosive acid in the mobile phase. The simultaneous detection of glycyrrhizin and glabridin in Glycyrrhizaglabra roots, rhizomes, and selected herbal formulations has been developed using a novel fast, easy, cheap, and environmentally friendly reversed-phase high-performance thin-layer chromatography (RPHPTLC) technique. The suggested technique is the first verified RP-HPLC method for determining the concentrations of glycyrrhizin and glabridin in G. glabra roots, rhizomes, and herbal preparations. The technique was carried out using RP-18 silica gel 60 F254S HPTLC glass plates with a mobile phase of methanol-water (7:3 v/v). The produced plates were scanned and densitometrically measured for glycyrrhizin and glabridin, respectively, at 256 and 233 nm. Glycyrrhizin and glabridin peaks were discovered by comparing their single spots at RF = 0.63 0.02 and RF = 0.28 0.01, respectively, from G. glabra roots and rhizomes and herbal formulations. The suggested technique will be beneficial for determining the therapeutic dosages of glycyrrhizin and glabridin in herbal formulations and bulk drugs. Statistical analysis demonstrates that the technique is precise, sensitive, repeatable, and selective for glycyrrhizin and

glabridin analysis. Additionally to being easy, environmentally benign, and requiring little sample preparation, the technique is inexpensive.

- **(Korhalkar et al.,2015):** Glyclyrrhizaglabra and its various biochemical fractions have been used for various pharmacological activities such as antioxidant effects, mitochondrial function protection, anti-inflammatory activity, antiadhesive properties, anticancer properties, immune stimulating effect, enhancer agent, melanogenesis stimulation, antiviral properties, and antimicrobial properties of Glyclyrrhizaglabra

- **(Hamed et al., 2015):** This scientific investigation found that 2.5 percent G. glabra cream was successful as a treatment for melisma in a 28-day period with minimal side effects after being formulated as a water in oil active cream (2.5 percent G. glabra)

- **(Damle et a., 2014):** The present study summarises the herbal application of Glyclyrrhizaglabra as well as the skin lightening action of glabridin. It also reviews the literature on Glyclyrrhizaglabra in terms of traditional usage, bioactive ingredients, and pharmacologic activities. This could be beneficial in identifying potential therapeutic effects and formulating novel medications.

- **(Kataria et al., 2013):**[1] The advantages, pharmacological action, and numerous aspects of the application were discussed, along with the many adverse effects and mechanisms of skin lightening activity.

- **(Deshmukh et al., 2012):** The goal of this study was to create and synthesise an appropriate form of glabridin using a microsponge drug delivery system.

  Animal experiments revealed that the glabridin had superior depigmenting activity than plain gel. The use of a Glabridinmicrosponge-loaded gel in the treatment of various hyperpigmentation diseases could be beneficial.

- **(Akhtar et al., 2011):** The goal of this study was to develop a stable w/o cream with Glyclyrrhizaglabra extract and investigate its effects on the skin pigment "melanin." The inner aqueous phase of the W/O emulsion was entrapped with Glyclyrrhizaglabra extract, which was made by concentrating the alcoholic extract of Glyclyrrhizaglabra roots. For four weeks, human volunteers were given both the base and the formulation. Different human skin parameters, such as melanin, were affected by the formulation, which resulted in a considerable drop in skin melanin.

Literature review on Skin Pigmentation:

- **(Rojekar et al., 2015)** summarizes several aspects related to skin pigmentation, including melanocyte abnormalities, pigmentation factors, and numerous systemic disorders with hyper or hypo-pigmentation as a symptom.
- **(Ebanks et al., 2019):** Explained the mechanism of colour production in the body and numerous external and internal factors responsible for hyperpigmentation, as well as several tactics utilised for hyperpigmentation therapy

- **(Jablonski et al., 2014):** The purpose of this review was to present a thorough yet cost-effective overview of the biology, development, and culture of human skin and skin colour, with a focus on current studies, particularly on skin colour evolution. The review begins with an introduction to the basic biology of skin, followed by talks of skin evolution and colour, as well as skin colour and race.

Literature Review on pellet formulations:

- **(Shinde et al., 2023) :** The purpose of this study was to determine the computational predictability of Metoprolol succinate modified-release multiple unit pellets systems (MUPS) tablets encapsulated in Polyethylene Glycol (PEG) (QbD). Researchers established the MUPS tablet's quality target product profile (QTPP) and key quality characteristics (CQAs). Plackett–Burman and Box– Behnken designs

were used to filter and improve the formulation. Pellets were produced utilizing a wruster coating technique with a variety of polymers, including hydroxypropyl methylcellulose (HPMC), ethyl cellulose (EC), and various excipients. The results of the ANOVA indicated that the chosen model was statistically significant. This study established that a PEG-encapsulated HPMC and EC polymer coated with an optimized curing time is capable of delivering a single dosage of metoprolol succinate through QbD. PEG- encapsulated modified-release metoprolol succinate MUPS tablets were produced in this research utilizing a QbD method. The Ishikawa diagram and FMEA analysis tool are used to identify possible risk factors that influence the quality of pharmaceutical goods. The important factor was identified using the Plackett- Burman screening design, and the variable range was optimized using the Box- Behnken optimization design. The CQAs for metoprolol succinate MUPS pills were recognized as drug release. PEG encapsulation maintains the film's integrity during the compression process. The results of the model confirmation tests indicated that the anticipated and experimental observations were consistent, confirming the model's correctness and resilience. This contextual study may serve as a guide for MUPS tablet manufacturers' future conventional product development.

- **(Nejati et al., 2018) :** The purpose of this research was to determine the feasibility of using pectin and chitosan polysaccharides in pellet formulation. These biopolymers offer several benefits, including biocompatibility, low toxicity, cheap cost, and ease of manufacturing, making them attractive candidates for drug administration. Controlling the porosity of the pellets is critical for achieving an adequate medication release profile. By substituting polysaccharides, particularly pectin, for microcrystalline cellulose (MCC), pellet porosity is enhanced. As model medicines, theophylline, dimenhydrinate, and ibuprofen were selected. It is critical to investigate potential ionic interactions between medicines and excipients to improve pellet formulations with adequate drug release. Differential scanning calorimetry of chitosan revealed an endothermic peak; however, this peak was not seen in thermograms of pectin, indicating that the polysaccharides do not interact. Fourier transform infrared

research revealed no evidence of drug-polymer interaction. The addition of MCC to the pellet formulation substantially increased the mean dissolving time, while substituting polysaccharides for MCC resulted in a quicker release of each of the three medicines – which had varying net charges – in both acidic and buffer conditions. These findings emphasize the potential use of polysaccharides in optimizing the drug delivery properties of pharmaceutical pellets. In general, adding MCC to the pellet formulation substantially increased the mean dissolving time, while substituting pectin or chitosan resulted in a quicker release of each of the three medicines with varying net charges in both acidic and buffer conditions.

- **(Farmoudeh et al., 2022) :** The research aimed to determine the efficacy of solid dispersion (SD) in increasing the dissolving rate of Deferasirox (DFX) in pellets. SDS was prepared using the solvent evaporation method with various drug-to-carrier ratios of polyethylene glycol 4000 (PEG 4000) and polyvinylpyrrolidone K25. The dispersion was then pulverized and combined with additional components, and the resulting material was pan coated onto sugar-based cores. The pellets' size distribution, morphology (standard error of the mean), and dissolving behavior were all examined. Differential scanning calorimetry (DSC), X-ray diffraction (XRD), and Fourier transformation infrared (FTIR) spectroscopy were used to investigate drug-polymer interactions. SD-coated pellets significantly increased the solubility of DFX compared to uncoated pellets. In comparison to other combinations, the dispersion with PVP K25 demonstrated a quicker dissolving rate. When the medication was distributed in the polymer, the DSC and XRD analyses showed that it was in an amorphous form. The FTIR tests showed that no interaction between the medication and polymer could be ruled out. The SEM analysis revealed that the pellets' surfaces were smooth. It is concluded that the SD technique significantly increased the rate of DFX dissolving in pellets, which may also be used for other weakly water-soluble medicines. In conclusion, a multiparticulate dosage form capable of accelerating the administration of poorly soluble medicines may be created by distributing the drug in a hydrophilic polymer matrix and coating the mixture layer on inert pellet cores.

Review of literature describes few reported methods of analysis for Apremilast drugs:

HPLC and HPTLC methods for apremilast estimation

- **(P. Kulkarni & Deshpande, 2023)** Apremilast is a nonsteroidal anti-inflammatory drug. It has been a thriving molecule in dermatology. Apremilast, a phthalimide derivative, is naturally non-hygroscopic. It is insoluble in water on a molecular level. Apremilast works by decreasing the activity of the intracellular enzyme phosphodiesterase 4 (PDE4). Analytical techniques are critical for deciphering the physiochemical properties of a pharmacological molecule. Due to the insoluble nature and limited permeability of the compound, developing analytical methods and formulations becomes difficult. There are currently no accessible standard test techniques for the analysis of Apremilast. As a result, a critical evaluation of Apremilast's analytical methods was conducted. UV spectroscopy, HPTLC, HPLC, X-ray diffraction, NMR, and LC-MS techniques were gathered and evaluated. Researchers spend an inordinate amount of time developing simple, accurate, resilient, and cost-effective techniques of analysis. This knowledge will help in the creation of novel delivery methods in pharmaceutical research. The review will demonstrate the value and benefit of pre-formulation research and will serve as a guide for formulation development. Apremilast is a successful chemical that was approved by the USFDA in 2014. Analysis of the medication is critical during formulation to identify the active ingredient and its metabolites. This review summarises the analytical techniques used to examine Apremilast. For selective and sensitive estimation, LC-MS and HPLC techniques have been primarily utilized. LC-MS methods have been used to examine not just bulk but also biological materials. Analytical methods that need less sample preparation and have a higher sensitivity must be developed to assist save both time and money.

- **(Khadse et al., 2023) :** Apremilast is used to treat psoriasis and psoriatic arthritis. It may also be helpful for other immune-related inflammatory disorders. The medication acts as a specific phosphodiesterase4 (PDE4) inhibitor, preventing the spontaneous production of TNF-alpha by human synovial rheumatoid cells. The current study discusses the various methods for evaluating apremilast in bulk and various formulations. The purpose of this brief study is to collect and analyze over 30 techniques for analyzing apremilast in biological matrices, bulk materials, and various dose formulations, including HPLC, HPTLC, UPLC, LC-MS, and UV-spectrophotometry. A brief review summarises and discusses 30 analytical techniques, including HPLC, HPTLC, UPLC, LC-MS, and UV-Spectrophotometry, that have been used to investigate apremilast in biological matrices, bulk materials, and various dose formulations. This comprehensive evaluation will be very beneficial to the researcher working on apremilast. The following review demonstrates the different analytical methods used to evaluate Apremilast. While liquid chromatography with UV detection has been extensively investigated for the measurement of Apremilastin in bulk and pharmaceutical dosage forms, hyphenated techniques such as LC-MS have been described for the identification of Apremilast and its metabolite in plasma and other biological fluids.
- **(Prajapati et al., 2020) :** For the development of a stability-indicating test technique for the acidic degradation kinetics of apremilast, a quality risk assessment and design of experiments (DoE)-based quality by design (QbD) methodology was used. The quality risk assessment approach began with the identification of method development risk factors via preliminary testing, the previous understanding of chromatography, and a brainstorming session. The risk variables discovered were correctly classified and listed in a fishbone diagram. Risk assessment was accomplished via the assignment of a risk priority number (RPN) to each risk element based on exploratory testing. The separation was carried out on an aluminum plate pre-coated with silica gel G F254 using toluene–ethyl acetate–methanol (7:1:2, v/v). The technique has been verified following the

International Conference on Harmonization's (ICH) Q2R1 standard. The proposed technique was used to investigate the kinetics of forced degradation and acidic degradation under various stress situations. The degradation kinetics were determined to be first order. The technique was verified under ICH standards and was determined to be accurate, exact, specific, and linear based on the data collected. The proposed technique was capable of isolating the active pharmaceutical ingredient from the breakdown products produced under various stress settings.

Additional literatures on analytical method developments of apremilast

- **(Kulsum et al., 2016)** Forced Degradation Studies by method development Solvent: Methanol

  Detection wavelength: 220 nm

- Method for the Estimation of Apremilast by Absorbance Maxima Method **(Lonkar et al., 2017)**

  Solvent: Methanol

  Detection wavelength: 230 nm

- Stability-Indicating Method for Determination of Apremilast **(Ravisankar et al., 2017)**

  Solvent: Acetonitrile

  Detection wavelength: 229.3 nm

- Estimation of Apremilast by Forced Degradation Studies-Stability Indicating RP-HPLC Method **(Lonkar et al., 2017)**

  Mobile phase:Methanol: Water (70:30 v/v)

  Colum: C18 column (250mm x 4.6ID, 5µm)

  Flow Rate: 0.8ml/min

  Detection wavelength: 230nm

- Apremilast Determination by Stability-Indicating RP-HPLC Method **(Ravisankar et al., 2017)**

  Mobile phase: water: Acetonitrile (25:75 v/v)

  Colum:eclipse XDB model C18 Column, 250 mm×4.6 mm, 5 µ

Flow Rate: 1.0 ml/min

Detection wavelength: 229nm

- Determination of Apremilast-An Antirheumatic drug by RP-HPLC method **(Annapurna et al., 2017)**

  Mobile phase:A mixture of 0.1% acetic acid and Acetonitrile(70:30 V/v)

  Colum:Intersil ODS3 C18 column (250 mm × 4.6 mm i.d., 5 µm particle size)

  Flow Rate: 0.8 ml/min

  Detection wavelength: 203 nm

- Method of Apremilast by HPLC -Stability-Indicating Related Substances **(Anerao et al.,2017)**

  Model no: Shimadzu HPLC system LC-2010 CHT

  Mobile phase: Acetonitrile: Water (25:75 v/v)

  Colum:Cosmosil C-18 column 250 mm x 4.6 mm, 5.0 µ

  Flow Rate: 1.0 ml/min

  Detection wavelength: 230nm

- HPLC study for Quantification for Impurities of Apremilast **(Xiong et al., 2022)**

  Mobile phase: water: Acetonitrile (30:70 v/v)

  Colum:Wondersil C18 (250mm×4.6mm, 5µm)

  Flow Rate: 1.0 ml/min

  Detection wavelength: 230 nm

- Drug analysis by Stability-Indicating HPTLC method**(Thorat et al., 2017)**

  Mobile phase-Chloroform: Methanol: Triethylamine in the ratio of (9.4:0.5:0.1v/v)

  Stationary phase- silica gel 60 F_{254}

  Detection wavelength- 261nm

- HPTLC for Estimation of Paracetamol and Etoricoxib in Combined Tablet Dosage Form

  Mobile phase- Toluene: Methanol: Chloroform (5:2:3 v/v/v)

  Stationary phase-silica gel 60 F_{254}

  Detection wavelength- 240 nm

Profile of Glabridin: (Kataria et al., 2013**,** Deshmukh et al.,2012, Naveed et al., 2011.**)** Glabridin is a chemical compound that found in root of Liquorices (Glycyrrhizin glabra) Glabridin is an isoflavane type of flavonoid. Insoluble in water, soluble in organic solvent.

Molecular Structure:

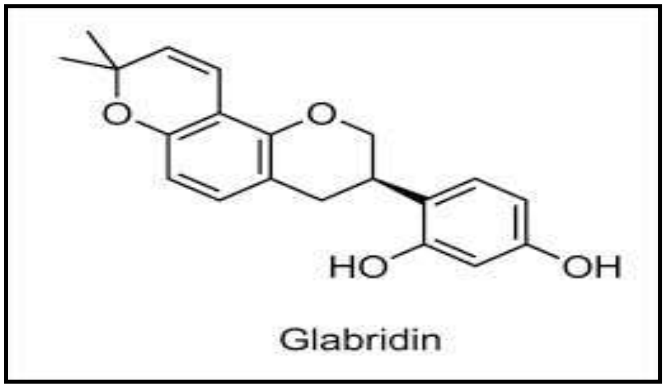

Figure 2.1: Structure of Glabridin.

IUPAC Name: 4-[(3R)-8, 8-dimethyl-3, 4-dihydro-2H-pyrano [2, 3- f] chromen -3-yl] -benzene- 1, 3-diol.

Empirical formula: $C_{20}H_{20}O_4$

Molecular weight: 324.37

Appearance: Yellow orange powder

Odors: Slight characteristic odour

Solubility: Insoluble in water, soluble in organic solvent.

Analytical Specifications:

Assay: 40.0% minimum

Loss on Drying: 5.0% maximum

Melting Point: 154-155° C

Mechanism of action

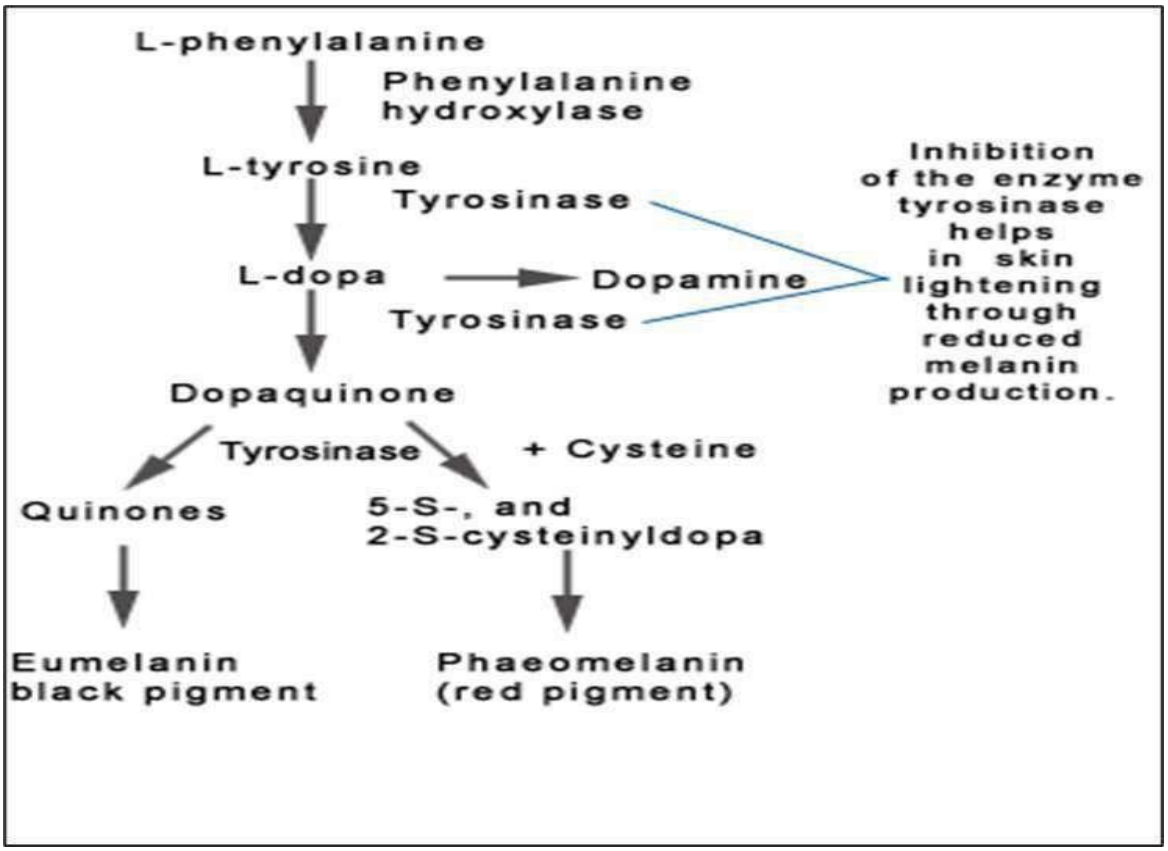

Figure 2.2: Mechanism of action of Glabridin

Glabridin inhibits melanogenesis by two mechanisms: inhibition of the synthesis of active oxygen species and inhibition of tyrosine. Human tyrosinase is an enzyme that regulates the production of melanin.

Adverse effect: inhibition of CYP3A4, nausea, vomiting, and dermatological reaction.

Pharmacology:

Absorption: Glabridin appears to be absorbed in vitro and enters the bloodstream when taken orally, either alone or as part of Liquorice root oil. According to serum levels of glabridin, absorption appears to be higher when eaten with Liquorice root oil. Glabridin has an oral bioavailability of about 7.5 percent, which is consistent across doses, and this low absorption rate is attributable to P-Glycoprotein transporters' high efflux (drug efflux transporters). Glabridin can be found in the liver (23-53nmol; 0.37-0.41% oral dosage),

kidney (0.02% oral dose; 1.14-2.21 nmol), and mesenteric body fat (0.02% oral dose; 1.14-2.21 nmol) after an oral dose (minimally)

Interaction

Glabridin inhibited the P450 enzymes CYP2B6, CYP2C9, and CYP3A4 and had a mild inhibitory effect on P-Glycoprotein. When licorice extract was incubated with CYP3A4 for 15 minutes at doses of 1.4, 6.9, 14, and 69ug/mL, activity was lowered to 73 percent, 45 percent, 25 percent, and 12 percent of control levels, respectively, and was totally inhibited with 50uM of pure Glabridin and was not reversible. Glabridin appeared to degrade the heme fragment of these P450 enzymes, which was thought to be related to its anti-oxidant abilities. The inhibition of CYP2B6 was less severe but followed a similar irreversible trend, and Glabridin appeared to degrade the heme fragment of these P450 enzymes, which was assumed to be related to its anti-oxidant abilities.When topically applied as a 0.5 percent cream to rodents, glabridin appears to slightly diminish erythema following UV-B exposure.

Application: Brightening, skin lightening, anti-aging.

Introduction of lipid, edge activators and polymers

Commonly utilised excipients include lipid, edge activators and polymers for the formulation of transfersomes and transfersomal gel respectively. These excipients will have an impact on the vesicle characteristics. You are here to talk to. PHOSPHOLIPON 90G & 90H, SPANS and TWEENS are employed as edge triggers for this project. The gel formulation of the transfersomal vesicles is developed using carbopol-934.

Lipid: (Raymond et al.,2006, Jacob et al.,2013)

Lipides are a wide variety of natural chemical compounds, including monoglycerides, diglycerides, triglyceride, phospholipids, and other, including fats, waxes, sterols, or fat-soluble vitamins (for instance vitamins A, D, E, and K). The principal biological activities of lipids include energy storage, signaling and the functioning of cell membranes as structural components. Lipids are used in cosmetics and the food and nanotechnology industries. The solubility of nonpolar organic solvents (e.g. ether, chloroform, benzene and acetone) and the overall water insolubility of these are associated with them.

A) Phospholipon 90 G:

Description Soya lecithin purified phosphatidylcholine, well established Lipoid brand is the natural and hydrogenated fraction of lecithin and soya phospholipids, eggs, milk, marine, cotton or sunflower sources.

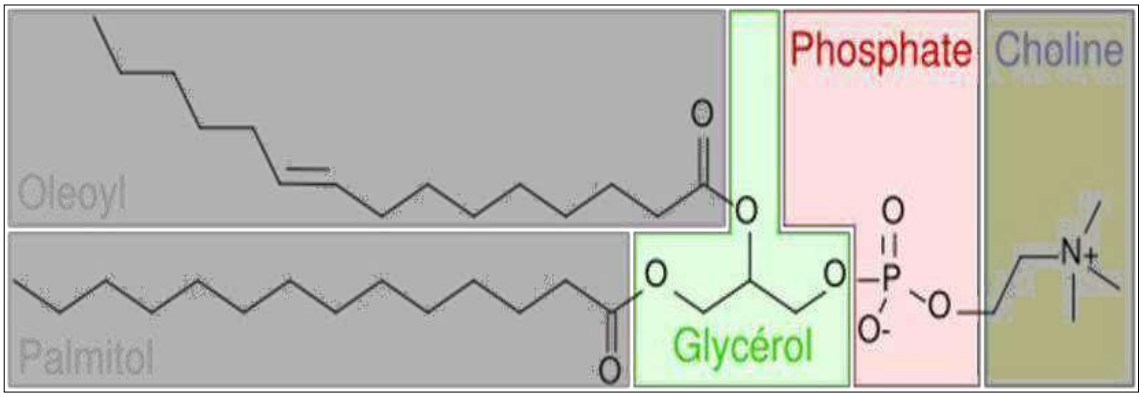

Figure 2.3: Structure of Phospholipids.

Molecular Formula: $C_{42}H_{80}NO_8P$

Regulatory status: The United States Food and Drug Administration approve lecithin (Phospholipid) for human ingestion with the designation "allgemeinalssicher" (GRAS). EU admission of lecithin as a food ingredient

pH Level: 5 to 8.

Phospholipids in Pharmaceutical Applications:

One significant area of application is drug delivery systems. The use of the following systems enables the release, absorption, bioavailability and efficiency of medicines and essential chemicals to be affected. Liposome, Emulsions, solid lipid nanoparticles, mixed micelles suspends, phospholipids-coated active ingredients are common drug delivery methods.

Storage:

Recommended storage: In dry conditions, enclosed in inert gas with a maximum of 80 degrees C, storage with -200C enhances the shelf life. A frozen product unit must not be opened without the packaging being conditioned to ambient temperatures in order to avoid adverse consequences for product quality due to dampness.

Edge activators: (Raymond et al., 2006;Jacob et al., 2013; Zaafarany et al., 2010.**)**

Belayed edge activators, such as biocompatible surfactants with an amphiphilic drug, are used to improve lipid bilayer flexibility and permeability. Tensile substances increase the membrane fluidity proportionately and improve penetration through the skin. Amphipathic molecules consisting of the non-polar hydrophobic part are often the straight or branched carbon or fluorocarbon chain comprising 8-18 hydrophilic atoms. The ionic, non-ionic, or zwitter-ionic hydrophilic part may be present. The phospholipid bilayer with a lipid makeup comparable to that of the skin is a key component of the vesicular system.

Edge activators are incorporated mostly in the lipid bilayer. An activator on the edge is generally a surfactant in a single chain that destabilises the fluid bilayer of the vesicles by decreasing the interface tension of the bilavian tube.

Interaction of edge activators with lipid bilayer:

The surfactant affinity for water and lipid is determined by HLB. Tween 80's HLB and span 80's HLB are 4.3 and 15, respectively. To analyse the distribution of surfactant between lipid and aqueous components of the vesicle, the molar ratio of surfactant to lipid should be established. Surfactant reduces interfacial tension, allowing it to concentrate and interact with skin. They have the ability to bind to the cell / cell membrane and alter the porosity/permeability of the cell membrane. Diffusion allows surfactant to reach the deeper corneal regions of the skin. Following diffusion, the surfactant begins denaturing the protein, causing the stratum corneum to expand. The concentration of surfactant can be raised up to CMC. Nonionic surfactants connect to the protein by hydrophobic interaction, whereas anionic/cationic surfactants interact with the protein via ionic interactions

A) Spans-80 (Sorbitanmonooleate):

Sorbitan is a combination of isomeric chemical molecules generated from sorbitol after it has been dehydrated. Sorbitan is generally used to make surfactants like polysorbates. Sorbitan is used as a step in the process of converting sorbitol to isosorbide. Sorbitan esters (also known as Spans) are lipophilic nonionic surfactants that are utilised as

emulsifying agents in pharmaceutical and cosmetic emulsions, creams, and ointments. In the food industry, sorbitan esters are employed as emulsifiers and stabilisers.

Category: Non-ionic surface active agent

Description: Amber-colored oily sticky liquid with a little odour, light cream to tan beads or flakes, or a hard, waxy solid..

Solubility: Soluble in ethanol, ether, ethylacetate, aniline, toluene, dioxane, petroleum ether, and carbon tetrachloride at temperatures over its melting point; insoluble in cold water; dispersible in warm water.

Molecular structure

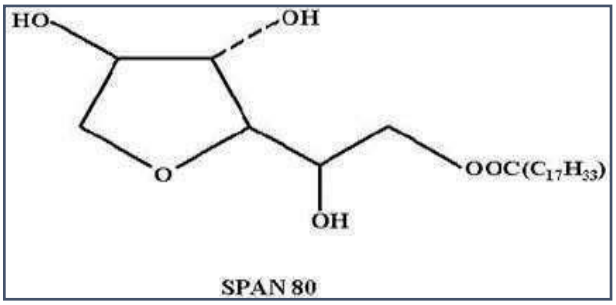

Fig. 2.4 Molecular structure- Spans-80

Molecular formula: $C_{24}H_{44}O_6$

Molar mass: 428.60

Density: 0.986 g/mL at 25 °C (lit.)

Flash point: >113 ℃

HLB value: 4.3

Usage: Emulsifier, stabilizer, used in food products and oral pharmaceuticals.

Storage: Store in a cool, dry location. Keep the container tightly shut in a dry, well-ventilated location

B) Tween-80 (polyoxyethylene-20 sorbitanmonooleate):

Tweens are sorbitan esters that have been polyethoxylated. They are ethoxylated spans, to put it simply. Tweens are naturally hydrophilic.

Tween-80 (polysorbate 80) is a nonionic surfactant and emulsifier that is commonly used in foods and cosmetics. This synthetic chemical is a thick yellow liquid that is water soluble. Polysorbate 80 is made up of oleic acid and polyethoxylatedsorbitan. Polyethers

act as hydrophilic groups in this molecule.

Molecular structure:

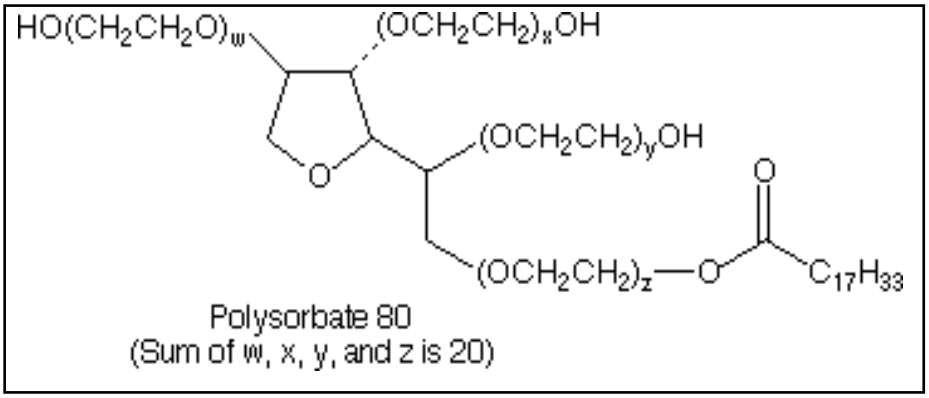

Fig. 2.5 Molecular structure- Tween-80

IUPAC name: Polyoxyethylene (20) sorbitanmonooleate

Molecular formula: $C_{64}H_{124}O_{26}$

Molar mass: 1310 g/mol

Description: Amber colored viscous liquid

Density: 1.06–1.09 g/mL, oily liquid

Boiling point: > 100 °C

pH: 7.0

Solubility: Water soluble, ethanol, cottonseed oil, corn oil, ethyl acetate, methanol, and toluene soluble

Viscosity: 300–500 centistokes at 25°C

Flash point: 113 °C (235 °F; 386 K)

HLB value: 15.0 points A high HLB value, such as 15.0, means the surfactant will enter the water phase.

Storage: Keep in a cool location. Keep the container tightly shut in a dry, well-ventilated location.

C) Sodium deoxycholate (SDC):

Sodium deoxycholate (deoxycholic acid) is a bile-acid, ionic detergent that is frequently employed in protein techniques. It's most commonly employed in cell lysis buffers, but it's also been utilised for liposome production, membrane protein and lipid isolation,

affinity chromatography non-specific binding prevention, and as a cell culture media supplement.

Sodium deoxycholic acid, deoxycholate, sodium salt are some of the other names for this substance.

Molecular Structure:

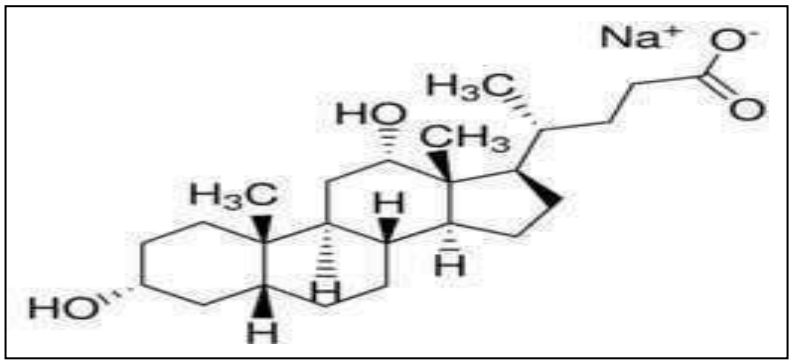

Fig. 2.6 Molecular structure- Sodium deoxycholate

Formula: $C_{24}H_{39}O_4Na$

HLB value:16.7

Application: The concentration of a detergent determines its efficiency in any application. Too much or too little detergent can have negative consequences. In your application, it is recommended that you test a variety of detergent concentrations. Cholate will form micelles with a MW of 2000 at concentrations greater than 2 mm. When needed, the small micelle size enables for simple removal via dialysis or gel filtering. Note that removing a detergent from a protein solution may cause precipitation and/or aggregation of the protein.

Polymers: (Raymond et al.,2006; Jacob et al., 2013)

Carbopol is made up of acrylic acid polymers that have been crosslinked with polyalkenyl ethers or divinyl glycol. They're made from primary polymer particles with an average diameter of 0.2 to 6.0 microns. When flocculated agglomerates are created, they cannot be broken down into the final particles. Each particle can be thought of as a network of polymer chains that are linked together by cross-linking

A) Carbopol-934 P.

Poly (acrylic acid) (PAA or Carbomer) is the generic name for acrylic acid polymers with a high molecular weight. Carbomer codes (910, 934, 940, 941, and 934P) indicate the polymer's molecular weight and specific components. A cross-linked polyacrylate polymer, carbopol-934 polymer is a cross-linked polyacrylate polymer. It creates thick formulations for opaque gels, emulsions, creams, and suspensions and has excellent viscosity stability. Carbopol-934 is a thickening agent that can be employed in viscous gels, thick emulsions, and heavy suspensions. It provides long-term stability at high viscosity.

Appearance:

Powder that is white and fluffy Carbomer-934 is an acrylic acid polymer with a high molecular weight that has been crosslinked with sucrose allyl ethers.

Molecular structure:

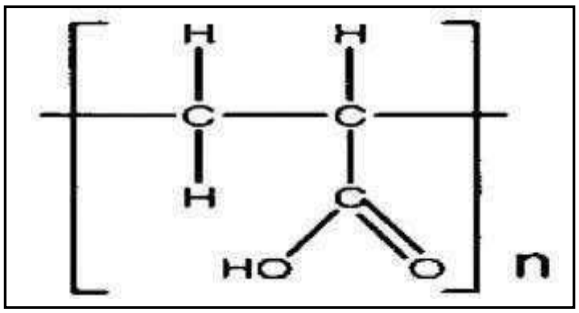

Fig. 2.7 Molecular structure-

Carbopol-934 P

Viscosity: Cp-250

Odour: Slightlyacetic

Applications of carbopol polymers:

useCarbomers are ideal for topical dosage form formulations in aqueous solutions. These polymers have been used in the formulation of many commercial topical treatments on the market today because they give several benefits to topical formulations.

Safe & Effective: Topical gels, creams, lotions, and ointments containing carbopol polymers have a long history of being safe and efficacious. Extensive toxicity studies back them up as well

Mechanism of Action:

Apremilast, a phosphodiesterase 4 (PDE4) Inhibitor that acts intracellularly, modulates a network of pro-inflammatory and anti-inflammatory mediators. PDE4 is the main PDE in inflammatory cells and is a cyclic adenosine monophosphate (cAMP)-specific PDE. Inhibition of PDE4 raises intracellular cAMP levels, which suppresses the inflammatory response by regulating TNF-, IL-23, IL-17, and other inflammatory cytokines production. Anti-inflammatory cytokines like IL-10 are likewise affected by cyclic AMP. Psoriatic arthritis and psoriasis have been linked to these pro- and anti-inflammatory mediators.

Metabolism:

Apremilast is extensively metabolised by both CYP and non-CYP mediated pathways, including oxidation, hydrolysis, and conjugation, implying that blocking a particular clearance mechanism is unlikely to result in a significant drug-drug interaction. Apremilast's oxidative metabolism is largely mediated by CYP3A4, with CYP1A2 and CYP2A6 playing minor roles. Following oral treatment, apremilast is the main circulating component. Apremilast is extensively metabolised, with just 3% and 7% of the parent molecule recovered in urine and faeces, respectively.Theglucuronide conjugates of O-demethylatedApremilast are the main circulating inactive metabolite (M12). Apremilast exposure is reduced when taken concurrently with rifampicin, a powerful inducer of CYP3A4, as Apremilast being a CYP3A4 substrate.

Excretion:

Apremilast has a plasma clearance of around 10 L/hr in healthy subject and a terminal elimination half-life of about 9 hours. Following oral administration of radiolabeled Apremilast, approximately 58 percent and 39 percent of the radioactivity is recovered in urine and faeces, respectively, with approximately 3% and 7% of the radioactive dose recovered as Apremilast in urine and faeces.

Pharmacokinetic and Pharmacodynamics properties of Apremilast:

Pharmacokinetics:

Pharmacokinetics parameters describe the action of the body on the drug.

Absorption:

Apremilast is well absorbed, with a 73 % absolute oral bioavailability and peak plasma concentrations (Cmax) reaching at a median time (tmax) of 2.5 hours. When Apremilast is given once a day, accumulation is negligible; when given twice a day, accumulation is around 53% in healthy participants and 68 percent in psoriasis patients. Because co-administration with food has no effect on bioavailability, Apremilast can be taken with or without meals.

Distribution:

Apremilast binds to roughly 68 % of human plasma proteins. Extravascular distribution is indicated by the mean apparent volume of distribution (Vd) of 87L.

Metabolism:

Apremilast is heavily metabolised, with just 3% and 7% of the given parent chemical being recovered in urine and faeces, respectively.

Excretion:

Apremilast has a plasma clearance of around 10 L/hr in healthy people and a terminal elimination half-life of about 9 hours.

CHAPTER-3
MATERIAL AND METHODS

3.1. Materials

The drug, excipients, chemicals and equipment's used for various experiments are enlisted as follows:

Table 3.1: List of drug and excipients

Sr.no	Chemicals	Manufacturer / Supplier
1	Glabridin	Novel Nutrient Pvt. Ltd. Bangalore.
2	Tween-80	Research lab, Mumbai
3	Span-80	Research lab, Mumbai
4	Sodium Deoxycholate	Research lab, Mumbai
5	Phospholipids	Jiiava Nutrient Pvt. Ltd,Pune
6	Ethanol	Research lab, Mumbai
7	Chloroform	Research lab, Mumbai
8	Carbopol 934P	Colorcon Asia Pvt. Ltd,Verna-Goa
9	Disodium hydrogen phosphate	Research lab, Mumbai
10	Potassium dihydrogen phosphate	Research lab, Mumbai
11	Apremilast	Lupin Research Park, Aurangabad
12	MCC (Avicel PH-101),	Loba chemicals, Mumbai
13	Lactose,	Loba chemicals, Mumbai
14	Crospovidone	Loba chemicals, Mumbai
15	Tamarind Kernel powder (TKP)	Research lab

All the chemicals and reagents used are of AR grade.

3.2. Equipments

Table 3.2: List of equipments

Sr. No.	Equipment/Instrument	Manufacturer/Brand
1	Mechanical stirrer	Remi, India
2	Weighing balance AE-100, PE-360	Shimadzu, India
3	Optical microscope	Carl Zeiss, USA
4	Rotary evaporator- EVATOR 1200	Medica, Mumbai
5	Digital melting point apparatus	Veego, Mumbai
6	Mechanical shaker	Remi, India
7	Ultrasonicator	Equitron, India.
8	Franz diffusion Cell	HemcoPharma, India
9	pH meterCL-54	Lab India, India
10	Viscometer RVDV-II + PRO	Brookfield, India
11	Stability testing cabinet	Neutronic, India
12	Refrigerator	Voltas, India
13	Digital camera S65	Sony, India
14	Computer Z50	Lenovo, India
15	Deep freezer	Voltas, India

Table 3.3: List of analytical equipments

Sr. no	Analytical equipment's	Manufacturer/Brand
1	UV-Visible spectrophotometer	Shimadzu 1700
2	FTIR spectrophotometer with diffused reflectance spectroscopy attachment	Shimadzu 8400S
3	Differential scanning calorimetry	Perkin Elmer DSC 7 (USA)
4	Particle size analyzer	HORIBA sz-100
	Zeta potential analyser	HORIBA sz-100 Zetasizer
5	Scanning electron microscopy	FESAM, Nova nano SEM- 450
6	Transmission electron microscopy	H-7500, Japan
7	HPLC	Shimadzu Lc-20 AD
8	Probe sonicator	PCI analytics 250F

3.3. Reagents and Chemicals Used for method development

In HPTLC method, methanol, toluene, Ethyl Acetate of AR grade were used.

Acetonitrile HPLC grade was used (Merck specialities Pvt. Ltd.). (30Mm) Potassium dihydrogen Phosphate buffer pH 3 Adj. with O- phosphoric acid was used. Double distilled water was used during the project work. Whatmann filter paper no. 42 and membrane filter (0.45 & 0.22 micron) was used for filtration.

Pure sample of drug i.e. Apremilast API was obtained as gift sample from pure drug, Apremilast are procured from Intas Pharmaceutical, Ahmadabad, India.

3.4. Methods:

Formulation and evaluation of transferosomes

3.4.1. Raw Material Analysis:

Reproducibility and reliability of results of formulation experimentation depends on the

quality of raw materials used. All ingredients used in the present study complied with respective specifications. Hence, they were used without any further purification.

Glabridin (Deshmukh et al, 2012; Naveed et al, 2011**)**

To confirm the purityof Glabridin the following tests were performed.

Melting point determination

Glabridin's melting point was obtained using an open capillary technique and a computerised melting point equipment (Make-Veego DM).

Differential Scanning Calorimetry

The thermograph of glabridin was obtained by DSC on Perkin Elmer DSC 7 (USA).

Specifications:

Sample Holder: Perforated Aluminium pans

Amount of sample: 2-5 mg

Temperature range: 35-300^{o}C

Heating rate: 10^{o}C/ min

Reference sample: Blank sealed Aluminium pans.

I.R. Spectrum

The sample (1.0 mg) was finely grounded to a particle size of 2 m or less and thoroughly mixed with 200.0 mg of dried KBr (kept at 100oC for 8 hrs). An agate mortar and pestle were used to grind and combine the ingredients. The mixture was then injected under high pressure into a transparent disc in an evacuable die. The entire procedure was carried out in a controlled environment, and the sample was scanned with a Shimazdu IR-8400S.

Determination of λmax in U.V. spectrophotometer

10.0 mg of Glabridin was weighed accurately on an analytical balance and was dissolved in ethanol in a 100.0 mL volumetric flask to give a 100 µg/mL stock solution. 1.0 mL of this stock solution was diluted with ethanol to 10.0 mL to give 10 µg/mL solution, the U.V. The wavelength range of 200-400 nm was used to record the spectrum. Using ethanol as a blank, the -max was measured using a UV-visible spectrophotometer

(Shimadzu 1700).

Solubility determination

The saturation solubility of Glabridin was determined by shaking method. In a 5.0 mL vial containing 2.0 mL distilled water, an excess quantity of medication was introduced. The vial was maintained in an orbital shaker at 251 ℃ for 72 hours after appropriate mixing with a cyclomixer. To establish equilibrium, the sample was centrifuged at 3000 rpm for 15 minutes and filtered through a 0.45 membrane filter. The filtrate was diluted appropriately, and the absorbance was measured at 226.0 nm with a UV-Visible spectrophotometer, and the solubility was calculated using a standard calibration curve.

Preparation of Standard Plot:

A) Analytical method for Glabridin in ethanol

Preparation of standard plot for Glabridin in ethanol

10.0 mg of Glabridin was weighed accurately on an analytical balance and was dissolved in ethanol in a 10 mL volumetric flask to give a 1000 µg/ml stock solution. 1.0 mL of this stock solution was diluted with ethanol to 10.0 mL to give 100 µg/mL solution. This was diluted adequately with ethanol to provide solutions of concentrations varying from 2-10 µg/mL. On a Shimadzu UV-Vspectrophotometer, the absorbance of these solutions was measured at 226.0 nm against ethanol as a blank. The standard plot presented in the next chapter was created using these absorbance values..

B) Analytical method for Glabridin in Phosphate buffer: ethanol (1:1) Mixture

a) Preparation of Phosphate Buffer (pH 6.8): Ethanol Mixture

The below tabulated ingredients were accurately weighed and mixed thoroughly in two third quantity of distilled water with the help of a bench stirrer and the volume made up to 1 liter. A pH metre was used to evaluate the pH of the solution, and if required, dilute HCl was used to adjust it to 6.8. The Phosphate buffer and ethanol were then combined in a 1:1 ratio as needed.

Table 3.4: Formula for Phosphate buffer pH 6.8

Ingredients	Quantity
Potassium dihydrogen phosphate	27.218g
Sodium hydroxide	0.09g
Distilled water	q.s to 1 liter

b) Determination of λ_{max} of Glabridin in Phosphate Buffer: Ethanol Mixture

A 10 µg/ml solution of Glabridin in Phosphate buffer:Ethanol (1:1) was scanned in the 200-400 nm range on Shimadzu UV-V 1700spectrophotometer against Phosphate buffer:Ethanol (1:1) as blank. The solution was found to exhibit maxima at 226.0 nm as shown in next chapter.

c) Preparation of standard plot of Glabridin in Phosphate Buffer: Ethanol Mixture

10.0 mg of Glabridin was weighed accurately and dissolved in phosphate buffer:ethanol (1:1) in a 10.0 mL volumetric flask to give a 1000 µg/mL stock solution. 1.0 mL of this stock solution was diluted with Phosphate buffer:ethanol mixture to 10.0 mL to give 100 µg/mL solution. This was diluted adequately with the same mixture to provide solutions of concentrations varying from 2-10 µg/mL. On a Shimadzu UV-V 1700spectrophotometer, the absorbance of these solutions was measured at 226.0 nm against a phosphate buffer:ethanol blank. The standard plot presented in the next chapter was created using these absorbance values.

Excipients:

All other materials used in the present study were checked for their conformance of specification with compendial standards or in house specifications in the absence of compendial standards. The material that complies with the standard was used for the study. Water used in the entire study was distilled water.

3.4.2. Pre-formulation Studies (Deshmukh et al, 2012)

Fourier Transform Infrared Spectroscopy (FTIR) and Differential Scanning Calorimetry (DSC) were used to investigate Glabridin's compatibility with the excipients that would

be contained in the transfersomal formulation. Glabridin and other excipients were measured in the 400-4000 cm-1 range using an FTIR Spectrometer (Schimadzu- 8400S). In the IR absorption spectra, the presence and absence of these bands, as well as the development of any new bands, were noted. DSC analysis was performed using Pyris software on a Perkin Elmer DSC 7 (USA) and calibrated with indium as per normal method. An auto-cooling attachment was included with the equipment for pre-programmed cooling. The sample (5.0 mg) was properly weighed into a standard aluminium pan, sealed, and heated at a constant rate of 100C/min from 400 to 3000C under continuous nitrogen purging at 20.0 mL/min. As a reference, an empty sealed pan was utilised. DSC graphs for glabridin,andglabridin containing transfersome were recorded as shown in chapter 9. The presence and absence of endothermic and exothermic peaks were observed in the DSC graphs.

Selection of Phospholipidsfortransfersome formulation depends on the basis of solubility of glabridin in lipid. The glabridin should be completely soluble in lipid for this Lipids such as Phospholipid plain, Phospholipon 90 H and Phospholipon 90 Gwere used to study the solubility of glabridin. Solubility of glabridin was determined by adding in small increments to known quantity of molten lipid. The amount of lipid required to solubilize glabridin was noted visually.

3.4.3. Preparation of Transfersomes:

Pre- formulation (Pre-optimization investigation)

In Pre- formulation study the 80:20 ratio of phospholipid & surfactant was found optimum in most of papers, this ratio was found well suited for transfersome without aggregation, in this study we also found 80:20 ratio of ethanol & chloroform was well suited for solublization of phospholipid and surfactant.(Table 3.5).

Table 3.5: Composition of Pre-formulation batches

F. no.	Lipid (Ph-90G) gm	Surfactant (Tween 80) gm	Solvent (eth: chlo) (80:20) mL	Phosphate buffer (pH-6.8) mL	Hydration time (min)	Sonication time (min)
T1	0.80	0.20	20.0	20.0	60.0	15.0
T2	0.80	0.20	20.0	20.0	60.0	20.0
T3	0.80	0.20	20.0	20.0	60.0	25.0
T4	0.80	0.20	20.0	20.0	60.0	30.0
T5	0.80	0.20	20.0	20.0	120.0	15.0
T6	0.80	0.20	20.0	20.0	120.0	20.0
T7	0.80	0.20	20.0	20.0	120.0	25.0
T8	0.80	0.20	20.0	20.0	120.0	30.0
T9	0.80	0.20	20.0	20.0	180.0	15.0
T10	0.80	0.20	20.0	20.0	180.0	20.0
T11	0.80	0.20	20.0	20.0	180.0	25.0
T12	0.80	0.20	20.0	20.0	180.0	30.0
T13	0.80	0.20	20.0	20.0	10.0	25.0
T14	0.80	0.20	20.0	20.0	10.0	30.0

3.4.4. Formulation Development (Deshmukh et al, 2012; Patel et al, 2014)

A) Experimental design

In the formulation of the drug loaded transfersomes two factors were varied.

1. **Factor A** – Ratio of phospholipid and surfactant

This variablewas included to optimize the proper ratio for formation of transfersome and Entrapment efficiency, vesicle size, deformability.

2. **Factor B** – Type of surfactant

This variable was included to optimize the vesicle size, entrapment efficiency and deformability.

Transfersomes were created using a 32 factorial design in which each of the two variables was examined at three levels. As a result, nine batches were made, as indicated in tables 3.6 and 3.7. The levels of the factors considered were:

Factor A: Ratio of phospholipid and surfactant (X_1 -Numeric factor)

Levels of factor A

Level	**Ratio**(lipid: surfactant)
Low (-)	70:30
Intermediate (0)	80:20
High (+)	90:10

Factor B: Type of surfactant (X_2 -Category factor)

Levels of factor B

Level	**Type**
Low (-)	Span-80
Intermediate (0)	Tween-80
High (+)	SDC

Response: **R_1**Entrapment efficiency (%)

R_2Vesicle size (nm)

Variables	
X_1= Ratio of lipid: surfactant	**Y_1**= Entrapment efficiency
X_2= Type of surfactant	**Y_2**= Vesicle size

Table 3.6: Factorial design employed (3^2)

Batch No.	Level of Factor A	Level of Factor B	Experimental code
B-1	(-)	(-)	**TF1**
B-2	(-)	(0)	**TF2**
B-3	(-)	(+)	**TF3**
B-4	(0)	(-)	**TF4**
B-5	(0)	(0)	**TF5**
B-6	(0)	(+)	**TF6**
B-7	(+)	(-)	**TF7**
B-8	(+)	(0)	**TF8**
B-9	(+)	(+)	**TF9**

The formulation of transfersome was based on 3^2 factorial design as shown below:

Table 3.7: Composition of Transfersome formulation.

Ingredients	**Batches**								
	TF 1	**TF 2**	**TF 3**	**TF4**	**TF5**	**TF6**	**TF7**	**TF8**	**TF 9**
Glabridin(mg)	100	100	100	100	100	100	100	100	100
Phospholipid (mg)	70	70	70	80	80	80	90	90	90
Span-80 (mg)	30	-	-	20	-	-	10	-	-
Tween-80 (mg)	-	30	-	-	20	-	-	10	-
SDC (mg)	-	-	30	-	-	20	-	-	10
Ethanol : Chloroform (80:20) mL	20	20	20	20	20	20	20	20	20
Phosphate buffer (pH-6.8) mL	20	20	20	20	20	20	20	20	20

B) Methodology for formulating Transfersome

Thin film hydration technique was employed for the preparation of transferosomes which comprised of three steps:

1. By dissolving the mixture of vesicles producing components, phospholipids and surfactant, in a volatile organic solvent (ethanol: chloroform (80:20)), a thin film was produced. After dissolving the glabridin in organic solvent, the organic solvent was evaporated with a rotary evaporator above the lipid transition temperature, and the leftover residues of solvent were removed overnight under vacuum.
2. At room temperature, the thin film was hydrated with buffer (pH 6.8) by rotating at 60 rpm for 3 hours. The resultant vesicles were enlarged at room temperature for 2 hours.
3. To make tiny vesicles, they were sonicated at room temperature for 25 minutes with a probe that had been sonicated at 4°C. The dialysis technique was used to filter the sonicated vesicles.

3.4.5. Evaluation (Wei et al; 2014**)**

Products from various batches were evaluated for the following criteria:

Entrapment Efficiency

A Dialysis membrane with a pore size of 2.4 nm and a molecular weight cutoff of 12,000–14,000 Dalton MW was used to determine drug entrapment efficiency. Before usage, the bags were soaked for 12 hours in the receiving phase (methanol: phosphate buffer (pH- 6.8) 50:50). The bag was filled with 1.0 mL of transfersomal dispersion and the two ends were tied together with thread. In a beaker, the bags were put, and 100.0 mL of receiving phase was added. At 37 °C, the beaker was stirred 100 times per minute using a magnetic stirrer. The5.0 ml of media from the beaker was taken via filtration for analysis at 12, 1,2 hours following the test, and new medium was added to maintain sink conditions. At 226.0 nm, unentrappedGlabridin was spectrophotometrically examined.

Vesicle Size Analysis

A nano particle analyzer (HORIBA sz-100) was used to evaluate the size of vesicles in formulations, and an aliquot was diluted in deionized water. All measurements were done

in triplicate at room temperature with the incoming laser beam at a constant angle of 90°. Data was analyzed by windows [z type-ver1.90] and the mean vesicle size and vesicle size distribution curve were recorded.

Percent Drug Content

The transfersomal dispersion (equivalent to 10.0mg of glabridin) was dissolved in ethanol, required dilutions were made and the glabridin content was analyzed by UV-spectrophotometer (UV-shimadzu 1700) at 226.0 nm using ethanol as blank.

***In - vitro* drug release**

The *in - vitro* drug release of transfersomal formulation were studied using Franz diffusion cell as shown in table 3.8.

Table 3.8: Diffusion test details for diffusion of formulation.

Sr. No	Specification	Standard values
1	Apparatus	Franz Diffusion cell
2	Speed	Magnetic stirrer (100 rpm)
3	Volume of diffusion medium	36 ml (phosphate buffer pH-6.8)
4	Membrane	Dialysis membrane
5	Temperature	37±1°c
6	Sampling interval	1 hr.
7	Total test time	8 hrs.
8	Sampling volume	1.0 ml

3.4.6. Selection of optimized formulation:

On the basis of appearance, drug content, entrapment efficiency and drug release profiles, vesicle size and zeta potentialthe optimized formulation of glabridin loaded TFs was selected and characterized for surface morphology, DSC, and Deformability.

On the basis of results formulation the TF-4batch was selected as optimized formulation.

3.4.7. Lyophilization:

The optimized formulation of glabridin loaded transfersomeswas lyophilized using a freeze dryer (Voltas SF25, India). 3.0% w/v Trehalose was added as a cyoprotectant to the TFs dispersion before freeze drying. The freezing was done at -20°C and freeze drying was done after 24 hrs of refrigeration.

3.4.8. Characterization of optimized formulation (Choi et al, 2015)

On the basis of above mentioned evaluation parameters, the optimized formulation of glabridin loaded transfersomes (TF-4) was selected. It was then characterized by the following parameters.

3.4.9. Morphological analysis of Transfersomes:

A) Vesicle Size Analysis

A nano particle analyzer (HORIBA sz-100) was used to assess the size of vesicles in formulations, and diluted in deionized water before testing. At room temperature and at 90° angle to the incoming laser beam, all measurements were made in triplicate. Data was analyzed by windows [z type-ver1.90] and the mean vesicle size and vesicle size distribution curve were recorded.

B) Zeta potential measurement

The Zetasizer (HORIBA sz-100) device was used to measure zeta potential. The sample was collected (1.0 mL) and dispersed in water. It was put in an ultra sonicator bath for 5.0 minutes to avoid agglomeration. The zeta potential of the sample was measured in a glass cuvette.

C) Scanning Electron Microscopy (SEM)

To examine the exterior morphology of solid TFs SEM is used (FESAM, Nova nano SEM-450). Powder was softly sprinkled over a double adhesive tape affixed to an aluminium stub to prepare the samples. Prior to SEM, sample were coated with 20 nm metal layer by auto fine coater to render them electrically conductive. Following that, the coated sample stubs were put in the scanning electron microscopy chamber. After that,

samples scanned and photomicrographs were obtained at a 10 kV acceleration voltage.

3.4.10. Differential Scanning Calorimetry

Differential scanning calorimetrythermogram analysis was used to determine the physical state of Glabridin in solid TFs. A Perkin-Elmer DSC equipment was used to record the DSC patterns. The sample analysis was carried out in an aluminium pan with a sample weight of approximately 2.0mg, Nitrogen Purging (20.0mL/min), Heating Range: 35-3000C, and Heating Rate: 100C/min.

3.4.11. I.R. Spectrum

At suitably high pressure, the freeze dried transfersome sample (1.0 mg) was transferred into a clear disc in an evacuable die. The entire procedure was carried out in a controlled environment, and the sample was scanned with a Shimazdu IR8400S.

3.4.12. Formulation of Gel Containing Glabridin Loaded Transfersome: (Wei et al, 2013)

Carbopol 934 P was used as the gelling agent. Three composition were prepared as shown in table 8.6.

Formulation of plain gel (Raymond et al, 2006; Jacob et al, 2013**)**

Carbopol was accurately weighed and distributed in a small amount of water before being allowed to soak. The remaining amount of water was dissolved in Methyl paraben and added to the soaked Carbopol, which was properly blended without trapping air bubbles. The final pH of the gel was then adjusted to a slightly acidic state by adding triethanolamine drop by drop.

Conversion of optimized liquid transfersome to gel

A mechanical stirrer was used to mix the required dose of TFs dispersion in Carbopol 934 P gel at a speed of 100 rpm. Triethanolamine was used to neutralise the dispersion. To eliminate entrapped air, the gel was left to stand overnight.

Table 3.9: Composition of gel formulation

Batch	**GT-1**	**GT-2**	**GT-3**
Ingredients	**% W/W**		
Carbopol 934	0.5	1.0	1.5
Triethanolamine	q.s.	q.s.	q.s.
Methyl paraben	0.18	0.18	0.18
Distilled water	q.s. to 100ml	q.s. to 100ml	q.s. to 100ml

Batches obtained were evaluated for different parameters to get the optimized batch of the gel formulation.

3.4.13. Evaluation of the gels (Wei et al, 2013)

Organoleptic characterization

- Clarity: Examined by unaided eye
- Odour: characteristics (bitter).

pH measurement

The pH of the formulation was measured using a pH meter (CL-54 Lab India). 5.0 gm of the formulation was suspended in 45.0 ml distilled water with intermittent agitation. The pH values were collected for three samples on average.

Spreadability studies

Mutimer, et al. proposed apparatus, which was built in-house. The apparatus consists of a wooden block with a fixed glass slide and a moveable glass slide, one end of which is attached to a weight pan moved on a pulley at a horizontal level. The "Slip and Drag" properties of the prepared gel were used to determine its spreadability.

Procedure

Two 2020 cm glass slides were chosen. One of the slides was covered with the gel formulations whoseSpreadability had to be assessed.The other slide was put on top of the gel, sandwiching the gel between the two slides in an area of 60.0 cm long by 100.0 cm

wide. A 100.0g weight was placed on the upper slide, pressing the gel between the two slides uniformly to produce a thin layer. The extra gels clinging to the slide were scraped off and the weight was removed. Only the top slide was allowed to fall off freely by the power of the weight connected to it since the two slides were fastened to a platform without the least disruption. A 20.0 g weight was securely attached to the upper slide. Under the direction of weight, the time it took for the top slide to travel 6.0 cm and separate from the lower slide was recorded. The tests were done in triplicate, with the average of three readings being recorded. The following formula was used to calculate spreadability:

$$S = M \times L/T$$

Where, S = Spreadability,

M = Weight in the pan.

L = Length moved by the glass slide,

T = Time taken to separate the slide completely from each other.

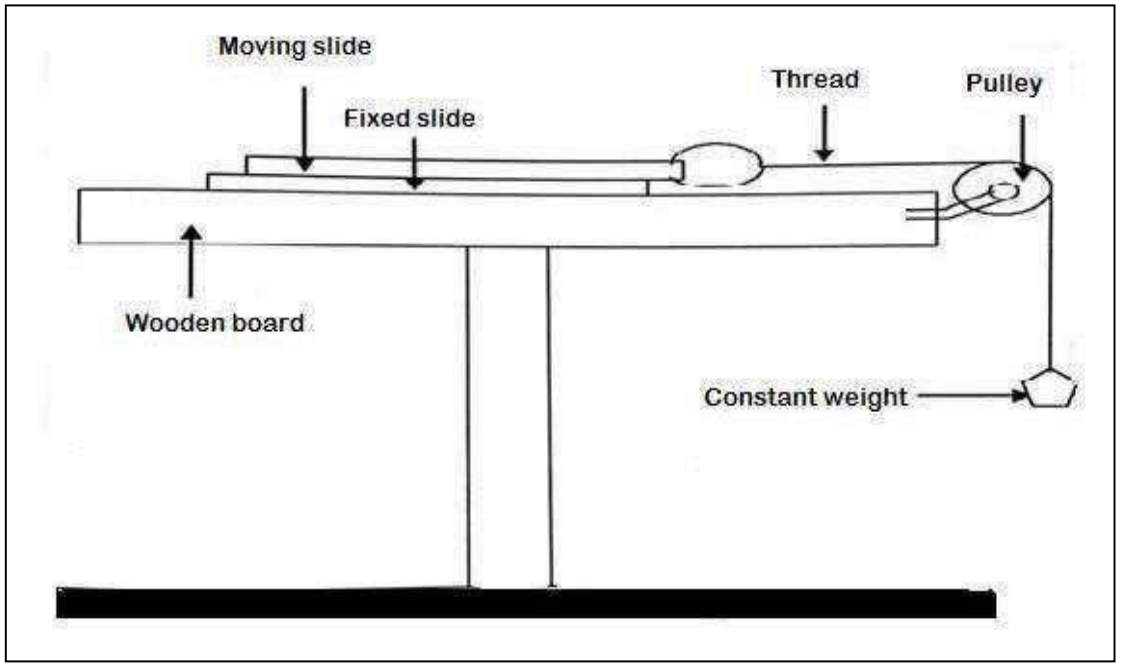

Figure 3.1: Schematic representation of apparatus for determination of spreadability of gels.

Rheological studies

Test condition

Type of equipment- Brookfield RVDV-II + Pro

Spindle- T-bar

Spindle code- S-95

Sample volume- 50.0g

Rpm- 1, 2,5,10,20,50,100.

Procedure

The spindle was attached to the lower shaft of the viscometer. The spindle was rotated within the beaker containing the required quantity of gels once the motor was turned on. Changing the angular velocity from 1 to 100 rpm was a typical run. The display pane was used to record the viscosity readings at each rpm.

Drug content by Spectrophotometric (UV) from gel

The transfersomal gel (equivalent to 10.0mg of Glabridin) was dissolved in ethanol, required dilutions were made and theglabridin content was analyzed by UV spectrophotometer (UV- shimadzu 1700) at 226.0 nm using ethanol as blank.

Diffusion studies (Deshmukh et al, 2012)

A) *In-vitro* diffusion studies

A Franz diffusion cell with a diffusion area of 0.785 cm2 was used to measure drug release from the gel. (See Figure 3.2).) The receptor chamber was filled with a 1:1 combination of phosphate buffer and ethanol, which was continuously swirled at 100 rpm by a tiny magnetic bar. A 12,000-14,000 Dalton MW cut-off dialysis membrane, soaked in the receptor medium overnight, was used to separate the donor and receptor chambers. To replicate in-vivo circumstances, the thermostat was adjusted to keep the membrane surface at 37°C. Before occluding the donor compartment with paraffin, 1.0 gm of each formulation was introduced. At the membrane/liquid contact, care was made to avoid air bubbles. 1.0 ml samples were taken at predefined intervals of 0.5, 1, 2, 4, 6, and 8 hours

for up to 8 hours. To maintain sink conditions, the medium is removed from the receptor and replaced with new media. Each formulation was tested in triplicate, and samples were evaluated using a UV spectrophotometer to measure absorbance at 226.0 nm.

B) *Ex-vivo* diffusion studies

Porcine skin is believed to closely mimic human skin in its responses as compared to other animal models. Thus it was used as the diffusion barrier. The porcine skin was freshly obtained. It was cleaned of all subcutaneous fat with care to avoid damaging epithelium.

It was then stored in sterile normal saline in a clean container and frozen at sub-zero temperature until use. Sections of the skin, of suitable size, were cut out using clean pair of scissors, and the container was closed immediately. The sections of the skin were allowed to equilibrate at 37+ 1^0C (physiological temperature) in deionized water. The skin specimens thus prepared were used in the diffusion experiment. The diffusion medium used was phosphate buffer: ethanol (1:1) mixture. The magnetic stirrer was put on top of the diffusion cell, and the receptor media was continually stirred at 100 rpm by the magnetic bar. Rubber latex tubes were used to link the reservoir outflow to the diffusion cell's water jacket, which was kept at 37°C. Fluid had flooded the receptor compartment. The prepared pig skin was then carefully mounted on the cell, avoiding the trapping of air bubbles beneath the mounted skin. Skin-to-receptor fluid contact was established by fastening it securely with a clamp. To keep the sink condition, 1.0 ml of sample was removed from the receptor compartment's sampling port at one-hour intervals and refilled with receptor fluid solution in the same volume. Sampling was continued for 8 hrs. The samples were appropriately diluted and the absorbance was measured at 226.0 nm using UV – V spectrophotometer. Results were expressed in percent drug release and the diffusion data was fitted in Higuchi and Korsmeyer-Peppas model. Diffusion coefficient and flux was found out.

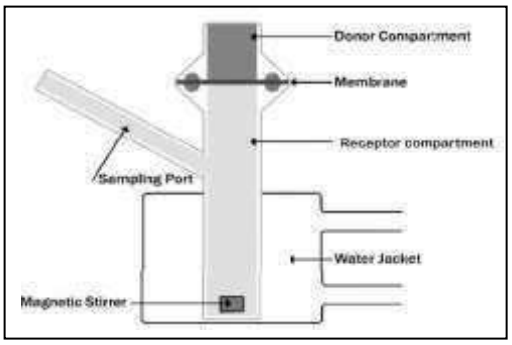

Figure 3.2: Franz diffusion cell

Stability Studies:[Patel et al , 2014]

To complete studies in less time, it would be more convenient to conduct stability tests on products that have been stored in extreme temperatures.Conditions were used 25 ± 2ºC (60 % ± 5 % RH) 40 ± 2ºC (75% ± 5 % RH) for a period of 3 months and evaluated for:

1) Organoleptic properties
2) Measurement of vesicle size
3) Measurement of pH
4) Drug content (%)
5) Drug release (%).

3.4.14. Formulation of Apremilast pellets

Extrusion and spheronization techniques were used to make apremilast pellets utilising the wet granulation process. All the powder ingredients like Apremilast, lactose, microcrystalline cellulose, crospovidone and TKP were weighed accurately mixed in mortar for 20 min and passed through sieve to insure uniform mixing. Then binding liquid water was added in sufficient quantity in order to get damp mass. To get the extrudates, the damp mass was passed through sieve no.16. To obtain pellets, the prepared extrudates were transferred to a spheronizer (Shakti Pharmatech) and processed at various spheronizing speeds. The pellets were dried for 30 minutes in a hot air oven at 40 degrees Celsius.

Table 3.10: Composition of core apremilast pellets

Batch No.	Ingredients								
	Drug (gm)	MCC (gm)	Lactose (gm)	Crospo-vidone (gm)	TKP (gm)	Water (ml)	Total Weight (gm)	Sphero-nization speed (RPM)	Sphero-nization time (sec)
F-1	1	10	7	1	1	q.s.	20	800	110
F-2	1	10	6	1	2	q.s.	20	900	100
F-3	1	10	5	1	3	q.s.	20	1000	90
F-4	1	12	5	1	1	q.s.	20	800	110
F-5	1	12	4	1	2	q.s.	20	900	100
F-6	1	12	3	1	3	q.s.	20	1000	90

Calibration curve of drug

Standard calibration curve of apremilast in 0.1N HCl (Muley et al., 2022)

In a 100ml volumetric flask, 10 mg of Apremilast was properly weighed and dissolved in 100 ml 0.1N HCL to obtain the solution of 100µg/ml concentration. Further dilutions with 0.1NHCl were prepared toget solutions with concentration ranging from1 to 10µg/ml. Absorbance of these solutions were measured at 230.5nm.

Standard calibration curve of apremilast in phosphate buffer pH-6.8 (Patil et al., 2017)

10 mg of Apremilast accurately weighed and was dissolved in 100 ml phosphate buffer pH-6.8in 100ml volumetric flask to obtain the solution of 100µg/ml concentration. Further dilutions withphosphate buffer pH-6.8were prepared toget solutions with concentration ranging from 1 to 10µg/ml. Absorbance of these solutions were measured at 230nm.

Evaluation of pellets (Muley et al., 2022; Law et al, 1998; Nandgude et al, 2011; Prashant et al, 2022; Lavanya et al, 2011)

As mentioned previously, pellets were evaluated for following characteristics- Bulk Density, Tapped Density, Hausner‘s ratio, Carr‘s Index, Angle of repose, Determination of % Yield, Hardness, Particle size, Drug Content, DSC

3.4.15. Glabridin Estimation in Transfersomal Gel Formulation Using a High Performance Liquid Chromatographic Method

Standard stock solution preparation:

10.0 mg of Glabridin, accurately weighed, was added to a 100.0 mL volumetric flask, followed by 25.0 mL of mobile phase, ultrasonicated for 10 minutes, and volume brought up to the mark with mobile phase to achieve a concentration of 100 µg/ml.

Choosing a mobile phase:

The mobile phase was used to dilute an aliquot of standard stock solution to achieve a final concentration of 30 µg/ml. A 0.2 membrane filter was used to filter the diluted standard solution.The filtrate was fed into the HPLC apparatus and run in several solvent systems (water, methanol, ACN, buffers). To find the best chromatographic conditions for generating a sharp peak of glabridin with minimum tailing, a mixture of various solvents with varied polarity was tested.In comparison to other mobile phases, it was discovered that a mixture of acetonitrile (ACN) and acetate buffer offers good results after numerous permutations and combinations. Finally, the optimum mobile phase composition, ACN: Acetate Buffer (70:30v/v) with a flow rate of 1.0 mL/min, was chosen because it produced a strong asymmetric peak for Glabridin with little tailing and the required elution duration. Glabridin's retention time was discovered to be 5.70 minutes.

Selection of analytical wavelength:

To produce a final concentration of 20 µg/ml of glabridin, an aliquot amount of standard stock solution was suitably diluted and scanned in the spectrum mode using UV- Visible Spectrophotometer against the mobile phase as a blank in the wavelength ranges of 400 to 200 nm.

Glabridin demonstrated substantial absorption at 228.0 nm thus that was chosen as the wavelength.

Optimum Chromatographic conditions:

HPLC Column : Kromasil C8, (250 mm X 4.6 mm, 5µm)

Column temperature : Ambient temperature

Mobile Phase : ACN: Acetate Buffer (70:30 v/v)

Flow rate : 1.0 mL/min

UV detection : 228.0 nm

Injection volume : 20 µl

Run time : 10 mins

System suitability parameters:

Method appropriateness factors such as the column efficiency (number of theoretical plates, N) and tailing factor (T) were examined. to determine the repeatability of the proposed chromatographic system for estimating glabridin in TFs gels.The standard stock solution was aliquoted and diluted with mobile phase to achieve a final concentration of 30 µg/ml, which was then utilised for analysis. A 0.2 membrane filter was used to filter the fluid.The filtrate (20µl) was put into the column, and the chromatographic conditions were tuned. At a wavelength of 228.0 nm, the chromatogram was taken.

Table 3.11: System suitability parameters

Parameter	**Standard values**	**Observed values**
Tailing factor	NMT 2	1.33
Theoretical Plates	NLT 2000	5842

NMT – Not more than NLT – Not less than

Study of linearity range:

Test solutions for Glabridin were prepared as follows:

A accurately weighed quantity (10.0 mg) of glabridin was put to a 100.0 mL volumetric flask, along with 25.0 mL of mobile phase, and ultrasonicated for 10 minutes.1.0, 2.0, 3.0, 4.0, 5.0, and 6.0 mL aliquots of the above solution were mobile phase diluted to 10.0 mL (concentration 10, 20, 30, 40, 50, and 60 µg/ml, respectively).

A 0.2 membrane filter was used to filter each solution.The diluted solutions were injected into the HPLC apparatus (20 l) and chromatographed under optimized condition. Glabridin's peak area was measured at 228.0 nm.

- ***Analysis of pellet formulation:***

The pellets were crushed to yield fine powder. An accurately weighed amount of powder equivalent to around 10 mg APL was transferred to a 100.0 ml volumetric flask, 50ml of mobile phase was inserted, and the volume was built up to the mark with mobile phase. The solution was then combined and filtered through No. 42 Whatmann filter paper. With mobile phase, 2.0 ml of the filtrate solution was diluted to 10.0 ml. A 0.22 membrane filter was used to filter the fluid.

In the HPLC system, an equal volume (20 L) of standard and sample solution was injected and chromatographic utilising optimal chromatographic conditions. Each solution was injected in triplicate and chromatographed.

3.4.16. Method validation:

Accuracy

Several characteristics were studied to verify the suggested technique, including limit of detection (LOD), limit of quantitation (LOQ), precision, linearity, accuracy, and robustness.

a) Preparation of standard solution

With mobile phase, a 1.0 mL solution was diluted to 10.0 mL.(30 µg/ml) from a standard stock solution (300 µg/ml).

b) Preparation of sample solution

The following nine samples were prepared and analysed:

A pre-analyzed TFs gel weighing about 5.0 mg of Glabridin was carefully weighed and

transferred to nine different 25.0 mL volumetric flasks. The following amounts of glabridin were added to each flask:

Flask No.	**Wt. of pure drug added**
1.	4.0 mg
2.	4.0 mg
3.	4.0 mg
4.	5.0 mg
5.	5.0 mg
6.	5.0 mg
7.	6.0 mg
8.	6.0 mg
9.	6.0 mg

The flasks were then filled with 15.0 mL mobile phase and ultrasonicated for 20 minutes, after which the volume was brought up to the mark. Individually mixed solutions were filtered via Whatman no. 42. A 5.0 mL solution was diluted to 10.0 mL from the filtrate. The mobile phase was then added to dilute the aforementioned solution to 3.0 mL. A 0.2 membrane filter was used to filter the diluted solution.

The column was injected with an equal amount of standard and sample solution (20µL) and chromatographed. Each solution was injected and chromatographed in triplicate. The peak area for glabridin was measured at 228.0 nm on the associated chromatograms.

By comparing the peak area, the total amount of drug estimated in the sample was obtained:

$$\text{Amount of drug estimated in sample(mg)} = \frac{PA_{Spl}}{PA_{Std}} \times C_s \times d_f$$

Where,

PA_{Spl} - Peak area of sample.

PA_{Std}- Peak area of standard.

C_s-Concentration of standard (mg/mL)

d_f - Dilution factor for sample.

The following equation was used to know drug recovered (mg) and the percent:

Amount of the drug recovered = Total amount of drug estimated in sample − Amount of drug contributed by TFs gel

$$\% \text{ Recovery} = \frac{\text{Amount of drug recovered}}{\text{Amount of pure drug added}} \times 100$$

Precision

a) Intermediateprecision (Intra- day and Inter- day precision)

TFs gel sample solutions were analysed at different time intervals on the same day and on three other days to assess intra-day and inter-day precision.

b) Solution stability study

The stability of the sample solution was tested by injecting TFs gel sample solution at varied time intervals (in triplicate). i.e., chromatographed under ideal circumstances at 0 minutes, 1, 3, 6, and 24 hours. At each time interval, a sample solution was injected and chromatographed in duplicate. The glabridin retention time and peak area were recorded.

Limit of detection(LOD) and limit of quantitation(LOQ)

The LOD and LOQ were calculated independently depending on the calibration curve's standard deviation of response. The slope of the calibration curves and the standard deviation of the y-intercept were used to compute the LOD and LOQ.

Robustness of method

Small but deliberate changes in the optimal technique parameters were performed to test the resilience of the suggested method. The impact of changing composition of mobile phase and flow rate on drug peak retention time and tailing factor was investigated.

In order to achieve optimal chromatographic conditions, the mobile phase composition

was altered in a 2.0 mL proportion and the flow rate was modified by 0.1 ml/min..

3.4.17. Analysis of Transfersomal gel formulation:

a) Preparation of standard solution

3.0 mL of standard stock solution (100 µg/ml) was diluted to 10.0 mL with mobile phase (30 µg/ml) from a standard stock solution (100 µg/ml).

b) Preparation of sample solution

Quantity of TFs gels accurately weighed (about 5.0 mg) Glabridin was volumetrically transferred to a flask, 15.0 mL of the mobile phase was added, and the flask was ultrasonicated for 20 minutes before the volume was brought up to the mark with the mobile phase. After mixing the solution and filtering the filtrate using Whatmann filter paper no. 42, the 5.0 mL solution was diluted to 10.0 mL with mobile phase. 3.0 mL of the aforementioned solution was diluted to 10.0 mL with the mobile phase.

In the HPLC system, an equal amount (20 L) of standard and sample solution was injected and chromatographed under optimal chromatographic conditions. Each solution was injected in triplicate and chromatographed. The peak area of glabridin was found at 228.0 nm in the chromatograms.

The amount of glabridin was calculated by comparing the sample's mean peak area to that of the standard using the following equation:

$$\text{Content of drug in sample (mg/gel)} = \frac{PA_{Spl}}{PA_{Std}} \times \frac{\text{Weight of std. d(mg)}}{\text{gel taken (g)}} \times \frac{d_{f2}}{d_{f1}} \times \frac{\text{Avg. weight of TFs}}{\text{Weight of TFs}}$$

Where, PA_{Spl} - *Peak area of sample,*

PA_{Std} - *Peak area of standard,*

d_{f1} - *Dilution factor for standard*

d_{f2} - *Dilution factor for sample*

Percent label claim was calculated using following equation:

Amount of drug estimated (mg) X 100

Percent label claim = Label claim

3.4.18. Forced degradation studies:

A amount of TFs gel corresponding to roughly 5.0 mg of glabridin was accurately weighed and transferred to six separate 50.0 mL volumetric flasks (flask no. 1, 2, 3, 4, 5 and 6). 3.0 mL methanol was added as a co-solvent to flasks 1, 2, and 3, followed by 3.0 mL 0.1 M HCl, 0.1 M NaOH, and 3 percent H2O2 to flasks 1, 2, and 3, respectively. 3.0 mL water was added to flask no.4 for neutral hydrolysis.

On a water bath at 800° C, the contents of flasks 1, 2, 3, 4, and 5 were heated for 3 hours. Flask No. 5 was placed in a 600° C oven for 24 hours to see how heat affected the TFs gel sample (thermal degradation).The forced degradation tests were carried out in complete darkness to rule out the possibility of light-induced deterioration. To investigate the influence of light on TFs gel samples, Flask no. 6 containing TFs gel was subjected to UV light for 24 hours (photo-degradation).All of the flasks were removed and cooled to room temperature after the specified time interval. The samples were then processed and evaluated in the same way as the TFs gel formulation was studied.

3.4.19. Estimation of Apremilast Pellet Formulation by High Performance Liquid Chromatographic Method

- ***Preparation of standard stock solution:***

10.0 mg Apremilast was carefully weighed and transferred to a volumetric flask with a capacity of 100.0 mL., followed by 50 ml of mobile phase and 20 minutes of ultrasonication. The volume was then made up to the mark with mobile phase. (A concentration of 100 µg/ml was obtained.)

- ***Selection of mobile phase:***

To achieve a final concentration of 100µg/ml, an aliquot quantity of standard stock solution was properly diluted with mobile phase. A 0.22 membrane filter was used to filter the diluted standard solution.The filtrate was injected into the HPLC apparatus, which was then run in various solvent systems. To establish the optimum

chromatographic conditions for obtaining a sharp peak with minimum tailing, a mixture of various solvents with varying polarity was tried. . After several permutation and combination, it was found that mixture of Acetonitrile and Water pH 3(Adjusted with Formic Acid) gives acceptable results when compared to other mobile phases. Finally, the mobile phase composition of Acetonitrile is a kind of acetonitrile (30mM) pH of potassium dihydrogen phosphate buffer (60:40 v/v) was adjusted to 3.0 using o-phosphoric acid and flow rate of 1.0ml/min was chosen because it produced a strong symmetric peak for APL with little tailing and a desirable elution graph. The APL retention time was noted to be 4.46 minutes.

- ***Selection of analytical wavelength:***

To produce a final concentration of 100 µg/ml of APL, an aliquot amount of standard stock solution was suitably diluted with mobile phase. The solution was scanned in the spectrum mode using a double beam UV-Visible Spectrophotometer-1700 against a blank mobile phase in the wavelength ranges of 400 nm to 200 nm.

- ***Optimum Chromatographic conditions:***

HPLC Column : C18 Neosphere, R (25 cm length, 4.6 mm inside diameter, 5 µm particle size)

Column temperature : Ambient temperature

Mobile Phase : Acetonitrile is a kind of acetonitrile (30mM) pH of potassium dihydrogen phosphate buffer (60:40 v/v) was adjusted to 3.0 using o-phosphoric acid.

Flow rate : 1.0 ml/min

UV detection : 236 nm

Injection volume : 20 µl

Run time : 10minutes

- ***System suitability parameters:***

System appropriateness characteristics such as column efficiency, number of theoretical plates (N), capacity factor (k), and tailing factor (T) were investigated to determine the

reproducibility of the proposed chromatographic system for estimating APL in pellets. A portion of the standard stock solution was diluted with mobile phase to achieve a final concentration of 10µg/ml, which was utilised for analysis. A 0.22 membrane filter was used to filter the fluid. Using optimal chromatographic conditions, the filtrate (10 µl) was injected into the column and chromatographed. At 236 nm, the chromatogram was read.

- ***Study of linearity range:***

Six linearity test solutions for APL were prepared as follows:

A accurately weighed quantity (10 mg) of APL was transferred to a 100.0 ml volumetric flask, 70 ml of mobile phase was added, and the flask was ultrasonicated for 20 minutes before the volume was brought up to the mark with mobile phase. With mobile phase, 1.0 ml of the above ementioned solution was diluted to 10.0 ml. From this 1.0, 2.0, 3.0, 4.0, 5.0, and 6.0 ml of this solution were diluted to 10.0 ml with mobile phase (concentration 10, 20, 30, 40, 50, and 60 µg/ml, respectively).After that, each solution was filtered using a 0.22 membrane filter. The diluted solutions were injected into the HPLC apparatus (20 µl) and chromatographed under ideal chromatographic conditions. APL's peak area was measured to be 236 nm. Mean peak areas were calculated for each drug concentration.

- ***Method validation:***

Method validation was done by studying, Precision, accuracy, linearity, limit of detection (LOD), limit of quantitation (LOQ), and resilience are all factors to consider.

- **Accuracy:**

Recovery experiments were carried out using the standard addition technique at 80, 100, and 120 % of the test concentration, as per ICH standards, to ensure the accuracy of the suggested procedures.

Preparation of standard solution:

Standard solution was prepared as discussed under analysis of marketed formulation.

Preparation of sample solution:

Nine samples were prepared and analysed in following manner:

Powder equivalent to 10mg of APL was weighed and transferred to nine different 100.0 ml volumetric flasks, to each of it following quantities were added:

Flask no.	1	2	3	4	5	6	7	8	9
Level of Recovery	80	80	80	100	100	100	120	120	120
Amount of APLadded (mg)	8.0	8.0	8.0	10.0	10.0	10.02	12.01	12.00	12.01

Followed by adding 50 ml of mobile phase to each flask and 20 minutes of ultrasonication. Then the volume was made upto the mark with mobile phase. The solutions were mixed individually and filtered -Whatmann filter paper no. 42.

With mobile phase, a 5.0 ml solution was diluted to 10.0 ml from the filtrate. 1 mL of the above mentioned solution was diluted to 10.0 mL with mobile phase. A 0.22 membrane filter was used to filter the diluted solution.

The column was injected with an equal volume of standard and sample solution (20L) and chromatographed under optimized chromatographic conditions. Each solution was injected in triplicate and chromatographed. The area of each peak for APL was measured at 236.0 nm on the corresponding chromatograms. Total amount of drug estimated using equation no. 16.

$$\text{Total amount of drug estimated in sample} = \frac{\text{Peak Area of sample/peak}}{\text{Area of Std. x conc. of Std (mg/mL) x DF}} \quad \text{---(16)}$$

Amount of the drug recovered (mg) was calculated by using equation 17, where percent recovery was calculated using equation no.18.

$$\text{Amount of drug recovered} = \text{Total amount of drug estimated in sample} - \text{Amt of drug contributed by tab powder} \quad \text{------ (17)}$$

$$\% \text{ Recovery} = \frac{\text{Amount of Drug Recovered}}{\text{Total Amount of Pure Drug Taken}} \times 100 \quad (18)$$

- **Precision:**

Same process used as discussed above for Intermediate precision (Intra - day and Inter - day precision), ***Limit of detection (LOD) and limit of quantitation (LOQ), Robustness of method***

- ***Forced degradation studies:***

Powder equivalent to 10 mg of APL was accurately weighed and transferred to six different 50.0 ml volumetric flask, (flask no. 1, 2, 3, 4, 5 and 6). To flask no. 1, 2 and 3, added 3.0 ml of 0.1N HCl, 0.1N NaOH, 3% H_2O_2 and H_2O to flask no.1, 2, 3, and 4 respectively. The contents of flasks 1, 2, 3, and 4 were heated for 2 hours on a water bath at 800°C. To investigate the effect of heat on APL bulk powder, Flask No. 5 was placed in a hot air oven at 600°C for 30 minutes (heat degradation).

The forced degradation tests were carried out in complete darkness to rule out the possibility of light-induced deterioration. Flask no. 6 containing bulk APL powder was exposed to UV light for 72 hours to investigate the effect of light on bulk APL powder (photo degradation). The entire flask was removed and brought to room temperature after the specified time span. The mobile phase was added to the 50.0 ml volumetric flask and ultrasonicated for 20 minutes, after which the volume was brought up to the mark with mobile phase. After that, the solution was mixed and filtered using No. 42 Whatmann filter paper. A total of 1.0 ml of solution was diluted with 10.0 ml of mobile phase from the filtrate. 20 µl of each of the standard and treatment sample solutions were injected and chromatographed under optimized chromatographic conditions.

3.4.20. Apremilast Estimation in Pellet Formulation Using a High-Performance Thin-Layer Chromatographic Method

Preparation of standard stock solution:

- **Preparation of standard stock solution:**

10.0 mg Apremilast was accurately weighed and put to a 10.0 ml volumetric flask.

5 ml methanol was added and ultrasonicated for 10 minutes, after which the volume was brought up to the mark with methanol (concentration attained 1000 g/ml)

- **Selection of mobile phase:**

Aliquots of standard stock solution 0.5µl were put on TLC plates in the shape of a band (size of a band should be: 6 mm), and the plates were run in various solvent systems.

Several trials were conducted utilizing different solvent systems comprising non-polar and some polar solvents in order to obtain the required Rf value range (0.2-0.5) with a dense band.

Solvent systems are tolune: methanol: chloroform methylacetate: hexane: ammonia chloroform:methanol:trie- thylamine toluene: methanol.The optimum conditions for the efficient separation of Apremilast were determined by experimenting with different concentration levels. Toluene: Ethyl Acetate (4:6 v/v) was the only mobile phase combination that produced compact bands with a symmetrical peak on the chromatogram and the necessary Rf value. Apremilast had an Rf value with a standard deviation of o 0.55 ± 0.02

- **Analytical wavelength Selected for densitometric evaluation**

With the aid of a CAMAG LINOMAT-V automated sample applicator, a standard stock solution was placed on to a 0.5µl TLC plate, and the plate was placed in a twin-through glass chamber saturated with mobile phase for 15 minutes.

The plate was removed and air dried after chromatographic development. The bands on the TLC plate were scanned in the 200-700 nm wavelength range.

Chromatographic conditions that have been optimised:

For densitometric analysis of Apremilast, the following chromatographic settings were optimised on a trial basis.

Stationary phase : Aluminium plates precoated with silica gel 60 F_{254} (Merck)

Mobile phase: Toluene: Ethyl Acetate (4: 6V/v)

Plate size : 10 cm X 10 cm,

Mode of application: Band

Band size: 6 mm (Distance between two bands: 5.6 mm)

Sample volume: 5µl

Development chamber: Twin-through glass chamber, 10 cm X 10 cm with Stainless steel lid.

Saturation time **:** 15minutes

Separation technique : Ascending

Migration distance **:** ≈ 80 mm

Temperature **:** 25 ± 5^0C

Scanning mode **:** Absorbance/Reflectance

Slit dimensions **:** 5 X 0.45 mm

Scanning wavelength: 236.0 nm

Study of linearity range:

10.0 mg Apremilast was carefully weighed and transported to a 10.0 ml volumetric flask, where it was combined with 5 ml methanol and ultrasonicated for 10 minutes. Methanol was then used to get the volume up to the required level. (A 1000 g/ml concentration was attained.)

With the aid of a micro syringe and the LINOMAT-V automated sample applicator, the above APL solution was applied to TLC plates in the range of 1- 6µl. Following that, developed plate scanned under ideal chromatographic conditions. The peaks recorded for APL were combined after scanning.

For APL, the calibration curve of Concentration vs. Peak area was developed by recording the peak area for each drug concentration

Analysis of Bulk drug:

Standard solution was prepared by diluting 1.0 ml of standard stock solution (Page no. 75) to 10.0 ml of methanol (concentration obtained 100 µg/ml).

The following six samples were produced and analysed in the following order:

Preparation of sample solution:

10.0 mg Apremilast was measured and added to a 10.0 ml volumetric flask, which was

then ultrasonicated for 10 minutes with 5 ml methanol. Methanol was then used to get the volume up to the mark. (A 1000 µg/ml concentration was attained.)

10.0 ml methanol was used to dilute 1.0 ml of the aforesaid solution. (A 100 µg/ml concentration was attained.) The TLC plate was coated with two bands of standard stock solution and four bands of sample solution, each containing 5l, and then developed and scanned under ideal chromatographic conditions. The peaks from the standard and sample bands were merged during scanning. Equations 13, 14, and 15 were used to calculate the drug content in mg and percent drug estimation, respectively.

Analysis of pellet formulation*:*

Preparation of a standard solution, as explained in the bulk drug analysis section.

The sample solution is prepared in the following manner:

The following procedure was used to produce and analyse six samples:

To achieve fine powder, pellets were weighed and crushed. Amount of powder corresponding to roughly 10.0 mg of APL was accurately weighed and transferred to a 10.0 ml volumetric flask, to which 5 ml methanol was added and ultrasonicated for 10 minutes before volume was brought up to the mark with methanol. Whatman filter paper no. 42 was used to filter the solution after it was mixed. Using methanol, a 1.0 ml solution was diluted to 10.0 ml from the filtrate.

Two bands of standard solution and four bands of sample solution, each containing 5µl, were applied to the TLC plate, which was then developed and scanned under ideal chromatographic conditions.

After scanning, the peak obtained for standard and sample bands were integrated.

Using equation no. 1, the quantity of APL in (in pellets) was determined by comparing the mean peak area of sample bands to that of standard bands (13) HPLC stands for high-performance liquid chromatography.

Equation no.15 was used to compute the percent label claim.

- ***Method validation***

The accuracy, precision, linearity, limit of detection (LOD), limit of quantitation (LOQ), and robustness of the proposed approach were all investigated.

- **Accuracy:**

Recovery tests were performed using the standard addition technique at 80, 100, and 120%of the test concentration, as per ICH standards, to determine the accuracy of the suggested procedures.

Preparation of standard solution:

As discussed under analysis of bulk drug.

Preparation of sample solution:

Amounts of pre-analyzed pellet powder corresponding to roughly 10.0 mg of APL were accurately weighed and transferred to nine separate 10.0 ml volumetric flasks. The following amounts of APL were added to each flask:

Flask no.	1	2	3	4	5	6	7	8	9
Level of Recovery	80%	80%	80%	100%	100%	100%	120%	120%	120%
Amount of APL added (mg)	8.0	8.0	8.0	10.0	10.0	10.0	12.0	12.0	12.0

After that, 5 mL methanol was added to each flask, ultrasonicated for 10 minutes, and methanol was used to make up the volume. Individually mixed solutions were filtered via Whatman filter paper no. 42. 5.0 mL of the above solution was diluted in 10.0 mL methanol. Using methanol, dilute 1.0 ml of the above solution to 10.0 ml.

Two bands of reference solution and four bands of sample solution were placed on the TLC plate.

5.0 µl were applied to each plate, which was then developed and scanned under ideal chromatographic conditions.

- **Precision:**

By testing pellet sample solutions at different time intervals on the same day and on three different days, intraday and interday precision were found.

- **Robustness**:

Small but systematic adjustments in the optimal technique parameters, such as mobile phase composition, chamber saturation time, time from spotting to development, scanning development , mobile phase volume, and solution stability, were used to determine the robustness of the proposed approach.

The mobile phase composition and chamber saturation duration of the employed optimum conditions were changed in the range of ±0.1 ml and ±5 min, respectively.

The mobile phase volume was varied by ±1 ml. The impact of these modifications on the Rf values as well as the peak area was investigated.

- **Limit of detection and limit of quantification:**

The LOD and LOQ were calculated independently depending on the calibration curve's standard deviation response.

The LOD and LOQ were calculated using SD of the y-intercept and the slope of the calibration curves.

- **Forced degradation studies:**

Separately, weighed pellet powder corresponding to 10.0 mg APL was transferred to five distinct 10.0 ml volumetric flasks (Flask no. 1, 2, 3, 4, 5 and 6) and 3.0 ml of flask no. 1, 2, 3 and 4, 0.1 N HCl, 0.1 N NaOH, 3% H_2O_2 and distilled water respectively.

For 2 hours, flasks 1, 2, 3, and 4 were maintained in a water bath at 800℃.

Flask no. 5 was maintained at 600℃ for 1 hour with 10 mg of APL in it. To investigate the effect of heat on the sample, it was placed in a hot air oven (heat degradation). dark to exclude the possible degradative effect of light. APL was maintained in Flask No. 6 for 72 hours in a UV chamber. The purpose is to study at the photolytic degradation of a bulk sample. After a certain time span, all of the flasks were removed, and the APL samples were handled and tested in the same way as the commercial formulation samples were.

CHAPTER -4
RESULT & DISCUSSION

I. Design and evaluate transfersomal gel for topical delivery of Glabridin

Raw Material Analysis:

The raw components utilized in this project were evaluated before being employed in the formulation. Tables 4.1 contain the results of these analyses. Unless otherwise specified, pharmacopeial processes were used to determine various parameters such as solubility, hydrophilic lipophilic balance (HLB), loss on drying, heavy metals, and so on.

4.1 Glabridin:

4.1.1 : Physicochemical characterization of Glabridin

Table 4.1: Physicochemical characterization of Glabridin

Tests	Specifications	Results
Description	Yellowish brown to reddish brown colored powder	Complied
Solubility	soluble in ethanol (95%), methanol, insoluble in water	Complied
Melting point	Melts between 154^0C and 155^0C	154ºC
Loss on Drying	Not more than 5%w/w, determined on 1.0 g by drying in an oven at 105^0C $\pm$ 2^0Cfor 3 hours	0.68%w/w
Assay	Contains not less than 97.0%w/w Glabridin	95.52%w/w
Total Viable Bacterial Count	Not more than 100cfu/gm	20cfu/gm

The Glabridin used in the formulation met all of the requirements, as evidenced by the results of the testing.

4.1.2 Differential Scanning Calorimetry

A single melting endotherm at 155.4^0C characterised the drug's thermogram. Figure 4.1 shows the thermogram, which was confirmed to be in agreement with the parameters.

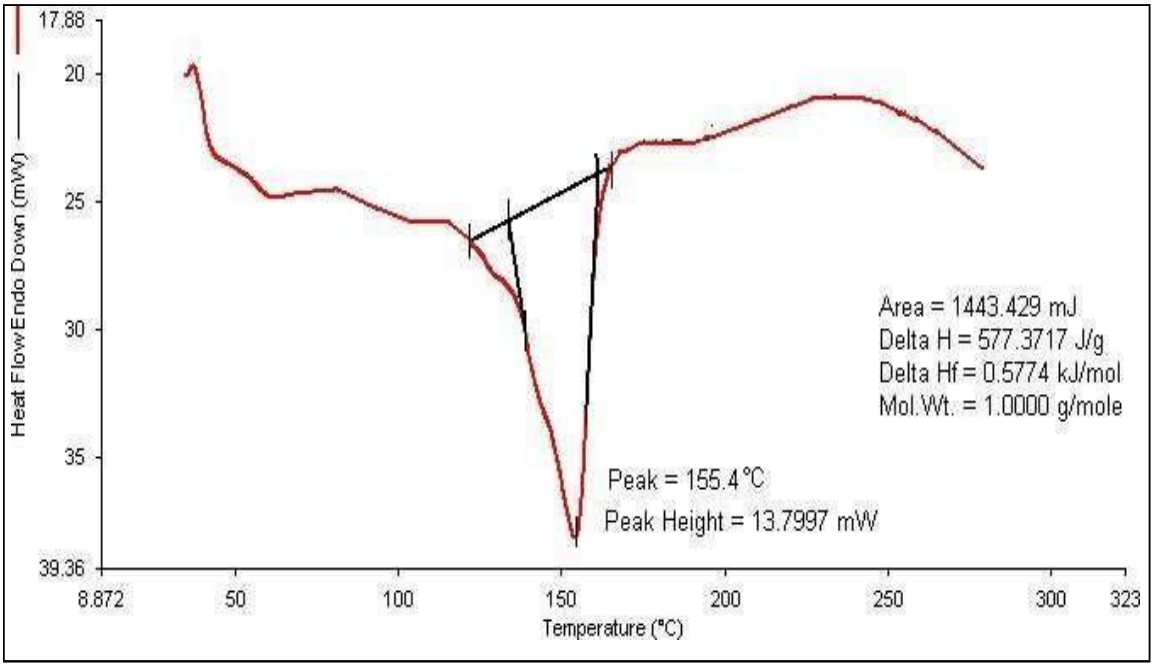

Figure 4.1: DSC graph of Glabridin

It is generally known that the presence of impurities in a sample causes the melting point to drop and the melting range to expand. Glabridin's DSC thermogram (Figure 4.1) revealed a sharp melting endothermic peak at 155.40C (H = 577.3717J/g), indicating that there were no traces of the impurity.

4.1.3 I.R. Spectrum of Glabridin

The sample (1.0 mg) was finely ground to a particle size of 2 m or less and well mixed with 200.0 mg dried KBr (kept at 100^{o}C for 8 hrs). The mixture was then injected under high pressure into a transparent disc in an evacuable die. The entire procedure was carried out in a controlled environment, and the sample was scanned with a Shimazdu IR-8400S. The I.R Spectrum is depicted in the diagram below (Figure 4.2). Table 4.2 summarises the findings.

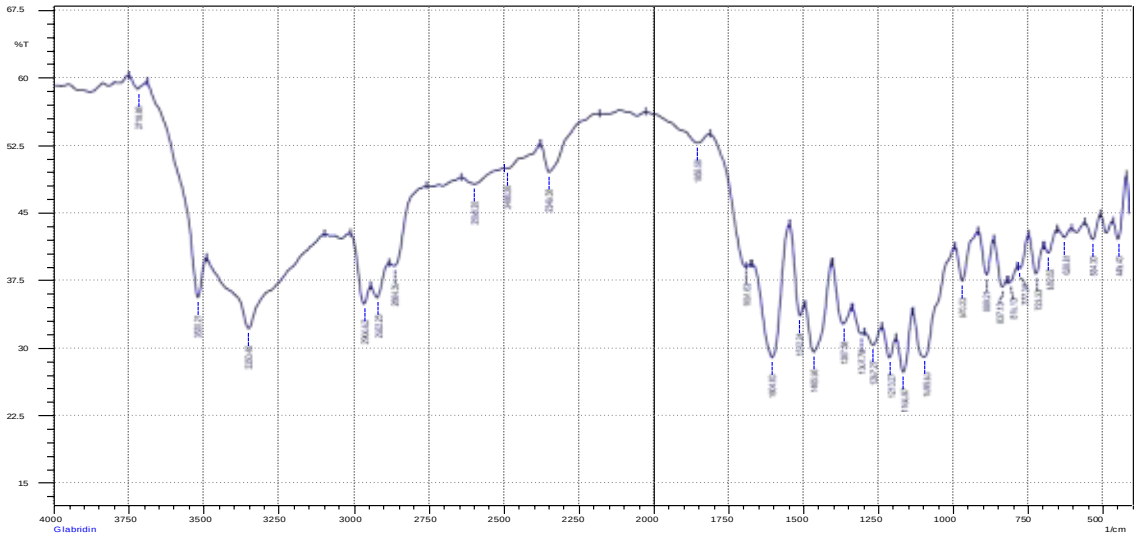

Figure 4.2: IR spectrum of Glabridin

Table 4.2: Results of IR interpretation of glabridin

Sr. no	Bond	Frequency range, cm^{-1}	Conclusion
1	O- H	3351 - 3528	Strong stretch
2	Benzene ring with C-H	2973, 2967, 2934, 2878	Medium stretch
3	C-O-C	1056, 1091, 1114, 1170	Strong stretch
4	C-H	1468, 1519	Strong bend

The IR spectrum of glabridin matches the criteria.

4.1.4 Determination of λmax for U.V absorption

In the region of 200-400 nm, a solution of 10g Glabridin in ethanol was scanned. The UV scan of the medication is depicted in figure 4.3.

File Name	**Glabridin**
Model	**Shimadzu UV- 1700**
Measurement Range	**400-200 nm**
Scanning Speed	**100 nm/min**
λ_{max}	**226.0nm**

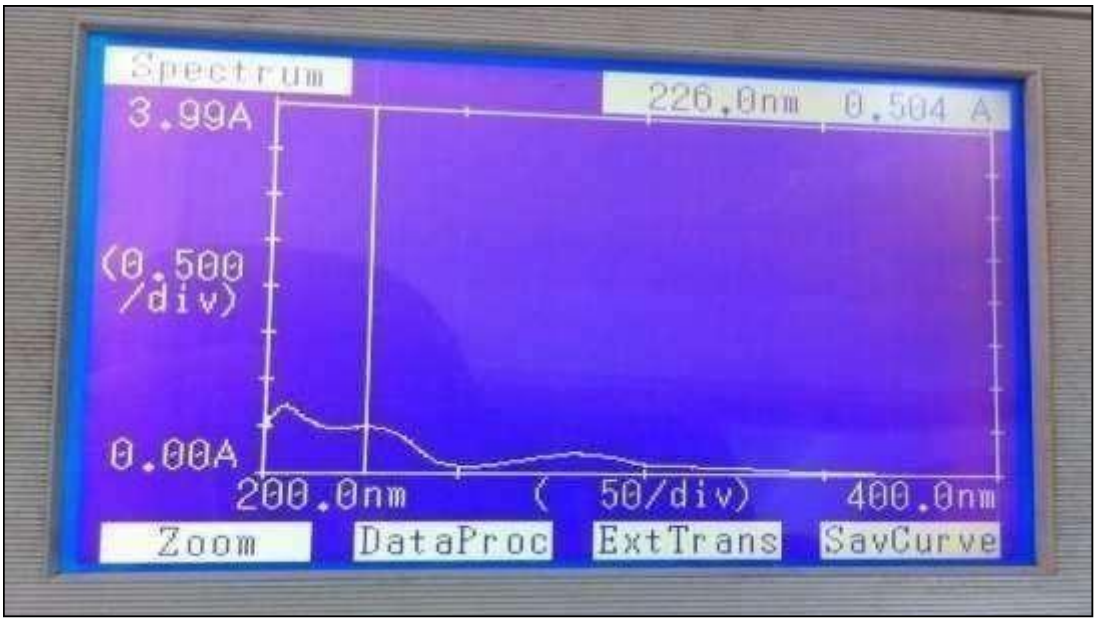

Figure 4.3: Absorption spectrum of Glabridin in ethanol

The λmax of the glabridin was found to be 226.0 nm in ethanol

4.1.5 Solubility of glabridin in water

In a 5.0 mL vial containing 2.0 mL water (distilled), an surplus amount of medication was added. The vial was placed in an orbital shaker at 251 °c for 72 hours after correct mixing with a cyclomixer. To attain symmetry, the sample was centrifuged at 3000 rpm for 15 minutes and filtered using a 0 .45µ membrane filter. The filtrate was diluted appropriately, and the absorbance was measured using a UV-Visible spectrophotometer at 226.0 nm. The saturation solubility of glabridin in distilled water was calculated and presented in table 4.3.

Table 4.3: Saturation solubility of Glabridin in Distilled water

Solvent system	**Solubility of Glabridin at 25±1^{0}C (µg/mL)**
Distilled water	3.80±0.0620

4.1.6 Analytical method Development by U.V. Spectrophotometry: A) Standard plot of Glabridin in ethanol

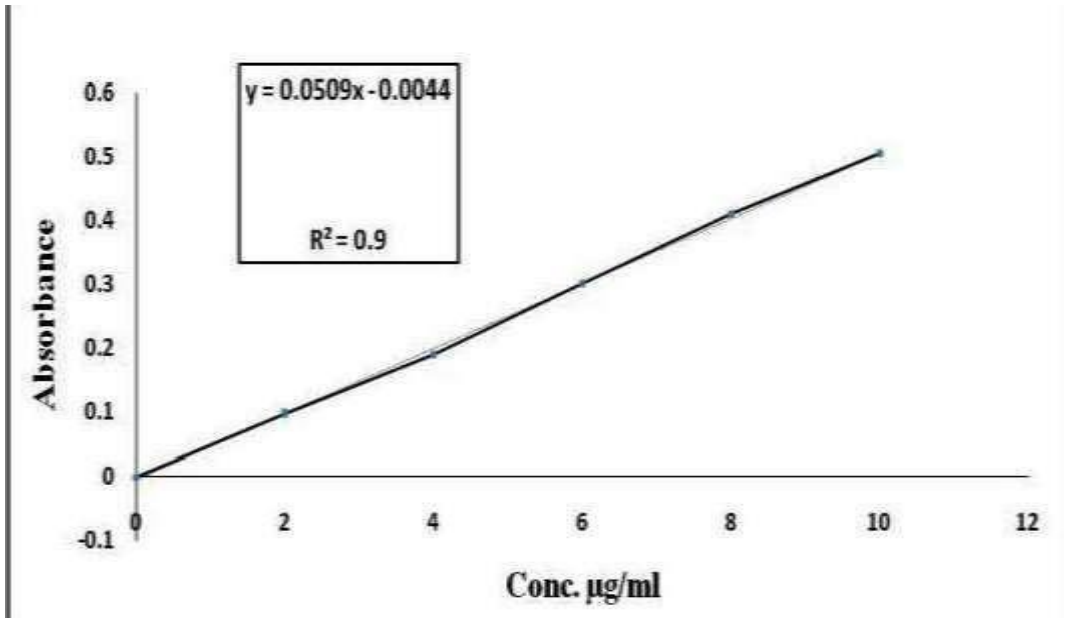

Figure 4.4: Standard plot of Glabridin in ethanol

The glabridin calibration curves in ethanol showed a good correlation of 0.9991. Over the full specified range of 2 – 10 µg/mL, the graph showed linearity.

Y= 0.0509x + 0.0044
R_2= 0.9991

Where, y= Absorbance

X= Concentration

R2= Correlation linearity coefficient

B) Absorption spectrum of Glabridin in ethanol: phosphate buffer (pH 6.8) mixture (1:1)

A 10mg glabridin solution in a phosphate buffer: ethanol combination (1:1) pH 6.8 was scanned in the 200-400 nm region. The drug's UV scan is illustrated in Figure 4.5. In phosphate buffer: ethanol, the drug's -max was measured at 226.0 nm.

Figure 4.5: Absorption spectrum of Glabridin in ethanol: phosphate buffer (pH 6.8) mixture (1:1)

File Name	**Glabridin**
Model	**Shimadzu UV- 1700**
Band width	**2.0 nm**
Measurement Range	**400-200 nm**
Scanning Speed	**100 nm/min**
λ_{max}	**226.0 nm**

Standard plot of Glabridin in ethanol: phosphate buffer (pH 6.8) mixture (1:1)

On a Shimadzu UV-V 1700 spectrophotometer, a 10 mg/ml solution of Glabridin in Phosphate buffer:Ethanol (1:1) was scanned in the 200-400 nm range against Phosphate buffer: Ethanol (1:1) as a blank. As illustrated in Figure

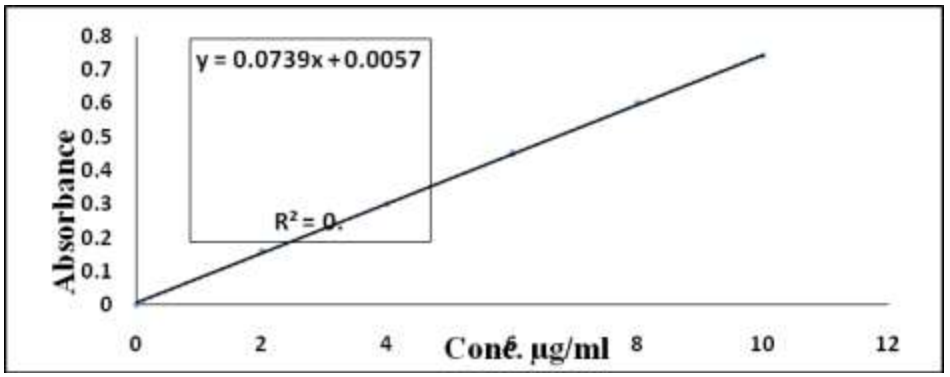

Figure 4.6: Standard plot of Glabridin in ethanol: phosphate buffer (pH 6.8) mixture (1:1)

Y= 0.0739x + 0.0057
R^2= 0.9997

Glabridin calibration curves in ethanol: phosphate buffer (pH 6.8) (1:1) revealed a 0.9997 correlation. Over the chosen range of 2 – 8 µg / mL, the curve revealed linearity. Glabridin is almost water insoluble. As a result, a polar oragnic solvent such as ethyl alcohol was combined with the phosphate buffer.

4.2 Characterization of Excipients:

4.2.1 Physicochemical characterization of Phospholipon 90G

Table 4.4 summarises the findings.

Table 4.4: Physicochemical characterization of Phospholipon 90G

Tests	**Specifications**	**Results**
Description	Yellowish waxy solid	Complied
Solubility	Soluble in ethanol, methanol and chloroform	Complied
LOD	Not more than 1.5 %	1.5%
Acid value	Not more than 0.5	0.5%

I.R. SpectrumofPhospholipon 90G

The I.R Spectrum is depicted in the diagram below. The results are listed in Table 4.5 below.

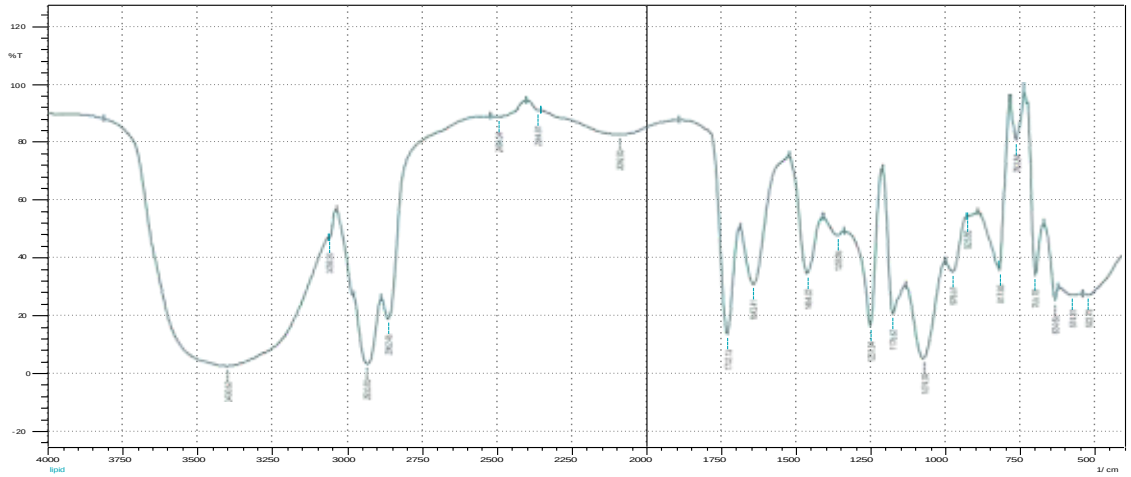

Figure 4.7: IR spectrum of Phospholipon 90G

Table 4.5: Results of IR interpretationofPhospholipon 90G

Sr. no	**Bond**	**Frequency range, cm^{-1}**	**Conclusion**
1	O-H	3400	Strong stretch
2	CH_3-CH_2	2933-2862	Symmetric stretch
3	C-O-C=O	1732	Strong stretch
4	CH_3-CH_2	2933-2862	Asymmetric stretch

IR spectrum of Phospholipon 90G is in within the specifications

4.2.2 Physicochemical characterization of Spans-80 (Sorbitanmonooleate)

The results are listed in Table 4.6 below

Table 4.6: Physicochemical characterization of Spans-80

Tests	Specifications	Results
Description	Amber colored oily viscous liquid	Complied
Solubility	Soluble in ethanol,ether, ethyl acetate, aniline,toluene, dioxane,petroleum ether	Complied
Density	0.950 - 0.986 g/ mL at 25 °C (lit.)	0.986 g / mL at 25°c
HLB value	Not more than 4.3	4.3

I.R. Spectrum of Spans-80

The I.R Spectrum can be seen in the diagram below. Table 4.7 summarizes the results.

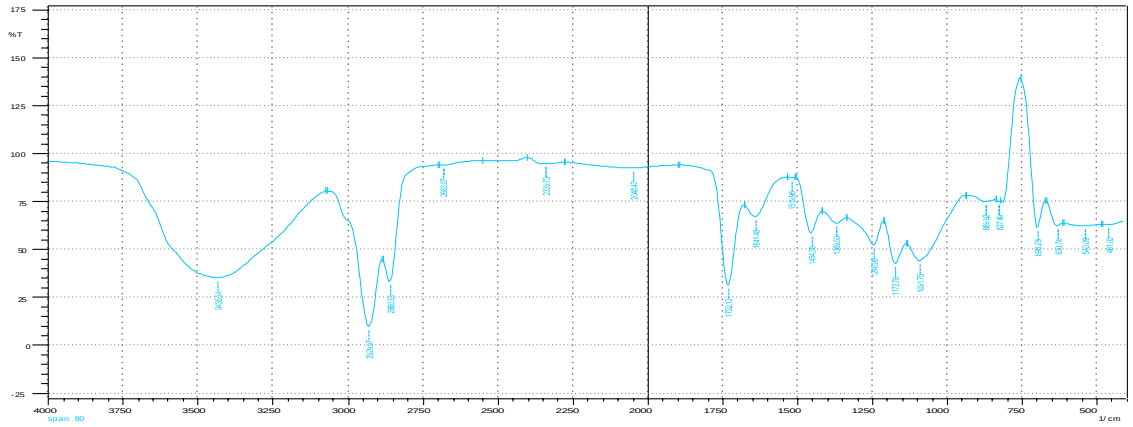

Figure 4.8: IR spectrum of Span-80

Table 4.7: Results of IR interpretation of Span-80

Sr. no	Bond	Frequency range, cm^{-1}	Conclusion
1	O-H	3455	Strong stretch
2	C-O	1091-1172	Medium stretch
3	C-O-C=O	1732	Strong stretch

IR spectrum of span-80 is in agreement with the specifications

4.2.3 Physicochemical characterization of Tween 80 (polyoxyethylene)

Table 4.8 summarises the findings.

Table 4.8: Physicochemical characterization of Tween 80

Tests	Specifications	Results
Description	Amber-coloured oily viscous liquid	Complied
Solubility	Very soluble in water soluble in ethanol and cottonseed oil	Complied
HLB value	Not more than 15.0	15.0

I.R. Spectrum of Tween 80

The I.R Spectrum is as shown below. The results are tabulated in Table 4.9

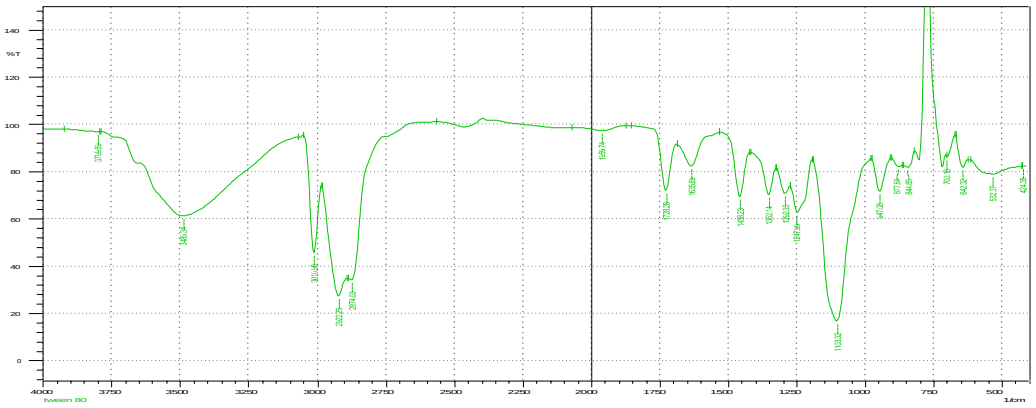

Figure 4.9:IR spectrum of Tween-80

Table 4.9: Results of IR interpretationofTween-80

Sr. no	Bond	Frequency range, cm^{-1}	Conclusion
1	C-O	1103	Strong stretch
2	C-O-C=O	1728	Medium stretch
3	CH_3-CH_2	2874-2922	Strong stretch
4	O-H	3489	Strong stretch

IR spectrum of Tween-80 is within the specifications.

4.2.4 Physicochemical characterization of Sodium deoxycholate (SDC)

The results are given below in Table 4.10

Table 4.10: Physicochemical characterization of Sodium deoxycholate

Tests	Specifications	Results
Description	White powder	Complied
Solubility	Solubility	Very soluble in water ,soluble in ethanol and cottonseed oil
Viscosity	Viscosity	400–500 centistokes at 25°C
Density	Density	1.08-1.09 g/mL at 25°c
HLB value	16.7	Not more than 18

I.R. Spectrumof Sodium deoxycholate

The I.R Spectrum is as shown below. The results are tabulated in Table 4.11

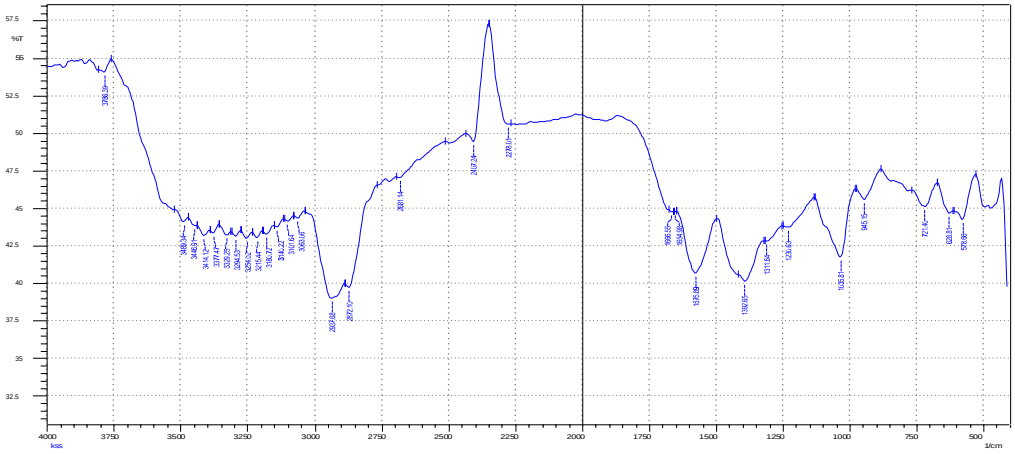

Figure4.10: IR spectrum of SDC

Table 4.11: Results of IR interpretation of Sodium deoxycholate

Sr. no	Bond	Frequency range, cm^{-1}	Conclusion
1	C-O	1035	Strong stretch
2	C-O-C=O	1666	Medium stretch
3	CH_3-CH_2	2872-2937	Strong stretch
4	O-H	3294-3329	Medium stretch

IR spectrum of SDC are in agreement with the specifications

4.2.5 Physicochemical characterization of Carbopol-934P

The results are tabulated in Table 4.12

Table 4.12: Physicochemical characterization of Carbopol-934P

Tests	Specifications	Results
Description	White, fluffy powder, hygroscopic	Complied
Solubility	Swells in water as well as in other polarsolvents after dispersion, Neutralization with sodium hydroxide solution.	Complied
Apparent nominal viscosity	The apparent viscosity of a product with a nominal apparent viscosity of 20 000 mPa•s or above is 70.0 to 130.0 percent of the value specified on the label.	112% of the value stated onthe label
Heavy metals	Not more than 20 ppm	11 ppm

I.R. Spectrum of Carbopol-934P

The I.R Spectrum is as shown below. The results are tabulated in Table 4.13

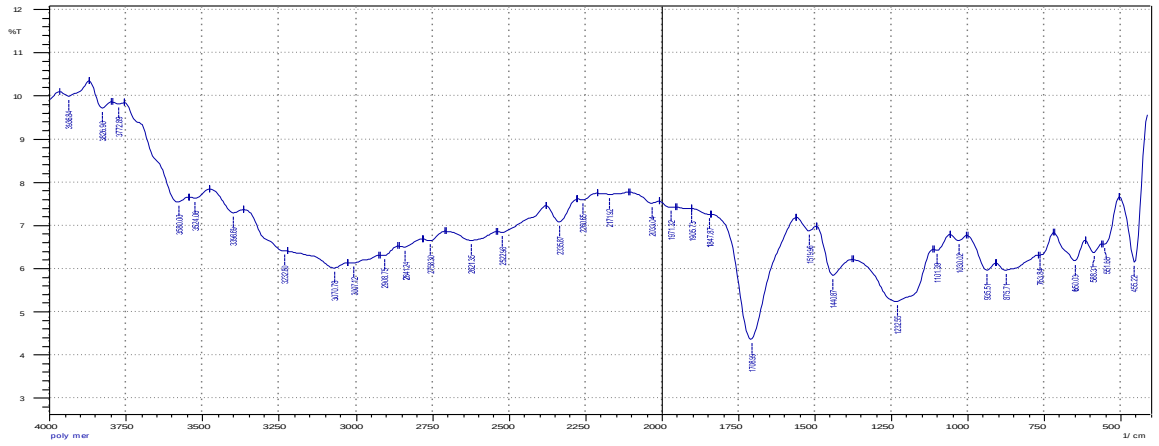

Figure 4.11:IR spectrum of Carbopol-934P

Table 4.13: Results of IR interpretationofCarbopol-934P

Sr. no	Bond	Frequency range, cm^{-1}	Conclusion
1	C-O	1232	Medium stretch
2	HO-C=O	1708	Strong stretch
3	CH_2-CH_3	2841-2908	Medium stretch
4	O-H	3070	Medium stretch

IR spectrum of Carbopol 934P is in agreement with the specifications.

All excipients were prepared in accordance with I.P. and U.S.P. specifications. To ensure the quality of the final product, all of the raw materials obtained were analysed before the experimental work began. The analysis reports that were kept revealed that the parameters were met (Tables 4.1-4.13). The work would be more reliable and reproducible if the raw materials were of higher grade.

4.3 Pre-Formulation Studies:

Drug-excipient compatibility tests are an important aspect of any preformulation study since even minor incompatibilities might result in the drug's or excipient's performance or degradation, lowering the dose form below the therapeutic level. Some incompatibility reactions produce obvious results, while others do not. As a result, using tools like FTIR spectrophotometry and DSC analysis, the compatibility of glabridin with excipients claimed to be contained in the Transfersome was investigated.

Figure 4.12 depicts a recorded FTIR spectrum that contains a number of absorption peaks, some narrow and others broad, with changing intensities throughout the spectrum (4000-500 cm-1). The presence or absence of certain group frequencies is frequently used to identify an unknown chemical molecule by looking at a specific region of the spectrum

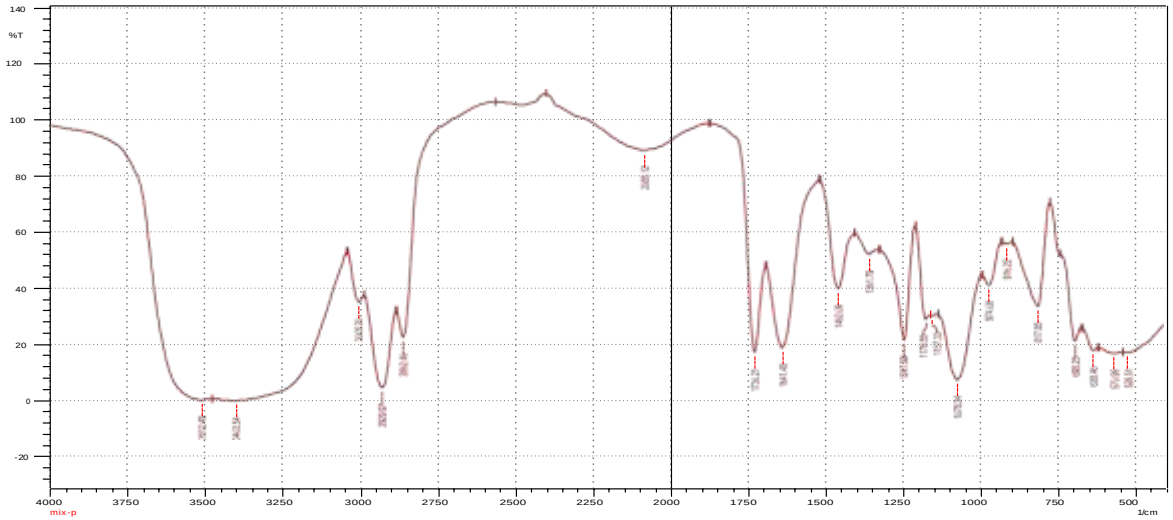

Figure 4.12: FTIR spectra of Glabridin- excipient mixture

Some absorption peaks clearly demonstrate the presence of specific groups that aid in the identification of a compound, thus when reading and interpreting spectra, keep in mind that no attempt should be made to assign for all peaks; instead, only the most essential distinctive spectra should be evaluated.

The physical mixture of glabridin and excipients was discovered to include the distinctive peaks representing respective functional groups for all of the constituents.

In the above spectrum (Figure 4.12), no new peaks were identified, indicating that Glabridin and other excipients are not incompatible.

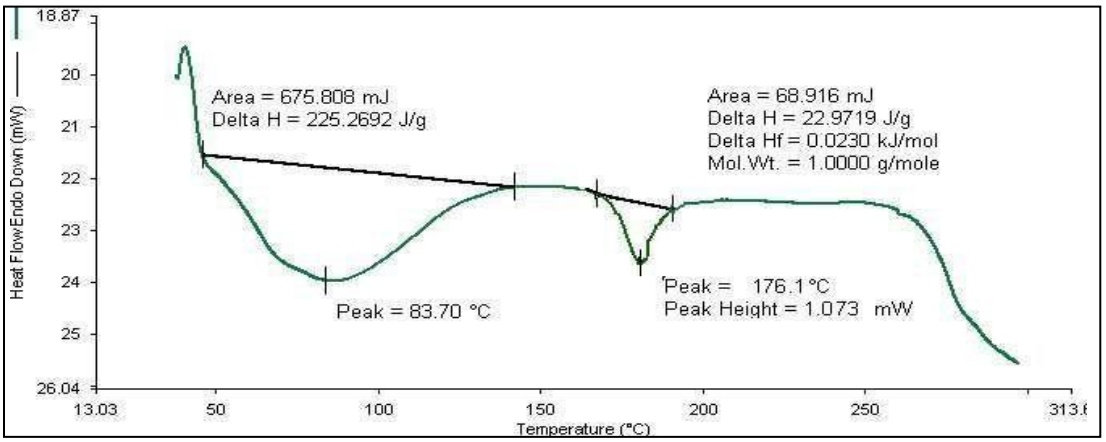

Figure 4.13. DSC thermogram of the formulation

The DSC thermogram of the formulation displays two endothermic peaks, as shown in Figure 4.13. Glabridin may be in the vesicular form in formulation, as endothermic peak was absent for melting. Additional effects were not found, leading to the conclude that the compatibility was in between the medicine and excipients.

Selection of Phospholipids

The solubility of the medication in lipid influences the use of phospholipids for transfersome formulation. For this reason, lipids like Phospholipid plain, Phospholipon 90 H, and Phospholipon 90 G were utilised to examine the solubility of glabridin. Phospholipids (PH) were chosen based on the greatest solubility of glabridin in lipids as well as the melting point of the lipids. The solubility of glabridin in various lipids is shown in Figure 4.14.

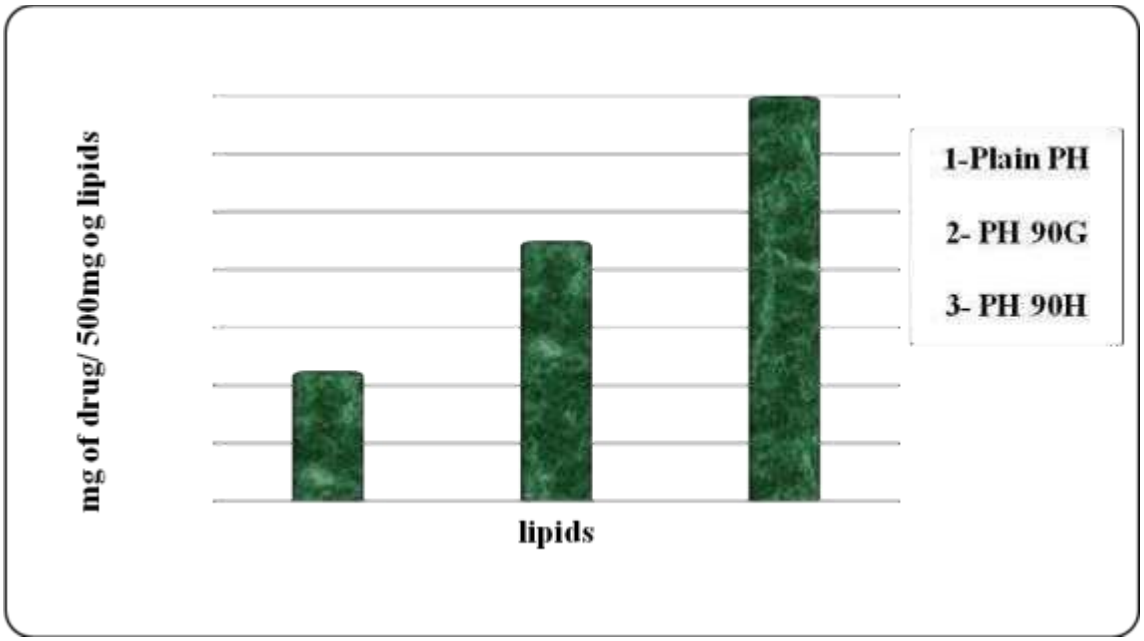

Figure 4.14. Solubility of Glabridin in different lipids

The solubility investigation revealed that glabridin was soluble in all of the phospholipids tested. Phospholipon 90G was chosen as the lipid phase for this study due to the maximum solubility of glabridin in this phospholipid (table 4.14) Phospholipon 90G was used as the lipid phase in the transfersomes.

Table 4.14: Solubility of Glabridin in different Phospholipids

1	Plain phospholipid	30± 1.1
2	Phospholipon 90H	90± 1.58
3	Phospholipon 90G	140± 1.35

4.4 Preparation of Transfersomes:

4.4.1 Pre-optimization investigation (Optimization of process variables)

The effect of numerous process variables like ultra-sonication time, hydration time, and vesicle size on % entrapment efficiency and vesicle size of subsequent transfersomal formulations were examined during the formulation of glabridin loaded transfersomes. For the pre-optimization experiment, fourteen formulations were produced and evaluated. Process factors were shown to have a considerable impact on entrapment efficiency and

vesicular size. Effect of various process variables on evaluation outcomes are shown in table 4.15.

For all batches, the lipid: surfactant ratio was 80:20, the solvent (ethanol: chloroform) was 20.0 mL, and the hydration medium (Phosphate buffer pH 6.8) was 20.0 mL.

Table 4.15: Optimization of Process Variables for Glabridin TFs.

F. no.	Hydration time (min)	Sonication time (min)	Vesicle size (nm)	Entrapment Efficiency (%)
T1	60.0	15.0	250.6	71.5±0.2
T2	60.0	20.0	245.2	71.6±0.8
T3	60.0	25.0	214.5	70.7±1.1
T4	60.0	30.0	214.5	68.1±0.5
T5	120.0	15.0	240.0	73.0±0.3
T6	120.0	20.0	238.5	69.7±1.6
T7	120.0	25.0	235.6	71.1±1.2
T8	120.0	30.0	236.2	70.3±1.8
T9	180.0	15.0	190.3	75.9±0.9
T10	180.0	20.0	190.3	68.3±0.3
T11	180.0	25.0	181.5	70.6±0.5
T12	180.0	30.0	183.6	69.8±0.4
T13	10.0	25.0	253.1	36.7±1.2
T14	10.0	30.0	287.4	32.0±1.8

A) Effect of Hydration time

t 37.00.5°C, the resulting dispersion was hydrated for 10, 60, 120, and 180 minutes. With increased hydration time, the vesicle size shrank. We learned from this research that hydration time has a significant impact on vesicle formation. Entrapment efficiency was only 36.7 percent at least 10 minutes after hydration. The entrapment efficiency was observed to rise as the hydration period was increased. Based on the foregoing findings and visual observation of Transfersomes, a hydration time of 180 minutes was chosen.

Based on the findings of the preliminary study, transfersome were created utilising span 80, tween 80, and SDCas surfactants, as well as Phospholipon 90G in various ratios and hydration duration of 180 minutes for the optimization of formulation factors.

B) Effect of Sonication time

After the dispersion was hydrated, an emulsion was created, which was sonicated for 15, 20, 25, and 30 minutes with a probe sonicator (PCI analytics 250F). With increasing sonication time, the % entrapment efficiency rose. Higher sonication time (30 minutes) resulted in vesicle agglomeration and increased vesicle size (250.6 nm) as well as percent entrapment efficiency (75.9%), whereas lower sonication time (15 minutes) resulted in vesicle agglomeration and decreased vesicle size (181.5 nm) and percent entrapment efficiency (75.9%). (68.1 percent).

However, when the sonication period was increased to 25 minutes, the entrapment efficiency increased to 71.1 percent and the vesicular size decreased (181.5 nm). Based on the above findings and a visual inspection of the product, a sonication time of 25 minutes was chosen.

4.4.2 Formulation Development

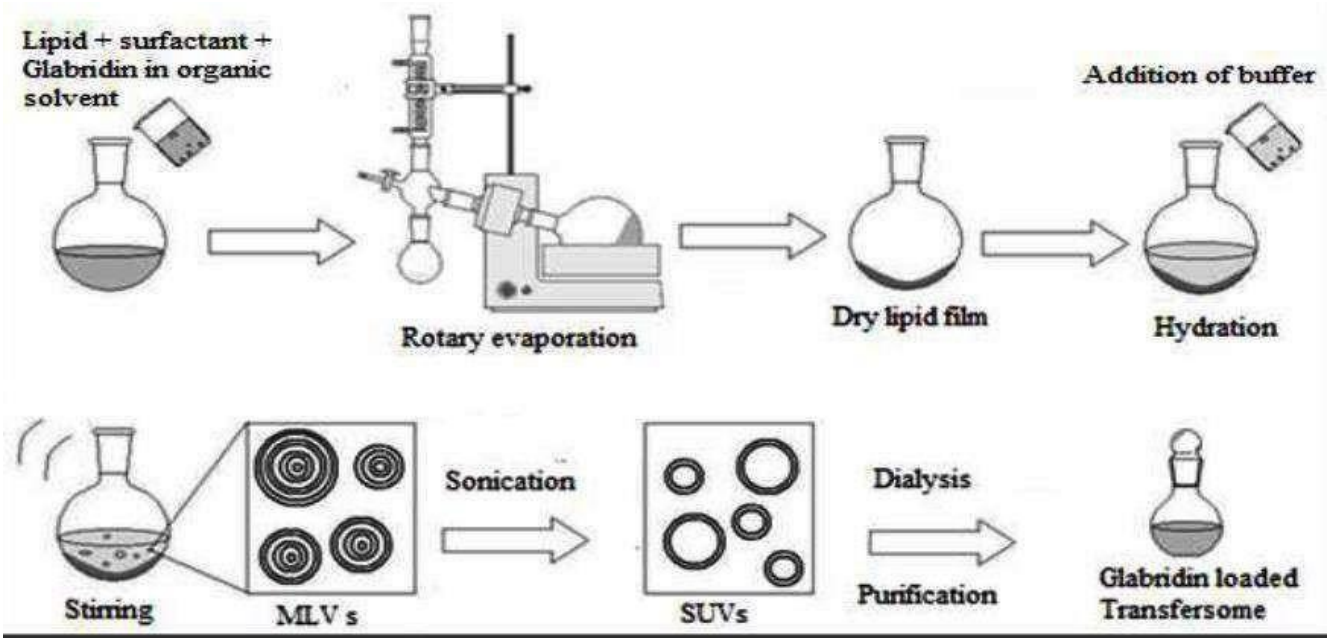

Figure 4.15: Preparation of transfersome by film hydration method

In order to make glabridin-loaded transfersomes, Transfersome was generated by hydrating the clear lipid film at 372℃ in the film hydration procedure (fig 4.15). In most cases, it's made up of lipid and surfactant, with an organic solvent that contains glabridin in the lipid phase. The film was hydrated for 3 hours while being stirred. The resultant dispersion was sonicated for 25 minutes using probe sonication. After sonication, dialysis was used to purify the water. The volume ratios of the lipid and surfactant were 90:10, 80:20, and 70:30, respectively. Because transfersome composition is such an important aspect in their manufacture, a variety of lipids and surfactants were tested.

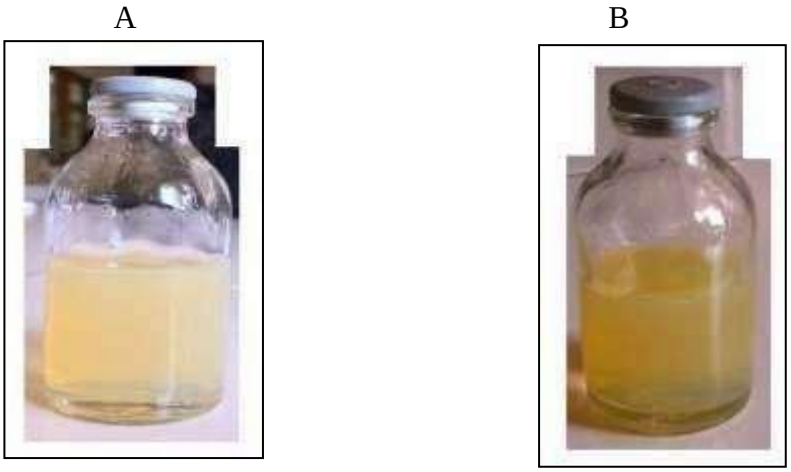

Figure 4.16: A) Formulation of empty Transfersome & B) Glabridin loaded Transfersomes

4.5 Evaluation:

A) Design of Experiment

A standard factorial design was used in this study to optimize the formulation variables of glabridin loaded transfersome. Design Expert® was used to examine the main, interaction, and comprehend the influence of multiple independent variables (X1&X2) using a two-factor model, as well as their interaction on dependent variables, 3-level General factorial design (Version 7.0 Stat-Ease Inc. Minneapolis, MN)

. There were two independent variables and three levels of each element in the design. At three levels, the first variable (X1) was treated as a numerical factor (-1, 0, 1). (X1) was the lipid: surfactant ratio, which had values of 70:30, 80:20, and 90:10. At three distinct levels, the second variable (X2) was considered a category factor (-1, 0, 1). (X2) was a surfactant with span-80, tween-80, and sodium deoxycholate levels (SDC). The description of all variables is shown in Table 4.16. Glabridin- loaded transfersomes (9 batches) were made and tested for entrapment efficiency (Y1), vesicle size (Y2), and other factors (Y2). The data for evaluating glabridin loaded transfersomes is shown in Table 4.18.

Table 4.16.Description of Independent variables.

Batch No.	Code value	
	X_1 **(Ratio of Lipid: Surfactant)**	**X_2** **(Type of surfactant)**
TF1	-1 **(70:30)**	-1 **(Span-80)**
TF2	-1 **(70:30)**	0 **(Tween-80)**
TF3	-1 **(70:30)**	1**(SDC)**
TF4	0 **(80:20)**	-1 **(Span-80)**
TF5	0 **(80:20)**	0 **(Tween-80)**
TF6	0 **(80:20)**	1 **(SDC)**
TF7	1 **(90:10)**	-1 **(Span-80)**
TF8	1 **(90:10)**	0 **(Tween-80)**
TF9	1 **(90:10)**	1**(SDC)**

Table 4.17: Measurement of Dependent Variables of the Glabridin TFs

Batch No.	Code value		E.E (%)	Vesicle size (nm)
	X_1	**X_2**	**Y_1**	**Y_2**
TF1	-1	-1	87.82±0.30	214.00
TF2	-1	0	79.65±0.56	189.24
TF3	-1	1	70.33±0.34	183.12
TF4	0	-1	92.24±0.25	130.60
TF5	0	0	82.06±0.20	129.32
TF6	0	1	76.41±0.52	110.00
TF7	1	-1	84.38±0.53	212.16
TF8	1	0	76.02±0.046	189.64
TF9	1	1	74.03±0.063	182.88

Mean ±SD (n=3)

Where, Table 4.17 shows the results of dependent variables such as percent entrapment efficiency (Y1) and vesicle size (Y2) from nine trials, which were used to create a polynomial equation using "Design Expert 7.0." to comprehend the impact of variables Design Expert Software's Statistical Analysis Traditional pharmaceutical formulation design is based on the time-consuming strategy of modifying one variable at a time, which ignores the combined influence of independent factors. As a result, factorial design can be a useful method for determining the complexity of pharmaceutical formulations. The outcomes can be stated as simple linear, second, or third order.

The responses were assessed using a statistical model that included interactive and polynomial variables.

$Y=b_0+b_1X_1+b_2X_2+b_1^2X_1X_2+b_{11}X1^2+b_{22}X_2^2$

Where Y is the dependent variable, b0 represents the arithmetic mean response of the nine runs, and b(b1, b2, b12, b11, and b22) represents the estimated coefficient for the corresponding factor Xi (X1, X2, X12, X11, and X22), this shows the average outcomes of altering one element at a time from a low to a high value.. When two factors are altered at the same time, the interaction term (X1X2) reflects the changes in the response. Nonlinearity is investigated using the polynomial terms (X12 and X22). Table 4.17 shows the responses of the formulations developed by 32 factorial design batche18 The data clearly shows that the values of entrapment and vesicle size are highly reliant on the independent variables chosen. In the equations, the fitted regression equations connecting the responses entrapment and vesicle size are shown. The equation provided the foundation for investigating the impacts of variables. The regression coefficient values represent the model fitting estimations. The r2 was high, showing that the quadratic model fit wells the amount of the co-efficient and the mathematical sign it conveys, i.e. positive or negative, can also be used to derive inferences using polynomial equations.

The Y1 answer had a positive coefficient, while the Y2 response had a negative coefficient. Variable X2 had a negative coefficient for both Y1 and Y2 responses.

Final Equation in Terms of Coded Factors: (E.E)

$E.E = + 82.49 - 0.56 * X_1 - 7.28 * X_2 + 1.78 * X_1 * X_2 - 4.87 * X1^2 + 1.63 * X2^2$

Final Equation in Terms of Actual Factors

E.E. =+ 82.49148-0.56000* Ratio of Lipid: Surfactant -7.27833* Type of Surfactant+1.78417 * Ratio of Lipid: Surfactant * Type of surfactant-4.86556* Ratio of Lipid: Surfactant^2+1.626 * Type of surfactant^2

Final Equation in Terms of Coded Factors: (vesicle size)

$\text{Vesicle size} = +121.49 - 0.28 * X_1 - 13.46 * X_2 + 0.40 * X_1 * X_2 + 71.87 * X_1^2 + 2.73 * X_2^2$

Final Equation in Terms of Actual Factors:

Vesicle size = +121.48889-0.28000* Ratio of Lipid: Surfactant -13.46000 * Type of surfactant+0.40000* Ratio of Lipid: Surfactant * Type of surfactant +71.86667 * Ratio of Lipid: Surfactant ^{2} +2.72667* Type of surfactant^2

B) ANOVA Study

The importance of evaluating and interpreting study findings is almost unquestionable, and the p-value plays a significant role in these outcomes. The ANOVA for the dependent variables Entrapment effectiveness and vesicle size is shown in Tables 4.18 and 4.19, respectively. The coefficients of X1 and X2 were determined to be significant at p0.05, indicating that both variables had a substantial effect on the selected replies. Overall, both variables influenced the responses in a considerable way. Stat-Ease Design Expert 7.0 was used to perform ANOVA and multiple regression analyses.

Table 4.18: Analysis of Variance for Entrapment efficiency

Source	Sum of squares	Degree of freedom	Mean square	F value	P value probe >F	Model significant/ Nonsignificant
Model	385.10	5	77.02	36.51	0.0069	significant
A-Ratio of Lipid: Surfactant	1.88	1	1.88	0.89	0.4146	
B-Type of surfactant	317.84	1	317.84	150.67	0.0012	
AB	12.73	1	12.73	6.04	0.0911	
A	47.35	1	47.35	22.44	0.0178	
B	5.29	1	5.29	2.51	0.2115	
Residual	6.33	3	2.11			
Cor Total	391.42	8				
R-Squared	0.9838					
Adj R-Squared	0.9569					
Pred R-Squared	0.8078					
Adeq Precision	17.410					

The 36.51 Model F-value indicated that the model was significant (fig. 4.17 a&b) Due to noise, there is only a 0.69 percent chance that a "Model F-Value" this large will occur. Model terms were significant if the "Prob> F" value was less than 0.0500. The 0.8078 "Pred R-Squared" was in reasonable agreement with the 0.9569 "Adj R-Squared." The signal-to-noise ratio was measured by "Adeq Precision." A ratio of more than four was taken into consideration. The ratio in this design was 17.410, indicating a sufficient signal

for entrapment efficiency. Variables facilitated the construction of a controlled release Transfersomal mechanism for glabridin, as evidenced by this.

Table 4.19: Analysis of Variance for vesicle size

Source	**Sum of squares**	**Degree of freedom**	**Mean square**	**F value**	**P value probe >F**	**Model significant/ Nonsignificant**
Model	11432.64	5	2286.53	40.68	0.0059	significant
A-Ratio of Lipid: Surfactant	0.47	1	0.47	8.369E-003	0.9329	
B-Type of surfactant	1087.03	1	1087.03	19.34	0.0218	
AB	0.64	1	0.64	0.011	0.9218	
A	10329.64	1	10329.64	183.77	0.0009	
B	14.87	1	14.87	0.26	0.6425	
Residual	168.63	3	56.21			
Cor Total	11601.28	8				
R-Squared	0.9855					
Adj R-Squared	0.9612					
Pred R-Squared	0.8793					
Adeq. Precision	16.249					

The Model F-value of 40.68 indicated that the model was statistically significant (fig. 4.18 a&b) Due to noise, there is only a 0.59 percent chance that a "Model F-Value" this large will occur.

Model terms are significant when "Prob> F" is less than 0.0500.

The 0.8793 "Pred R-Squared" is in reasonable agreement with the 0.9612 "Adj R-Squared." The signal-to-noise ratio was measured by "Adeq Precision." A ratio of more than four was taken into consideration. The ratio in this design was 16.249, indicating that the signal was adequate for the vesicle size. This revealed that certain variables supported the creation of a controlled release glabridin transfersomal mechanism

The response plots and counter plot in figure 4.17 show that the lipid: surfactant ratio and type of surfactant have a net influence on transfersome entrapment efficiency.

There was no significant change in E.E. at any level of lipid: surfactant concentration, even when lipid and surfactant concentrations were increased. However, E.E. changed as the type of surfactant changed. When the type of surfactant was adjusted from a lower to a higher level, the E.E of TFs decreased at all levels of surfactant type. The type of surfactant had a greater impact than the concentration of surfactant.

Surfactants of various types had varying E.E percents. TFs-Span 80 had the highest E.E percent (92.24%), followed by TFs-Tween 80 (82.06%), and TFs-SDC (82.06%). (76.30 percent). The results suggest that the drug distribution coefficient between the lipid phase and the aqueous solution aided the entrapment of lipophilic drugs into lipid vesicles. Span 80, Tween 80, and SDC have HLBs of 4.3, 15.0, and 16.7, respectively. Surfactant affinity for lipids reduced in sequence as a result of this. Glabridin would be more widespread dispersing in double-layer lipids formed by Span-80 and PC, based on surfactant affinity for lipids and the drug's strong lipophilic characteristic (logP 3.96). SDC is a non-profit organisation that the macrolide has a steroidal structure. Glabridin could be vying for a spot in the bi-layered vesicles. To some extent, this extinction of species resulted in low E.E.

C) Effect of lipid : surfactant ratio & type of surfactant on the entrapment Efficiency

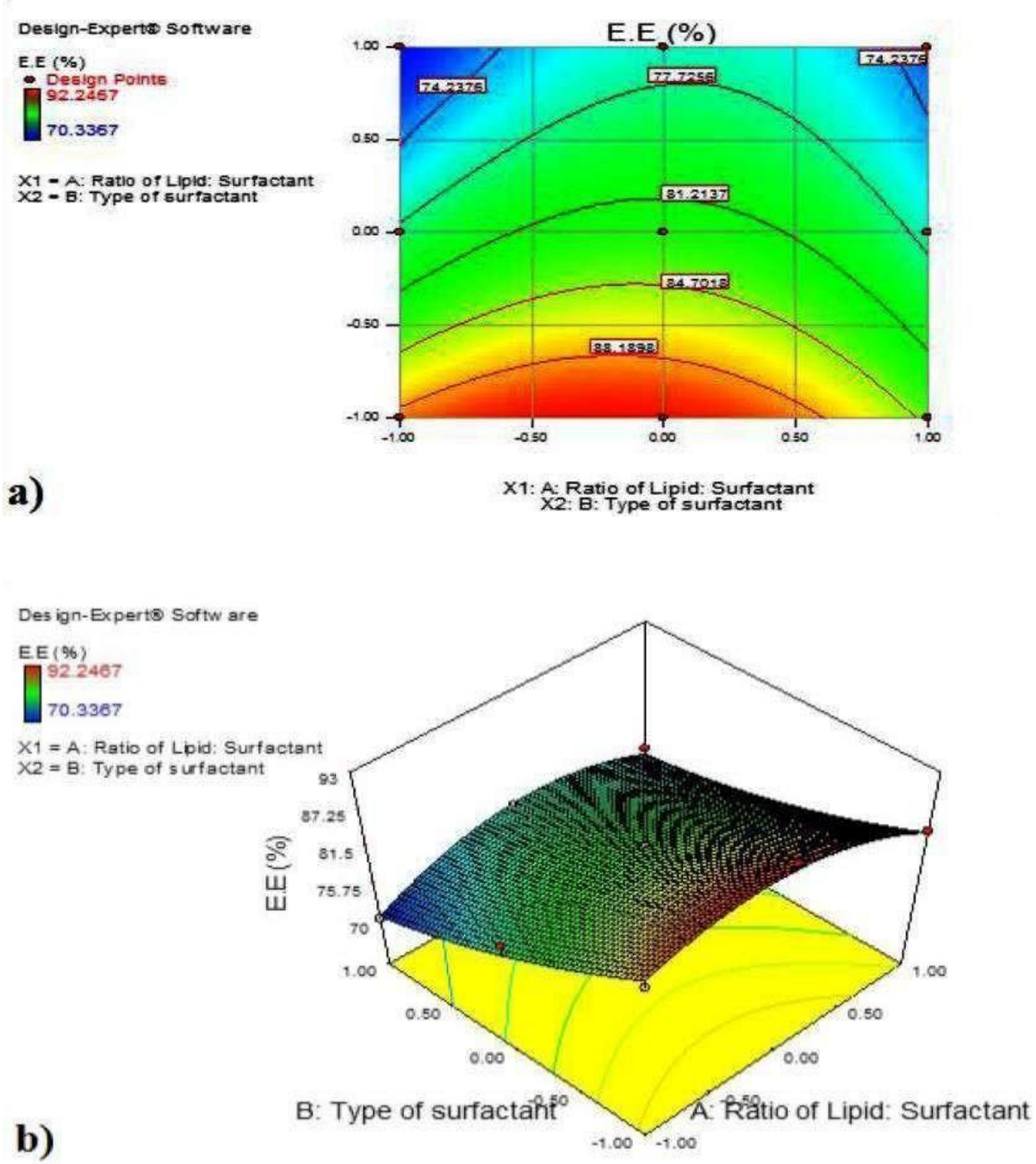

Figure 4.17: a) Contour plot b) Response surface showing

D) vesicle size - effect of lipid: surfactant ratio and type of surfactant

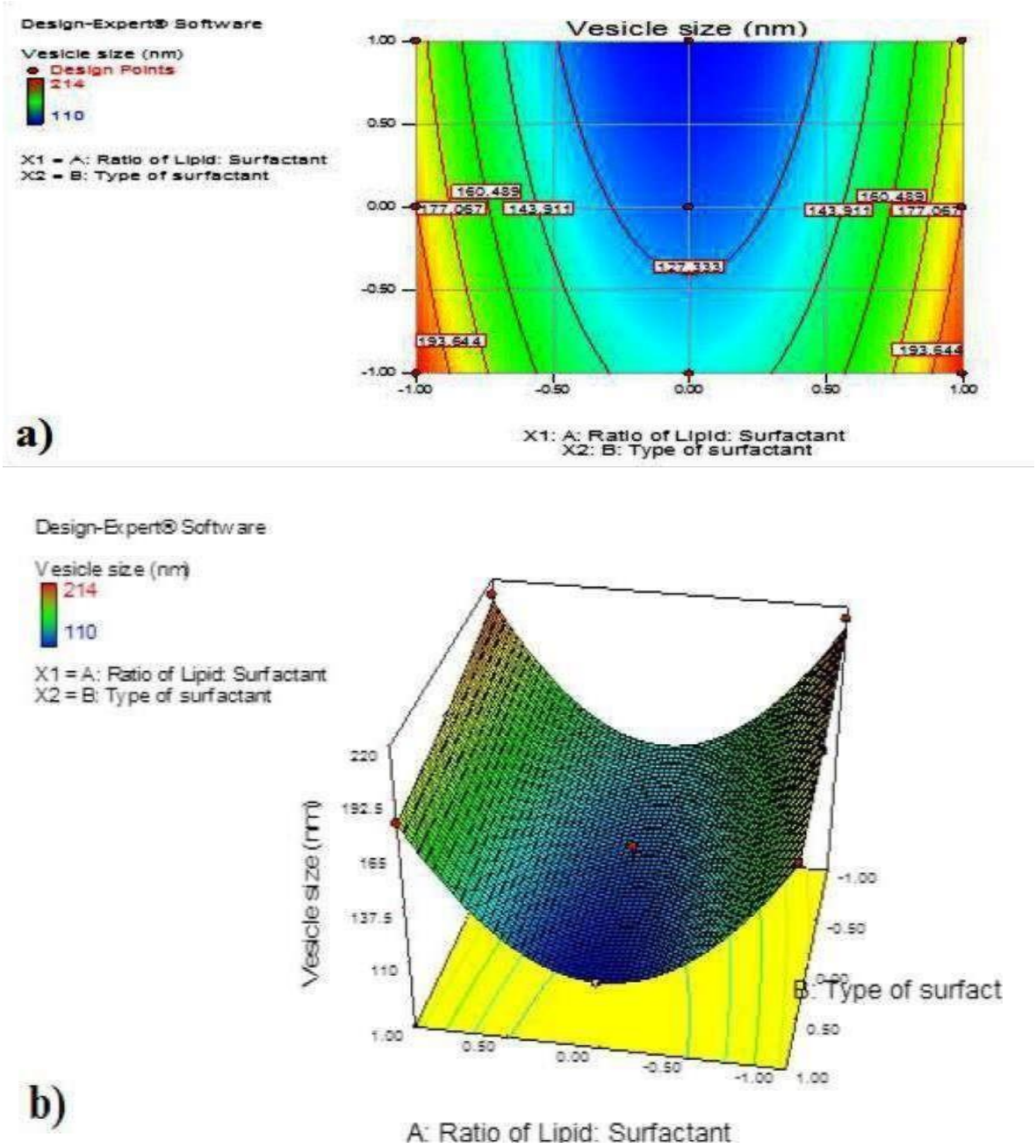

Figure 4.18 Effect of lipid: surfactant ratio and type of surfactant on the vesicle size a) Contour plot b) Response surface

The response plots and contour plots in figure4.18 show that the lipid: surfactant ratio and kind of surfactant have a combined effect on the size of transfersome vesicles. When

lipid concentration was increased at lower and middle levels of lipid: surfactant concentration, vesicle size shrank. However, vesicle size increased marginally as lipid concentration increased at greater levels. However, the kind of surfactant had a greater impact on vesicle size than the concentration, as the type of surfactant changed. Particle size differences between TFs containing different surfactants were considerable. SDC, Tween-80, and Span-80 have mean particle sizes of 110.0, 129.32, and 130.6 nm, respectively. Particle size increased in general when surfactants with reduced HLB were used.

E) Drug content determination:

All the glabridin loaded Transfersome formulation prepared was evaluated for drug content. The drug content found is given in Table. 4.20.

Table 4.20: Drug content of Glabridin loaded TFs formulation

F. code	Drug content (%)	F. code	Drug content (%)
TF1	95.36±0.36	TF6	90.36±0.88
TF2	94.21±0.68	TF7	93.35±1.48
TF3	93.21±1.10	TF8	93.97±0.92
TF4	94.36±1.89	TF9	91.25±0.23
TF5	90.15±0.45		

F) *In-vitro* drug release and diffusion studies

The Franz diffusion cell was used to investigate the in-vitro drug diffusion profile of a liquid transfersome formulation. The diffusion media was used a combination of ethanol and phosphate buffer pH 6.8. (1:1) the main issue with water insoluble medications is the lack of sink conditions. In the case of soluble chemicals, completely aqueous medium can

be used by adjusting the pH. Alternative tactics, on the other hand, must be tried for insoluble pharmacological compounds like glabridin. In such circumstances, a co-solvent that improves drug solubility in aqueous-based diffusion medium has been used to establish sink condition

The *in-vitro* drug release data showed in table 4.21 &*in-vitro* drug release profiles for the TFsbatches are represented in figure 4.19 to 4.21.

Table: 4.21.Cumulative percent drug release of Glabridin loaded liquid Transfersome

Batch code	Glabridin Diffusion (%)						
	30 min	**60 min**	**120 min**	**180 min**	**240 min**	**300 min**	**360 min**
TF1	17.7±1.4	24.9±0.2	34.6±0.5	52.2±1.1	55.1±1.5	71.1±0.9	82.4±0.8
TF2	13.9±1.4	20.5±1.1	32.7±0.4	40.8±1.2	48.0±1.5	59.19±1.06	77.0±1.4
TF3	22.0±0.6	25.1±0.4	34.9±0.7	42.8±1.8	47.9±0.9	52.9±0.6	61.3±0.2
TF4	21.9±1.3	28.6±1.1	38.2±0.2	46.4±1.6	56.2±0.9	68.3±1.2	74.2±1.6
TF5	17.1±0.9	23.7±1.4	32.2±0.6	37.7±1.5	41.0±1.2	51.9±0.8	59.4±1.7
TF6	16.6±0.9	24.2±0.9	39.6±1.8	53.1±0.9	62.1±0.9	74.4±0.4	78.3±0.5
TF7	17.3±1.2	23.2±1.8	32.6±0.4	49.0±1.4	57.2±0.9	62.6±0.8	67.4±1.1
TF8	19.5±0.6	31.8±0.3	40.8±1.9	47.6±1.7	61.7±1.7	73.2±1.3	87.2±1.8
TF9	25.2±1.0	32.4±1.4	45.1±1.7	52.2±1.5	67.8±1.5	73.2±1.0	82.8±0.3

Mean ±SD (n=3)

The drug diffusion profile of the TFs formulation demonstrates that there was little difference in drug diffusion across the various TFs formulations. Drug diffusion was seen in all formulations in a burst pattern for around 30 minutes and then continued for another 8 hours.

The drug released within the first 30 minutes was in the range of 13.901.4 to 25.201.0. The drug diffusion ranged from 59.41.7 to 87.201.8 after 6 hours of diffusion.

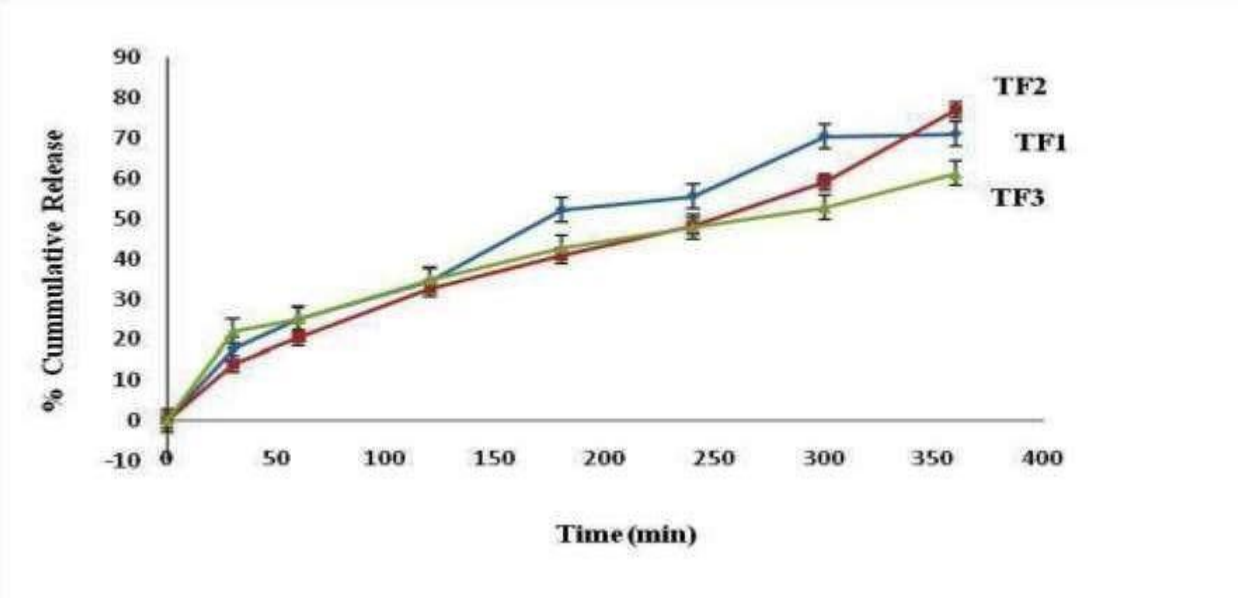

Figure 4.19: Drug release patterns for TFs formulation batches from TF1 to TF3

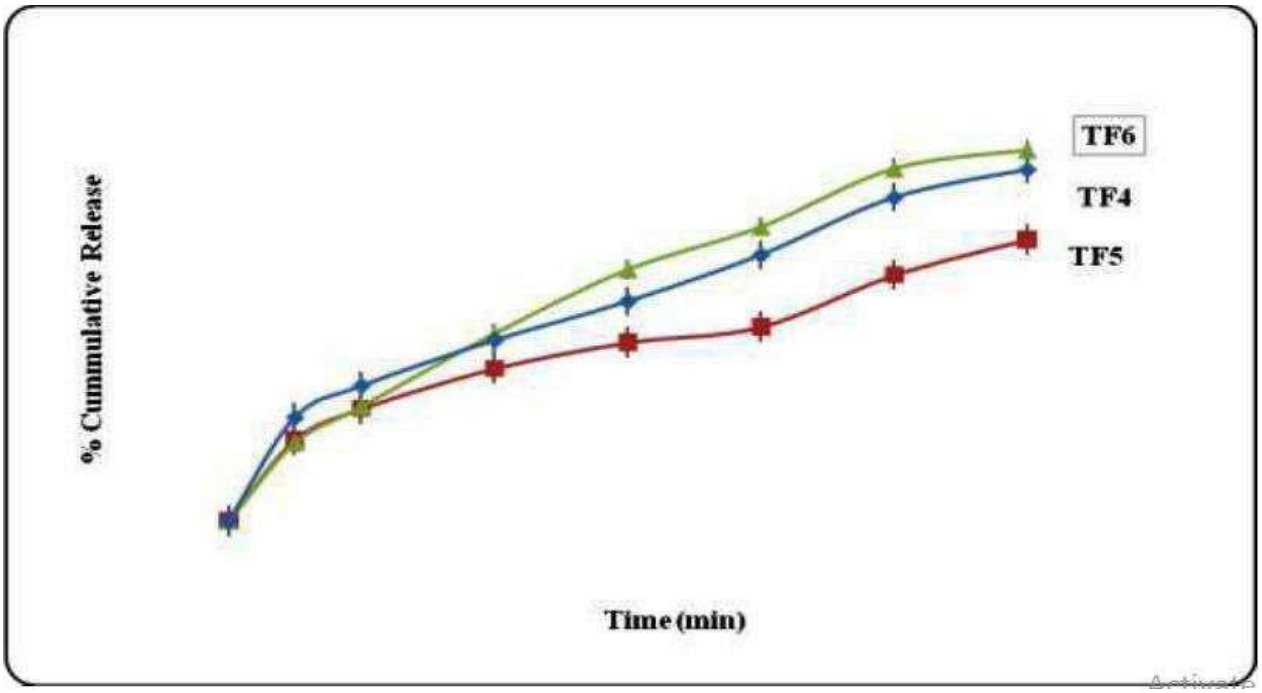

Figure 4.20: Drug release patterns for TFs formulation batches from TF4 to TF6

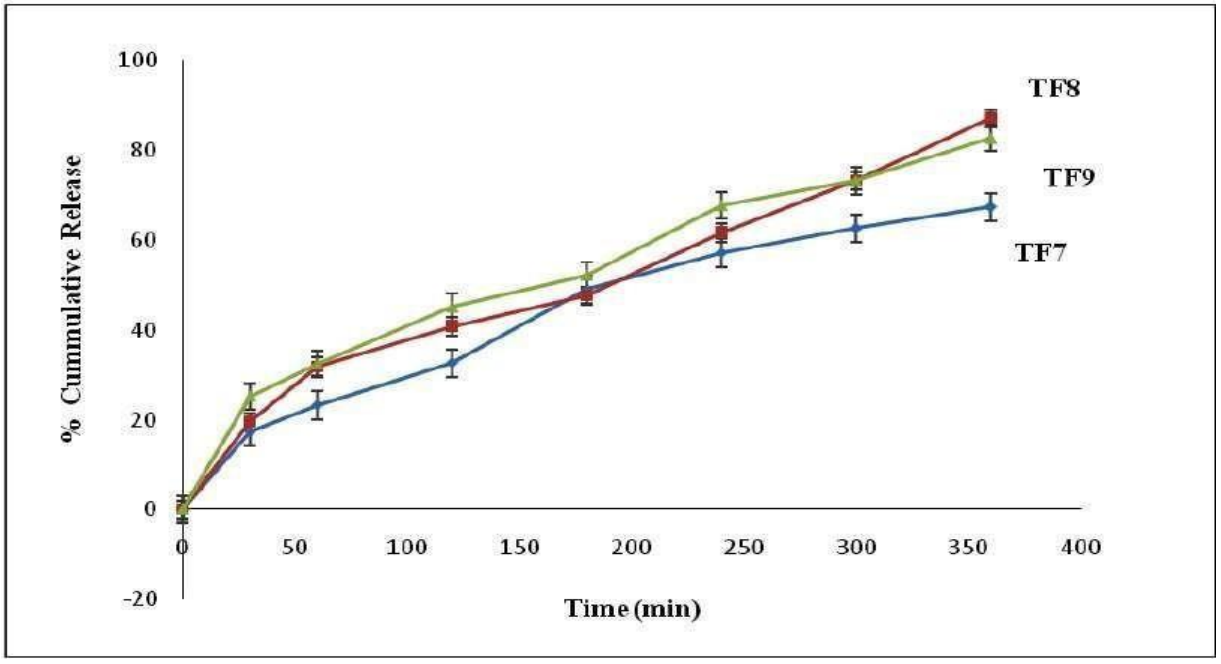

Figure 4.21: Drug release patterns for TFs formulation batches from TF8 to TF9

- When compared to the second-hour rate of drug release, the first hour had a larger drug release rate. This could be because the formulations contain free glabridin.
- In a pH 6.8 phosphate buffer, the total drug release increased from the TF1 to the TF9 formulation. The release rate was shown to increase as the concentration of surfactants increased.
- When span 80 was utilised as a surfactant, medication release increased as the lipid concentration increased. When SDC was utilised as a surfactant, however, drug release was shown to be reduced at the same time as lipid concentrations increased.
- When tween 80 was utilised as a surfactant, medication release was shown to be increased at high and low lipid levels, but decreased at the medium level.
- Because of the increased surface area, the higher rate of drug release was for the batch with smaller particle size.
- As a result, the concentration of fat and surfactants was discovered to alter the

release rate.

- According to the drug release profile, all of the batches had a sustained release rate, however the TF-4 batch had the best drug entrapment and vesicle size compared to the others.

- **4.6 Optimum Formulations:**

Physical stability, entrapment efficiency, vesicle size, and the formulation's drug release profile were the main criteria for selecting an optimum batch. In light of the findings, the formulation TF-4 was chosen as the best. Tables summarise the composition of optimised batches.4.22

Table 4.22: Composition of optimized formulation

Composition	**Batch code**
	TF-4
Glabridin	50.0 mg
Type of surfactant	Span-80
Lipid: surfactant ratio	80:20
Phosphate buffer (pH 6.8)	20.0 mL

4.7 Lyophilization:

Lyophilization is an industrial technique that involves sublimation and desorption under vacuum to remove water from a frozen material. For the morphological investigation of formulations, TEM freeze drying is necessary for characterization utilising SEM. Various strains are generated throughout the freezing and drying stages of this process. To protect the transfersome against freezing and dessication stressors, cyoprotectants such as Trehalose and sucrose were added to the formulation. The characteristics of lyophilized formulation are shown in Table 4.23.

Table 4.23 Lyophilized Transfersome

Batch code	**Description**
TF-4	Brownish dry powder, Odourless

4.8 Characterization:

4.8.1 Morphological analysis of Transfersome

A) Vesicle size determination:

The size of the vesicles in the formulations was determined using a nano particle analyzer (HORIBA sz-100). In deionized water, the transfersome was diluted before the tests. At room temperature, with the incident laser beam at a fixed angle of 90°, all measurements were made in triplicate. Data was analysed using Windows [z type-ver1.90], and mean vesicle size and vesicle size distribution curve values were calculated. Figure 4.22 and Table 4.24 show the results of the vesicle size analysis.

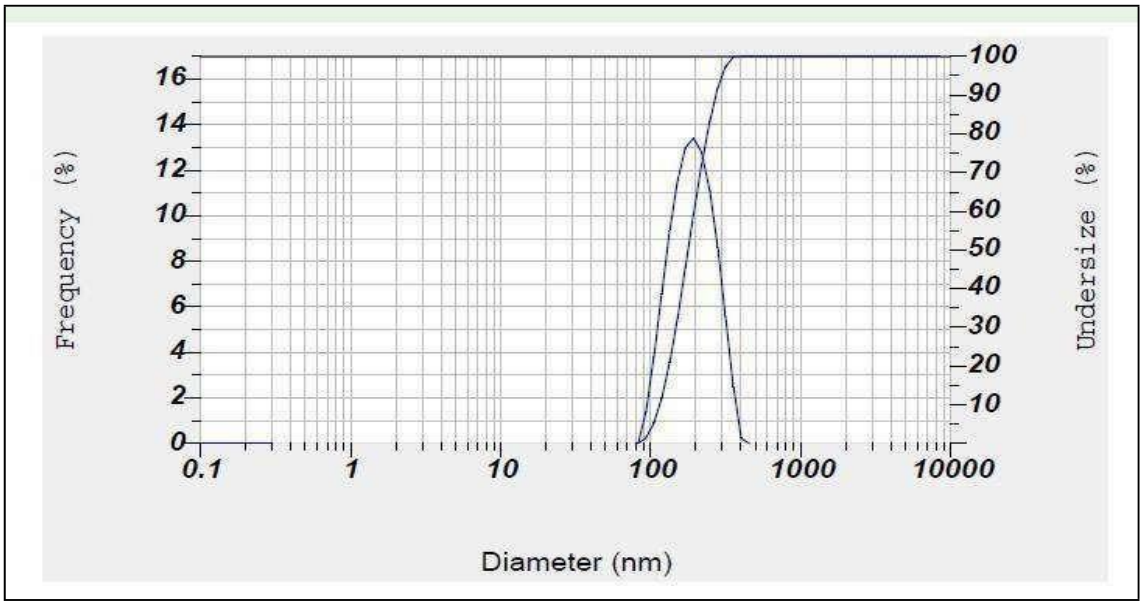

Figure 4.22: Particle size analysis of TF4 formulation

Table 4.24: Particle size analysis of TF4 formulation

Batch code	Vesicle size (nm)	Poly dispersity index (PDI)
TF4	130.60 nm	0.375

B) Zeta potential analysis

The Zetasizer (HORIBA sz-100) equipment was used to measure zeta potential. A 1.0 mL sample was obtained and dissolved in double distilled water. The dispersed solution was immersed in an ultra sonicator bath for 5.0 minutes to prevent agglomeration. The zeta potential of the sample was measured in a glass cuvette.The charge on the vesicles is represented by the zeta potential. Negative zeta potential values were discovered during zeta potential investigation (-25.1).It's possible that the zeta potential value was discovered to be sufficient to maintain the vesicles stable as presence of nonionic surfactants in the formulation. The data for the zeta potential is shown in graph 4.23.

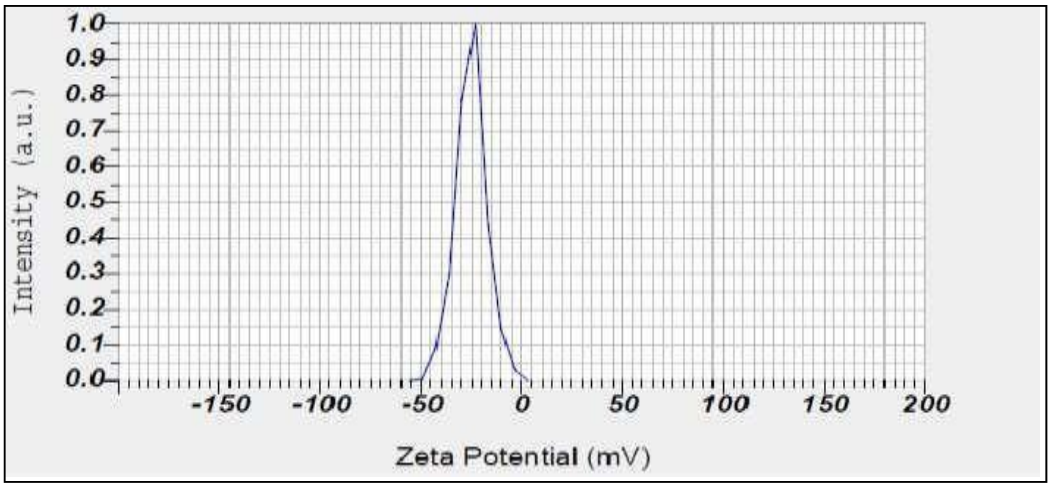

Figure 4.23: Zeta potential analysis of TF4 formulation

C) Scanning Electron microscope (SEM)

Scanning electron microscopy was used to examine the exterior morphology of lyophilized TFs (SEM). Powder was softly sprinkled on a double adhesive tape adhered to an aluminium stub to prepare the samples for SEM. To make the samples electrically conductive, they were coated with a 20 nm metal layer by an auto fine coater prior to estimate. The coated samples were then placed on stubs and placed in the scanning electron microscopy chamber. The samples were then scanned at random and photomicrographs were made at a 10 kV acceleration voltage, with the SEM results shown in figure 4.24.

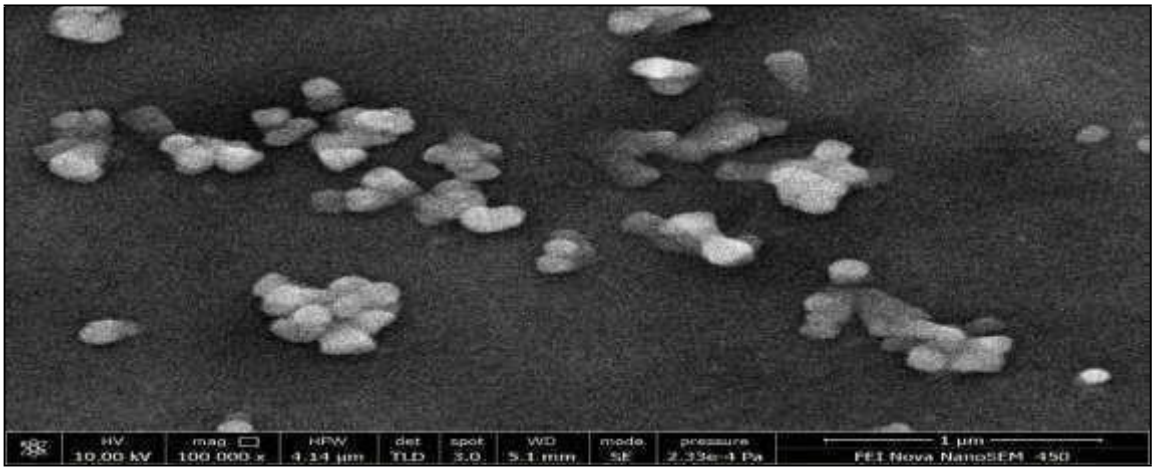

Figure 4.24: Scanning electron micrographs of Glabridin loaded TFs.

D) Transmission Electron Microscopy (TEM)

The transfersomal vesicle was seen using a TEM (H-7500, Japan). Negatively stained

magnification was achieved using a 100Kv accelerating voltage and 10% aq. Phototungustic acid solution after drying, the sample was examined under a microscope at a magnification of 10-100k, and the TEM results are shown in figure 4.25.

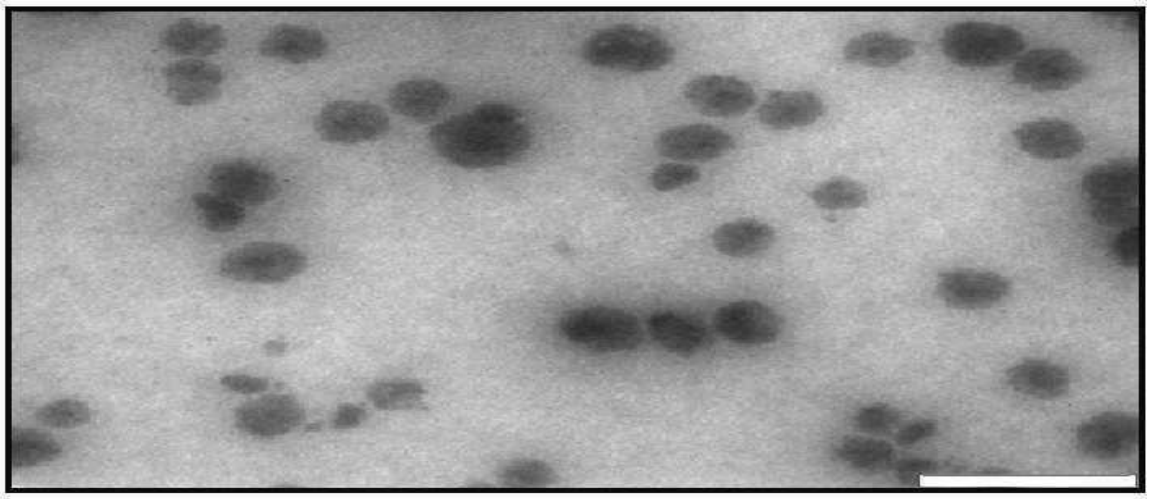

Figure 4.25: Transmission Electron Microscopy of Glabridin loaded TFs

4.8.2 Differential Scanning Calorimetry

The DSC thermogram of the formulation reveals endothermic peaks, as seen in Figure 4.26. Glabridin may be present as vesicle in the transfersomal formulation because there was no endothermic peak for melting.

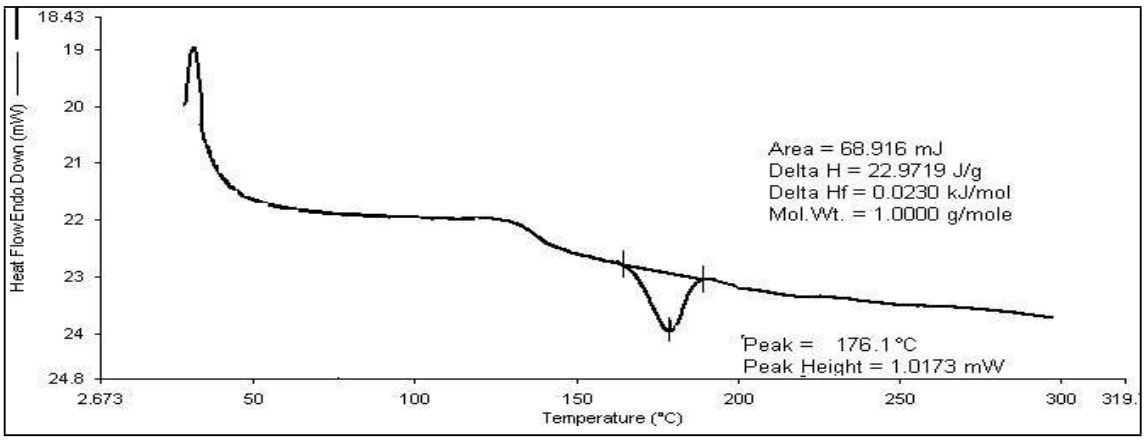

Figure 4.26: DSC thermogram of TFs formulation

Thus, it could be concluded that Glabridin was found to be compatible with all the excipients and stable.

4.8.4 I.R. Spectrum

Lyophilized Transfersome with the characteristic peaks representing respective functional

groups for all the ingredients were found to be present in the spectrum (Figure 4.27)

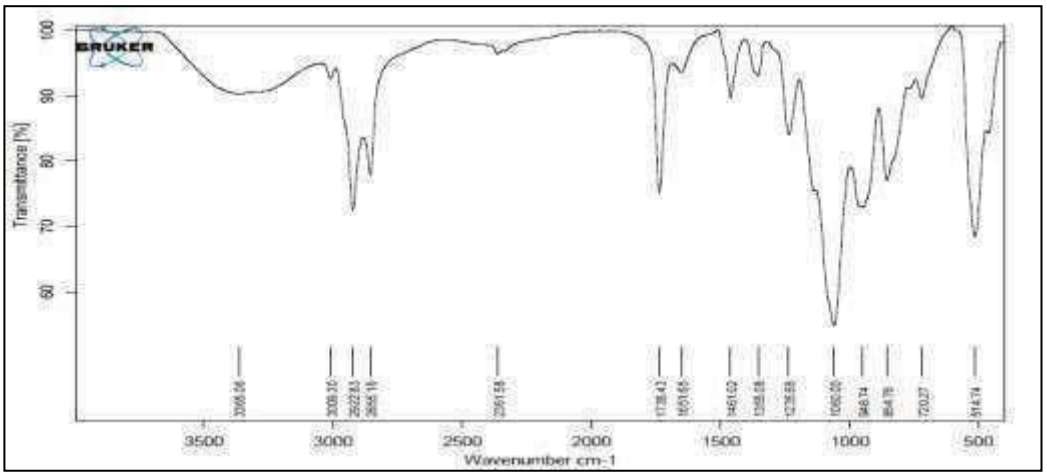

Figure 4.27: FTIR spectra of Transfersome formulation

No additional peak was found in the above spectrum (Figure 4.27) which indicates there is absence of any incompatibility between glabridin and other excipients.

4.9 Formulation of Gel Containing Glabridin Loaded Transfersome:

Because the transfersome was in liquid form, it was less appropriate for use as a topical to achieve therapeutic efficacy. As a result, the optimum batch of Glabridin loaded transfersome (TF-4) was determined to be taken ahead for topical formulation for a successful application. For the same, carbopol gel compositions were examined (TF-4). The transfersome was enhanced with the gelling agent Carbopol 934P at the appropriate concentrations [0.5 percent, (GT-1) 1 percent, (GT-2) and 1.5 percent (GT-3)]. As a result, each batch was exposed to a well-designed examination for several criteria.

Preparation of glabridin loaded transfersomal gel

The 50.0 mg of glabridintransfersomes were dispersed in 9.50 g of 1 percent carbopol plain gel to make the gel. The 0.5 percent transfersome-loaded gel was tested for the parameters listed below.

4.10 Evaluation of the gel formulation:

4.10.1 Product appearance

Unaided eyes were used to inspect the gels for clarity, lumps, and entrapped air bubbles. Table 4.25 shows that all of the batches were clear, with no lumps or air bubbles.

4.10.2 pH measurement

pH was measured with pH meter(Lab India). All the batches showed pH between 6.0 and 6.5 (Table 4.25).

4.10.3 Spreadability study

Spreadability tests were performed on all batches of gels with different Carbopol 934P concentrations. Table 4.25 & Figure 4.28 show the results. The gel with a concentration of 0.5 percent was very thin, resulting in uneven spreadability, whereas the gel with a concentration of 1.5 percent was very viscous and sticky, resulting in very limited spreadability. The batch with 1.0 percent Carbopol had adequate spreadability and was equally spread.

Table 4.25: Spreadability study of TFs gel

Batches	Transparency	Odour	Clarity	pH	Spreadability	
					(g.cm/sec)	cm
GT-1	Transparent	Odourless	Clear	6.0	11.250	6.4
GT-2	Transparent	Odourless	Clear	6.2	11.388	6.3
GT-3	Transparent	Odourless	Clear	6.1	14.575	5.5

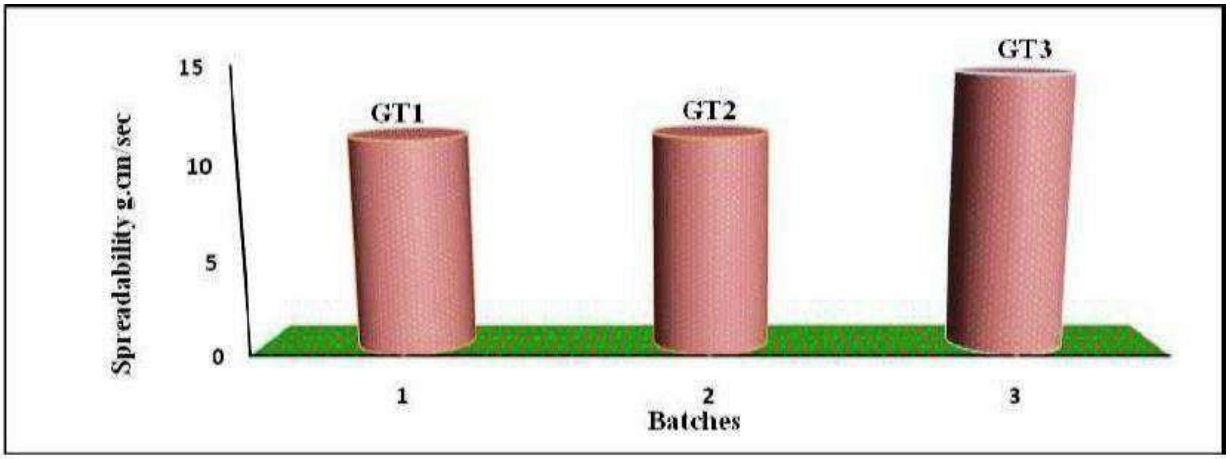

Figure 4.28: Spreadability study of TFs gel formulation

4.10.4 Rheological studies

Rheological studies were conducted for all the batches of gels of varying concentrations of Carbopol 934P. Results are tabulated in table 4.26 Type of equipment- Brookfield RVDV-II + Pro Spindle- T-bar, Spindle code- S95 , Sample volume- 50.0g , Rpm- 1, 2,5,10, 20, 50,100.

Table 4.26: Viscosity of formulation

RPM	GT-1	GT-2	GT-3
1	130,500	180,400	230,650
2	80,000	135,400	180,000
5	50,700	85,300	130,750
10	39,500	50,700	90,000
20	14,800	20,200	49,000
50	8,600	12,600	22,250
100	4,800	6,500	10,500

The results of viscosity measurements revealed that as the concentration of Carbopol 934P was raised, the viscosity values of formulations TF-4 increased marginally. It was also discovered that as the Carbopol concentration climbed from 0.5 percent to 1.5 percent, viscosity values increased.When the rate of shear (rpm) was raised in the rheological examination of the TF-4 gel, the viscosity dropped, indicating pseudoplastic flow. Figure 4.29 depicts this.

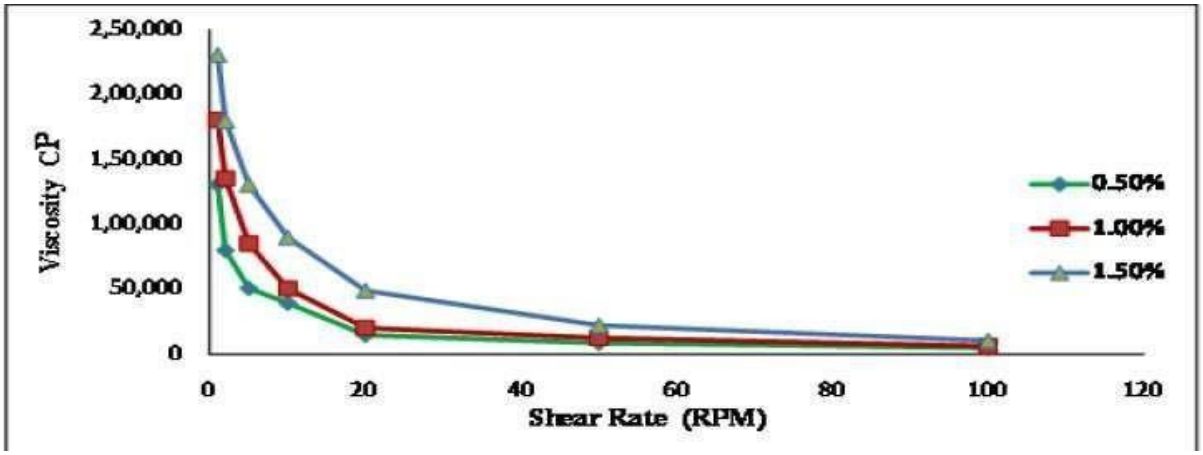

Figure 4.29: Plot of shear rate versus viscosity of formulation

4.10.5 Drug content determination of TFs gel formulation

Glabridin loaded transfersome gel prepared were evaluated for drug content. The drug content found is given in Table. 4.27

Table 4.27:Drug content of Glabridin loaded TFsgel formulation

Batch code	Drug content (%)
TF-4	**98.39±0.71**

4.10.6 Diffusion studies

A) In-vitro diffusion studies

The Franz diffusion cell was used to investigate drug diffusion over the dialysis membrane in vitro. The rate of drug release during the first two hours was higher than the rate of drug release during the next two hours. This could be because the formulations contain free glabridin. The size of the vesicle has been shown to have a substantial impact on the ingredient's release rate. Figure 4.30 shows the data collected by diffusion.

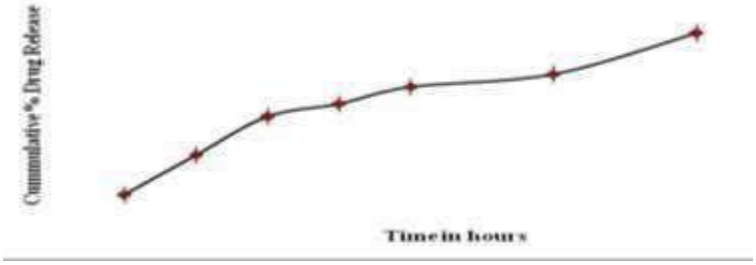

Figure 4.30: Drug release profile of batch TF4 gel: zero order plot

I) Average cumulative drug release of optimized formulation containing Carbopol

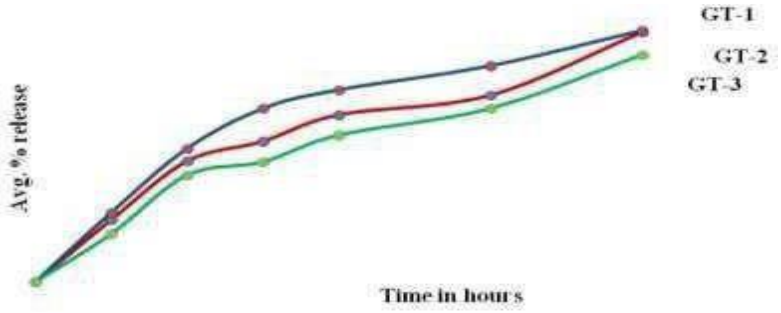

Figure 4.31: Average cumulative drug release of optimized formulation containing Carbopol 934P

As the content of Carbopol 934P increased, the average cumulative release of TFs based glabridin gel reduced (Figure 4.31). This could be due to increased viscosity of the formulation with increased Carbopol 934P concentration, as the difference was minimal up to 2 hrs in the beginning, and the 0.5 percent and 1.0 percent gels showed relatively smoother release. This observation, combined with the rheology, e.g. Spreadability (Table 4.25), performed well.

II) Comparative evaluation of *in-vitro* drug release of Glabridin TFs gel formulation Over Conventional gel.

Figure 4.32: Comparative evaluation of *in-vitro* drug release of GlabridinTFsgel formulation Over Conventional gel.

- The rate of drug release over the first two hours in the TF gel formulation was higher than the rate of drug release over the following hours. This could be because the formulations contain free glabridin.
- In a plain gel formulation, the rate of drug release was steady but slow; nonetheless, there was fluctuation in drug release, which could be attributable to a solubility issue or a lack of glabridin in molecular form.
- Drug release was sustained and higher in the glabridin-loaded transfersome than in the ordinary gel, possibly due to the greater availability of glabridin in molecular form.
- The TFs gel exhibits a burst release lasting up to 2 hours, followed by almost constant release, whereas the plain gel exhibits a similar constant release without the burst, and the plain gel also showed 22 percent release at the end of 8 hours. The TFs gel exhibits release at a rate that is almost double that of the plain glabridin gel.

III) Comparative evaluation of *in-vitro* drug release of Transfersome gel formulation with Transfersome dispersion.

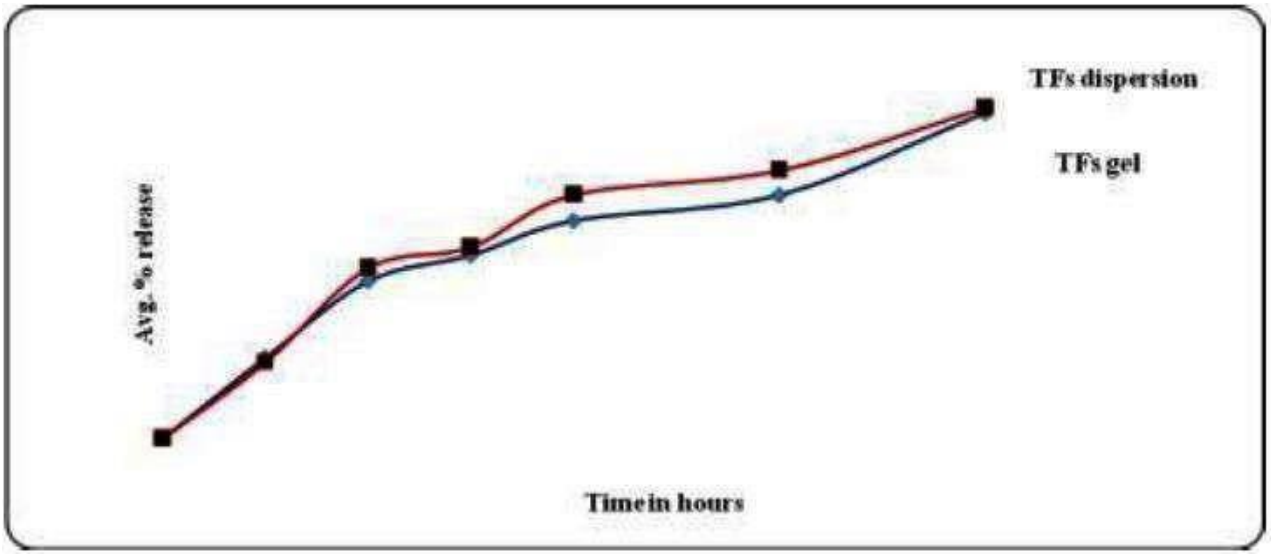

Figure4.33: Comparative evaluation of *in-vitro* drug release of Transfersome gel formulation with Transfersome dispersion.

- Both products have a brief burst followed by continual release. The brief burst could be owing to the presence of unbound glabridin molecules near the diffusion membrane. In the case of TFs dispersion, the release rate was determined to be 11.87 percent per hour, while in the case of gel, it was around 11.60 percent per hour. The difference of 0.27 percent was seen due to the gel's diffusion resistance.
- The gel would have better skin retention due to its rheology, though the release duration would be more or less the same, indicating that using a 1.0 percent gelling agent did not result in a significant increase in sustaining action, and any sustaining observed was due to the nature of transfersome.

4.12 Stability studies:

Stability tests were performed on optimised TF formulations (TF-4). These formulations were investigated for accelerated stability testing as well as a three-month long-term investigation. Entrapment efficiency, vesicle size, and drug release experiments were all performed on samples that were withdrawn and retested. The results, as given in table 4.28, revealed that the formulation had maintained its stability

Table 4.28. Effect of composition on various parameters.

Batch code	Ratio of lipid: surfactant	Type of surfactant	E. E (%)	Vesicle size (nm)	Deformability index
TF4	80: 20	Span-80	92.24±0.25	130.60	16.31±0.68
TF5	80: 20	Tween-80	82.06±0.20	129.32	17.40±0.45
TF6	80: 20	SDC	76.41±0.52	110.0	13.53±0.40

II. Develop and validate an HPLC method for determining Glabridin in transfersomal formulations

Choosing a mobile phase:

It was discovered that a mixture of acetonitrile (ACN) and acetate buffer offers good results after numerous permutations and combinations. Finally, the optimum mobile phase composition, ACN: Acetate Buffer (70:30v/v) with a flow rate of 1.0 mL/min, was chosen because it produced a strong asymmetric peak for Glabridin with little tailing and the required elution duration. Glabridin's retention time was discovered to be 5.70 minutes.

Selection of analytical wavelength:

The solution was scanned in the spectrum mode using a double beam UV-Visible Spectrophotometer against the mobile phase as a blank in the wavelength ranges of 400 to 200 nm. Glabridin demonstrated substantial absorption at 228.0 nm thus that was chosen as the wavelength.

System suitability parameters:

Method appropriateness factors such as tailing factor (T) and column efficiency (number of theoretical plates, N) were investigated to determine the repeatability of the proposed chromatographic system for estimating glabridin in TFs gels. At a wavelength of 228.0 nm, the chromatogram was taken given in Figure 4.34 As Table 4.29 shows the system

appropriateness parameters that were computed.

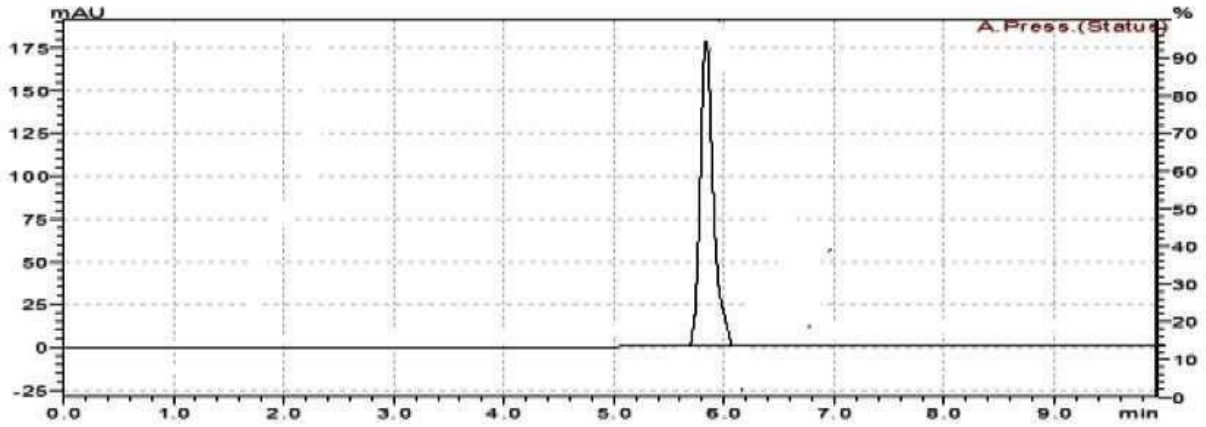

Figure 4.34: Typical Chromatogram of Glabridin

Table 4.29: System suitability parameters

Parameter	Standard values	Observed values
Tailing factor	NMT 2	1.33
Theoretical Plates	NLT 2000	5842

NMT – Not more than NLT – Not less than

Study of linearity range:

The mean peak areas for each medication concentration were computed and are given in Table 4.30 Figure no. 4.35 shows the standard calibration curve of Peak area vs. Concentration.

Table 4.30: Calibration data ofGlabridin

S.No.	Concentration(µg/mL)	Peak area*
1.	10	336092
2.	20	590877
3.	30	996261
4.	40	1342457
5.	50	1819566
6.	60	2126032

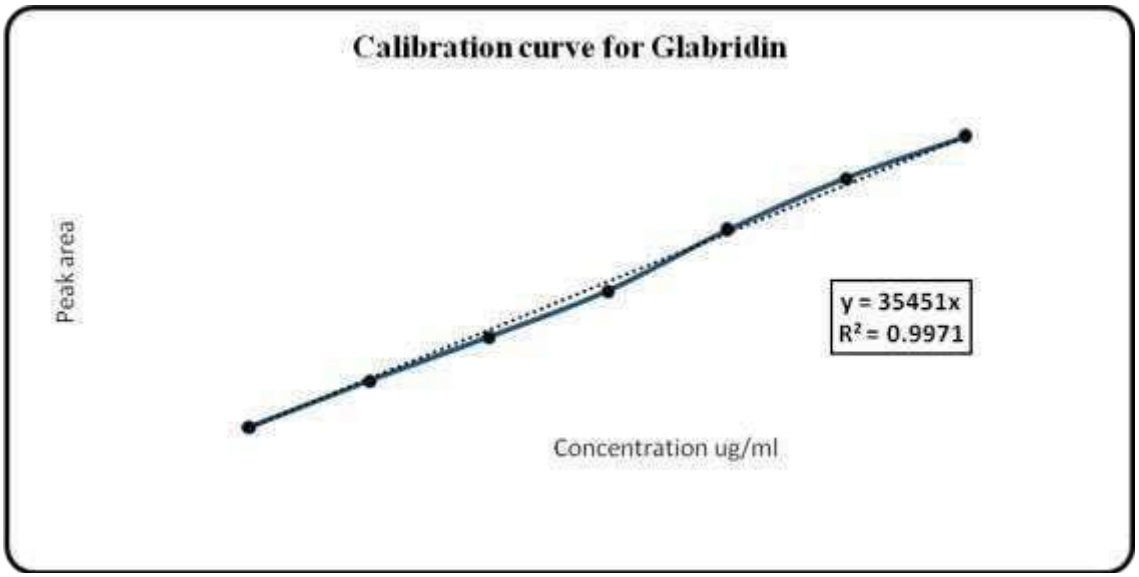

Figure 4.35: Calibration curve for Glabridin

Method validation

Accuracy

The column was injected with an equal amount of standard and sample solution (20µL) and chromatographed under optimum chromatographic conditions. Each solution was injected and chromatographed in triplicate. The area of each peak for glabridin was measured at 228.0 nm on the associated chromatograms.

Results of recovery studies and its statistical evaluation are shown in Table 4.31.

Table 4.31: Results of recovery studies

Sr. No.	Level of recovery	Weight of TFs gel taken (mg)	Amount of drug added (mg)	Amount of drug recovered (mg)	% Recovery
1.	80 %	2000	4.0	3.96	99.0
		2000	4.0	3.98	99.5
		2000	4.0	3.92	98.0
2.	100%	2000	5.0	4.91	98.2
		2000	5.0	4.95	99.0
		2000	5.0	4.97	99.4
3.	120%	2000	6.0	5.95	99.16
		2000	6.0	6.1	101.66
		2000	6.0	5.93	98.83

Statistical validation for recovery study

Level of recovery	% Recovery*	S.D. (±)	% R.S.D.
80%	98.83	0.03055	0.03091
100%	98.86	0.03055	0.03090
120%	99.88	0.09291	0.09302

*****Mean of three determinations

Precision

a) Intermediate precision (Intra-day and Inter-day precision)

TFs gel sample solutions were analysed at different time intervals on the same day and on three other days to assess intra-day and inter-day precision. Table 4.32 shows the results of the intra-day and inter-day precision analyses.

Table 4.32: Result of Intra-day and Inter-day precision of HPLC method

Drugs	Intra-day Precision			Inter-day Precision		
	% Label claim*	S.D. (±)	R.S.D.	% Label claim*	S.D. (±)	R.S.D.
Glabridin	100.37	0.7692	0.7663	99.31	0.2478	0.2468

* Mean of three determinations

b) Solution stability study

The stability of the sample solution was tested by injecting TFs gel sample solution at varied time intervals (in triplicate). i.e., chromatographed under ideal circumstances at 0 minutes, 1, 3, 6, and 24 hours. At each time interval, a sample solution was injected and chromatographed in duplicate. The glabridin retention time and peak area were recorded and are given in Table 4.33.

Table 4.33: Result of solution stability study

Sr. No.	Time intervals (hr)	Retention time (min)	Peak area*
1.	0	5.70	995498
2.	1	5.73	995510
3.	3	6.10	995663
4.	6	6.15	995710
5.	24	6.13	995698
* Mean of three determinations		**Mean**	995607
		S.D. (±)	103.59
		R.S.D.	0.01040

4.6.3 Limit of detection (LOD) and limit of quantitation (LOQ)

The LOD and LOQ were calculated independently depending on the calibration curve's standard deviation of response. The LOD and LOQ were calculated using the standard deviation of the y-intercept and the slope of the calibration curves. Table 4.34 shows the results.

Table 4.34: Result of Limit of Detection (LOD) and Limit of Quantitation (LOQ)

Parameters	**Glabridin**
LOD (µg/ml)	0.1260
LOQ (µg/ml)	0.4199

4.6.4 Robustness of method

Glabridin solution was injected into the HPLC system and chromatographed under different conditions. The results of robustness studies are shown in Table 4.35

Table 4.35: Result of robustness study

Chromatographic Changes			
Factor **Flow Rate (ml/min)**	**Level**	**Retention time (mins)**	**Tailing factor**
0.9	-0.1	5.2	1.1
1.0	0	5.6	1.0
1.1	+0.1	5.1	1.0
	Mean	5.3	1.03
	S.D. (±)	0.264	0.057
Mobile Phase (v/v)		6.1	1.1

68:32	-2	5.6	1.2
70:30	0	5.8	1.1
72:28	+2	5.83	1.13
	Mean	0.251	0.057
	S.D. (±)	6.1	1.1

4.7 Analysis of Transfersomal gel formulation:

In the HPLC system, an equal amount (20 L) of standard and sample solution was injected and chromatographed under optimal chromatographic conditions.Each solution was injected in triplicate and chromatographed. The peak area of glabridin was found at 228.0 nm in the chromatograms.

Results of analysis of TFsgel formulation and its statistical validation are shown in Table.4.36

Table 4.36: Result of analysis of TFs gel formulation

TFs-gel	**Avg. wt. 1000 mg**	**Label Claim: 0.5 %**		
Sr. No.	**Weight of gel taken (mg)**	**Peak area**	**Amount Estimated (mg)**	**% label claim**
1.	1000.1	995632	5.06	101.2
2.	1000.0	996021	5.06	101.2
3.	1000.3	981698	4.99	99.82
4.	1000.3	979654	4.98	99.61
5.	1000.0	989910	4.99	99.89
6.	1000.1	976490	4.96	99.29
		Mean	5.006	100.16
		S.D. (±)	0.0427	0.825
		R.S.D.	0.854	0.823

4.8 Forced degradation studies:

Figures 4.36, 4.37, 4.38, 4.39, 4.40, and 4.41 show representative densitograms for acid, alkali, oxide, and neutral hydrolysis, thermal, and photolytic treated materials, respectively. Table 4.37 shows the results of investigations on forced (stress) degradation.

Table 4.37: Result of degradation studies

Sr. No.	Stress Condition	Temperature and Time	% assay of active substance	Retention time of degraded product
1.	Acid (0.1 M HCl)	80^0C for 3 hrs	95.12	5.59
2.	Alkali (0.1 M NaOH)	80^0C for 3 hrs	96.24	5.58
3.	Oxide (3 % H_2O_2)	80^0C for 3 hrs	83.36	2.72, 5.62
4.	Neutral	80^0C for 3 hrs	Stable	-
5.	Heat	80^0C for 24 hrs	Stable	-
6.	Photo degradation	24 hrs	Stable	-

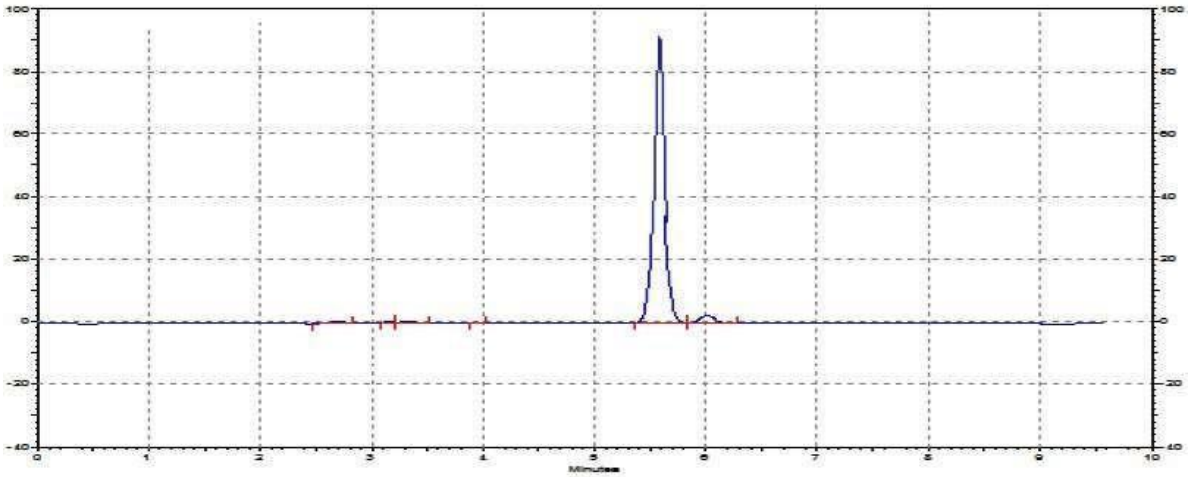

Figure 4.36: Chromatogram of acid (0.1M HCl) treated sample

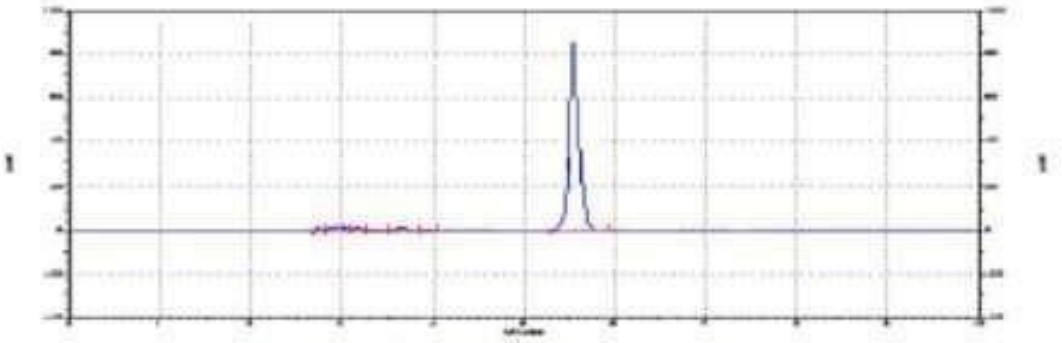

Figure 4.37: Chromatogram of alkali (0.1 M NaOH) treated sample

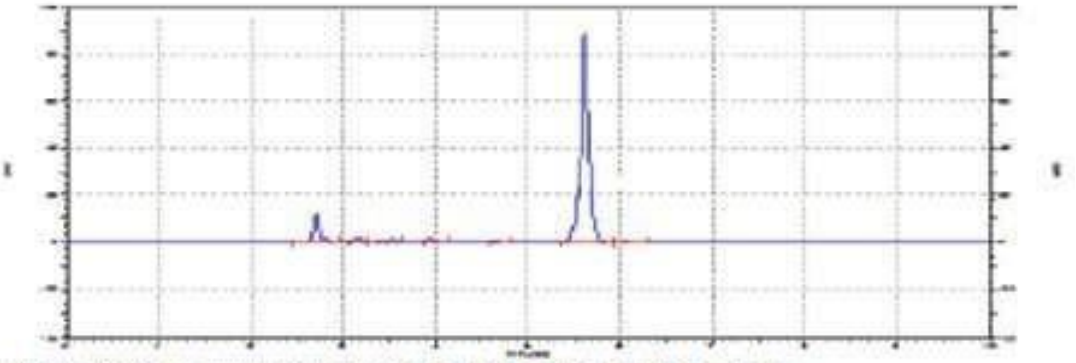

Figure 4.38: Chromatogram of Oxide (3% H_2O_2) treated sample

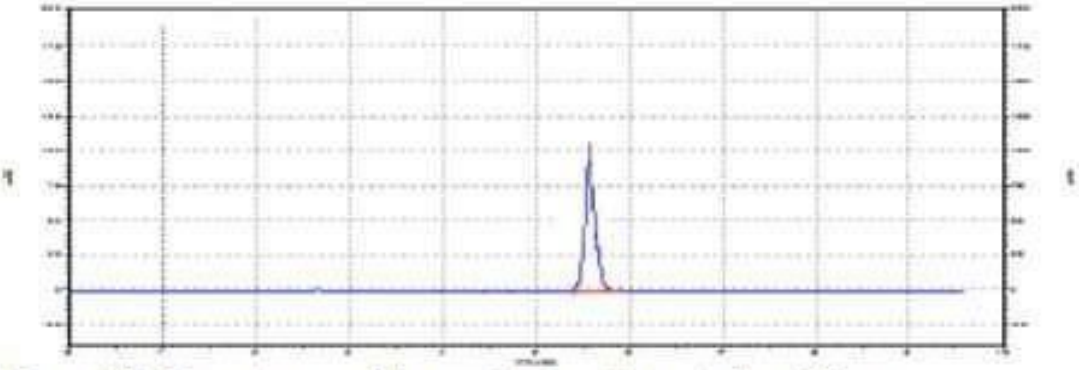

Figure 4.39: Chromatogram of the sample exposed to neutral condition

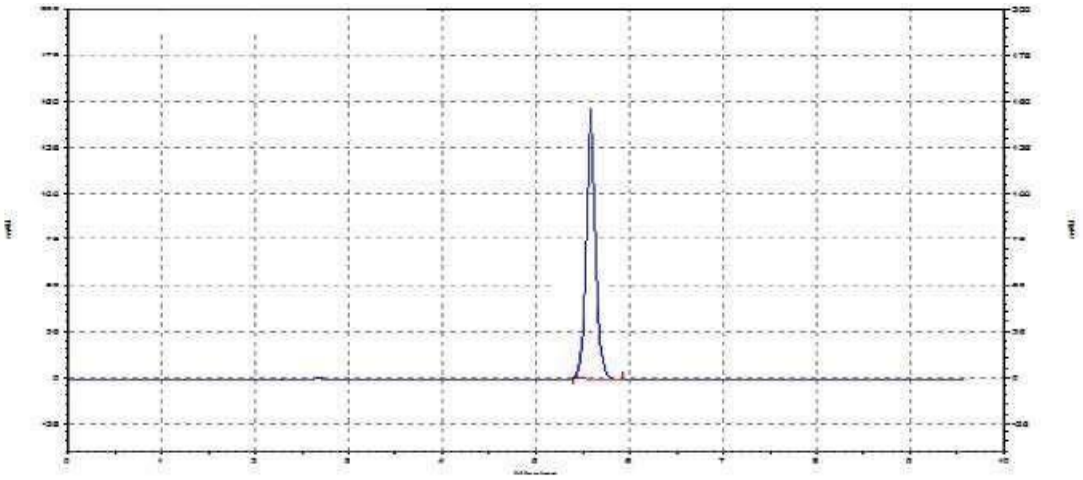

Figure 4.40: Chromatogram of heat treated sample for 24 hrs.

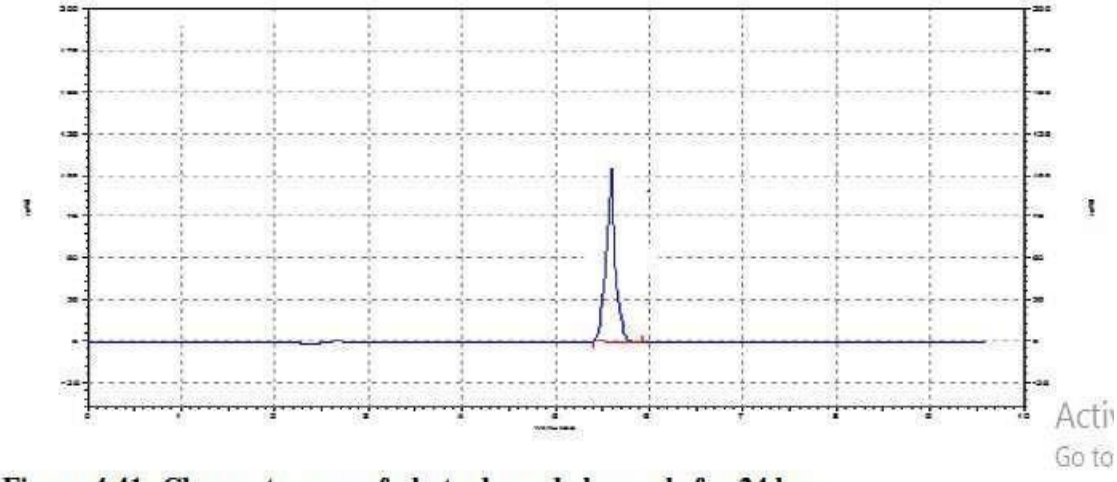

Figure 4.41: Chromatogram of photo degraded sample for 24 hrs.

III. Formulate & evaluate apremilast pellets

Standard calibration curve of Apremilast

Calibration curves of Apremilast in 0.1N HCl and Phosphate buffer pH 6.8 are shown in Figure.4.42

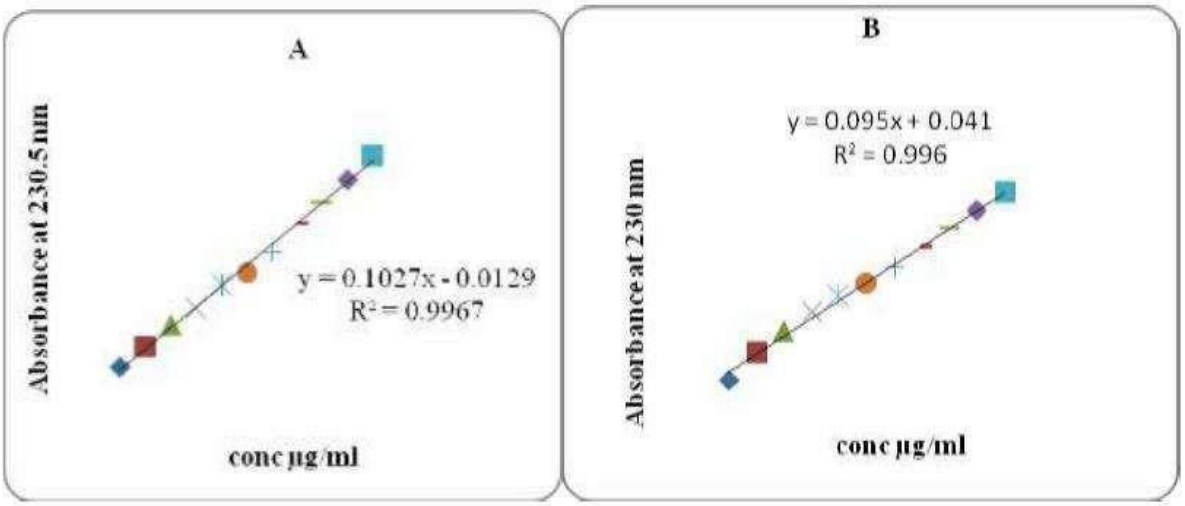

Figure 4.42: Calibration curve of apremilast in 0.1N HCl and pH 6.8

Drug Polymer compatibility study by FT-IR

The FTIR spectra of pure drug (Figure 4.43-A) showed functional peak at 3638.00, 3513.18, 2984.87,2209.65, 1703.20, 1386.86,657.25cm-1,while physical mixture (Figure 4.43-B) shows peaks at 3661.51, 3517.30, 2917.29, 2208.57, 1692.68, 1387.66, 656.42 cm-1with negligible shift in wave number indicated no interaction between drug and excipients.

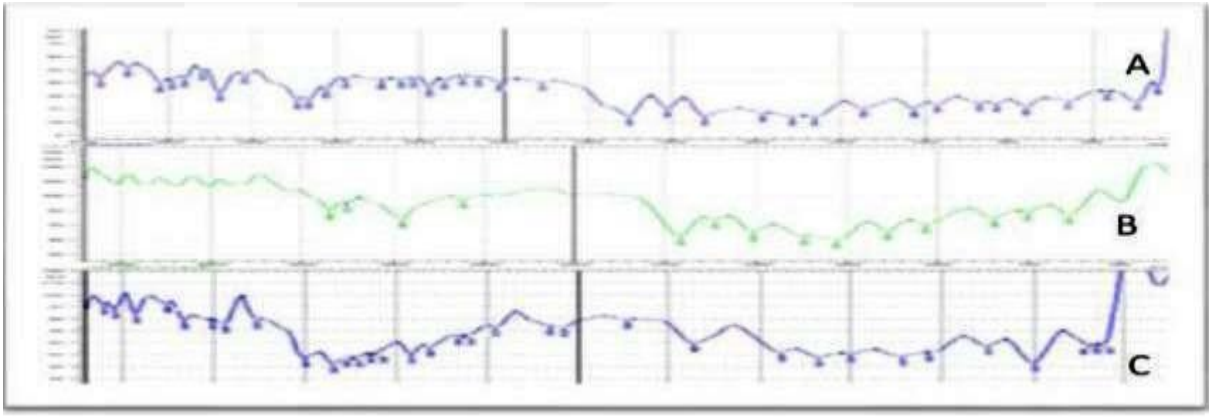

Figure 4.43: FTIR spectrum of apremilast (A), apremilast and EudragitL100 (B), and apremilast Formulation(C)

Table 4.38: Evaluation of Apremilast pellets

Batch No.	% Yield	Diameter (mm)	Hardness (kg/cm2)	Bulk density (g/ml)	tapped density (g/ml)	Hausner's ratio	Carr's index (%)	Angle of repose(°)	Drug content (%)
F-1	85.72	0.97	0.131	0.528	0.632	1.197	16.45	25.24	80.32
F-2	89.21	0.93	0.146	0.552	0.641	1.161	13.88	27.32	83.22
F-3	82.65	0.91	0.185	0.673	0.712	1.057	5.47	28.15	79.15
F-4	86.18	0.89	0.231	0.427	0.523	1.224	18.35	27.88	81.23
F-5	87.03	0.94	0.258	0.432	0.519	1.201	16.76	29.13	84.11
F-6	94.72	0.95	0.297	0.531	0.627	1.181	15.31	26.01	90.13

Differential scanning calorimeter (DSC)

The endothermic peak obtained in thermogram of pure apremilast at 152.4ºC can attribute to melting point of Apremilast, slightly change was observed in thermogram of formulation while addition endoderm at182.8º C represent peak of eudragitL100. Thus the thermogram showed that the Apremilast, eudragitL100 are compatible with each other since there is no significant difference in endothermic of pure drug and formulation batch. As shown in Figure 4.44.

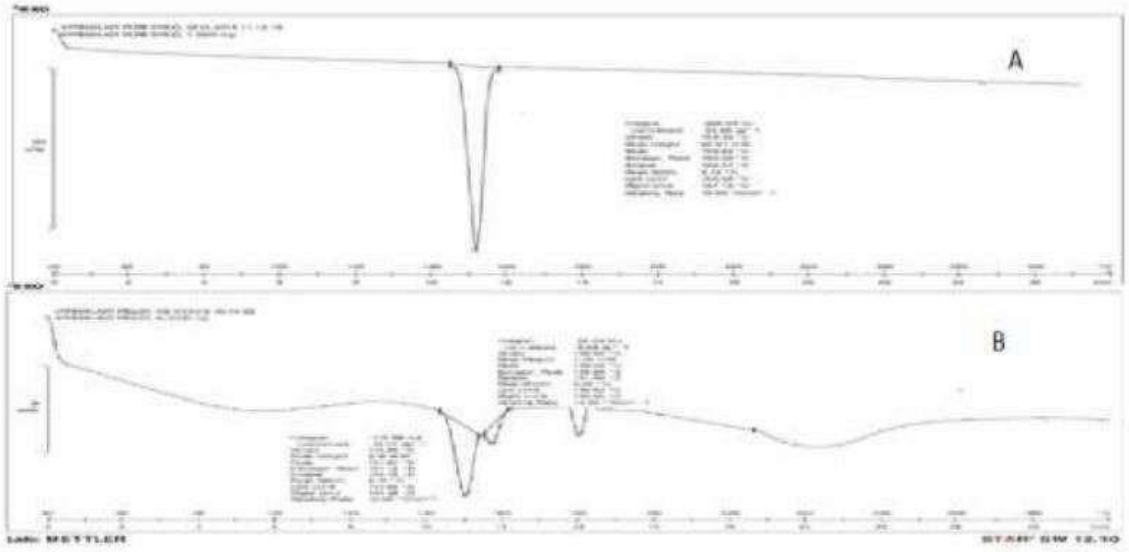

Figure 4.44: DSC thermogram of pure Apremilast (A) and F-6 batch (B)

Scanning electron microscopy (SEM)

From the SEM study the pellets are having spherical shape with outer smooth surface. Pellets size ranging from 0.8-1.1mm**.**As shown in Figure 4.45(A) & normal images of material & pellets with formulation process shown in Figure 4.45 (B)

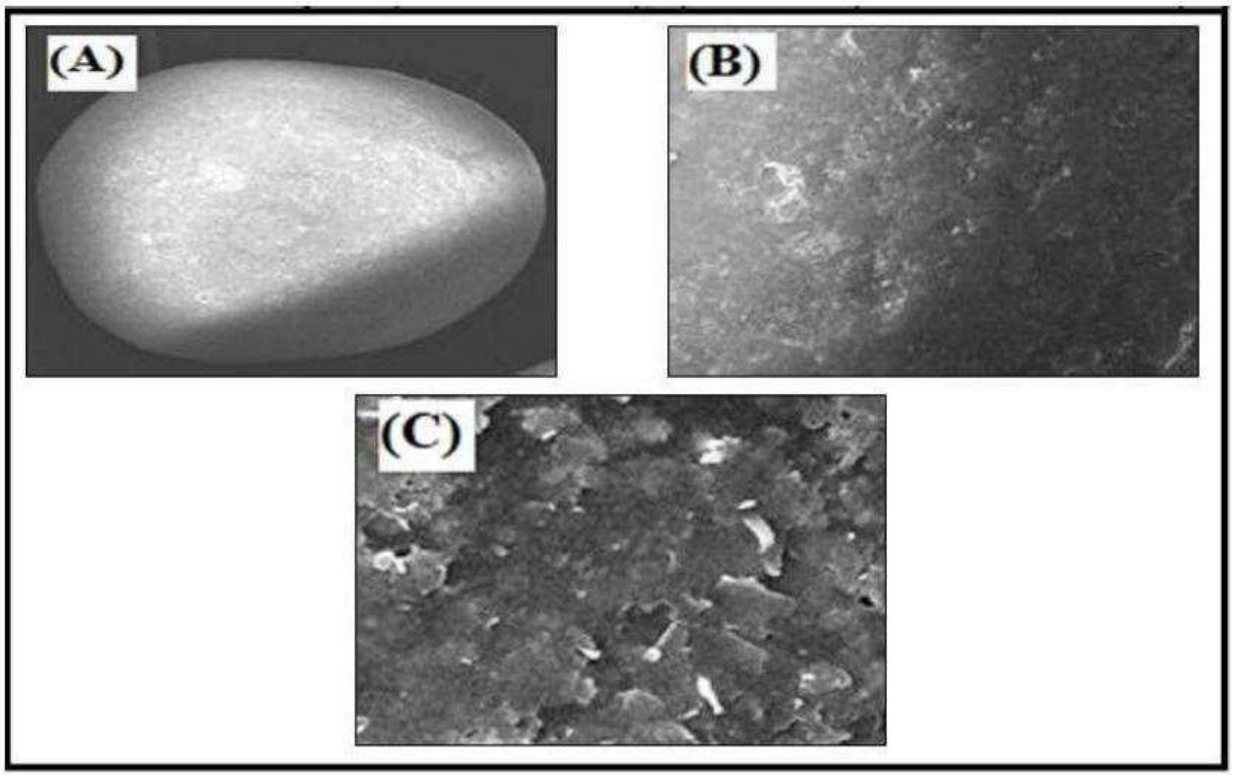

Figure 4.45 (A): SEM images of F-6 batch; A) at 105X, B) at 500X, C) at 1000X.

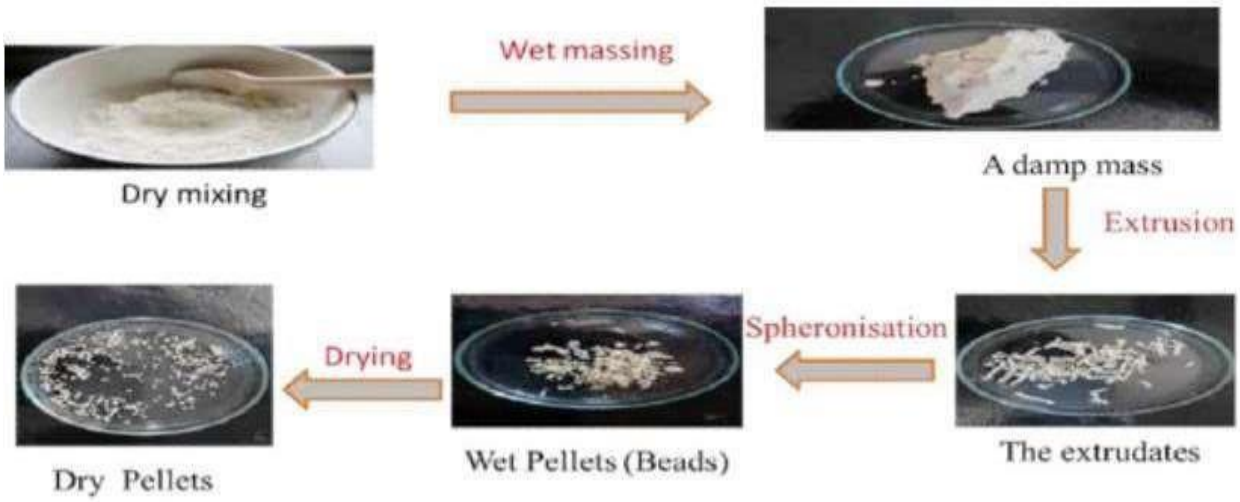

Figure 4.45 (B): Images of material change during formulation and pellets.

IV. Develop and evaluate a stability-indicating RP-HPLC and HPTLC method for estimating Apremilast

A reversed phase HPLC method is used to estimate Apremilast in formulation has been developed and validated. As the stationary phase, a Neosphere C18 (250 mm X 4.6 mm, 5m) column was used. After various permutations and combinations, it was observed that a mixture of Acetonitrile and (30Mm) potassium dihydrogen phosphate buffer pH-3 (adjusted with o-phosphoric acid) produces acceptable results when compared to other mobile phases. Finally, the best mobile phase composition, Acetonitrile: (30mM) potassium dihydrogen phosphate buffer pH-3 (adjusted with o-phosphoric acid) (60: 40 v/v), and flow rate 1.0ml/min, was chosen because it produced a sharp symmetric peak for APL with minimum tailing and the desired elution period.. APL solution in suitable dilution was scanned in spectrum mode using a double beam UV-Visible Spectrophotometer-1700 in the wavelength ranges of 400 nm to 200 nm against mobile phase as a blank. The wavelength of 236 nm was chosen since APL indicated considerable absorption at this wavelength. APL retention time was discovered to be
4.46 minutes (Fig. 4.46).

Optimized chromatographic conditions are as follows:

HPLC Column: Neosphere C18 R, (250 mm X 4.6 mm,
5µm)Column temperature:Ambient temperature
Mobile Phase: Acetonitrile: (30mM) potassium dihydrogen phosphate
buffer*pH*-3(Adjusted with o-phosphoric acid), (60: 40 V/v)
Flow rate: 1.0ml/min
UV detection: 236 nm
Injection volume: 20 µl
Run time: 10 minutes.

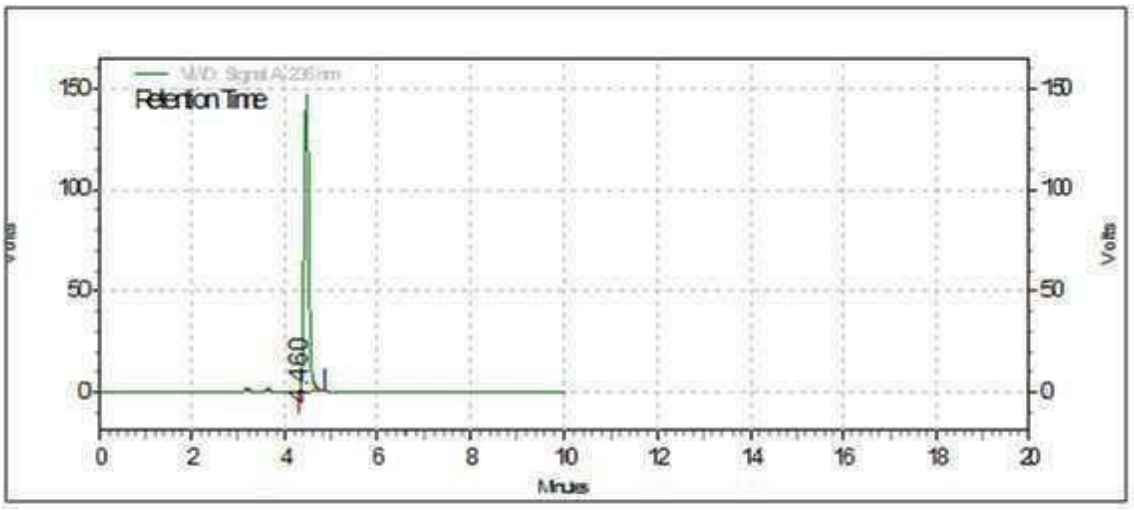

Fig. 4.46: Typical chromatogram of Apremilast (RT = 4.460 min).

System suitability parameters

System suitability studies were performed to determine the repeatability of the chromatographic conditions listed above. The measured tailing factor and theoretical plates values were 1.00 and 9606, respectively. Table 4.39 shows the system suitability parameters that were determined.

Table 4.39: System suitability parameters

Parameter	Standard values	Observed values
Theoretical Plates	NLT 2000	9606
Capacity Factor	2-5	3.46
Tailing factor	NMT 2	1.0

NMT – Not more than **NLT –** Not less than

Linearity range

Mean peak areas were calculated for each drug concentration and are shown in Table 4.40. The standard calibration curve of Peak area vs. Concentration is depicted in Fig. 4.47. The linearity of detector response was tested by producing a concentration vs. peak area graph. APL was shown to have a linear response at concentrations ranging from 10 to 60 µg/ml. The correlation coefficient for the APL calibration curve was determined to be 0.994 (Fig. 4.47).

Table 4.40: Standard calibration data of Apremilast

Sr. NO	**Concentration (µg/ml)**	**Peak area***
1.	10.0	9141770.0
2.	20.0	17721753.0
3.	30.0	24874329.5
4.	40.0	30566339.0
5.	50.0	41595925.5
6.	60.0	48816289.5

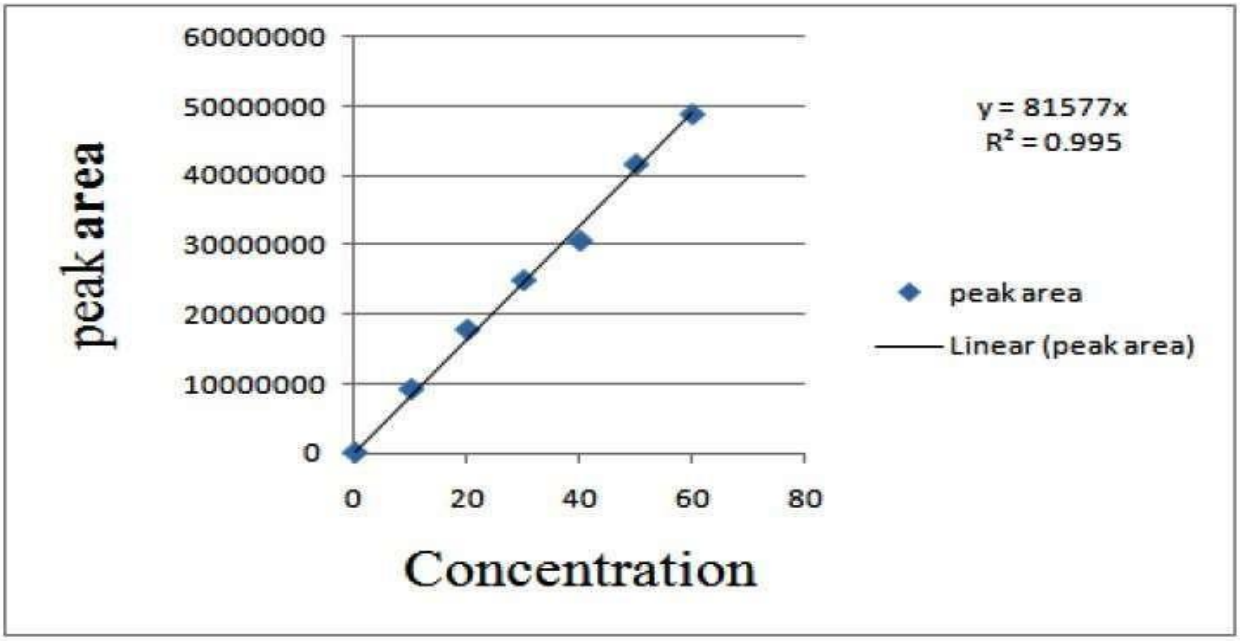

Fig. 4.47: Standard calibration curve for Apremilast

Analysis of pellet formulation

The chromatograms were recorded, and the peak area of APL at 236 nm was measured. The amount of APL (equivalent to given amount) and percent added in formulation were calculated by comparing the sample's mean peak area to that of the standard. Table 4.41 summarizes the results of the formulation analysis.

Table 4.41: Results of analysis of formulation

Otezla	Avg. wt. 103.2 mg		Label Claim: 10 mg	
Sr. No.	Weight of pellet powder (mg)	Peak area*	Amount Found (mg/tab)	% label claim
1.	103.2	16159518	9.809	98.09
2.	103.2	16351182	9.926	99.26
3.	103.3	16535735	10.03	100.3
4.	103.3	16537510	10.03	100.3
5.	103.3	16582842	10.06	100.6
6.	103.4	16671456	10.12	101.2

Table 4.42: Statistical validation

Amount Found (mg/tab)*	% Label claim*	S.D. (±)	R.S.D.
9.99	99.973	0.61	0.610

* Mean of six determinations

Method validation

Accuracy

Recovery studies were performed using the usual addition method at three different levels to determine the correctness of the proposed approaches (80 percent, 100 percent & 120 percent). Table 4.43 summaries the findings of recovery research. The percent recovery was observed to be about 100 percent, demonstrating that the suggested method is accurate and that the excipients had no influence in the estimation.

Table 4.43: Results of recovery studies

Sr. No.	Level of recovery	Weight of pellet powder taken (mg)	Amount of drug added (mg)	Amount of drug recovered (mg)	% Recovery
1.	80 %	103.2	8.0	7.96	99.60
		103.1	8.0	7.90	98.75
		103.3	8.0	7.95	99.52
2.	100 %	103.1	10.0	9.84	98.4
		103.2	10.0	10.0	100.0
		103.2	10.0	9.96	99.64
3.	120 %	103.1	12.0	11.92	99.33
		103.2	12.0	11.93	99.41
		103.2	12.0	12.0	100.0

Statistical validation for recovery study (*Mean of three determinations)

Level of recovery	% Recovery*	S.D. (±)	R.S.D.
80%	99.29	0.251	0.251
100%	99.34	0.442	0.442
120%	99.58	0.190	0.190

- ***Precision:***

Intermediate precision (Intra-day and Inter-day precision)

The method's precision was investigated by analysing formulation at three distinct times of day (intra-day precision) and on three separate days (inter-day precision). Table 4.44 summarises the results of intra-day and inter-day precision. The findings of the percent label claim and the standard deviation of the set of results suggested that the approach was repeatable and reproducible under diverse situations.

Results of intra-day and inter-day precision are shown in Table 4.44.

Table 4.44: Result of Intra-day and Inter-day precision of RP– HPLC method

Drug	Intra-day Precision			Inter-day Precision		
	% Label claim*	S.D. (±)	R.S.D.	% Label claim*	S.D. (±)	R.S.D.
APL	99.98	0.2254	0.2254	98.31	0.3221	0.3221

* Mean of six determinations

- ***Limit of detection(LOD) and limit of quantitation(LOQ):***

The LOD and LOQ were calculated using the standard deviation of the y-intercept and the slope of the calibration curve. Table 4.45 & 4.46 summarizes the findings of LOD and LOQ studies.

Table 4.45: Result of LOD and LOQ

Parameters	APL
LOD (µg/ml)	0.93
LOQ (µg/ml)	2.82

Table 4.46: Summary of results of method validation

		RP-HPLC Method
Linearity Range (µg/ml)		5.0-30
Accuracy	**Percent Recovery*S.D. (±)**	99.40 ± 0.75
Precision	**Intra-day Precision**	
	% Label Claim ** **S.D. (±)**	99.98 ± 0.225
	Inter-day Precision	
	% Label Claim ** **S.D. (±)**	99.31±0.322
LOD (µg/ml)	0.93	
LOQ (µg/ml)	2.82	

* Mean of nine determinations **Mean of six determinations

Robustness of method

The robustness of the suggested technique was investigated by making tiny but deliberate changes to the optimum method parameters. The effect of flow rate and mobile phase ratio changes on retention time and tailing factor was investigated. Table 4.47 displays the results of robustness studies. The standard deviation of the set of results was determined to be less than 2, showing the method's robustness.

Table 4.47: Result of robustness study

Chromatographic Changes			
Factor	**Level**	**Retention time**	**Tailing factor**
Flow Rate (ml/min)	**(±0.1)**		
0.9	– 0.1	4.53	1.04
1	0	4.46	1.00
1.1	+ 0.1	4.07	1.05
	Mean	**4.35**	**1.37**
	S.D. (±)	**0.13**	**0.015**
Mobile Phase (v/v)	**(±2)**		
58:42	-2	4.73	1.07
60:40	0	4.46	1.00
62:38	+2	4.18	1.06
	Mean	**4.45**	**1.04**
	S.D. (±)	**0.14**	**0.017**

Forced degradation studies

In forced degradation studies, pellet powder equivalent to 10 mg of APL was subjected to the following stress conditions: acidic (0.1 NHCl), alkaline (0.1 N NaOH), oxidation (3% H2O2), neutral hydrolysis, heat (60℃ for 30 minutes), and UV exposure (for 72hrs). The contents of the flasks were held in a water bath at 80℃ for 2 hours for acidic, alkaline, oxide, and neutral conditions. All of the flasks were withdrawn and allow it to cool after the specified intervals. The samples were then evaluated in the same manner as indicated in the section on analysing marketed formulations.The results of forced (stress) degradation studies are summarized in Table 4.48. The typical chromatograms are shown in Fig. 4.48, 4.49, 4.50, 4.51, 4.52

and 4.53 for acid, alkali, neutral and Oxide hydrolysis, thermal and photolytic treated samples, respectively. % assay of active substance calculated by using equation no. 13 & 14.

Table 4.48: Result of degradation studies

Sr. No.	Stress Condition	Temperature and Time	% assay of active substance	Rt of degraded product
1.	Acid (0.1 NHCl)	80^0C for 2hr	82.95	2.073, 2.420
2.	Alkali (0.1NNaOH)	80^0C for 2hr	84.20	2.090, 2.437
3.	Neutral	80^0C for 2hr	88.23	2.403
4.	Oxide (3 % H_2O_2)	80^0C for 2hr	93.00	2.240
5.	Thermal	60^0C for 30 min	94.12	2.317
6.	Photo degradation	72hr	100	-

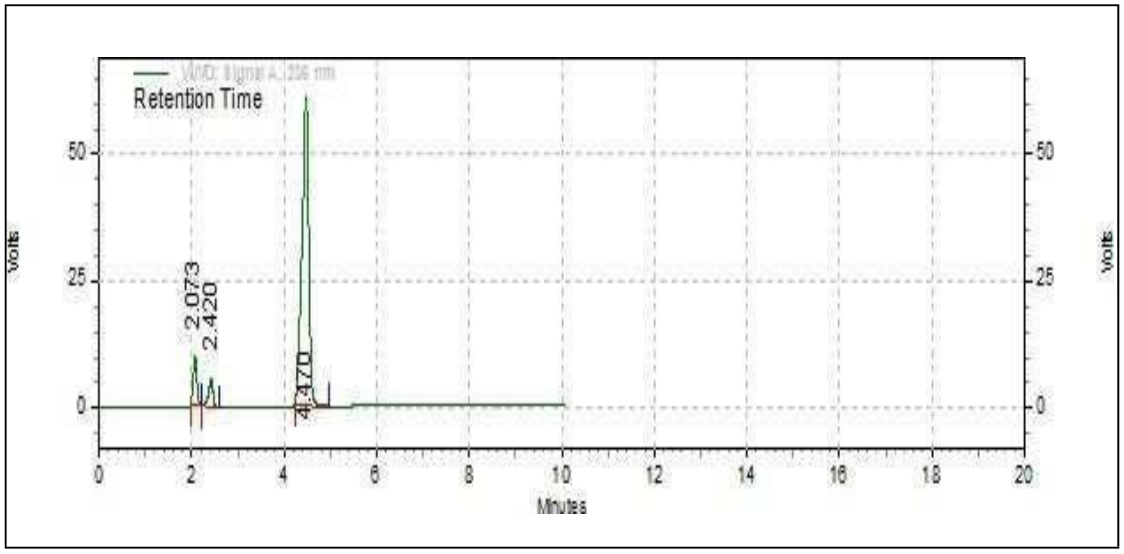

Fig. 4.48**: Chromatogram of Acid (0.1 NHCl) treated sample**

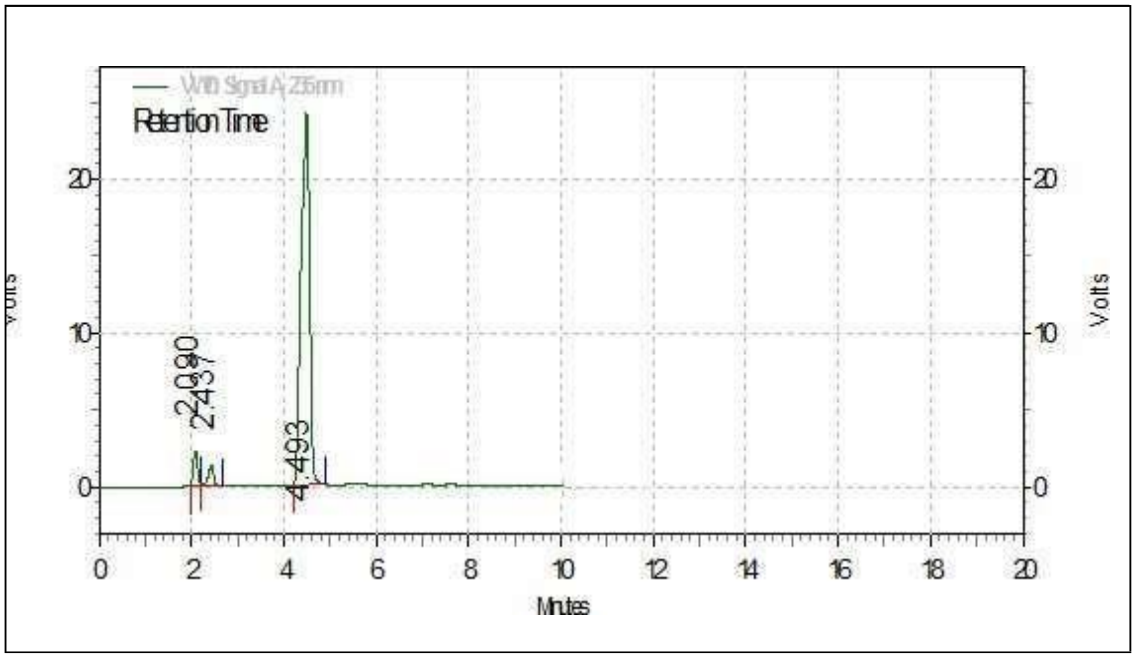

Fig. 4.49: Chromatogram of Alkali (0.1N NaOH) treated sample

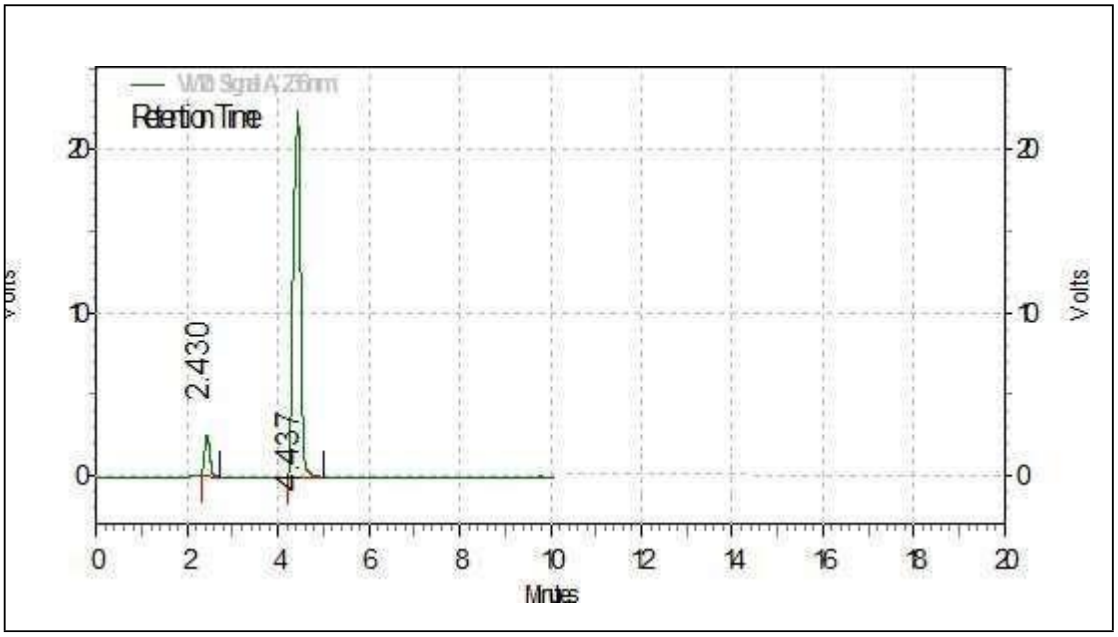

Fig. 4.50: Chromatogram of Neutral (H_2O) treated sample

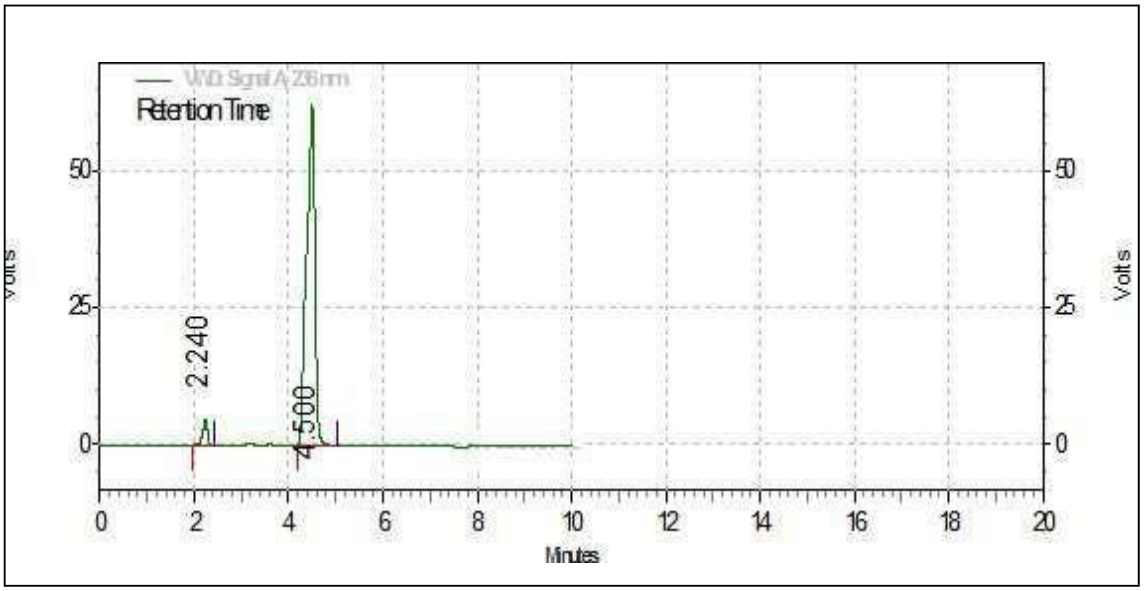

Fig. 4.51: Chromatogram of Oxide (3 % H_2O_2) treated sample

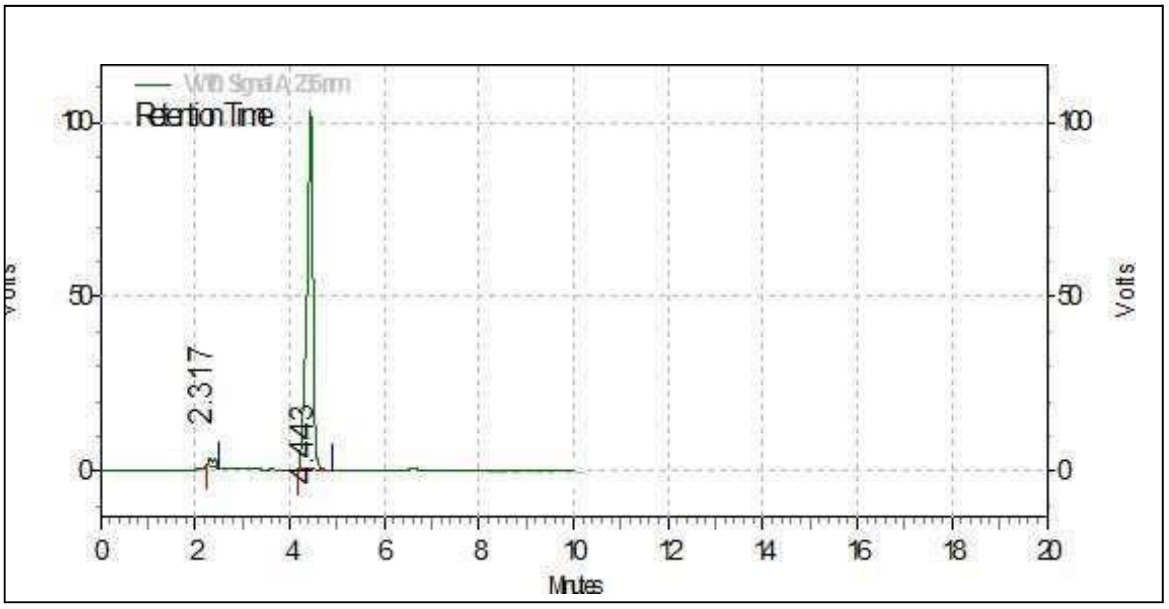

Fig. 4.52: Chromatogram of Thermal (heat) treated sample

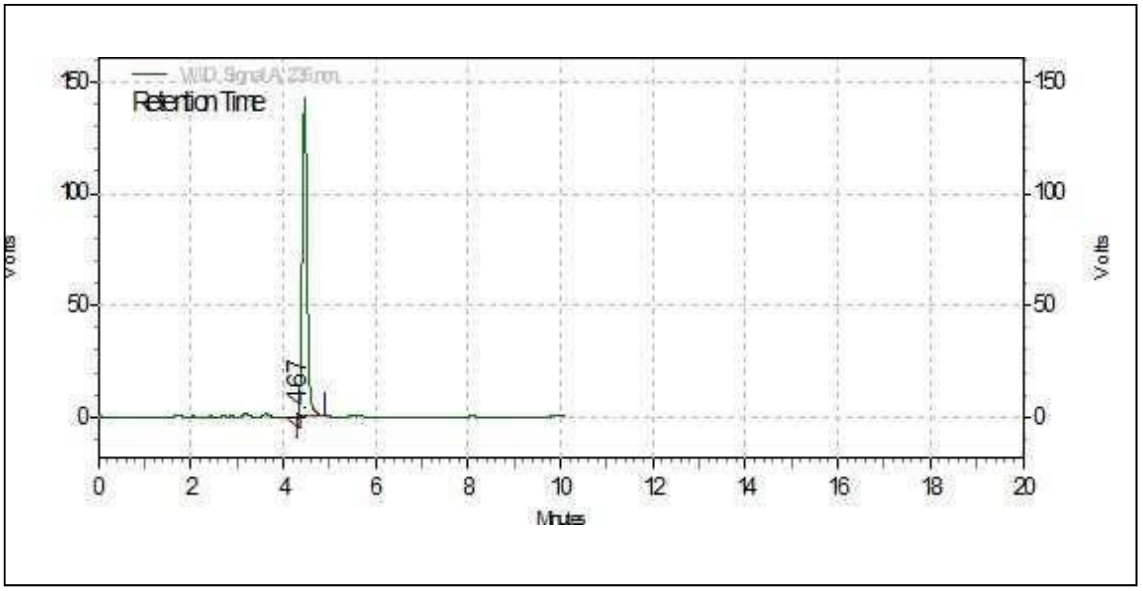

Fig. 4.53: Chromatogram of photo degraded sample

Mobile phase: Toluene: Ethyl Acetate (4: 6V/v)

Plate size : 10 cm X 10 cm,

Mode of application: Band

Band size: 6 mm (Distance between two bands: 5.6 mm)

Sample volume: 5µl

Development chamber: Twin-through glass chamber, 10 cm X 10 cm with Stainless steel lid.

Saturation time : 15minutes

Separation technique : Ascending

Migration distance : ≈ 80 mm

Temperature : 25 ± 5^0C

Scanning mode : Absorbance/Reflectance

Slit dimensions : 5 X 0.45 mm

Scanning wavelength: 236.0 nm

The typical densitogram obtained for APL is shown in Fig. 4.55.

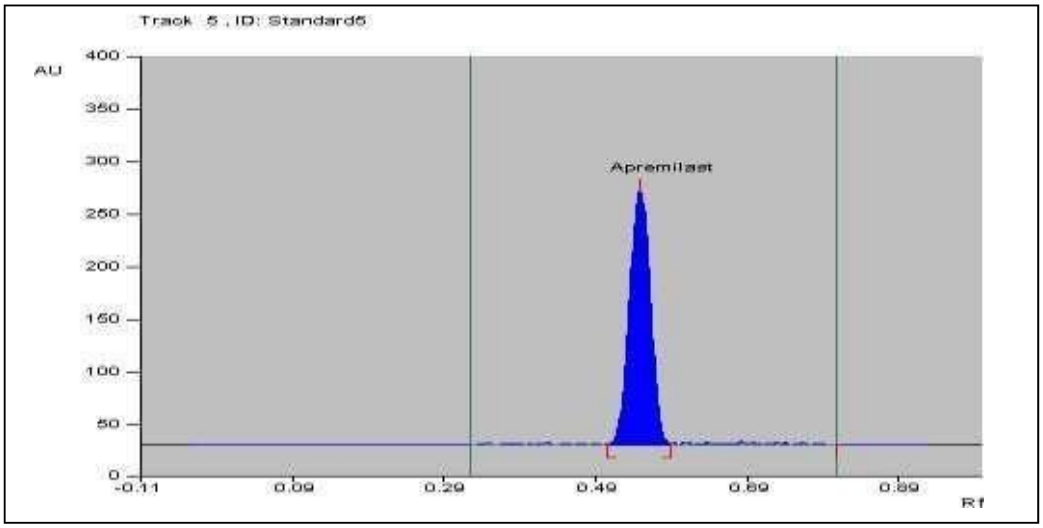

Fig. 4.55: Typical densitogram of Apremilast.

Retention factor: 0.55(±0.02) for Apremilast.

Linearity range

For APL, the calibration curve of Concentration vs. Peak area was developed by

recording the peak area for each drug concentration. Table 4.49 shows the standard calibration data Figures 4.60 and 4.61 illustrate the linearity and 3D graph of APL, respectively. For the concentration range 100-600 ng/band, linearity was observed. The coefficient of correlation for the APL calibration curve was found to be 0.998, as shown in Fig 4.56 & 4.57.

Table 4.49: Standard calibration data for Apremilast.

Sr. No.	**Concentration ng/band**	**Mean peak area**
		236.0 nm
1.	100	1666.99
2.	200	2917.52
3.	300	3878.55
4.	400	4638.22
5.	500	5476.6
6.	600	6586.90

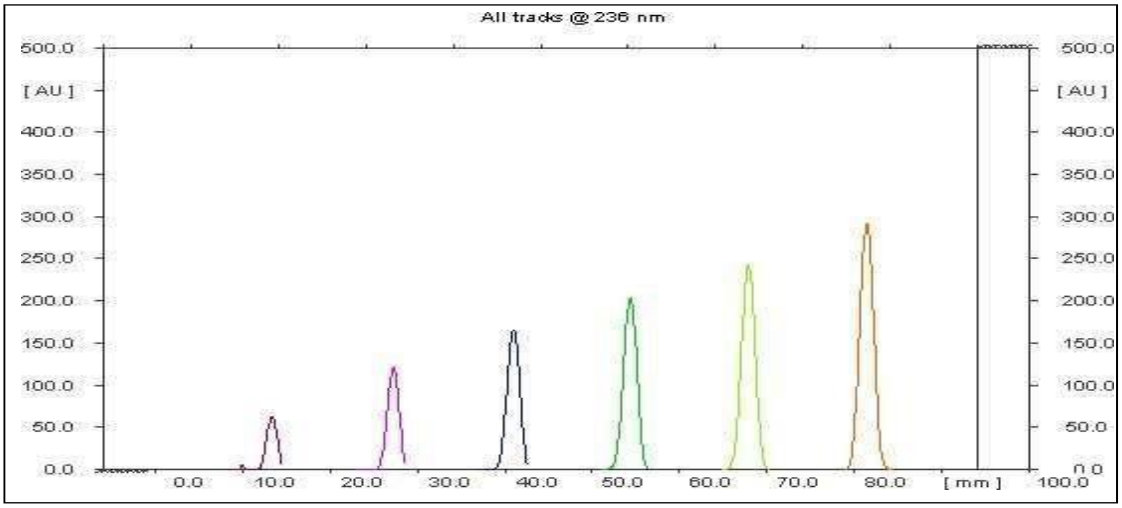

Fig. 4.56: Linearity of Apremilast at 236.0 nm

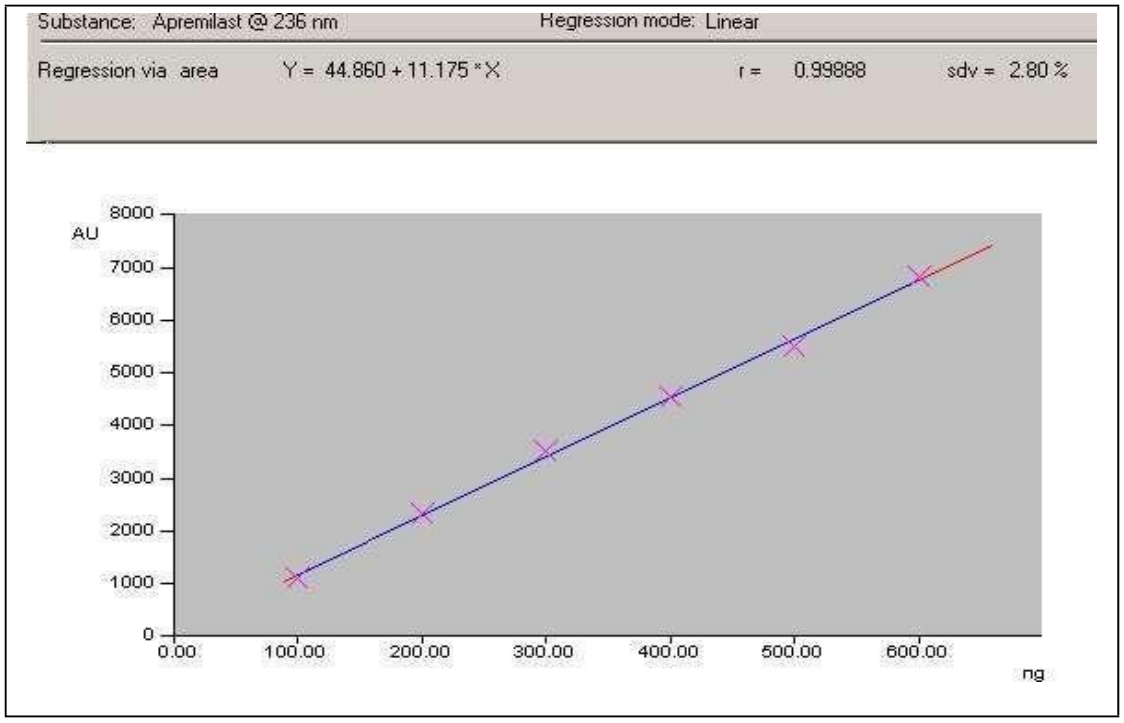

Fig. 4.57: Calibration curve of Apremilast at 236.0 nm

Analysis of Bulk drug

On the TLC plate, two bands of standard stock solution and four bands of sample solution, each with a volume of 5 µl, were applied., and the plate was developed and scanned under ideal chromatographic conditions. After scanning, the peaks for the standard and sample bands were combined. By comparing the peak area of the sample bands to that of the standard bands, the drug content in stock solution (mg) and percent drug estimation were computed. The results of analysis standard bulk drug and its statistical validation are shown in Table 4.50 and 4.51.

Table 4.50: Results of analysis of standard Bulk drug

Sr. No.	Weight of drug taken(mg)	Peak area*	Amount drug estimated (mg)	% Estimation
1.	10.00	5360.1	9.87	98.47
2.	10.01	5376.4	10.0	100.0
3.	10.02	5486.0	9.97	99.97
4.	10.00	5465.8	9.94	99.36
5.	10.01	5376.2	9.97	99.94
6.	10.00	5376.4	10.0	99.07

Table 4.51: Statistical validation for analysis of standard Bulk drug

% Estimation*	S.D. (±)	R.S.D.
99.53	0.3709	0.3664

*Mean of six determinations

Analysis of pellet formulation

Two bands of standard stock solution and one band of standard stock solution were placed on the TLC plate. 5 µl were added to each plate, and the plate was developed and scanned under optimal chromatographic conditions. After scanning, the peaks for the standard and sample bands were combined. By comparing the mean peak area of sample bands to that of standard bands, the amount of APL and percent drug added were calculated. The results of the pellet formulation study and statistical evaluation are presented in Table4.52 and 4.53.

Table 4.52: Results of analysis of formulation

Otezla-10 mg	**Avg. wt. 103.2 mg**		**Label Claim: 10 mg**	
Sr. No.	**Weight of pellet powder (mg)**	**Peak area***	**Amount Found (mg/tab)**	**% label claim**
1.	103.3	5369.2	9.96	98.81
2.	103.4	5347.7	9.28	98.83
3.	103.2	5476.6	10.1	100.0
4.	103.3	5396.4	10.0	101.0
5.	103.1	5342.2	9.74	99.36
6.	103.1	5446.1	10.0	100.1

Table 4.53: Statistical validation for analysis of marketed formulation

Amount Found (mg/tab)*	**% Label claim***	**S.D. (±)**	**R.S.D.**
9.95	99.78	0.56	0.56

***** Mean of six determinations

Method validation

The proposed technique was validated in accordance with the ICH guidelines. Recovery studies were performed using the usual addition method at three different levels to determine the correctness of the proposed approaches (80 percent , 100 percent & 120 percent). Table 4.54 summarises the findings of recovery research. The percent recovery was found to be about 100 percent, showing that the proposed procedures are accurate and that the excipients had no interference in the estimation. The method's precision was investigated by analysing formulation at three distinct times of day (intra-day precision) and on three separate days (inter-day precision).

Table 4.54: Results of recovery studies

Sr. No.	Level of recovery	Weight of pellet powder taken (mg)	Amount of drug added (mg)	Amount of drug recovered (mg)	% Recovery
1.	80 %	103.1	8.0	7.98	99.81
		103.2	8.0	7.90	98.92
		103.1	8.0	7.96	99.59
2.	100 %	103.2	10.0	10.0	100.0
		103.2	10.0	9.97	99.02
		103.1	10.0	9.87	99.00
3.	120 %	103.2	12.0	11.99	99.99
		103.2	12.0	11.95	99.50
		103.2	12.0	11.99	99.96

Table 4.55: Statistical validation for recovery study

Level of recovery	% Recovery*	S.D. (±)	R.S.D.
80%	99.44	0.245	0.24
100%	99.86	0.312	0.31
120%	99.81	0.149	0.14

* Mean of three determinations

Precision

Table 4.56 displays the results of intra-day and inter-day accuracy. The findings of the percent added drug and the standard deviation of the set of results suggested that the approach was repeatable and reproducible.

Table 4.56: Statistical evaluation of precision of developed method.

Drugs	Intra-day Precision			Inter-day Precision		
	% Label claim*	S.D. (±)	R.S.D.	% Label claim*	S.D. (±)	R.S.D.
APL	99.36	0.296	0.296	99.62	0.326	0.326

*Mean of three determinations

Robustness

To assess the robustness of the suggested approach, small but deliberate modifications in optimum process parameters such as mobile phase composition, chamber saturation time, time from spotting to development, time from development to scanning, and mobile phase volume were performed. The mobile phase composition and chamber saturation time were changed within the optimal parameters of 0.1 ml and 2 min, respectively. The volume of mobile phase was changed by 1 ml, as were the times from spotting to development and development to scanning. The impact of these adjustments on Rf values and peak area was studied.

Under all of the systematically modified technique parameters, the relative standard deviation for peak area was determined to be less than 2. The Rf values of both drugs did not vary significantly because they were within 0.05 Rf units of standard values. The fact that there was no substantial change in Rf value or peak area for either treatment illustrates the method's robustness.

Table 4.57 : Result of robustness study

Factor	Level	Peak area	Rf
Mobile phase composition (± 0.1 ml)			
3.9:6.1	– 0.1	5434.12	0.52
4 : 6	0	5476.90	0.55
4.1:5.9	+ 0.1	5497.38	0.57
	R.S.D.	**0.30**	**--**
Duration for chamber saturation (± 5 min)			
10 min	- 5	5215.41	0.53
15min	0	5476.40	0.55
20 min	+ 5	5492.72	0.56
	R.S.D.	**1.06**	**--**
Spotting to development			
5 min	-	5496.13	0.58
15 min	-	5476.20	0.55
30 min	-	5570.45	0.53
1 hr	-	5642.83	0.52
	R.S.D.	**0.76**	**--**
Development to scanning			
5 min	-	5462.70	0.56
15 min	-	5476.62	0.55
30 min	-	5492.80	0.54
1 hr	-	5689.31	0.52
	R.S.D.	**1.01**	**--**
Volume of mobile phase (± 1 ml)			
9.0	– 1	5395.3	0.53
10.0	0	5476.70	0.55
11.0	+ 1	5645.20	0.56
	R.S.D.	**1.19**	**--**

Limit of detection and limit of quantification

The LOD and LOQ were calculated individually based on the calibration curve's standard deviation of response. The LOD and LOQ were calculated using the standard deviation of the y-intercept and the slope of the calibration curve.

Table 4.58: Results of LOD and LOQ

Parameters	APL
LOD (µg/band)	0.77
LOQ (µg/band)	2.35

Forced degradation studies:

Accurately weighed tablet powder equivalent to10.0 mg APL was separately transferred to five different 10.0 ml volumetric flasks (Flask no. 1, 2, 3, 4, 5 and 6), added 3.0 ml of flask no. 1, 2, 3 and 4,0.1 N HCl, 0.1 NNaOH, 3 % $H_2O_2$and distilled water respectively. The flask no. 1, 2, 3 and 4 were kept in water bath at 80^0C for 2 hrs. Flask no. 5 containing 10 mg of APL was kept at 60^0C for1hrs. In hot air oven to study the effect of heat on sample (heat degradation). The forced degradation studies were performed in dark to exclude the possible degradative effect of light. Flask no. 6 containing APLwas kept under UV chamber for 72hrs.To study the photolytic degradation of bulk sample. All the flasks were removed after stipulated time interval; the APL samples were treated and analyzed in similar manner as described under analysis of marketed formulation.

The typical densitogram of APL for acid, basic water, Oxide, thermal and photo degradation samples are shown in Fig. 4.58, 4.59, 4.60, 4.61, 4.62 and 4.63 respectively.

Results of forced (stress) degradation studies are shown in Table 4.59.

Table 4.59: Result of degradation studies

Sr. No.	Stress Condition	Temperature and Time	% assay of active substance	R_f of degraded product
1.	Acid (0.1 N HCl)	80^0C for 2hr	80.62	0.12, 0.19, 0.24, 0.61
2.	Alkali (0.1N NaOH)	80^0C for 2hr	83.14	0.12, 0.19, 0.24, 0.61
3.	Neutral	80^0C for 2hr	90.44	0.12, 0.26, 0.30, 0.81, 0.88
4.	Oxide (3% H_2O_2)	80^0C for 2hr	94.85	0.10, 0.29, 0.49, 0.51, 0.75
5.	Thermal	60^0C for 1hr	93.87	0.32
6.	Photo degradation	72 hr	100	-

CHAPTER-5
CONCLUSION

There is a need for the formulation and development of drug delivery system that has the ability to deliver glabridin, and skin lightening agent, at controlled rate and provide high topical bioavailability. Therefore the objective of this study was to investigate the feasibility of encapsulating glabridin in transfersome carrier (TFs), which could be used as a potential vehicle to deliver Glabridin. The formulation showed improved drug retention and the drug diffusion from the formulation was suitable. Transfersome based gel formulations would be more effective with enhanced patient compliance.

The proposed RP-HPLC method for estimating Glabridin in transfersomal gel formulation is sensitive, accurate, precise, and reproducible. The RP-HPLC method was developed successfully for glabridin loaded transfersome formulation, the optimal composition of the mobile phase, acetonitrile: Acetate Buffer (70 : 30 v/v), and the flow rate of 1.0 mL / min was chosen because it produced a strong asymmetric peak for Glabridin with little tailing and elution time that was needed. Retention time of glabridin was found to be 5.70 minutes. Hence, this said method can be used for regular quality control of pharmaceutical formulations containing these drugs.

The apremilast pellets optimized batch prepared with MCC (12gm) and TKP (3gm) spheronized at 1000rpm for 90 sec showed good results in terms of yield, drug content, particle size.

The developed RP-HPLC and HPTLC method was verified in accordance with ICH Q2 (R1) requirements for the estimation of APL, providing useful information regarding the degradation behaviour of APL under various stress settings. Furthermore, the procedure was discovered to be simple, accurate, and reproducible. The established HPTLC approach aids in the separation of the medicine as well as the entire breakdown products of Apremilast, showing its stability. The proposed HPLC and HPTLC methods were discovered to be more sensitive, precise, efficient, and time and cost effective. The RP-HPLC and HPTLC methods developed were capable of quantifying APL when degradation products presents. As a result, the established RP-HPLC and HPTLC procedures can be employed for routine quality monitoring of APL-containing pharmaceutical formulations.

BIBLIOGRAPHY

- Akhtar, N. (2020). Vesicles: A Recently Developed Novel Carrier for Enhanced Topical Drug Delivery, Current Drug Delivery, (11) 87-97.
- Anerao, A., Telange, V., Bondre, N., John, S., Gadhave, T., Api, D.(2017). International Journal of Current Medical and Pharmaceutical Stability-Indicating Related Substances Method of Apremilast by HPLC and Synthesis and Chracterization of Related Impurities Using MASS and NMR Spectroscopy. International Journal of Current Medical and Pharmaceutical Research, Feb 28; 3(02): 1378-1385.
- Annapurna, M.M., Pradhan, D.P., Sushmitha, M. (2017). A new Stability indicating RP-HPLC method for the determination of Apremilast-An Antirheumatic drug. Research Journal of Pharmacy and Technology, Apr 1; 10(4):1160.
- Atila, H. A., Suhelya, K. H., (1994) Preparation of Micropellets by Spray Congealling. In: Multiparticulate Oral Drug Delivery, Marcel DekkarInc, New York, 17-34.
- Attimarad, M., Ahmed, K.M., Aldhubaib, B.E., Harsha, S. (2021). High-performance thin layer chromatography: A powerful analytical technique in pharmaceutical drug discovery. Pharmaceutical methods; 2(2), 71.
- Barankin, B., Silver, S. G., & Carruthers, A. (2002). The skin in pregnancy. Journal of Cutaneous Medicine and Surgery, 6(3), 236–240.
- Basrur, V., Yang, F., Kushimoto, T., Higashimoto, Y., Yasumoto, K., Valencia, J., Muller, J., Vieira, W. D., Watabe, H., Shabanowitz, J., Hearing, V. J., Hunt, D. F., & Appella, E. (2003). Proteomic analysis of early melanosomes: Identification of novel melanosomal proteins. Journal of Proteome Research, 2(1), 69–79.
- Beckett, A. H., Stenlake, J. B., (2002). Practical Pharmaceutical Chemistry. Part-2. 4th ed. New Delhi: CBS Publishers and Distributors; 275-325.
- Berson, J. F., Harper, D. C., Tenza, D., Raposo, G., & Marks, M. S. (2001). Pmel17 initiates premelanosome morphogenesis within multivesicular bodies. Molecular Biology of the Cell, 12(11), 3451–3464.
- Bhalaria, M. K., Naik, S., & Misra, A. N. (2019). Ethosomes: A novel delivery

system for antifungal drugs in the treatment of topical fungal diseases. Indian Journal of Experimental Biology, 47(5), 368–375.

- Bhole, R. P., Shinde, S.S., Chitlange, S. S., Wankhede, S. B., (2021). A high-performance thin layer chromatography (HPTLC) method for simultaneous determination of diphenhydramine hydrochloride and naproxen sodium in tablets. An Alytical chemistry insight.10: ACI-S31506.
- Bhole, R. P., Tamboli, F. R., (2018). Development and Validation of Stability Indicating HPTLC-MS Method for Estimation of Empagliflozin in Pharmaceutical Dosage Form. Analytical Chemistry Letters. 8(2), 244-56.
- Bhole, R. P., Wankhede, S. B., Pandey, M., (2017). Stability Indicating HPTLC Method for Simultaneous Estimation of Empagliflozin and Linagliptin in Pharmaceutical Formulation. Analytical Chemistry Letters. 2;7(1), 76-85.
- Bhoomi, P. (2020). Design and Development of Transferosome of Fluconazole for Topical Drug Delivery System, Pharma Science Monitor, 5(2), Sup-1, 298-312.
- Biju, S. S., Talegaonkar, S., Mishra, P. R., & Khar, R. K. (2006). Vesicular systems: An overview. Indian Journal of Pharmaceutical Sciences, 68(2).
- Boissy, R. E., & Nordlund, J. J. (1997). Molecular basis of congenital hypopigmentary disorders in humans: A review. Pigment Cell Research, 10(1–2), 12–24.
- Brenner, M., & Hearing, V. J. (2008). The Protective Role of Melanin Against UV Damage in Human Skin. Photochemistry and Photobiology, 84(3), 539–549.
- Brookfield DV-II, Programmable viscometer operating instruction manual No. M/03-165-A0404.
- Brues, A. M. (1992). Biological perspectives on human pigmentation. By A. H. Robins. xiii + 253 pp. New York: Cambridge University Press. 1991. $69.50 (cloth). American Journal of Human Biology, 4(4), 557–558.
- Cayce, K. A., Feldman, S. R., & McMichael, A. J. (2004). Hyperpigmentation: A review of common treatment options. Journal of Drugs in Dermatology: JDD, 3(6), 668–673.
- Cevc, G. (1991). Isothermal lipid phase transitions. Chemistry and Physics of Lipids,

57(2–3), 293–307.

- Cevc, G., & Blume, G. (1992). Lipid vesicles penetrate into intact skin owing to the transdermal osmotic gradients and hydration force. Biochimica et Biophysica Acta (BBA) - Biomembranes, 1104(1), 226–232.
- Cevc, G., Blume, G., Schätzlein, A., Gebauer, D., & Paul, A. (1996). The skin: A pathway for systemic treatment with patches and lipid-based agent carriers. Advanced Drug Delivery Reviews, 3(18), 349–378.
- Chatwal, G. R., Sharma, A. K. (2002). Instrumental Methods of Chemical Analysis. 5th ed. Mumbai: Himalaya Publishing House; 2.149-2.184.
- Chitlange, S. S., Tawargeri, S. R., Bhole, R. P.,(2023). Stability indicating HPTLC method for simultaneous estimation of Amoxicillin trihydrate and Ambroxol hydrochloride in bulk and pharmaceutical dosage form. Pharm Anal Acta., 4(261), 2.
- Choi, J.H., Cho, S. H. (2021). Ethosomes and Transfersomes for Topical Delivery of Ginsenoside Rh1 from Red Ginseng: Characterization and In Vitro Evaluation, Journal of Nanoscience and Nanotechnology, Volume 15, 8.
- Choi, Jae-Hwan Cho., (2021). Ethosomes and Transfersomes for Topical Delivery of Ginsenoside Rh1 from Red Ginseng: Characterization and In Vitro Evaluation, *Journal of Nanoscience and Nanotechnology*, Vol 15, 8.
- Chopra, R., Alderborn, G., Podczeck, F., Newton, J. M., (2002). The influence of pellet shape and surface properties on the drug release from uncoated and coated pellets. International journal of pharmaceutics. 239(1-2):171-8.
- Christen, G. D. (2003). Analytical Chemistry. 5th ed. John Wiley and Sons; 35-42, 131-132.
- Costin, G.-E., & Hearing, V. J. (2007). Human skin pigmentation: Melanocytes modulate skin color in response to stress. The FASEB Journal, 21(4), 976–994.
- Cotsarelis, G., Sun, T. T., & Lavker, R. M. (1990). Label-retaining cells reside in the bulge area of pilosebaceous unit: Implications for follicular stem cells, hair cycle, and skin carcinogenesis. Cell, 61(7), 1329–1337. https://doi.org/10.1016/0092-8674(90)90696-c
- Couse, J. F., Lindzey, J., Grandien, K., Gustafsson, J. A., & Korach, K. S. (1997).

Tissue distribution and quantitative analysis of estrogen receptor-alpha (ERalpha) and estrogen receptor-beta (ERbeta) messenger ribonucleic acid in the wild-type and ERalpha-knockout mouse. Endocrinology, 138(11), 4613–4621.

- Damle, M. (2020). Glycyrrhiza glabra (Liquorice) - a potent medicinal herb, International Journal of Herbal Medicine,; 2(2): 132-136.
- Deshmukh,K., and Poddar,S.S. (2022). Tyrosinase inhibitor-loaded microsponge drug delivery system: new approach for hyperpigmentation disorders, Journal of Microencapsulation, 29(6): 559–568.
- Dong, M. W. (2006). Modern HPLC for practicing scientists. New Jersey: John Wiley & Sons, Inc., Publication, Hoboken; 1-9.
- Ebanks, J. P., Wickett, R. R., & Boissy, R. E. (2019). Mechanisms regulating skin pigmentation: The rise and fall of complexion coloration. International Journal of Molecular Sciences, 10(9), 4066–4087.
- Eldem, T., Speiser, P., Hincal, A., (1991) Optimization of spray-dried and-congealed lipid micropellets and characterization of their surface morphology by scanning electron microscopy. Pharmaceutical research. ; 8(1):47-54.
- Elias, P. M. (2005). Stratum corneum defensive functions: An integrated view. The Journal of Investigative Dermatology, 125(2), 183–200.
- Embil, K., Nacht, S., (1996). The microsponge delivery System (MDS): A topical delivery system with reduced irritancy incorporating multiple triggering mechanisms for the release of actives. *J Microencapsul*,;13: 575–78.
- Enmark, E., Pelto-Huikko, M., Grandien, K., Lagercrantz, S., Lagercrantz, J., Fried, G., Nordenskjöld, M., & Gustafsson, J. A. (1997). Human estrogen receptor beta-gene structure, chromosomal localization, and expression pattern. The Journal of Clinical Endocrinology and Metabolism, 82(12), 4258–4265.
- Fitzpatrick, T. B., & Breathnach, A. S. (1963). [The Epidermal Melanin Unit System]. Dermatologische Wochenschrift, 147, 481–489.
- Friedmann, P. S., Wren, F. E., & Matthews, J. N. (1990). Ultraviolet stimulated melanogenesis by human melanocytes is augmented by di-acyl glycerol but not TPA. Journal of Cellular Physiology, 142(2), 334–341.

- Gandhi, B., Baheti, J., (2023). Multiparticulate Drug Delivery System: A Review, International Journal of Pharmaceutical and Chemical Science, 2(3):1620-1626.
- Garland, C. F., Garland, F. C., & Gorham, E. D. (1993). Rising trends in melanoma. An hypothesis concerning sunscreen effectiveness. Annals of Epidemiology, 3(1), 103–110.
- Gaur, A., Mittal, K.V.(2023). Formulation and Evaluation of Ketoprofen Loaded Protransfersome by Using Sodium Deoxycholate and Brij 35 International Journal of Current Pharmaceutical Review and Research, 4(3), 80-87.
- Gavali,S.M., Pacharane,S.S.,. Jadhav,K.R., and Kadam,V.J. (2021). Clinical Transfersomes: A new technique for Transdermal Drug Delivery. Indian Journal of Research in Pharmacy and Chemistry. 1(3): 735-738.
- Ghanbarzadeh,S., and Arami,S. (2023). Enhanced Transdermal Delivery of Diclofenac Sodium via Conventional Liposomes, Ethosomes, and Transfersomes Hindawi Publishing Corporation Bio Med Research International Volume, Article ID 616810, 7.
- Gilchrest, B. A., Park, H. Y., Eller, M. S., & Yaar, M. (1996). Mechanisms of ultraviolet light-induced pigmentation. Photochemistry and Photobiology, 63(1), 1–10.
- Goh, C. L., & Dlova, C. N. (1999). A retrospective study on the clinical presentation and treatment outcome of melasma in a tertiary dermatological referral centre in Singapore. Singapore Medical Journal, 40(7), 455–458.
- Gordon, P. R., & Gilchrest, B. A. (1989). Human melanogenesis is stimulated by diacylglycerol. The Journal of Investigative Dermatology, 93(5), 700–702.
- Grimes, P. E., Yamada, N., & Bhawan, J. (2005). Light microscopic, immunohistochemical, and ultrastructural alterations in patients with melasma. The American Journal of Dermatopathology, 27(2), 96–101.
- Gupta, P. N., Singh, P., Mishra, V., Jain, S., Dubey, P. K., & Vyas, S. P. (2004). Topical immunization: Mechanistic insight and novel delivery systems. IJBT Vol.3(1) [January 2004].
- Hadgraft, J. (2001). Modulation of the barrier function of the skin. Skin

Pharmacology and Applied Skin Physiology, 14 Suppl 1, 72–81.

- Hamed,A., Hamad,E.S. (2021). Evaluation of Glycyrrhiza glabra Cream as Treatment for Melasma, Evidence-based Strategies in Herbal Medicine, Psychiatric Disorders and Emergency Medicine. Intech, 252.
- Harris, S. M., Tazeen, J., Merchant, H. A., Yousuf, R. I.(2006). Evaluation of drug release kinetics from ibuprofen matrix tablets using HPMC. *Pak J Pharm Sci*,19 (2):119–24.
- Hideya, A., Atsuko, R., Akira, H., Masahiro, O., Masamitsu, I., (1998). Linoleic acid and a-linoleic acid lightens ultraviolet-induced hyperpigmentation of the skin. *Arch dermatology Res***;** 290:375–81
- High Performance Liquid Chromatography & Capillary Electrophoresis—1st Edition. (n.d.). Retrieved October 19, 2023, from https://www.elsevier.com/books/high-performance-liquid-chromatography-and-capillary-electrophoresis/weston/978-0-12-136640-7
- Higuchi, T., (1963). Mechanism of sustained action medication: Theoretical analysis of rate of release of solid drugs dispersed in solid matrices. *J Pharm Sci*,; 52:1145–9.
- ICH, Q1A (R2), (2003). Harmonized Tripartite Guideline, Stability guidelines on stability testing of new drug substances and products, IFPMA. Geneva.
- ICH, Q1B, (1996). Harmonized Tripartite Guideline, Stability testing: Photostability testing of new drug substances and products, IFPMA. Geneva1-8.
- ICH, Q2 (R1), (2005). Harmonized Tripartite Guideline, Validation of analytical procedures: Text and Methodology, IFPMA. Geneva;1- 13.
- ICH, Q2A, (1994). Harmonized Tripartite Guideline, Text on Validation of Analytical Procedures. IFPMA. Geneva;1-5.
- ICH, Q2B, (1996). Harmonized Tripartite Guideline, Validation of Analytical Procedures: Methodology, Text and Methodology, IFPMA. Geneva;1-8.
- Ito, M., Liu, Y., Yang, Z., Nguyen, J., Liang, F., Morris, R. J., & Cotsarelis, G. (2005). Stem cells in the hair follicle bulge contribute to wound repair but not to homeostasis of the epidermis. Nature Medicine, 11(12), 1351–1354.
- Jablonski, N. G. (2004). The Evolution of Human Skin and Skin Color. Annual

Review of Anthropology, 33(1), 585–623.

- Jablonski,N.G. (2004). The Evolution of Human Skin and Skin Colour, Annu. Rev. Anthropol.,33:585–623.
- Jacob, L., Anoop, K. R., (2023). A review on surfactants as edge activators in Ultadeformable vesicles for enhanced skin delivery. *Int. J. Pharm. Bio. Sci*.; 4(3): 337 – 344.
- Jadhav, S. M., Morey, P., & Karpe, M. M. (n.d.). NOVEL VESICULAR SYSTEM: AN OVERVIEW. Journal of Applied Pharmaceutical Science, 10.
- Jee, S. H., Lee, S. Y., Chiu, H. C., Chang, C. C., & Chen, T. J. (1994). Effects of estrogen and estrogen receptor in normal human melanocytes. Biochemical and Biophysical Research Communications, 199(3), 1407–1412.
- Jeffery, G. H, Bassett, J, Mendham, J, Denney R. C. (1989). Vogel's Textbook of Quantitative Chemical Analysis. 5th ed. New York: John Wiley and Sons, Inc.; 3-4.
- Jerajani, H. R., Amladi, S. T., Bongale, R., Adepu, V., Tendolkar, U. M., Sentamilselvi, G., Janaki, V. R., Janaki, C., Vidhya, S., Marfatia, Y. S., Patel, K., Sharma, N., & Cooverji, N. D. (2000). Evaluation of clinical efficacy and safety of once daily topical administration of 1% oxiconazole cream and lotion in dermatophytosis: An open label, non comparative multicentre study. Indian Journal of Dermatology, Venereology and Leprology, 66(4), 188–192.
- Jimbow, K., Obata, H., Pathak, M. A., & Fitzpatrick, T. B. (1974). Mechanism of depigmentation by hydroquinone. The Journal of Investigative Dermatology, 62(4), 436–449.
- Jody, P., Ebanks, R.(2019). Mechanisms Regulating Skin Pigmentation: The Rise and fall of Complexion Coloration, Int. J. Mol. Sci. 10, 4066-4087.
- Kang, W. H., Yoon, K. H., Lee, E.-S., Kim, J., Lee, K. B., Yim, H., Sohn, S., & Im, S. (2002). Melasma: Histopathological characteristics in 56 Korean patients. The British Journal of Dermatology, 146(2), 228–237.
- Katare, O. P., Raza, K., Singh, B., & Dogra, S. (2010). Novel drug delivery systems in topical treatment of psoriasis: Rigors and vigors. Indian Journal of Dermatology, Venereology and Leprology, 76(6), 612–621.

- Kataria,R., Singh,G. (2023). Pharmacological activities on Glycyrrhiza glabra –a review, Asian Journal of Pharmaceutical and Clinical Research, ,1(6), 02-09.
- Khopkar, S. M. (1998). Basic Concept of Analytical Chemistry. 2nd ed. New Delhi: New Age International Ltd. Publishers; 1, 178-179.
- Kippenberger, S., Loitsch, S., Solano, F., Bernd, A., & Kaufmann, R. (1998). Quantification of tyrosinase, TRP-1, and Trp-2 transcripts in human melanocytes by reverse transcriptase-competitive multiplex PCR--regulation by steroid hormones. The Journal of Investigative Dermatology, 110(4), 364–367.
- Kirti, D. and S. S. Poddar. (2022). Tyrosinase inhibitor-loaded microsponge drug delivery system: new approach for hyperpigmentation disorders, *Journal of Microencapsulation*, 29(6), 559–568
- Korhalkar, A., Deshpande, M., Lele, P. (2021). Critical Review of Various Pharmacological Activities of Glycyrrhiza Glabra (Yashtimadhu): In Vitro Studies. Asian Journal of Biochemical and Pharmaceutical Research, 1(5) 12-22
- Kothapalli, L. P., Shahane, R. R., Nanda, R. K., Thomas, A. B., (2022). Development and validation of stability indicating HPTLC method for simultaneous estimation of Domperidone maleate and Naproxen sodium in pharmaceutical formulations. Asian Journal of Research in Chemistry.28;9(7), 350-6.
- Kothapalli, L. P., Vighne, K. S., Nanda, R. K., Thomas, A. B., (2021). Development and Validation of a Stability Indicating HPTLC Method for Estimation of Artemether in Bulk and Formulated dosage form. Asian Journal of Research in Chemistry. 28;8(10), 635-42.
- Kuiper, G. G., Carlsson, B., Grandien, K., Enmark, E., Häggblad, J., Nilsson, S., & Gustafsson, J. A. (1997). Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. Endocrinology, 138(3), 863–870.
- Kulsum, S., Sagar, G.V., Butul, A., Fatima, S., Uddin, S. (2022). Method development and validation of forced degradation studies of Apremilast by using UV spectrophotometric method. World Journal of Pharmacy and Pharmaceutical Sciences, 5:1595-1601.

- Kushimoto, T., Basrur, V., Valencia, J., Matsunaga, J., Vieira, W. D., Ferrans, V. J., Muller, J., Appella, E., & Hearing, V. J. (2001). A model for melanosome biogenesis based on the purification and analysis of early melanosomes. Proceedings of the National Academy of Sciences, 98(19), 10698–10703.
- Lei,W., Yu,C., Lin,H., Zhou,X. (2023). Development of tacrolimus-loaded transfersomes for deeper skin penetration enhancement and therapeutic effect improvement in vivo, Asian journal of pharmaceutical sciences, 8, 336-345.
- Levantine, A., & Almeyda, J. (1973). Drug induced changes in pigmentation. The British Journal of Dermatology, 89(1), 105–112.
- Levy, H. (1982). Chloroquine-induced pigmentation. Case reports. South African Medical Journal = Suid-Afrikaanse Tydskrif Vir Geneeskunde, 62(20), 735–737.
- Lin J. W., Chiang H. M., Lin Y. C., Wen K. C., (2008). Natural products with skin – Whitening effects. *J Food Drug Anal*, 16 (2):1–10.
- Lindelöf, B., Sigurgeirsson, B., Tegner, E., Larkö, O., Johannesson, A., Berne, B., Ljunggren, B., Andersson, T., Molin, L., Nylander-Lundqvist, E., & Emtestam, L. (1999). PUVA and cancer risk: The Swedish follow-up study. The British Journal of Dermatology, 141(1), 108–112.
- Lonkar, N.A., Dole, M.N., Sawant ,S.D. (2017). Development and Validation of Uv-Spectrophotometric Method for the Estimation of Apremilast in Bulk Form by Absorbance Maxima Method. World Journal of Pharmacy and Pharmaceutical Sciences, 6(7): 758-766.
- Lonkar, N.A., Sawant, S.D., Dole, M.N. (2017). Development and Validation of Stability Indicating RP-HPLC Method for the Estimation of Apremilast by Forced Degradation Studies. World Journal of Pharmacy and Pharmaceutical Sciences, Feb 2; 6(04):1493-1502.
- McLeod, S. D., Ranson, M., & Mason, R. S. (1994). Effects of estrogens on human melanocytes in vitro. The Journal of Steroid Biochemistry and Molecular Biology, 49(1), 9–14.
- Michael, B., Michael, E. A., (1991). Fluidised-bed granulation: A chronology. Drug development and industrial pharmacy.17(11):1437-63.

- Mohammed, I., Verma,S., Alpana,R. (2022). Preparation and characterization of ibuprofen loaded transferosomes as a novel carrier for transdermal drug delivery system, Asian J Pharm Clin Res, 3 (5), 162-165.
- Molokhia, M. M., & Portnoy, B. (1973). Trace elements and skin pigmentation. The British Journal of Dermatology, 89(2), 207–209.
- Müller-Goymann, C. C. (2004). Physicochemical characterization of colloidal drug delivery systems such as reverse micelles, vesicles, liquid crystals and nanoparticles for topical administration. European Journal of Pharmaceutics and Biopharmaceutics: Official Journal of Arbeitsgemeinschaft Fur Pharmazeutische Verfahrenstechnik e.V, 58(2), 343–356.
- Nanda, R. K., Bhagwat, V. V., Potawale, S. E., Hamane, S. (2010). Development and validation of a HPTLC method for simultaneous densitometric analysis of cefotaxime sodium and sulbactam sodium as the bulk drugs and in the pharmaceutical dosage form. J Pharm Res.3:1667-9.
- Nanda, R. K., Kulkarni, A. A., Ranjane, M.N., Ranjane, P.N.,(2010) .Simultaneous estimation of Nadifloxacin and Mometasone Furoate in topical cream by HPTLC method. Der PharmaChemica. 2(3), 25-30.
- Nanda, R. K., Potawale, S. E., Bhagwati, V. V., Deshmukh, R. S., Deshpande, P. B., (2010). Development and validation of HPTLC method for simultaneous densitometric analysis of ranitidine hydrochloride and dicyclomine hydrochloride as the bulk drugs and in the tablet dosage form. J Pharm Res. 3(8), 1997-9.
- Nash, R.A., Wachter, A. H.,(2003). Pharmaceutical process validation. 3rd ed. New York: Marcel Dekker Inc.; 1-10.
- Naveed, A., Haji, M. K., (2021). Glycyrrhiza glabra extract cream: effects on skin pigment melanin, International Conference on Bioscience, *Biochemistry and Bioinformatics* vol.5, 434-439.
- Naveed, A., Haji, M.K. (2022). Glycyrrhiza glabra extract cream: effects on skin pigment melanin, International Conference on Bioscience, Biochemistry and Bioinformatics vol.5, 434-439.
- Nishizuka, Y. (1986). Studies and perspectives of protein kinase C. Science (New

York, N.Y.), 233(4761), 305–312.

- Palumbo, A., d'Ischia, M., Misuraca, G., & Prota, G. (1991). Mechanism of inhibition of melanogenesis by hydroquinone. Biochimica Et Biophysica Acta, 1073(1), 85–90.
- Pandey, S., Goyani, M., Devmurari, V., & Fakir, J. (2019). Transferosomes: A novel approach for transdermal drug delivery. Der Pharmacia Lettre, 1, 143–150.
- Pandey, S., Goyani, M., Devmurari, V., Fakir, J. (2019). Transferosomes: A Novel Approach for Transdermal Drug Delivery, Der Pharmacia Lettre, 1 (2) 143-150.
- Parashar,B., Kaur,A., Gupta,N., Singh,B., and Yadav.V. (2022). Transfersomes: An approach for vascular drug delivery system. International Pharmaceutical Science, 2(2): 86-92.
- Patel, B. (2020). Design and Development of Transferosome of Fluconazole for Topical Drug Delivery System, Pharma Science Monitor, 5(2), Sup-1, 298-312.
- Pathak, M. A., & Stratton, K. (1968). Free radicals in human skin before and after exposure to light. Archives of Biochemistry and Biophysics, 123(3), 468–476.
- Pathak, M. A., Fitzpatrick, T. B., & Kraus, E. W. (1986). Usefulness of retinoic acid in the treatment of melasma. Journal of the American Academy of Dermatology, 15(4), 894–899.
- Prasanthi,D.,.Lakshmi,P.K. (2022). vesicles – mechanism of transdermal permeation: a review, Asian Journal of Pharmaceutical and Clinical Research, 1(5), 18-25.
- Publishers, C. B. S. (n.d.). Sethi's High Performance Liquid Chromatography Quantitative Analysis Of Pharmaceutical Formulations Vol.7 (Hb 2021). Default Store View. Retrieved October 19, 2023, from https://www.cbspd.co.in/sethi-s-high-performance-liquid-chromatography-quantitative-analysis-of-pharmaceutical-formulations-vol-7-hb-2021
- R. S. Murthy,. (2010). Vesicular & particulate drug delivery system, *career publication*, 1st edi, **;**25-65
- Ramesh. (1997). Transdermal delivery of drugs. Indian Journal of Pharmacology, 29(3), 140.
- Ranson, M., Posen, S., & Mason, R. S. (1988). Human melanocytes as a target tissue

for hormones: In vitro studies with 1 alpha-25, dihydroxyvitamin D3, alpha-melanocyte stimulating hormone, and beta-estradiol. The Journal of Investigative Dermatology, 91(6), 593–598.

- Ravisanka, P., Sulthana, S., Sri, K.H., Ravisankar, P. (2017).Development and Validation of a Stability-Indicating Reversed Phase HPLC Method for Determination of Apremilast in Bulk and Pharmaceutical Dosage Form. Scholars Research Library, 9(06):63–73.
- Ravisankar, P., Sulthana, M.S., Babu, P.S. (2017). Development and validation of stability-indicating UV spectrophotometric method for determination of Apremilast in bulk and pharmaceutical dosage form. Indian Journal of Research in Pharmacy and Biotechnology. 5(01): 47.
- Raymond, C. R., Paul, J. S., and Sian, O., (2006). Hand Book of Pharmaceutical Excipients, edi-5. *Published by pharmaceutical press*, London, UK.
- Raymond, C. R., Paul, J. S., and Sian, O., (2006). Hand Book of Pharmaceutical Excipients, edi-5. *Published by pharmaceutical press*, London, UK.
- Reddy,Y.D., Reddy,S.R.(2021). Transferosomes A Novel Vesicular Carrier for Transdermal Drug Delivery System, JIPBS, 2 (2), 193-208.
- Robins, A. H. (1972). Skin melanin concentrations in schizophrenia. The British Journal of Psychiatry, 121(565), 613–617. https://doi.org/10.1192/bjp.121.6.613
- Rojekar, M. V., Sawant, S.D. (2021). A Short Review of Pigmentation Disorders in Systemic Diseases, Journal of Pigmentary Disorders, 2-4.
- Seiji, M., Shimao, K., Birbeck, M. S., & Fitzpatrick, T. B. (1963). Subcellular localization of melanin biosynthesis. Annals of the New York Academy of Sciences, 100, 497–533.
- Serup, J., Kluger, N., & Bäumler, W. (2021). Tattooed Skin and Health. Karger Medical and Scientific Publishers.
- Sethi, P. D. (2001). High Performance Liquid Chromatography, Quantitative Analysis of Pharmaceutical Formulations. 1st ed. New Delhi: CBS Publishers and Distributors; 3-11, 116-120.
- Sharma, B. K. (2005). Instrumental Methods of Chemical Analysis. 24th ed. Meerut:

Goel Publication House; S-3-S-8.

- Sharma, S. K., Chauhan, M., & Anilkumar, N. (2019). Span-60 Niosomal Oral Suspension of Fluconazole: Formulation and In Vitro Evaluation. Asian Journal of Pharmaceutical Research and Health Care, 1(2), 142–156.
- Sies, H., & Stahl, W. (2004). Nutritional protection against skin damage from sunlight. Annual Review of Nutrition, 24, 173–200.
- Singh, S., Bakshi, M., (2002) Guidance on The conduct of Stress tests to determine Inherent Stability of Drugs, Pharmaceutical Technology.
- Sinico, C., & Fadda, A. M. (2019). Vesicular carriers for dermal drug delivery. Expert Opinion on Drug Delivery, 6(8), 813–825.
- Skoog, D. A., Holler, F. J., Crouch, S. R., (2004). Principle of Instrumental Analysis. 5th ed. Thomson Brooks/Cole; 1-2, 678-688.
- Skoog, D. A., Holler, F. J., Nieman, T. A. (2007). Principles of Instrumental Analysis. 6th ed., Thomson Brooks/Cole.; 762-63,816-827.
- Smith, A. G., Shuster, S., Thody, A. J., & Peberdy, M. (1977). Chloasma, oral contraceptives, and plasma immunoreactive beta-melanocyte-stimulating hormone. The Journal of Investigative Dermatology, 68(4), 169–170.
- Stern, R. S. & PUVA Follow up Study. (2001). The risk of melanoma in association with long-term exposure to PUVA. Journal of the American Academy of Dermatology, 44(5), 755–761.
- Stern, R. S., Nichols, K. T., & Väkevä, L. H. (1997). Malignant melanoma in patients treated for psoriasis with methoxsalen (psoralen) and ultraviolet A radiation (PUVA). The PUVA Follow-Up Study. The New England Journal of Medicine, 336(15), 1041–1045.
- Studies on Solubility, Bioavailability and Hypoglycemic Activity of Gliclazide β-cyclodextrin Complexes. (n.d.).
- Swadesh J. K. (2002). HPLC Practical and Industrial Applications. 2nd ed. USA: CRC Press; 141-143.
- Swarbrick, J., Boylan, J. C. (1997). Encyclopedia of Pharmaceutical Technology. Vol-1., 3rd ed. New York: Marcel Dekker Inc.; 538-540.

- Thoma, K., Ziegler, I. (1998). The pH-independent release of fenoldopam from pellets with insoluble film coats1. European journal of pharmaceutics and biopharmaceutics. 1; 46(1):105-13.
- Thorat, S. G., Chikhale, R. V., Tajne, M. R, (2017). A validated stability-indicating high-performance thin-layer chromatographic method for the analysis of methotrexate in bulk drug and marketed injection. JPC-Journal of Planar Chromatography-Modern TLC; 30(1),75-9.
- Thorat, S.G., Chikhale,R.V., Tajne, M.R. (2017). A validated stability-indicatinghigh-performance thin-layer chromatographic method for the analysis of methotrexate in bulk drug and marketed injection. JPC-Journal of Planar Chromatography-Modern TLC, Feb; 30(1):75-9.
- Thornton, M. J. (2005). Oestrogen functions in skin and skin appendages. Expert Opinion on Therapeutic Targets, 9(3), 617–629.
- Tomita, Y., Iwamoto, M., Masuda, T., & Tagami, H. (1987). Stimulatory effect of prostaglandin E2 on the configuration of normal human melanocytes in vitro. The Journal of Investigative Dermatology, 89(3), 299–301.
- Touitou, E. (2002). Drug delivery across the skin. Expert Opinion on Biological Therapy, 2(7), 723–733.
- V, M., & Sangeetha, S. (2023). Transfersomes: A Novel Vesicular Drug Delivery System for Enhanced Permeation through Skin. Research Journal of Pharmacy and Technology, 13(5), 2493–2501.
- Verdier-Sévrain, S., Bonté, F., & Gilchrest, B. (2006). Biology of estrogens in skin: Implications for skin aging. Experimental Dermatology, 15(2), 83–94.
- Vishwanathan,V., Mukne,A.P. (2022). Development and validation of HPLC and HPTLC methods for estimation of glabridin from the extract of Glyclyrrhiza glabra, journal of AOAC International, 99, 2, 374-379.
- Vivek, V., Alka, M., (2022). development and validation of HPLC and HPTLC methods for estimation of glabridin from the extract of Glyclyrrhiza glabra, *journal of AOAC International*; 99, 2, 374-379.
- Wang, S. Q., Setlow, R., Berwick, M., Polsky, D., Marghoob, A. A., Kopf, A. W., &

Bart, R. S. (2001). Ultraviolet A and melanoma: A review. Journal of the American Academy of Dermatology, 44(5), 837–846.

- Wei Lei, Chuqin Yu, Huaqing Lin, Xiaoyuan Zhou, (2023). Development of tacrolimus-loaded transfersomes for deeper skin penetration enhancement and therapeutic effect improvement in vivo, *Asian journal of pharmaceutical sciences*, 8; 336-345.
- Weston, A., Brown, P. R. (1997). High Performance Liquid Chromatography, and CE – Principles and practice. 1st ed. California: Academic Press; ,1-4, 8-11, 24-26, 29-32, 71.
- Willard, H. H., Meritt, L. L., Dean, J. A., Settle, F. (2001). Instrumental Method of Analysis, 7th ed. Delhi: CBS Publishers and Distributors, 14.
- Williams Textbook of Endocrinology—12th Edition. (n.d.). Retrieved October 19, 2023, from https://www.elsevier.com/books/williams-textbook-of-endocrinology/melmed/978-1-4377-0324-5
- Wolff, K., Goldsmith, L., Katz, S., Gilchrest, B., Paller, A., & Leffell, D. (2008). Fitzpatrick‘s Dermatology in General Medicine. 7th Edition. McGraw-Hill.
- Xiong, K., Ma, X., Cao, N., Liu, L., Sun, L., Zou, Q., Wei, P. (2022). Identification, characterization and HPLC quantification of impurities in Apremilast. Analytical Methods, 8(8):1889-97.
- Yoshioka, T., Sternberg, B. F., Lorence A. T., (1994). Preparation and properties of vesicles (niosomes) of sorbitan monoesters (Span 20, 40, 60 and 80) and a sorbitan triester (Span 85*). Int J Pharm***;*** 105:1-6.

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CONFERENCE Sunday 29 Oct. 2023

CERTIFICATE OF RECOGNITION

This is to certify that

Prof./ Dr./Mr./Ms. **STUTI PANDEY, RESEARCH SCHOLAR, P K UNIVERSITY, SHIVPURI (M.P)**

has participated as Delegate in International Conference **IC-23**

on ' Global Prospective of Pharmacy Education: Bridging the Gap Between Academia and Industry Under NEP-2020 '

organised by **SHREE KRISHNA COLLEGE OF PHARMACY, Sitapur (U.P.) India**

on Sunday, 29th October 2023.

He/She has also presented Paper/ Poster entitled as

RP-HPLC TECHNIQUE FOR QUANTIFICATION OF GLABRIDIN'S EVOLUTION IN POLYHERBAL FORMULATION

Vivek Agarwal	Dr. Rakesh Saini	Dr. Neelkanth Mani Pujari	Dr. Satyendra Singh	Dr. Anurag Mishra
Patron/ Manager	*Convenor*	*Co-Convenor*	*Co-Organising Secretary*	*Director & Organising Secretary*

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STUTI PANDEY

RESEARCH SCHOLAR, FACULTY OF PHARMACY, P K UNIVERSITY, SHIVPURI (M.P) INDIA.

has participated in the conference. He/She has also presented a paper entitled

CREATIVITY AND APPROVAL OF AN INVERTED PHASE HPLC METHOD THAT CONSTANTLY INDICATES FOR APRIMILLAST SOLVING IN BULK AND PHARMACEUTICAL DOSAGE FORM

Prof. S.S. Modi	Dr. Snigdha Sharma	Dr. Jolly Sahni	Dr. Ravi Kant Modi	Dr. Gaurav Jain
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RP-HPLC TECHNIQUE FOR QUANTIFICATION OF GLABRIDIN'S EVOLUTION IN POLYHERBAL FORMULATION

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ABSTRACT

A technique for evaluating glabridin in unrefined medications and blends of polyherbal remedies has been created and approved. It is simple, affordable, focused, accurate, and durable. Using a gradient elution approach and acetonitrile and water as the mobile phases at a flow rate of 1 mL/min, phase-reversal chromatography is performed on a C18 column. At 230 nm, glaciridin is identified, and a prominent peak is seen with a retention time of 14.9+0.02 min. A good linear correlation between response and concentration in the 1–500 mg/mL range was found by the calibration plot's linear regression analysis; the linear regression equation is y 5 26.683 xs – 142.17, and the regression coefficient is 0.9992. The approach is validated for robustness, accuracy, precision, repeatability, and quantification restrictions, in accordance with the guidelines provided by the International Conference on Harmonization. Statistics show that the process for glabridin analysis is precise, repeatable, selective, and accurate. Application of the high-performance liquid chromatography method, which has been developed, validated, and suggested for glabridin measurement, can be used for quality control and standardization of licorice (Glycyrrhiza glabra Linn.) and various herbal formulations containing licorice as an ingredient.

Key Words-: Harmonisation, Glabridin, Glycyrrhiza glabra,

INTRODUCTION

For almost 4,000 years, people have been consuming licorice, sometimes referred to as "sweet root," which is the root of the leguminous Glycyrrhiza plant species. [4-6[(3R)-8,8-dimethyl-3,4-dihydro-2H-pyrano][6, 5-f]chromen-3-yl] Glabridin[Benzene-1,3-diol] A significant polyphenolic flavonoid unique to Glycyrrhiza glabra L. is shown in Figure 1. Recent investigations have demonstrated the anticancer efficacy of glabridin by blocking the focal adhesion

kinase/Rho signalling pathway, which in turn prevents the migration, invasion, and angiogenesis of human non-small cell lung cancer A549 cells and MDA-MB-231 human breast adenocarcinoma cells (1, 2). It has also been observed that glabridin exhibits a number of pharmacological properties, including antioxidant (3), anti-Helicobacter pylori (4), antifungal (5), estrogenic, anti-proliferative (6, 7) and antinephritic activity (8). Furthermore, glabridin suppresses inflammation (10), melanogenesis (9), and serotonin reuptake (10).

In India and many other nations, the Unani medical method is widely used. Various herbal formulations, including solid (Qurs, Habbs, Safoof), semi-solid (Khamira, Itrifal, Majoon), and liquid (Sharbat) preparations, are employed in the Unani system (11, 12). The tablet form Qurs-e-Gul is listed in the National Formulary of Unani Medicines (NFUM). It is frequently given in the Unani medical system as a deobstruent and to treat cardiac-related issues and jaundice. Glycyrrhiza glabra (28.57% w/w), Pistacia lentiseus (4.7% w/w), Bamboosa bambo Druce (4.76% w/w), Nardostachys jatamansi (14.28% w/w), Rosa damascene (47.6% w/w), and excepients are its five constituents (13).

Glabridin has been quantified using a number of analytical techniques, including capillary electrophoresis (17), liquid chromatography–mass spectrometry (LC–MS) (18), and high-performance liquid chromatography (HPLC) (14–16). The isocratic HPLC technique that was previously reported was found to have a relatively narrow linearity range (10–100 mg/mL) and to have a poor recovery rate of 92%. Additionally, it was discovered that the isocratic approach that was previously published was unsuitable for separating the primary ingredients in polyherbal formulations from their immediate contaminants. The development and validation of a gradient reversed-phase (RP)-HPLC method was deemed worthwhile in order to quantify glabridin in both crude drug and polyherbal formulations. This method is capable of effectively separating glabridin in multicomponent formulations with improved validation parameters. Comparatively speaking, the recently developed and verified HPLC method is easier to use and more effective for quantifying glaciridin in polyherbal mixtures than previously documented techniques.

METHODOLOGY

Reagents and chemicals

Glabridin (98%) was obtained as a gift sample from Sami Labs (Bangalore, India). HPLC-grade acetonitrile and methanol were purchased from Merck (India). Milli-Q water used throughout

the experiment was prepared using a Millipore water purification system.

Instrumentation and general conditions

Chromatographic experiments were conducted on a YL9100 HPLC system (South Korea) that comprised quaternary YL9110 pumps, a variable wavelength programmable YL9120 ultraviolet (UV)-visible detector, YL9130 column oven and a system controller. The instrument was controlled by use of YL-Clarity software installed with the equipment. Samples were injected by using a rheodyne injector fitted with a 20-mL fixed loop. Standard and sample solutions were filtered through a 0.22-mm syringe filter before injection. The separation was achieved by using a C18 reversed-phase column (Merck Lichrocart 250-4, Lichrosphere 100 RP-18e, 5 mm, Sorbent lot number L57020637). The mobile phase consisted of acetonitrile and water in gradient elution method from 50 to 80% in 20 min. The flow rate was kept at 1.0

mL/ min. All the analyses were performed at room temperature and detection was carried out at a wavelength of 230 nm using a UV-visible detector.

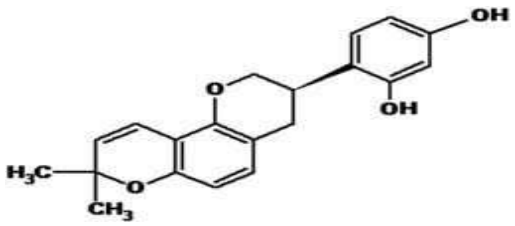

Figure 1. Chemical structure of glabridin

Preparation of sample solutions

Preparation of sample for crude One gram of the powdered crude drug was extracted with 25 mL of 30% aqueous ethanol by soaking overnight and then refluxing on a water bath for 45 min. It was filtered and evaporated to dryness under reduced pressure. The obtained residue was then reconstituted in HPLC-grade methanol and the volume was adjusted to 25 mL, which was filtered through a 0.22-mm syringe filter before injecting into the HPLC column.

Preparation of sample for polyherbal formulation twenty tablets were randomly selected from the formulation and average weight was determined. The tablets were triturated to get a uniform fine powder. Two grams, accurately weighed, of the powdered sample was extracted with 25 mL

of 30% aqueous ethanol, as discussed previously. The obtained residue was then reconstituted in HPLC-grade methanol and volume was adjusted to 25 mL, which was filtered through a 0.22-mm syringe filter before injecting into the HPLC column.

Validation methodology

Method validation was carried out to confirm the suitability of the proposed analytical method for its intended use. The proposed method was validated as per International Conference on Harmonization (ICH) (19) guidelines for different parameters such as linearity, accuracy, precision, limit of detection (LOD) and limit of quantification (LOQ) and robustness, similar to the previously reported laboratory methods .

Calibration curve for glabridin

A stock solution of glabridin with a known concentration of 1,000 mg/mL was prepared in methanol and different aliquots were made to get six different desired concentrations from 1 to 500 mg/mL, which were injected (20 mL each) by rheodyne injector and chromatographed as per the previously mentioned protocol. The stock solution was kept in dark for storage to avoid possible degradation that may result from exposure to light.

Accuracy as recovery

The accuracy of the method was determined by recovery studies using the standard addition method. Preanalyzed samples were spiked with standard glabridin at three different concentration levels, i.e., 50, 100 and 150%, and the mixtures were reanalyzed by the proposed method. Data obtained was analyzed for percent recovery.

Precision

The precision of the method was carried out by performing repeatability and intermediate precision. In repeatability, six different injections of the same standard sample (three concentrations) were injected and calculated in the assay. The percent relative standard deviation (%RSD) of the area and retention time (Rt) were calculated. In intermediate precision, intra-day, inter-day, and inter-system precisions were performed. Intra-day and inter-day precisions were performed by preparing and applying three different concentrations of standard in triplicate six times a day and on six different days, respectively. Inter-system precision was performed by repeating the same procedure in a different HPLC system. Assay for each analysis was calculated and %RSD was determined.

LOD and LOQ

The LOD and LOQ were determined based on the basis of signal-to-noise ratio. The concentration of the sample with a signal-to-noise ratio of three was fixed as the LOD. The concentration of the sample with a signal-to-noise ratio of ten was fixed as the LOQ.

Robustness of the method

Robustness of the method was performed by introducing very small changes in the analytical methodology at a single concentration level (100 mg/mL). Robustness of the proposed method was determined in two different ways, i.e., by making deliberate changes in the flow rate and by changing the detection wavelength of the analysis. In the present study, the robustness was evaluated by using the Box-Behnken response surface design (24). The design simultaneously evaluated the effects of the three important parameters on peak area: flow rate of the mobile phase, detection wave length and temperature of the column oven. Design Expert version 7.0.0.1

(Stat-Ease, Minneapolis, MN) was used to evaluate the results.

Three-dimensional graphs represented peak area dependence on flow rate of the mobile phase and detection wavelength temperature of the column and flow rate of the mobile phase and temperature of the column and detection wavelength. Effects of the selected factors were evaluated over a range of conditions by determining the maximum area response of the glabridin peaks.

RESULTS AND DISCUSSION

Method development

In order to create an HPLC method suited for glabridin analysis in crude drug and polyherbal formulations, a range of mobile phases were explored. The acetonitrile-phosphate buffer (pH 3.5 adjusted with orthophosphoric acid), 80:20 (% v/v), methanol–water, 50:50 (% v/v), acetonitrile–phosphate buffer (pH 3.5 with orthophosphoric acid), 60:40 (% v/v), acetonitrile–water, 50:50 (% v/v), and acetonitrile–water, 60:40 (% v/v) were among the investigated mobile phases. At a retention period of 9.1+0.02 minutes, a strong peak of glabridin was seen when acetonitrile and water were mixed 60:40 (% v/v). Unfortunately, due to inadequate separation from the immediate contaminants, this mobile phase proved ineffective in effectively separating the chemical in the polyherbal formulation. In order to achieve good separation from the contaminants, a strong peak at the Rt of 14.9+0.02 min was obtained by creating a gradient elution system with an acetonitrile concentration of 50 to 80% within 20 minutes (Figure 2). Additionally, YL Clarity software was

used to optimise chromatographic settings and determine system appropriateness parameters such as theoretical plates (7,739), tailing factor (0.982), and asymmetry (0.958) of glabridin. These calculations demonstrated the applicability of the suggested approach.

Validation of analytical method

Linearity

A stock solution containing 1,000 mg/mL was made, and aliquots ranging from 1 to 500 mg/mL were taken from it in order to evaluate the linearity of the procedure. For storage, the stock solution was maintained in the dark. With an excellent regression coefficient value of 0.9992, the linearity of the calibration for glabridin was evaluated in the range of 1–500 mg/mL; the regression equation is y ¼ 26.683x – 142.17. It was discovered that the slope+SD and intercept+SD were, respectively, 25.13633+0.52 and 141.11+1.2.

Accuracy

The accuracy of the method was determined by recovery studies. The preanalyzed samples were spiked with standard at three different concentration levels, i.e., 50, 100 and 150%. The mixtures were reanalyzed by the proposed method and found to be within the limit of 97.39–103.25%, which is better than the method reported by Shanker et al. (14). The values of recovery percent and %RSD are listed in Table I.

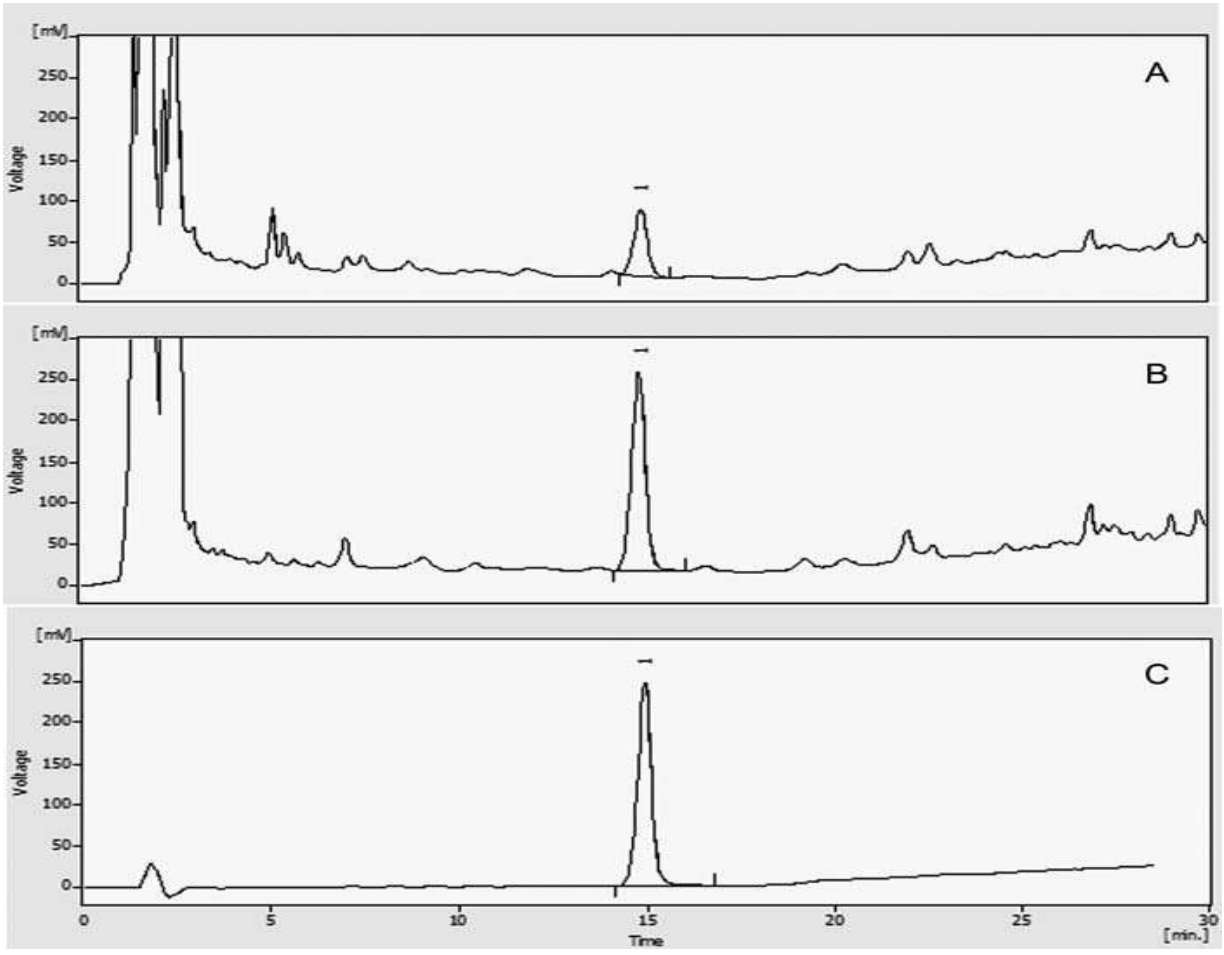

Figure 2. HPLC chromatogram of glabridin at 230 nm: standard (A); Glycyrrhiza glabra (B); Qurs-e-Gul tablet formulation (C).

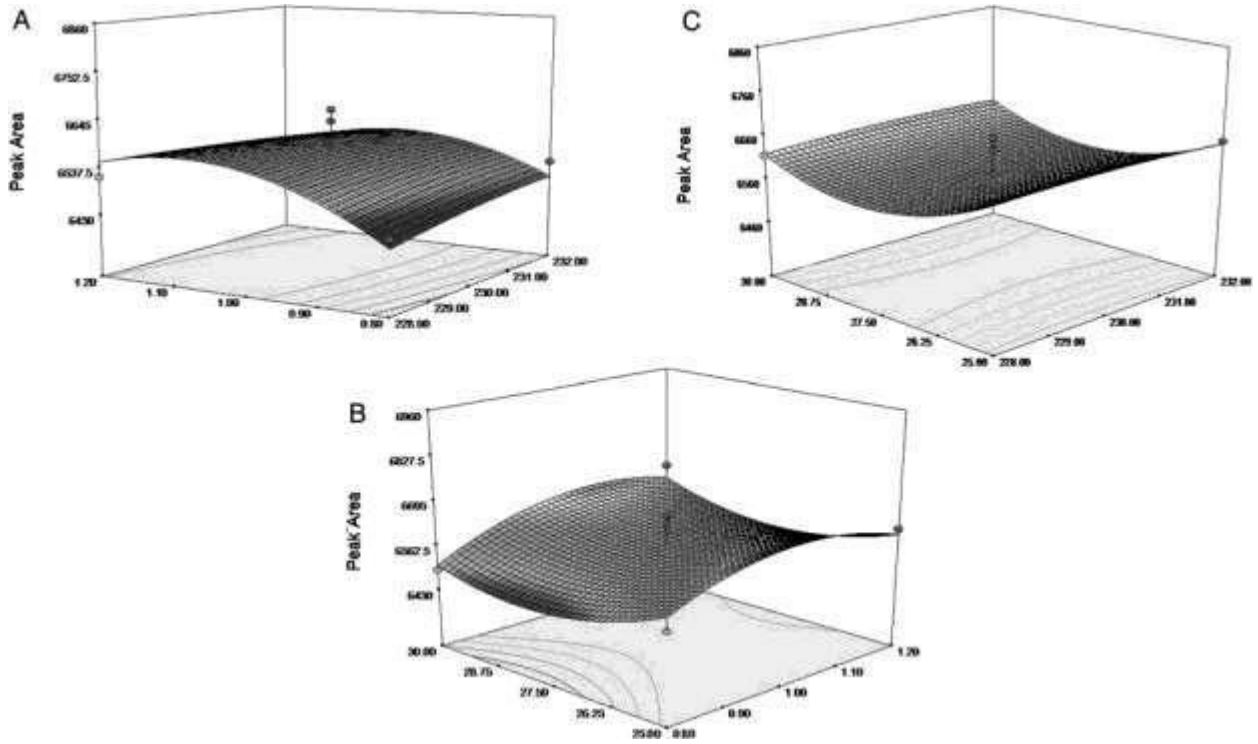

Figure 3. Three-dimensional graphs: Peak area ¼ f (flow rate of the mobile phase, detection wave length) (A); peak area ¼ f (temperature of the column, flow rate) (B); peak area ¼ f (temperature of the column, detection wave length) (C).

Precision

According to the ICH guidelines, repeatability and intermediate precision were used to determine the suggested method's precision. The assay was calculated using six distinct injections of the same standard sample (three concentrations) in order to test repeatability. Rt and the area's %RSD were computed. Three distinct sample concentrations were prepared and applied on three separate days, respectively, to achieve inter-day and intra-day precisions. To achieve inter-system precision, the same process was carried out again using a different system.

LOD and LOQ

The limits of quantification and detection were calculated by using the linearity curve method by using the formula LOD ¼ 3.3s/S and LOQ ¼ 10s/S, where s is the standard deviation of the response and S is the slope of the calibration plot. For the developed method, LOD was found to be 0.35 mg/mL and LOQ was calculated at 1 mg/mL. Once the LOD and LOQ were determined, six replicates of blank and standard solutions at the levels of LOD and LOQ were applied and the %RSD was calculated.

Robustness

The robustness was evaluated by using the Box-Behnken response surface design. The Design Expert software proposed the following polynomial equation for peak area: Peak area ¼ 6572.00 þ 0.76A þ 35.22B – 11.42C – 24.68AB þ 3.16AC

þ 16.77BC – 7.38A2 – 73.39B2 – 69.63C2, where A is the detection wavelength (nm), B is the flow rate (mL/min) and C is the temperature (8C). According to the equation, flow rate appeared to have more effect on the peak area than detection wavelength and temperature. As the flow rate increases, peak area also increases. The detection wavelength was also found to increase the peak area, as indicated by the positive coefficient value. However, the lower magnitude of the coefficient indicated that the effect of detection wavelength is less than the flow rate on peak area.

CONCLUSION

It was possible to develop and validate an RP-HPLC–UV approach that is simple, inexpensive, accurate, precise, repeatable, resilient, and robust for the analysis of glabridin in crude drug and polyherbal formulations over a wide concentration range. Neither the standard nor the samples showed any interference when glabridin was being eluted.The validation results displayed good precision and accuracy together with the method's dependability. With LOQ and LOD of 0.019 and 0.065 mg/mL, respectively, the developed method is significantly more sensitive for glabridin detection than the existing HPLC method as reported by Shanker et al. (16). The method's strong sensitivity is demonstrated by the low LOD (0.35 mg/mL) and LOQ (1 mg/mL) values. The proposed method worked pretty well for routine testing of glabridin in crude medicine and polyherbal formulation.This simple and well-established analytical method will help with future research on this important anticancer moiety to be measured in different herbal formulations. For quality control of glycyrriza root and numerous polyherbal products including it, the approach can be used instead of glycyrrhizin and glycyrrhetinic acid, which are less specific markers than glabridin.

REFERENCES

1. Tsai, Y.M., Yang, C.J., Hsu, Y.L., Wu, L.Y., Tsai, Y.C., Hung, J.Y., et al.;Glabridin inhibits migration, invasion, and angiogenesis of human non-small cell lung cancer a549 cells by inhibiting the FAK/Rho signaling pathway; Integrative Cancer Therapies, (2021); 10: 341–349.
2. Hsu, Y.L., Wu, L.Y., Hou, M.F., Tsai, E.M., Lee, J.N., Liang, H.L., et al.; Glabridin, an isoflavan from licorice root, inhibits migration, invasion and angiogenesis of MDA-MB-231 human breast adenocarcinoma cells by inhibiting focal adhesion kinase/Rho signaling pathway; Molecular Nutrition and Food Research, (2021); 55: 318–327.
3. Carmeli, E., Harpaz, Y., Kogan, N.N., Fogelman, Y.; The effect of an endogenous antioxidant glabridin on oxidized LDL; Journal of Basic and Clinical Physiology and Pharmacology, (2018); 19: 49–63.
4. Fukai, T., Marumo, A., Kaitou, K., Kanda, T., Terada, S., Nomura, T.; Anti-helicobacter pylori flavonoids from licorice extract; Life Science, (2022); 71: 1449–1463.

5. Fatima, Gupta, V.K., Luqman, S.,Negi, A.S., Kumar, J.K., Shanker, K., et al.; Antifungal activity of Glycyrrhiza glabra extracts and its active constituent glabridin; Phytotherapy Research, (2009); 23: 1190–1193.
6. Somjen, D., Katzburg, S., Vaya, J., Kaye, A.M., Hendel, D., Posner, G.H., et al.; Estrogenic activity of glabridin and glabrene from licorice roots on human osteoblasts and prepubertal rat skeletal tissues; Journal of Steroid Biochemistry and Molecular Biology, (2004); 91: 241–246.
7. Choi, E.M.; The licorice root derived isoflavan glabridin increases the function of osteoblastic MC3T3-E1 cells; Biochemical Pharmacology, (2005); 70: 363–368.
8. Toshio, F., Kazue, S., Taro, N., Hiroshi, S.; Antinephritis and radical scavenging activity of prenylflavonoids; Fitoterapia, (2023); 74: 720–724.
9. Ofir, R., Tamir, S., Khatib, S., Vaya, J.; Inhibition of serotonin re-uptake by licorice constituents; Journal of Molecular Neuroscience, (2023); 20: 135–140.
10. Yokota, T., Nishio, H., Kubota, Y., Mizoguchi, M.; The inhibitory effect of glabridin from licorice extracts on melanogenesis and inflammation; Pigment Cell Research, (1998); 11: 355–361.
11. The Unani Pharmacopoeia of India. Ministry of Health and Family Welfare. Department of AYUSH, Government of India, New Delhi, (2017); pp. 32–33.
12. Ahmad, S., Rehman, S., Ahmad, M.A., Siddiqui, K.M., Shaukat, S., Khan, M.S., et al.; Khamiras, a natural cardiac tonic: An overview; Journal of Pharmacy and BioAllied Sciences, (2010); 2: 93–99.
13. National Formulary of Unani Medicine. Ministry of Health and Family Welfare, Department of AYUSH. Government of India, New Delhi, (2006); pp. 30–31.
14. Shanker, K., Fatima, A., Negi, A.S., Gupta, V.K., Darokar, M.P., Gupta, M.M., et al.; RP-HPLC method for the quantitation of glabridin in Yashti-madhu (Glycyrrhiza glabra); Chromatographia, (2017); 65: 771–774.
15. Tian, M., Yan, H., Row, K.H.; Extraction of glycyrrhizic acid and glabridin from licorice; International Journal of Molecular Science, (2018); 9: 571–577.
16. Jirawattanapong, W., Saifah, E., Patarapanich, C.; A validated stability-indicating HPLC method for analysis of glabridin prodrugs in hydrolysis studies; Drug Discoveries and Therapeutics, (2009); 3: 97–103.
17. Rauchensteiner, F., Matsumura, Y., Yamamoto, Y., Yamaji, S., Tani, T.; Analysis and comparison of Radix Glycyrrhizae (licorice) from Europe and China by capillary-zone electrophoresis (CZE); Journal of Pharmaceutical and Biomedical Analysis, (2005); 15: 594–600.

18. Aoki, F., Nakagawa, K., Tanaka, A., Matsuzaki, K., Arai, N., Mae, T.; Determination of glabridin in human plasma by solid-phase extraction and LC-MS/MS; Journal of Chromatography B, (2005); 15: 70–4.
19. International Conference on Harmonization (ICH). Technical Requirements for the Registration of Pharmaceuticals for Human Use. Validation of Analytical Procedures: Methodology. Geneva, Switzerland, (1996).
20. Ahmad, S., Rizwan, M., Parveen, R., Mujeeb, M., Aquil, M.; A validated stability-indicating TLC method for determination of forskolin in crude drug and pharmaceutical dosage form; Chromatographia, (2018); 67: 441–447.
21. Ansari, M.J., Ahmad, S., Kohli, K., Ali, J., Khar, R.K.; Stabilityindicating HPTLC determination of curcumin in bulk drug and pharmaceutical formulations; Journal of Pharmaceutical and Biomedical Analysis, (2005); 39: 132–138.
22. Alam, P., Ali, M., Singh, R., Madhurima, Ahmad, S., Shakeel, F.; A validated HPLC method for estimation of cordifolioside A in Tinospora cardifolia Miers and marketed formulations; Journal of Chromatographic Science, (2009); 47: 910–913.
23. Rabea, R., Baboota, S., Ali, J., Ahuja, A., Ahmad, S.; Stability-indicating HPTLC method for quantitative estimation of silybin in bulk drug and pharmaceutical dosage form; Biomedical Chromatography, (2010); 24: 639–647.
24. Kuntic, V., Pejic, N., Ivkovic, B., Vujic, Z., Ilicc, K., Micic, S., Vukojevicd, V.; Isocratic RP-HPLC method for rutin determination in solid oral dosage forms; Journal of Pharmaceutical and Biomedical Analysis, (2017); 43: 718–721.
25. Akinloye, B.A., Adalumo, L.A.; Abrus precatorius leaves—A source of glycyrrhizin; Nigerian Journal of Pharmacy, (1981); 12: 405–410.

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CREATIVITY AND APPROVAL OF AN INVERTED PHASE HPLC METHOD THAT CONSTANTLY INDICATES FOR APRIMILLAST SOLVING IN BULK AND PHARMACEUTICAL DOSAGE FORM

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ABSTRACT

This paper describes the newly developed stability-indicating Reversed Phase High-Performance LC method for estimating and determining Apremilast (APR) in bulk and pharmaceutical formulation. An Eclipse XDB model C18 Column (based on 99.999% ultra high purity silica) and an Agilent Technologies model SPD 20A conspicuous UV-Vis detector were used to carry out the chromatographic separation. This study used a sample that measured 250 mm by 4.6 mm and had a particle size of 5 µ. The mobile phase used in the experiment was acetonitrile, which was injected at a volume of 20 µL and flowed at a rate of 1 ml/min. The separation was carried out at room temperature, and the eluents were seen using a laser diode array detector adjusted at 229 nm. The measured APR retention duration was 2.488 minutes. The calibration curve for APR was linear (r2=0.9989) for the concentration range that was examined, with 0.003 µg/ml and 0.001 µg/ml for LOD and LOQ, respectively. In tablet format, APR was found to recover between 99.18 to 101.61%. The bulk medication and APR pills (Otezla) yielded % assay findings of 99.90 ± 0.001 and 99.80 ± 0.002, respectively. The stability of the method was demonstrated by forced degradation trials of the medication under acidic, alkaline, oxidative, photolytic, and thermal stress settings, in accordance with ICH Q1A (R2) requirements. As a result, it was shown that the proposed method for estimating APR could be used to both bulk and pharmaceutical dose form.

Key Words: *Apremilast; RP-HPLC;Validation;Forced degradation.*

INTRODUCTION

The chemical name for APR is N-[2-[(1S)-1-(3-ethoxy-4-methoxyphenyl)-2-(methylsulfonyl) ethyl]-2, 3-dihydro-1,3-dioxo- 1H-isoindol-4-yl] acetamide. It is soluble in many organic solvents such as acetonitrile and DMSO, but insoluble in aqueous media. It is utilized for the healing of certain types of Psoriasis [1] and Psonatic arthritis. It may also be utilized for other immune system associated inflammatory diseases [3]. APR is a selective inhibitor of the enzyme phosphodiesterase 4 [4-5] andstops spontaneous production of TNF-alpha from human rheumatoid synovial cells is taken by mouth.

Literature Survey shows that the APR has been determined by UV spectrophotometric method [6-7], Ultra HPLC- Mass spectroscopy [UHPLC -MS] [8] in biological fluids like human and rat plasma. However no stability indicating High Performance Liquid Chromatography has been reported for the estimation of APR in bulk and pharmaceutical dosage forms hitherto. Hence the major objective of the present research is to develop and validate a simple, precise, sensitive liquid chromatography method for APR in its bulk and tablet dosage form and stress degradation studies of APR as per International Conference on Harmonization (ICH) Q2 (R2) guidelines. (Figure 1) shows the chemical structure of Apremilast.

METHODOLOGY

Chemicals and Reagents

APR pure drug was supplied as gift sample by Hetero Drugs Ltd., Hyderabad, Telangana, India. The marketed formulation Otezla tablets containing 30 mg of APR tablets were obtained from local market. Acetonitrile was obtained from E. Merck specialties private Ltd., Mumbai, India (Table 1).

Table1: Calibration data of the proposed method for the estimation of Apremilast

S.NO	Concentration (µg/mL)	Peak area
1	2	571428
2	4	982067
3	6	1450540
4	8	1959690
5	10	2463855

Instrument

The HPLC system utilized was a Agilent technologies 1260 infinity system supplied with a gradient pump connected to Photo diode Array DAD VL detector set at 229 nm. Ezchrome elite software was utilized for data acquisition. An digital balance (Essae vibra AJ (0.001g)) and a sonicator (Model no-91250 mode) were

utilized in this study.

Method development

Chromatographic conditions:

Accurately weighed 10 mg quantity of APR transferred to a 10 ml volumetric flask, dissolved and filled up to the mark with acetonitrile and was ultra-sonicated for 5 minutes.

Preparation of standard working solution

It was prepared by taking 1 ml of APR stock solution into 10 ml volumetric flask and the final volume was made up with diluent (100 µg/ml). The solution was filtered and then diluted immediately before use to appropriate concentration levels by utilizing mobile phase.

Analytical Method validation

The developed method was validated for different parameters like linearity, precision, accuracy, specificity, ruggedness, robustness, LOD and LOQ as per Q1A(R2) and ICH Q2(R1) guidelines.

System suitability

The system suitability test was carried out on freshly prepared Apremilast standard solution (100 %) was used for the evaluation of the system suitability parameters such as Area, retention time, unique selling proposition peak tailing, and the number of theoretical plates, asymmetry factor, LOD and LOQ. The system suitability data and the optimum chromatographic conditions are reported in (Table 2).

Table 2: Optimum chromatographic conditions and system suitability data

Parameter	Chromatographic conditions
Instrument	Agilent Technologies.
Column	eclipse XDB model C_{18} Column (4.6 mm i.d. X 250 mm, 5 µm particle size)
Detector	1260 DAD VL detector
Flow rate	1 mL/min.
Detection wave length	229 nm
Run time	Five minutes
Temperature	Ambient temperature (30 ºC)

Volume of injection loop	20 µL
Retention time (Rt)	2.488 minutes
Theoretical plates	8191
Asymmetry factor	1.231

Linearity

Under developed experimental conditions the relationship between the peak area and concentration of APR was studied. The calibration curve was plotted against concentration vs peak area by the prepared different aliquots i,e., (2-10 µg/ml at 229 nm) of stock solution, and r^2 value was determined. Five replicate of prepared 10 µg/ml solution of APR taken from different stock solution and measured area. The relative standard deviation was determined. **(**Figure 2) shows the linearity curve of Apremilast and overlain spectra of is shown in (Figure 3).

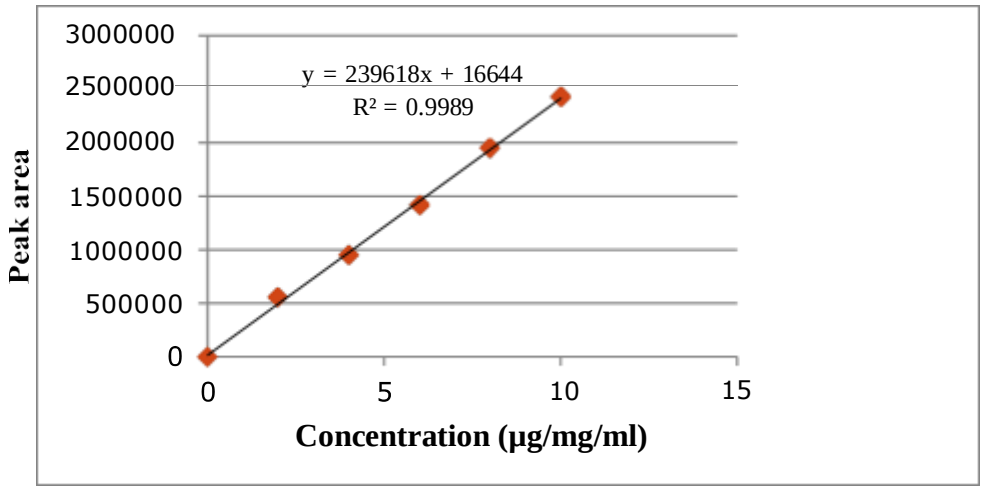

Figure 2: Linearity curve of Apremilast

Accuracy

Accuracy of the method was resolved by standard addition method in which standard addition of pure APR at three different concentration levels of 50 %, 100 % and 150 % was performed in triplicate. Accuracy of the method is calculated in the terms of % recovery of the APR. **Robustness**

Robustness of the method was determined by varying the method parameters such as change in flow rate (± 0.2 mL/min), temperature (± 2 %) and wavelength (± 1 nm).

Precision

Precision of the method was determined by evaluating repeatability, intraday and interday precision. Repeatability was confirmed by injecting same concentration in six replicates and corresponding areas were calculated. Intra-day and Inter-day variation APR was analyzed by selecting three concentrations which were 4, 6 and 8 µg/ml from linearity range. Intraday analysis was carried on same day whereas interday analysis was carried on three different days in replicates of three. The respective peak areas for different concentrations were reported.

Ruggedness

Ruggedness of the method was determined by carrying out the analysis by two different analysts and the respective peak areas were noted.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

A limit of detection (LOD) and a limit of quantification (LOQ) were calculated according to the formula:

LOD = 3.3 σ/s

LOQ = 10 σ/s

Where, _σ‘ is the standard deviation of _y‘ intercept of regression line and _s‘ is the slope of the calibration curve.

Force degradation studies

To conduct the force degradation study, 10 mg APR was subjected to acidic, alkaline, oxidising, thermal, and UV light conditions. For acidic degradation, 10 mg APR was dissolved in 5 ml of acetonitrile to which 5 ml of 0.1 N HCl was added and heated under reflux at 70 ^{0}C for eight hours. The mixture was neutralized by the addition of 1 M NaOH. For alkaline degradation, 10 mg drug was dissolved in 5 ml of Acetonitrile to which 5 ml of 1 M NaOH was added and heated under reflux at 70 ^{0}C for eight hours before the mixture was neutralized by the addition of 0.1 N HCl.

For degradation under oxidising conditions the drug was heated under reflux with 3 % H2O2 (v/v) at 40 ^{0}C for 2 days. For thermal degradation the powdered drug was exposed at 80 ^{0}C for five days. Regarding UV light degradation, powdered APR was exposed to UV light for five days. Pharmaceutical APR dosage forms were also subjected to the same stress conditions to determine whether any peaks arose from the degraded excipients. After completing the treatments, the APR solutions were left to return to room temperature diluted with solvent mixture to obtain 10 µg/ml solutions.

Assay of marketed formulation

Twenty OTEZLA® (APR) tablets were weighed, average weight was calculated, and was made to fine powder. APR powder proportionate to 10 mg was taken in a 10 ml volumetric flask to which small amount of acetonitrile (ACN) was added. The flask is then ultra-sonicated for fifteen minutes and volume is made up with ACN. The tablet APR solution is then filtered through whatman filter paper (No. 42) to get rid of insoluble materials. From the above solution 10 mL is added to 100 mL with diluent so as to attain concentration of 100 µg/mL for the assay. It was further diluted according to the need and then analyzed following the proposed procedure. The content of the Otezla was calculated either form the previously plotted calibration graph or utilizing regression equation.

Determination of APR in Bulk drug

For the analysis of bulk drug accurately weighed 10 mg APR was taken in a 100 ml volumetric flask and the volume was filled up to the mark with mobile phase to get 100 µg/ml concentration. From this 1ml was taken and transferred to a 10 ml volumetric flask and the volume was made up to the mark with mobile phase to get 10 µg/ml concentration. The concentration of the bulk drug was calculated from the linear regression equation.

RESULTS AND DISCUSSION

Several mobile phases of different compositions were tested so as to develop an optimization of chromatographic conditions such as tailing factor, decorous peak shape, and theoretical plates. For the selection of the mobile phase primarily methanol, acetonitrile, CH3OH: water, ACN: water has been tried in different compositions. Eventually only acetonitrile used at a flow rate of 1 ml/min was found to be satisfactorily and decorous system suitability parameters. The average retention time (Rt) got for Apremilast was at 2.488 min. The tailing factor and theoretical plates for APR were found to be 1.231and 8191 respectively. Accuracy of APR was determined by calculating the % recovery. The method was found to be accurate with % recovery between 99.18 -101.61 %. Accuracy is shown in (Table 3). Intra and interday precision was calculated. Infact the method was precise with percentage RSD > 2%. Intra and interday precision are shown in (Table 4 and 5) respectively. The % RSD value of robustness which is less than 2% for Apremilast reveals that the proposed method is robust (Table 6). (Change in flow rate, temperature and wavelength). The % RSD values of ruggedness for Apremilast reveal that the proposed method is quite rugged which is shown in (Table 7). The LOD and LOQ of Apremilast were found 0.003 µg/ml and 0.001 µg/ml respectively. The % assay of the bulk was found to be 99.90 ± 0.001. The average content of APR was 99.80 ± 0.002, which was in good agreement with labelled claim. (Table 8 and 9) displays the assay of the APR tablets and Bulk drug. The method was specific and has no interference observed when the APR were

estimated in presence of excipients.

Table 3: Accuracy studies of Apremilast

Level (%)	Sample concentration (µg/ml)	Amount of standard added (µg/ml)	Total concentration (µg/ml)	Found concentration (µg/ml)	% RSD*	% recovery
50	6	3	9	8.98	0.329	99.81
	6	3	9	8.99		
	6	3	9	8.98		
100	6	6	12	11.89	0.563	99.05
	6	6	12	11.88		
	6	6	12	11.89		
150	6	9	16	15.96	0.716	99.85
	6	9	16	15.98		
	6	9	16	15.99		
Note: *Average of three determinations (n=3).						

Table 4: Intra-day precision studies of Apremilast

Amount of Standard taken (µg/ml)	Area (mAU)	Retention Time (Rt) (minutes)	Asymmetry factor	Theoretical plates	% Relative Standard Deviation (n =3)
Day-1 (Morning)					
4	682056	2.48	1.19993	8171	0.0031
6	1450532	2.48	1.12340	8084	0.0015
8	1959693	2.48	1.16215	8200	0.0009
Day-1 (Afternoon)					
4	682074	2.48	1.19991	8170	0.0026
6	1450556	2.50	1.12343	8086	0.0012
8	1959674	2.48	1.16216	8204	0.0006
Day-1 (Evening)					
4	682099	2.48	1.19995	8168	0.0021
6	1450576	2.48	1.12341	8082	0.0009
8	1959656	2.48	1.16212	8205	0.0002

Table 5: Inter-day precision studies of Apremilast

Amount of Standard taken (µg/ml)	Area (mAU)	Retention Time (Rt) (in minutes)	Asymmetry	Theoretical plates	% Relative Standard Deviation (n =3)
Day-1 (Morning)					
4	682054	2.48	1.19993	8166	0.002
6	1450543	2.48	1.12340	8082	0.001
8	1959692	2.50	1.16214	8201	0.0008
Day-1 (Afternoon)					
4	682066	2.48	1.19997	8178	0.001
6	1450567	2.48	1.12343	8084	0.01
8	1959667	2.51	1.16215	8206	0.0004
Day-1 (Evening)					
4	682082	2.48	1.19994	8175	0.002
6	1450 587	2.48	1.12346	8080	0.02
8	1959673	2.50	1.16218	8203	0.001

Table 6: Robustness studies of Apremilast

S. No	Parameter	Optimized	Used	Retention time (Rt), min	Plate count	Peak asymmetry	% RSD
1	Flow rate (±0.2 mL/min)	1.0 mL/min	0.8 mL/min	2.48	8120	1.135980	0.011
			1.0 mL/min	2.50	8111	1.135987	0.010
			1.2 mL/min	2.50	8118	1.135982	0.012
2	Detection wavelength (±1 nm)	229 nm	228 nm	2.47	8079	1.19997	0.010
			229 nm	2.48	8084	1.19992	0.010
			230 nm	2.50	8080	1.19995	0.099
3	Change in Temperature (±2°C)	30 ^{0}C	28 ^{0}C	2.45	8382	1.19989	0.012
			30 ^{0}C	2.48	8388	1.19990	0.010
			32 ^{0}C	2.50	8174	1.19982	0.011

Table 7: Ruggedness studies of Apremilast

Analyst	Area (mAU)	Retention Time (Rt) (minutes)	Asymmetry factor	Theoretical plate	% Relative Standard Deviation
Analyst 1	1959693	2.48	1.16210	8082	0.010
Analyst 2	1959689	2.50	1.16214	8080	0.011

Table 8: Stability studies of Apremilast

Stress condition	Mean peak area	Drug recovered (%)	Drug decomposed (%)	Theoretical plates	Asymmetry factor
Standard drug	583239	100	Nil	8111	1.35987
Acidic degradation	195198	33.46	66.54	7306	1.11105
Alkaline degradation	203454	34.83	65.17	3718	1.22746
Oxidative degradation	531910	91.19	9.01	3114	1.56435
Thermal degradation	473260	81.14	18.86	3477	1.55941
UV light degradation	220502	37.80	62.20	3253	1.29968

Table 9: Assay of Apremilast tablets

Name of the Formulation	Concentration taken	Amount obtained	% assay ± S.D.	% RSD (n=6)
OTEZLA 30 mg	10 µg/ml	9.98 µg/ml	99.80 ± 0.002	0.010
Bulk drug	10 µg/ml	9.99 µg/ml	99.90 ± 0.001	0.012

Degradation behaviour of APR under various stress conditions is studied. The percent of the degradation products of APR were calculated and found to be 66.54 %, 65.17 %, 9.01 %, 18.86 %, 62.20 % in case of acid hydrolysis, alkaline hydrolysis, oxidation, UV light and thermal stability respectively. In acidic conditions for eight hours (0.1 N HCl) 66.54 % of APR drug was degraded with generation of one novel peak in addition to the peak of the APR. (Figure 4) More degradation of APR observed when conducted different stress conditions such as acidic, alkaline, UV light and thermal degradation so these stress conditions particularly interfere with detection of APR. Degradation behaviour of APR under various stress

conditions are shown in (Table 8) and degradation figures are shown in (Figure 4- 8). The assay method of APR in pharmaceutical formulation was successfully developed and validated for its intended purpose. Infact there was no particular precaution necessary during manufacturing and storage of APR formulation because there was no degradation studied at room temperature.

CONCLUSION

The creation of a stability-indicating RP-HPLC method for determining APR in pharmaceutical dosage form and bulk is the focus of the current study. The LOD, LOQ, robustness, ruggedness, accuracy, and precision values were all within the acceptable ranges. APR is unstable in alkaline, acidic, oxidative, thermal, and UV radiation conditions due to its high sensitivity. The statistical analysis of the data shows unequivocally that the approach is appropriate for determining APR in tablet and bulk forms without interference, and that no extra care is required when storing and formulating the material at room temperature. This investigation has led to the conclusion that the new RP-HPLC method for determining APR in a bulk and tablet formulation was effectively created and proven to work as planned.

REFERENCES

[1] Papp, K., Cather, JC., Rosoph, L., et al. *Lancet*, **2022.** 380:738–746.

[2] Schafer, P., *Biochem Pharmacol*, **2022.** 83: 1583–1590.

[3] Schett, G., Sloan, VS., Stevens, RM., et al. *Ther Adv Musculoskelet Dis*, **2010.** 2: 271–278.

[4] Paul, C., Cather, J., Gooderham, M., et al. *Br J Dermatol*, **2015.**173:1387–1399.

[5] Papp, K., Reich, K., Leonardi, CL., et al. *J Am Acad Dermatol*, **2015.** 73:37–49.

[6] Syeda, K., Vidya Sagar, G., Afreen, B., et al. M *World journal of pharmacy and pharmaceutical sciences*, **2016.** 5: 1595- 1601.

[7] Ravisankar, P., Sulthana, S., Srinivasa Babu, P., *Indian Journal of Research in Pharmacy and Biotechnology*, **2017.** 5: 47- 53.

[8] Lian-guochen, Zhewang, et al. *Oxford journals*, **2016.** 4:1336-1340.

[9] Xiong, K., Ma, X., Liu, L., et al. *Publishing book journals and database*, **1997.** 20: 1-56.

[10] Q1A (R2) stability testing of new drug substance and products. International conference on harmonization,**2023**.

[11] ICH Q2 (R1) Validation of analytical procedures, Text and methodology International conference on Harmonization, Geneva, **2005.** 1-17.

[12] Ravisankar, P., Naga Navya, Ch., Pravallika, D., et al.. *IOSR Journal of Pharmacy*, **2015.** 5: 10, 7-19.

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