

# NOVEL CARRIER SYSTEMS FOR TARGETED DRUG DELIVERY IN THE TREATMENT OF ARTHRITIS

A Thesis submitted to Gujarat Technological University

for the Award of

Doctor of Philosophy

in

Pharmacy

By

Prachi Pandey

Enrollment No-119997290028

under supervision of

Dr. S.S Pancholi



**GUJARAT TECHNOLOGICAL UNIVERSITY  
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# ABSTRACT

Arthritis is a major cause of disability and morbidity, particularly in older individuals. The symptoms and signs of arthritis and related conditions include pain, stiffness, swelling, muscle weakness, and limitation of movement of the joints.

Nonsteroidal anti-inflammatory drugs are most widely used and effective drugs for treatment of arthritis. Nonsteroidal anti-inflammatory drugs (NSAIDs) that act by inhibiting cyclooxygenase and the formation of prostaglandins, are known to cause gastrointestinal toxicity, leading to peptic ulcers, cardiotoxicity, renal toxicity and anaphylactic reactions in selected patients. The currently available oral dosage forms of NSAIDs like tablet and capsules etc. are more likely to produce above mentioned adverse effects of these drugs. The intravenous administration of these drugs leads to distribution throughout the whole body and rapid clearance, thus a high and frequent dosing is necessary to achieve an effective concentration of drug at inflamed target sites. Moreover, the activities of drug in many different tissues increase the risk of adverse effects in patients. Transdermal delivery of drugs can be a suitable option and is associated with advantages such as avoidance of hepatic first-pass metabolism and GI adverse effects, improved patient compliance and ease of access, a means to quickly terminate dosing, sustained therapeutic drug levels, possible self administration, non-invasive (no needles or injections needed), avoidance of food related interaction, reduction of doses as compared to oral dosage forms and intravenous therapy.

The transdermal drug delivery also suffers from some shortcomings such as poor permeability through skin, unpredictable drug release and skin irritation. These shortcomings can be overcome if we develop a drug delivery system in drug carriers which can provide enhanced localization to the target site and sustained drug release.

The objective of the present research work was to develop a drug delivery system for localized action of drugs used in treatment of arthritis to the affected tissues for prolonged period and to avoid side effects in non-target organs. To achieve this, the drug loaded liposomes and transferosomes were prepared for nonsteroidal anti-inflammatory drugs and further incorporated in transdermal gel formulation. The

formulations were prepared by experimental design using screened factors and their levels and by optimized process parameters.

The process of rotary vacuum evaporation at 50° C temperature and 90 rpm, for 20 minutes was found to produce a thin film which was uniform and translucent in appearance. Probe sonication for 5 cycles each of 2 minutes at amplitude of 60% using 13 mm standard probe produced transparent vesicular dispersion with reproducibility and uniformity in vesicle size.

Among phospholipids , 1,2-disteroyl -sn- glycerol-3-Phospho-ethanolamine,Na salt was found to be better for liposome preparation and among Surfactants ,Span 60 was screened as suitable surfactant for transferosome preparation on the basis of size, drug entrapment efficiency and drug release. The excipients were found to be compatible with the drug based on the results of drug excipient compatibility studies. The transferosomes and liposomes batches were prepared based on experimental design using minitab software 16. Both the drug carriers were found in the nanometric range with size uniformity with good zeta potential value indicating stability of drug carrier suspension.

The optimized transferosomal gel and liposomal gel of aceclofenac showed better drug permeation in ex-vivo studies through rat skin as compared to its available marketed gel. The transdermal drug permeation was found to be highest for transferosomal gel, whereas drug was found to be slightly retained in skin during permeation from liposomal gel formulation. The reason may be fusion of phospholipids during diffusion through skin. Both the transferosomal gel formulation and liposomal gel formulation showed sustained release of drug for more than 6 hrs.

Based on the pharmacokinetic studies, both the liposomal gel and transferosomal gel were found to have better bioavailability as compared to available marketed formulation of aceclofenac.

The anti-inflammatory activity measured by rat paw edema method revealed that aceclofenac transferosomal gel showed maximum anti-inflammatory action followed by liposomal gel. Both the transferosomal and liposomal gel showed better anti-inflammatory action and analgesic action than marketed gel.

The radioactive labelling experiment also showed greater permeation and deposition of drug in skin when released through transferosomal gel as compared to marketed gel. The experiment also suggested that, the drug can be released in a sustained manner from the skin from transferosomal gel.

The drug indomethacin, which is of the same therapeutic class of drug aceclofenac, also shows better efficacy when formulated as transferosomal gel for arthritis. The transferosomal gel of indomethacin showed better skin permeation, analgesic activity and anti-inflammatory activity in rats as compared to plain drug gel of indomethacin.

The rheological properties of gel formulation indicated a potential to withstand stress conditions of handling and packaging. The pH of the gel formulation was found to be compatible with the skin and the formulation was also found to be free of any toxic organic solvent. The spreadability, gel strength and extrudability of the formulation were found to be satisfactory for ease of application.

The transferosomal gel formulations of both aceclofenac and indomethacin were found to be stable till 6 months in accelerated stability testing.

The prepared formulation of drug carriers incorporated in gel can be a novel approach for treatment of arthritis using transdermal route through which the nonsteroidal anti-inflammatory drugs can permeate through skin and also show sustained release characteristics. The localized action of drug through novel drug carriers containing gel for prolonged period at the site of pain can provide more relief to patients as well as can replace the oral route thereby avoiding the gastrointestinal side effects of drug associated with conventional oral route.

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**Ms. Prachi Pandey**

**Date-**

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## LIST OF SYMBOLS

Symbol	Name
%	Percentage
±	Positive or Negative
°C	Degree Celsius
μg	Micrograms
cm	Centimeter
conc.	Concentration
gm	Gram
mg	Milligram
min	Minute
hr	Hour
s	Second
λ	Lambda
mMol	Millimole
rpm	Rotations per minute

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- Appendix B : [Thesis originality report from turnitin]

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# CHAPTER- I

## Introduction

### 1.1 Introduction to arthritis:-

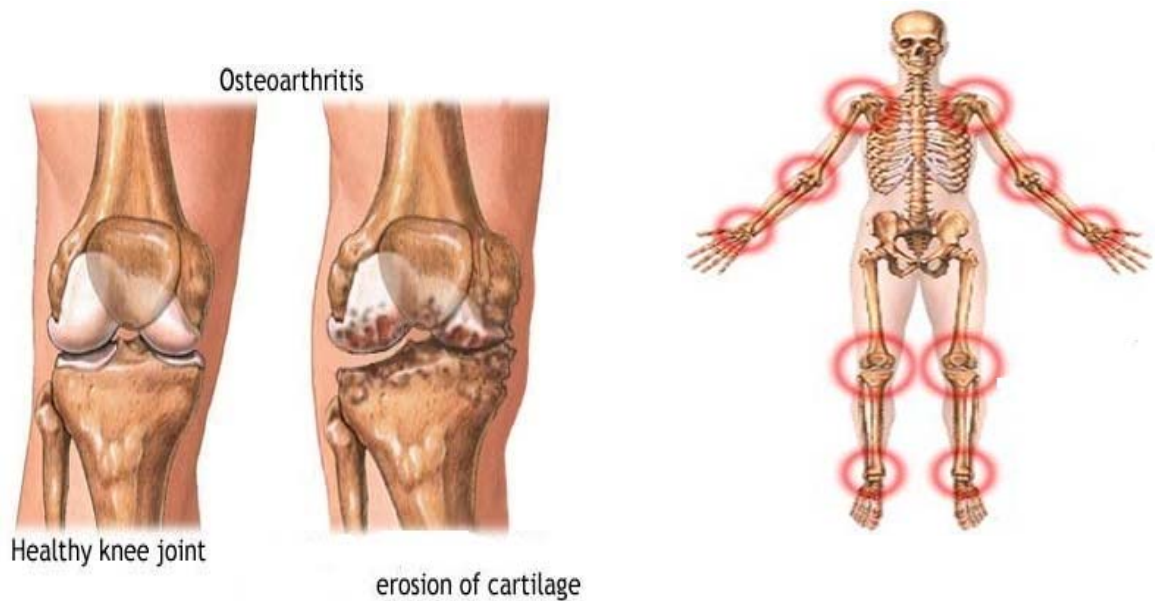
Arthritis is a major cause of disability and morbidity, particularly in older individuals. The symptoms and signs of arthritis and related conditions include pain, stiffness, swelling, muscle weakness, and limitation of movement of the joints. More than 30 percent of females have some degree of osteoarthritis by age 65.

There are four key warning signs of arthritis that include-

- **Pain-** Pain from arthritis can be constant, or intermittent. Pain might be isolated to one place or felt in many parts of the body.
- **Swelling-** Some types of arthritis cause the skin over the affected joint to become red and inflamed. The inflammation leads to discomfort to the patient.
- **Stiffness-** Stiffness is a typical arthritis symptom, which is felt upon waking up in the morning, after sitting at a desk, or after sitting in a car for a long time, also after exercise or even felt as persistent stiffness.
- **Difficulty in moving a joint-** The symptom of difficulty in moving a joint or feeling pain in getting up from a chair indicate arthritis or other joint problem. Arthritis can be categorized into two major groups: degenerative and inflammatory.

**Osteoarthritis:-**

Osteoarthritis is a chronic disease of the joint cartilage and bone, often thought to result from wear and tear on a joint, although there are other causes such as congenital defects, trauma and metabolic disorders. In an inflammatory osteoarthritis, the cartilage in joints breaks down. Cartilage is the slippery tissue that covers the ends of bones in a joint. Healthy cartilage absorbs the shock of movement. When cartilage is lost, bones rub together. Over time, this rubbing can permanently damage the joint.<sup>2,4</sup>



**Osteoarthritis**

**Rheumatoid Arthritis**

**Figure 1.1 Types of Arthritis**

Source:-Health Information, Medical Reference Guide, In-Depth Patient Education Reports, University of Maryland medical centre.USA

**1.1.2 Rheumatoid arthritis:** In an inflammatory arthritis such as rheumatoid arthritis, there is a systemic illness with inflammation. Rheumatoid arthritis is an autoimmune disorder which is chronic and progressive and its manifestations are chronic inflammation of the synovial joint and also the joint destruction. Inflammation is associated with the body's immune system and attacks the tissue around the joints. Synovial lining layer surrounds the connective tissue in the joints and consists of a few cell layers of mainly fibroblast-like and macrophage-like

synoviocytes. In RA, the synovial lining layer expands as a result of newly arrived macrophages from the periphery.<sup>4,5</sup>

Macrophages in the synovial lining of arthritic joints have been shown to produce many pro-inflammatory cytokines, attract new inflammatory cells and produce enzymes that can damage the cartilage. Inflammatory cells and enzymes responsible for inflammation is caused by release of chemicals from tissues and migrating cells. Most strongly implicated are the prostaglandins (PGs), leukotrienes (LTs), histamine, bradykinin, and, more recently, platelet- activating factor (PAF) and interleukin-1<sup>5, 6, 7</sup>

### 1.2 Treatment of osteoarthritis and rheumatoid arthritis:-

Arthritis treatment focuses on relieving symptoms and improving joint function. The medications used to treat arthritis vary depending on the type of arthritis.

**Table 1.1 Comparison of treatment of osteoarthritis and rheumatoid arthritis**

Osteoarthritis	Rheumatoid arthritis
<ul style="list-style-type: none"> <li>Weight loss</li> </ul>	<ul style="list-style-type: none"> <li>PT/OT, vocational rehabilitation</li> </ul>
<ul style="list-style-type: none"> <li>Orthotics/assistive device</li> </ul>	<ul style="list-style-type: none"> <li>Exercise – strengthening, flexibility, aerobic</li> </ul>
<ul style="list-style-type: none"> <li>NSAIDs</li> </ul>	<ul style="list-style-type: none"> <li>NSAIDs</li> </ul>
<ul style="list-style-type: none"> <li>Glucocorticoids</li> </ul>	<ul style="list-style-type: none"> <li>Glucocorticoids</li> </ul>
DMARDs <ul style="list-style-type: none"> <li>Methotrexate, sulfasalazine</li> <li>IL-1 antagonist – Anakinra, Diacerein</li> </ul>	DMARDs <ul style="list-style-type: none"> <li>Methotrexate, sulfasalazine</li> <li>TNF antagonists – infliximab, adalimumab</li> <li>IL-1 antagonist – Anakinra, Diacerein</li> <li>B-Cell depletion – Rituximab</li> </ul>
<ul style="list-style-type: none"> <li>Non-acetylated salicylate</li> </ul>	<ul style="list-style-type: none"> <li>Intra-articular, I/M or oral steroids</li> </ul>
<ul style="list-style-type: none"> <li>Viscosupplementation - hyaluronic acid, glucosamine sulfate, chondroitin sulfate</li> </ul>	<ul style="list-style-type: none"> <li>Viscosupplementation - hyaluronic acid, glucosamine sulfate, chondroitin sulphate</li> </ul>
<ul style="list-style-type: none"> <li>Surgical therapy – osteotomy, arthroscopy, joint replacement, arthroplasty.</li> </ul>	<ul style="list-style-type: none"> <li>Surgical therapy – osteotomy, arthroscopy, joint replacement, arthroplasty.</li> </ul>

### **1.2.1 Nonsteroidal anti-inflammatory drugs (NSAIDs):-**

Nonsteroidal anti-inflammatory drugs, or NSAIDs are the most prescribed medications for treating conditions such as arthritis. These are widely used drugs that act by inhibiting cyclooxygenase and the formation of prostaglandins.

Therefore, inflammation, pain, and fever are reduced. Since the prostaglandins that protect the stomach and promote blood clotting also are reduced, NSAIDs that block both COX-1 and COX-2 can cause ulcers in the stomach and intestines, and increase the risk of bleeding.

They are known to cause GI toxicity, leading to the formation of peptic ulcers, unwanted antiplatelet effects (nonselective inhibitors of cyclooxygenase), cardio toxicity, renal toxicity and anaphylactic reactions in selected patients.<sup>1,2,3</sup> This class of drugs include diclofenac Na, aceclofenac, naproxen, ibuprofen, ketoprofen etc. The side effects of indomethacin, such as ulceration of the kidney and central nervous system (CNS) toxicity, limit its use as a drug for rheumatoid arthritis. Encapsulation of this drug in liposomes may reduce the toxic effects.<sup>3,4</sup>

### **1.2.2 Corticosteroids:-**

These are medications that suppress the immune system and symptoms of inflammation. These are often injected into painful osteoarthritis joints. Steroids are used to treat autoimmune forms of arthritis. Steroids have multiple side effects, including upset stomach, gastrointestinal bleeding, high blood pressure, thinning of bones, cataracts, and increased infections. The risks are most pronounced when steroids are taken for long periods of time or at high doses. Close supervision by a physician is essential.<sup>4,5</sup>

Besides the poor safety profile, also the poor pharmacokinetic behavior limits the usefulness of corticosteroids in systemic therapy. Corticosteroids are drugs with a relatively high clearance rate and a large volume of distribution. This implies that to reach pharmacologically active drug levels at the site of inflammation, high and frequent doses must be administered. The majority of these systemically administered doses localizes in healthy non-target tissues if not rapidly excreted from the body.<sup>4,5,6</sup>



### **Disease-modifying anti-rheumatic drugs:-**

These have been used traditionally to treat rheumatoid arthritis and other autoimmune causes of arthritis. These drugs include gold salts, penicillamine, sulfasalazine, and hydroxychloroquine. More recently, methotrexate has been shown to slow the progression of rheumatoid arthritis and improve quality of life. Methotrexate itself can be highly toxic and requires frequent blood tests for patients on the medication.<sup>2,3</sup>

### **1.3 Need of Study:-**

Arthritis is a major cause of disability and morbidity, particularly in older individuals. The symptoms and signs of arthritis and related conditions include pain, stiffness, swelling, muscle weakness, and limitation of movement of the joints.

The currently available oral dosage forms of NSAIDs like tablet and capsules etc. are more likely to produce above mentioned adverse effects of these drugs. The intravenous administration of these drugs leads to distribution throughout the whole body and rapid clearance, thus a high and frequent dosing is necessary to achieve an effective concentration of drug at inflamed target sites. Moreover, the activities of drug in many different tissues increase the risk of adverse effects in patients. Topical delivery of drugs can be a suitable option and is associated with advantages such as avoidance of hepatic first-pass metabolism, improved patient compliance and ease of access, provides a means to quickly terminate dosing, sustained therapeutic drug levels, possible self administration, non-invasive, avoids food related interaction, reduction of doses as compared to oral dosage forms and intravenous therapy and most important is avoidance of gastrointestinal adverse effects.

The topical drug delivery also suffers from some shortcomings such as poor permeability through skin, unpredictable drug release and skin irritation. These shortcomings can be overcome if we develop a transdermal drug delivery system in nanoscaled drug carriers, with enhanced localization to the target site and sustained drug release.

## **1.4 Approaches for targeted drug delivery :-**

To increase the amount of drug at the target site after systemic administration and to decrease localization at non target tissues, drug targeting approach may offer perspective through passive or active drug targeting.

Drug targeting makes use of colloidal carrier systems in which the drug is incorporated or to which the drug is attached. Distribution of the carrier-associated drug to organs/tissues is reduced, as the carrier cannot diffuse into extravascular tissues.

### **1.4.1 Passive targeting:-**

Passive targeting is the drug targeting that can be achieved without any integration of a specific targeting moiety on the surface of particle. Interestingly, the approach of passive drug targeting can effectively be employed in inflammatory diseases. Inflammation generally results in locally increased vascular permeability. It has been observed that, nanoparticles accumulate at the sites of inflammation in arthritic joints probably due to enhanced local capillary permeability.

The immune-related cells like macrophages is commonly present at the site of inflammation and it has been observed that microspheres and nanoparticles can be taken up efficiently by the macrophages, usually by the process of phagocytosis.<sup>4, 5, 6</sup>

Therefore, uptake of particles into such immune related cells and also by the disruption of the epithelium may lead to the selective accumulation of the nanocarrier based drug delivery system in the desired area of inflammation.<sup>4, 9</sup>

### **1.4.2 Active targeting:-**

Active targeting of refers to the conjugation of site-directing ligands to the surface of Colloidal drug carriers to obtain specific binding to cell receptors on the surface of the target cells. Ligands may be antibodies, glycoproteins and glycolipids. The US patent bearing no- US 20070286896 in page no-38 says that the sugar chain present in glycolipids and glycoproteins play an important role in active targeting.

Active targeting aims at improving the therapeutic availability of colloidal drug carriers to target cells within the pathological site and to minimize undesired side effects to non-target cells within the pathological tissue.<sup>10</sup>

The most remarkable advantage of nanoscaled drug carriers over conventional drug delivery systems is the ability to improve selective drug delivery to the site of action by drug targeting which can be classified further into the active and passive drug targeting approaches.

### **1.5 Transdermal delivery of antiarthritic drugs:-**

Transdermal delivery of drugs has many advantages such as avoidance of hepatic first-pass metabolism, improved patient compliance and ease of access, provide a means to quickly terminate dosing sustained therapeutic drug levels, possible self-administration, non-invasive (no needles or injections needed), avoids food related interaction, reduction of doses as compared to oral dosage forms and intravenous therapy. Transdermal route allows drug to diffuse out of its vehicle onto the surface tissues of skin.

In fact ease of applicability makes this route more comfortable for the patient which results in better patient compliance.<sup>11,12,13</sup>

Transdermal drug administration is a localized drug delivery system in which drug can be applied through any part of skin of body. Skin is main route of transdermal drug delivery system. It is important to know the structure of skin and its barrier properties, which allowed the development of more easily applicable and novel transdermal formulations. Several drugs can be given by transdermal route with proved successfully safety and efficacy for both transdermal as well as systemic action. The pH of the skin varies from 4 to 5.6. Generally, the oral route is considered as the most common route of delivery of drugs. Though oral route has the benefit of ease of administration, it also suffers from shortcomings like poor bioavailability, the tendency to produce rapid blood level spikes leading to a need for high and /or frequent dosing. To overcome these difficulties there is a need for the development of new drug delivery system; which can improve the therapeutic efficacy and safety of drugs, for that purpose one has to select such a delivery in which is drug is given by

transdermal route. Skin is main route of transdermal drug delivery system. Most of transdermal formulations are applied by the skin so, basic knowledge of skin anatomy and physiology is very important. The skin is the largest single organ of the body. The success of transdermal delivery depends on the ability of the drug to permeate the skin in sufficient quantities to achieve its desired therapeutic effect.<sup>14, 15</sup>

### 1.5.1 Advantages of transdermal drug delivery system:-

- Provides targeted and localized action
- Avoid gastrointestinal drug absorption difficulties caused by gastrointestinal pH and enzymatic activity and drug interaction with food and drinks.
- Avoidance of the first pass effect.
- Non-invasive and have patient compliance.
- Can be easily removed from the skin.
- Economic.
- Reduction of doses as compare to oral dosage forms.
- Localized effect with minimum side effects.<sup>12, 13</sup>

### 1.5.2. Ideal properties of drug candidate for transdermal Drug Delivery and marketed compositions:-

Table 1.2 Ideal properties of drug for TDDS

Parameter	Properties
Dose	Should be low (20-50 mg/day)
Molecular weight	<500 Daltons
Partition coefficient	Log P (octanol-water) between 1-4
Skin permeability coefficient	$>0.5 \times 10^{-3}$ cm/hr
Melting point	$<200^{\circ}$ C
Skin reaction	Non irritating and non-sensitizing <sup>11, 12, 13</sup>

**Table 1.3 Marketed transdermal compositions of NSAID's**

S.no	NSAID	Strength	Trade name	Manufacturing company
1.	Diclofenac Sodium	1%Topical gel	Diclofenac sodium	Amneal Pharmaceuticals, USA
2.	Diclofenac Sodium	3%Topical gel	Diclofenac sodium	Actavis Mid Atlantic, New Zealand
3.	Diclofenac Sodium	3%Topical gel	Diclofenac sodium	Taro Pharmaceuticals, USA
4.	Diclofenac Sodium	3%Topical gel	diclofenac sodium	Tolmar Inc. northern Colorado
5.	Diclofenac Sodium	3%Topical gel	Solaraze	Fougera Pharmaceuticals, US
6.	Diclofenac Sodium	1%Topical gel	Voltaren	Glaxo Smithkline, UK
7.	Acelofenac	1.5% Topical gel	hifenac gel	Intas Pharmaceuticals Ltd, India
8.	Acelofenac	1.5% Topical gel	Accept gel	Vivid Biotech,India
9.	Indomethacin	1.5% Topical gel	VI-Gel	Diamond Lab, Philippines
10.	Indomethacin	1.5% Topical gel	Elmetacin	Medinova , Switzerland
11.	Indomethacin	1.5% Topical gel	M-CIN	Macro Pharma, India,
12.	Indomethacin	0.75% Topical gel	Satogesic	Sato Pharma, Japan
13.	Indomethacin	1.0% Topical gel	Indobene gel	Merckle GmbH, Blaubeuren, Germany

## **1.6 Skin:**

The skin is one of the most extensive and readily accessible organs of the human body. The skin of the average human being cover an area of about 2 square meter and weighs 4.5-5 kg, about 16 % of total body weight. It also receives 1/3rd of the total blood supply of the body.

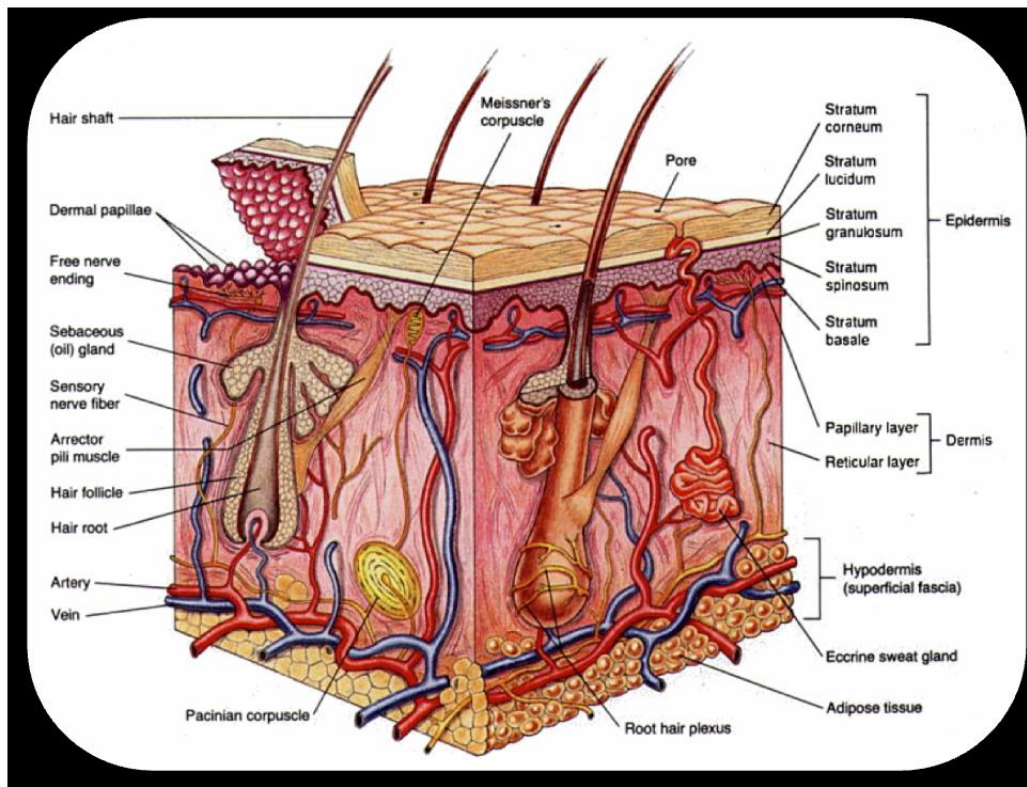
The thickness of the human skin ranges from 0.5 mm on the eyelids to 4 mm on the heels. The skin separates the underlying blood circulation network from the outside environment and serves as barrier against physical and chemical attacks, acts as thermostat in maintaining body temperature and shields the body from microbial invasion.<sup>14, 15</sup>

### **1.6.1 Anatomy of the skin:**

The skin is a multi-layered organ and anatomically has many histological layers. Skin is an anatomical barrier between the body and its environment and contributes to about 16-18 % of normal body weight.

Skin is composed of three primary layers such as:

- Epidermis
- Dermis
- Hypodermis or subcutaneous fat



**Figure 1.2 Longitudinal section of skin**

Source:-Ross and Wilson Anatomy and Physiology in health and illness by Waugh A and Grant A in chapter- "The skin".

➤ **Epidermis:**

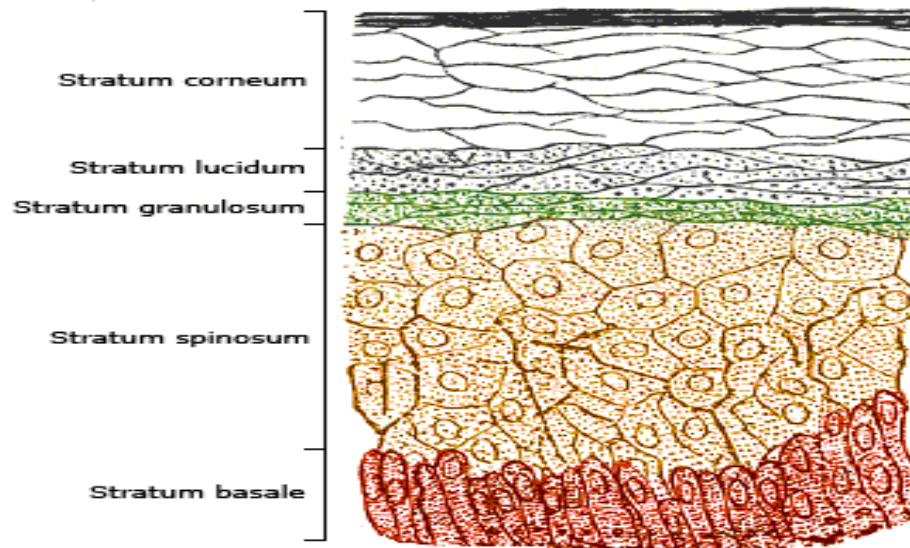
It is the most superficial layer of the skin and is composed of stratified squamous epithelial cells, which varies in thickness in different parts of the body. It is thickest on the palm of the hand and soles of the feet. There are no blood vessels or nerve ending in the epidermal layer.

The epithelial cells are held together mainly by highly convoluted interlocking bridge, which are responsible for the unique integrity of the skin. The main types of cells which make up the epidermis are keratinocytes, melanocytes, langerhans cells and merkels cells. The epidermis helps the skin to regulate body temperature. The outermost layer of the epidermis consists of 25 to 30 layers of dead cells.<sup>14, 15, 16, 17</sup>

The Epidermis is divided into five anatomical layers:-

- Stratum corneum (Horny Layer)
- Stratum lucidum

- Stratum granulosum (Granular Layer)
- Stratum spinosum (Prickly cell Layer)
- Stratum basale (Growing Layer)



**Figure 1.3** The skin showing the main layers of the epidermis

Source:- The Dermatology Nurses' Association by Paul Kolarsick and Carolyn Goodwin in chapter Anatomy and Physiology of the Skin.

➤ **Dermis:-**

This layer lies, just below the epidermis. The dermis is 1/8th of centimeter thick and constitutes the main mass of the skin. The thickness of the dermis ranges from 2000  $\mu\text{m}$  to 3000  $\mu\text{m}$ . It consists of matrix, loose connective tissue, composed of fibrous protein embedded in an amorphous ground substance. Collagen imparts elasticity to the dermis.

Beneath the dermis the fibrous tissue opens out and merges with the fat containing subcutaneous tissue. The upper portion of the dermis is formed into ridges or papillae projecting into the epidermis.<sup>14, 15, 16, 17</sup>

The dermis of the skin consists of the following structures:

- a. Blood vessel
- b. Lymph vessel
- c. Sensory nerve ending
- d. Sweat glands and their ducts



- e. Hair roots, hair follicles and hairs
- f. Sebaceous gland

The dermis is structurally divided into two areas: a superficial area adjacent to the epidermis, called the papillary region, and a deep thicker area known as the reticular region.<sup>15, 16</sup>

➤ **Hypodermis:-**

It is composed of loose, textured white fibrous connective tissue in which fat and elastic fibres are intermingled. Water content is about 30%. The thickness is variable but it is much thicker than the dermis. It is richly supplied with blood and lymph vessels. The base of the hair follicle, the secretory portion of sweat gland, and cutaneous nerves are also present in this layer. The main cell types are fibroblasts, macrophages and adipocytes (the hypodermis contains 50% of body fat). Fat serves as padding and insulation for the body.<sup>15, 16, 17</sup>

**1.6.2 Functions of skin:-**

- Protection of body fluids and tissues.
- Protection from external stimuli like chemicals, light, heat, cold and radiation.
- Reception of stimuli like pressure, heat, pain etc.
- Biochemical synthesis.
- Metabolism and disposal of biochemical wastes.
- Regulation of body temperature.
- Control of blood pressure.
- Prevention of penetration of toxic foreign material & radiation.
- Cushions against mechanical shock.
- Interspecies identification.<sup>16, 17, 18</sup>

### 1.6.3 Absorption through skin:-

Percutaneous absorption is defined as penetration of substances into various layers of skin and permeation across the skin into the systemic circulations. This is a step-wise process and can be divided into three parts:-

- Penetration is the entry of a substance into a particular layer.
- Permeation is the penetration from one layer into another, and is different both functionally and structurally from the first layer.
- Absorption is the uptake of a substance into systemic circulation.<sup>19,20,21,22</sup>

#### ❖ Routes of drug permeation:

##### A) Transepidermal route:

The transepidermal route across the continuous stratum corneum comprises transport via intracellular and intercellular spaces. Both polar and non-polar substances diffuse via transcellular and intercellular routes by different mechanisms.

The polar molecules mainly diffuse through the polar pathway consisting of bound water within the hydrated stratum corneum, whereas the non polar molecules dissolve and diffuse through the non-aqueous lipid matrix of the stratum corneum.<sup>20, 21, 22</sup>

##### B) Transappendageal route:

The transappendageal route, transport substances via the sweat glands and the hair follicles with their associated sebaceous glands. This route is considered to be of minor importance because of their relatively small.<sup>16, 18, 20</sup>

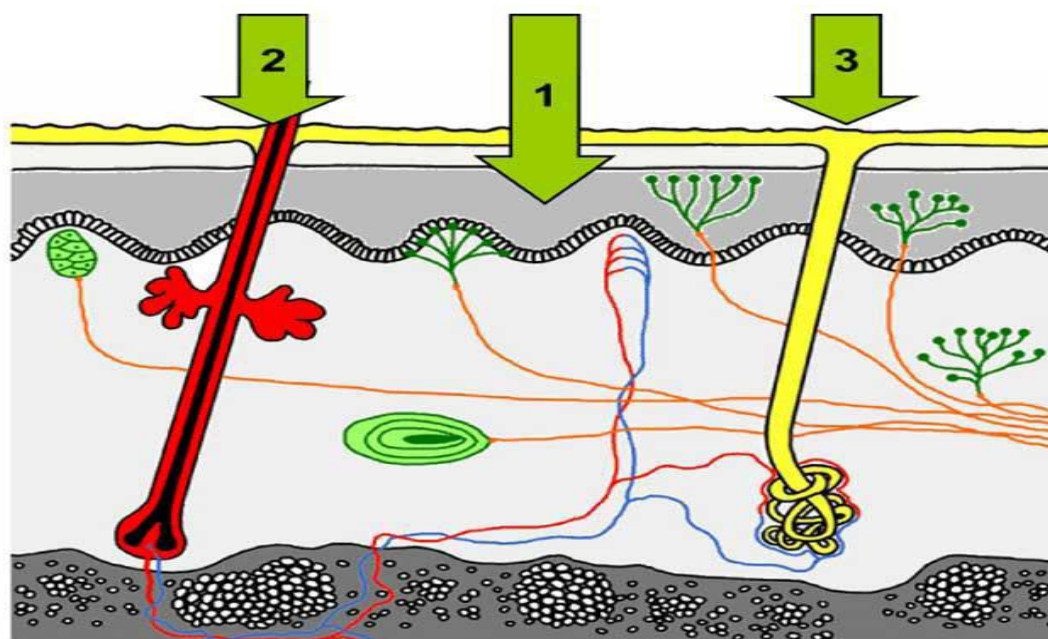


Figure 1.4 Routes of drug permeation

- 1) Transepidermal route: across continuous stratum corneum
- 2) Transappendageal route: via hair follicles
- 3) Transappendageal route: via sweat gland

Source: Ansel's Pharmaceutical dosage form and drug delivery System by Allen L.V, Popovich NG and Ansel HC in chapter Transdermal Drug Delivery System.

When a drug is placed onto the skin surface it will first partition into the stratum corneum. The major route of penetration is through the intercellular channels, which contain a complex mixture of structured lipids.<sup>11, 12, 13</sup> The partitioning behavior of the drug is therefore an important determinant in this first step of the absorption process. Also the solubility characteristics of the penetrant in the lipids of the stratum corneum should be taken into account. The solubility may be influenced by the components that diffuse out of the formulation into the skin.

The second stage for the molecule is diffusion through the structured lipids. Molecular weight, size, shape and polarity also influence the diffusion rate.<sup>20, 21</sup>

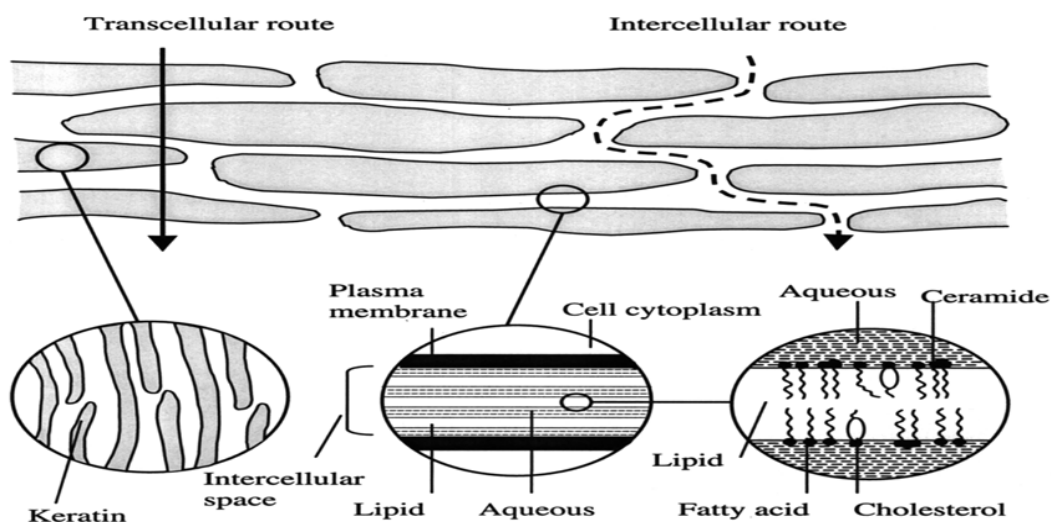


Figure 1.5 Permeation routes through stratum corneum

- 1) Transcellular
- 2) Intercellular

Source:-Ansel's Pharmaceutical dosage form and drug delivery system by Allen L.V,Popovich NG and Ansel HC in chapter Transdermal Drug Delivery System.

#### 1.6.4 Principles of drug permeation through skin:-

In the initial transient diffusion stage, drugs molecules may penetrate the skin along the hair follicles or sweat ducts and then be absorbed through the follicular epithelium and sebaceous glands. When the steady state has been reached, diffusion through stratum corneum becomes the dominant pathway.<sup>20, 21, 22, 23</sup>

The membrane-limited flux ( $J$ ) under steady condition is described by the following expression.

$$J = - \frac{D K_0}{h} \frac{C}{h}$$

Where:

$J$  = Amount of drug passing through the membrane system per unit area, per unit area per unit time.

$D$  = Diffusion coefficient of drug molecule

$A$  = Area of the diffusion membrane

$C$  = Concentration gradient

$K_0/w$  = Partition coefficient of drug

$h$  = Thickness of the membrane

### **1.7 Semi solid preparations:-**

Semi-solid formulations are widely used for transdermal delivery. Semisolids constitute various pharmaceutical dosage forms which aids for transdermal delivery through the skin, cornea, rectal tissue, nasal mucosa, vagina, buccal tissue, urethral membrane, and external ear lining. Semisolids have peculiar rheological behavior, they can adhere to the application surface for sufficiently long periods before they are washed off. This property helps prolong drug delivery at the application site. A semisolid dosage form is advantageous in terms of its ease of application, rapid formulation, and ability to topically deliver a wide variety of drug molecules.<sup>18, 19, 20</sup>

Semi-solid dosage forms are normally available in the formulations of creams, gels, ointments, pastes etc. They consist of one or more active pharmaceutical ingredients dissolved or uniformly dispersed in a suitable base and may contain other acceptable excipients such as emulsifiers, viscosity-increasing agents, antimicrobial agents, antioxidants, or stabilizing agents. Formulations susceptible to microbial contamination should contain suitable antimicrobial agent in an appropriate concentration for rendering them safe for use. Adequate studies must be conducted to ensure that any added excipient do not adversely affect the stability or efficacy of the final product and all the excipients must be compatible with each other in the dosage form.<sup>20, 21, 22</sup>

The selection of appropriate base for semi-solid dosage forms is governed by several factors such as the nature of the incorporated active ingredients, the desired therapeutic effectiveness, the release and availability of the active ingredients at the site of application, the stability and shelf-life of the finished product in the normal environmental conditions. Sometimes, the selection of base depends more on stability aspects as compared to efficacy aspects. For example, drugs that prone to hydrolysis are considered to be more stable in hydrophobic bases as compared to water-containing bases, even though their effectiveness may be more in the hydrophilic base.<sup>19, 20, 21</sup>

The base must be non-irritating and non-sensitizing to the skin. It also should not delay the wound healing. It should be inert in nature, smooth in texture, odourless, physically and chemically stable and compatible with the skin and with the other

active ingredients incorporated in the formulation. It should be of such rheological properties that it has the ease of application and also can withstand the stress of handling, packaging and transportation.

A transdermal semi-solid dosage form should be necessarily sterile and can also be applied on large open wounds or broken skin.<sup>20, 21</sup> Semisolids are available as a wide range of dosage forms, each having unique characteristics.

### **1.7.1 Ointments:-**

These semi-solid preparations are homogeneous and used for external application on the skin or mucous membranes. They are used either for emollient action or for the application of pharmaceutical active ingredients to the skin for protective, therapeutic, or prophylactic purposes and for occlusive action on skin if desired. They are difficult to be removed from skin and clothing. The release of medicament is unpredictable and there are chances that the formulation may go rancid and cause allergies.<sup>18, 19, 20, 21</sup>

### **1.7.2 Creams:-**

These semisolid dosage forms consist of one or more active ingredients dissolved or uniformly dispersed in a suitable base. The formulation is usually an oil in-water emulsion or aqueous microcrystalline dispersion of long-chain fatty acids or alcohols that are water washable and are cosmetically and aesthetically acceptable. They also are difficult to remove from skin since they are greasy and hence less patient compliance and can go rancid. They may easily break down if exposed to excessive heat or sudden changes in temperature.<sup>19, 21</sup>

### **1.7.3 Pastes:-**

These semi-solid preparations are homogeneous which contains high concentrations (not less than 20%) of insoluble powdered substances dispersed uniformly in a suitable base. The pastes are usually possess less greasiness, more absorptive properties, and stiffer consistency as compared to ointments because of the large concentration of powdered ingredients present in the formulation. The pastes should adhere well on the skin surface. In many cases they form a protective covering film that prevents the evaporation of water from the skin surface and imparts an occlusive effect. The major disadvantage of such dosage forms is the non-uniformity in dose as there are chances of solid particles getting aggregated after a period of time.<sup>19, 21</sup>

#### **1.7.4 Gels:-**

These are transparent to opaque semisolids containing a high ratio of solvent to gelling agent merge or entangle to form a three-dimensional colloidal network structure. This network limits fluid flow by entrapment and immobilization of the solvent molecules. The network structure is also responsible for a gel resistance to deformation and therefore, its viscoelastic properties. Gels tend to be smooth, elegant, non greasy and produce cooling effect and utilize better drug release as compared to other semi-solid formulation.<sup>16,19,20</sup>

#### **❖ Introduction to gels:-**

Gels are becoming more popular due to ease of application and better percutaneous absorption.

The term gel was introduced in the late 1800 to name some semisolid material according to pharmacological, rather than molecular criteria. The USP defines gels as semisolid systems consisting of either suspensions made up of small inorganic particles, or large organic molecules interpenetrated by a liquid, where the gel mass consists of a network of small discrete particles, the gel is classified as a two-phase system.<sup>16,19,20</sup> Gels are usually clear transparent semisolids containing the solubilized active substance. Single-phase gels can be described as three-dimensional networks formed by adding macromolecules such as proteins, polysaccharides, and synthetic macromolecules to appropriate liquids. In pharmaceutical applications, water and hydro alcoholic solutions are most common.<sup>19, 25</sup>

Many polymer gels exhibit reversibility between the gel state and sol, which is the fluid phase containing the dispersed or dissolved macromolecule. However, formation of some polymer gels is irreversible because their chains are covalently bonded. The term gel represents a physical state with properties intermediate between those of solid and liquids. However, it is often wrongly used to describe any fluid system that exhibits some degree of rigidity.<sup>20, 25</sup>

It is therefore recommended that the term should be restricted to those systems that satisfy the following criteria:-

1. These should be coherent colloidal system of at least two components (the gelling agent and a fluid component).
2. They should exhibit mechanical properties of the solid state.
3. Each component should be continuous throughout the system.

The term gels is broad, encompassing semisolid of a wide range of characteristics from fairly rigid gelatin slabs, to suspensions of colloidal clays, to certain greases. A gel can be looked upon as being composed of two interpenetrating phase, the gelling agent and a fluid component. Gels are semisolid dosage forms, that are either suspensions of small inorganic particles or large organic molecules interpenetrated with liquid vehicle. In the first case, the inorganic particles form a three-dimensional structure throughout the gel. This forms a biphasic system, as the inorganic particles are not soluble but dispersed uniformly throughout the continuous phase. Large organic molecules tend to exist in solution form as randomly coiled flexible chains.<sup>18, 25</sup>

These molecules which consist of natural or synthetic polymers, tend to entangle with each other because of their random motion. It is interaction between the units of the colloidal phase, inorganic or organic, that sets up the structural viscosity immobilizing the liquid continuous phase. Thus gels exhibit characteristics intermediate to those of liquid and solids.<sup>24</sup>



❖ **Classification of Gels:-**

Gels are classified mainly by two methods based on:

**a) Nature of colloid phase**

- i) Inorganic gels
- ii) Organic gels

**b) Based on nature of solvent**

- i) Aqueous gels
- ii) Non aqueous gels

**c) Based on their microstructure**

- i) Covalently bonded polymer networks with completely disordered structures.
- ii) Physically bonded polymer networks, predominantly disordered but containing ordered loci.
- iii) Well-ordered lamellar structures, including gel mesophases formed by inorganic clays.<sup>24, 25, 26</sup>

❖ **Gel forming substances:-**

Gels forming hydrophilic polymers are typically used to prepare lipid-free semisolid dosage forms, including dental, dermatological, nasal, ophthalmic, rectal, and vaginal gels and jellies. Polymers produce a structural network, which is necessary for the preparation of gels.<sup>24, 25</sup>

❖ **Benefits of transdermal gel over other semi solid dosage forms:-**

- Gels have high water content so they hydrate the skin and reduce the skin irritation.
- Hydrophilic gels are easily removed by gentle rinsing or natural flushing with body fluids reducing the propensity of mechanical abrasion.
- They show thixotropic behavior and have good spreadability.
- Gels are non staining and compatible with number of excipients.<sup>20,25,26</sup>

❖ **Limitations of transdermal gel:-**

- Skin irritation of contact dermatitis may occur due to the drug.
- Poor permeability of some drugs through the skin.
- Possibility of allergenic reactions.
- Drugs of larger particle size not easy to absorb through the skin.
- The drug release is unpredictable and there is probability of loss of drug.<sup>18,20</sup>

**1.8 Approaches to overcome the skin barrier:-**

A number of approaches have been used to cross the stratum corneum, the major skin barrier layer. These include the following physical and chemical methods:-

**1.8.1 Physical methods:** The physical methods to overcome the skin barrier includes the following:-

- Iontophoresis
- Sonophoresis
- Thermal modulation
- Stripping of stratum corneum
- Hydration of stratum corneum

Physical enhancement techniques have been studied that involve the use of an energy source to overcome the barrier properties of the skin. Iontophoresis and electroporation have been used typically for delivery of large molecular weight compounds. Low frequency sonophoresis has been demonstrated. Among all these methods chemical permeation enhancers are widely used for the permeation of drug across the skin since, the physical methods causes painful destruction of the skin with high current settings.

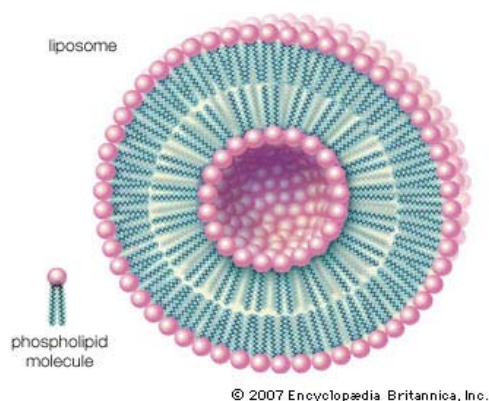
**1.8.2 Chemical methods:** The chemical agents can also interfere with the barrier mechanism of skin and can enhance the skin permeation of drugs. Some of the methods are listed below:-

- Delipidization of stratum corneum:-by organic solvents, surfactants in topical drug delivery.
- Synthesis of lipophilic analogues:- Liposomes, transferosomes, niosomes, Solid lipid nanoparticles etc.
- Chemical penetration enhancers: Agents capable of modifying the barrier to penetration presented by the skin are called as penetration enhancers or the chemical penetration enhancers are the substances which reversibly reduce the barrier resistance of the stratum corneum without damaging the viable cells. Examples are- Sulphoxides, Azone, Pyrrolidones, Essential oil, terpenes and terpenoids etc.

## 1.9 Introduction of novel carrier systems in transdermal gels:-

### 1.9.1 Liposomes:-

Liposomes are spherical vesicles made of phospholipid bilayer comparable to mammalian cell membrane. Liposomes contain an aqueous compartment which can carry molecules that are protected from the external environment.



**Figure 1.6 Schematic diagram of liposomes**

Source :- Encyclopedia Britanica,Inc,2007,USA

The different types of liposomes include small unilamellar vesicles made of a single bilayer, large unilamellar vesicles and multilamellar vesicles that contain several bilayers in a concentric manner. Methods of forming liposomes include dispersing phospholipids in aqueous medium, sonication, high pressure extrusion , detergent dialysis etc.<sup>27,28</sup>

The liposomes are supposed to enhance the permeation of drugs through skin and also can provide sustained release due to following theories:-

- They may adsorb and fuse with skin surface, the collapse of Formulation on tissues increases the driving force for permeation of liberated drug molecules and hence increase penetration<sup>28</sup>
- They may provide a reservoir of drug from which the slow release of drug allows the drug content within Arthritic tissues to increase. Through transdermal route, a lower systemic blood levels is reached as the drug is localized within skin membrane.<sup>29</sup>

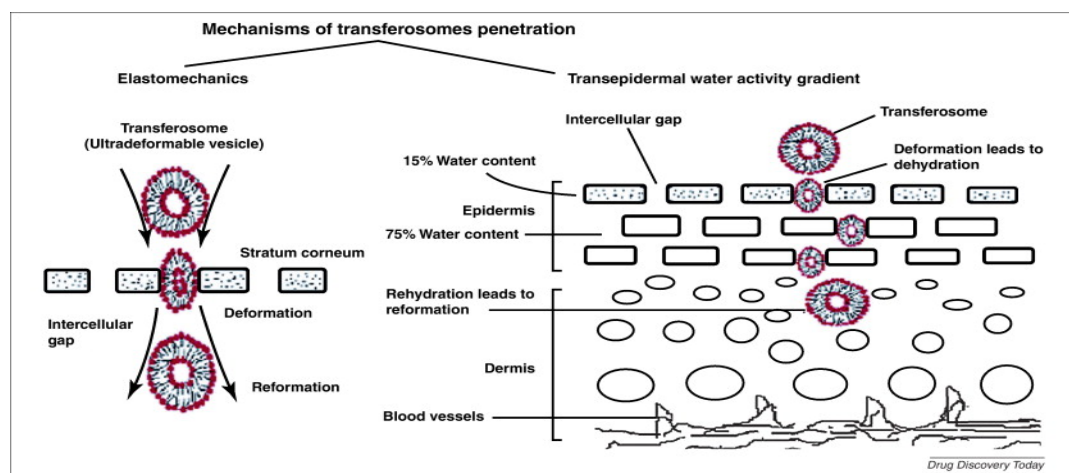
Strategies to optimize liposome formulation for higher drug loading and sustained drug release in localized region of the body need to be developed and the uptake of anti-inflammatory drugs to these cells need to be quantified.<sup>27,28,29</sup>

Liposomes still faces major deficiencies including:-

- Lack of control over drug release rate.
- Unsatisfying chemical stability of lecithin has an unfavorable influence on drug and liposome stability.
- Fast penetration through skin is difficult as liposomes cannot penetrate through narrow skin pores.<sup>28,29</sup>

Liposome encapsulation of different groups of anti-inflammatory agents, i.e., glucocorticosteroids, nonsteroidal anti-inflammatory drugs show an improved anti-inflammatory effect upon local administration and decreased toxic side effects<sup>32</sup>

### 1.9.2 Transferosomes:-



**Figure 1.7 Assumed mechanism of skin permeation of transferosomes**

Source:- Kumar A., Pathak K. and Bali V; Ultra adaptable nanovesicular systems: a carrier for systemic delivery therapeutic agents; Drug Discovery Today; 2012; 17; Numbers 21/ 22; 1233-1241.

Transferosomes are composed of a phospholipids component along with surfactants such as sodium cholate, spans and tweens. Ultradeformable vesicles are deformable or elastic in nature which can squeeze themselves through narrow pores even many times smaller ( $1/10^{\text{th}}$ ) than their size owing to their elasticity. The moisture seeking tendency hydrotaxis of transferosomes permits the carrier to bring more than 50% of the epicutaneously administered drug across the skin barrier. The transferosomes are

applied in a non-occlusive way to the skin and permeate through the stratum corneum lipid lamellar regions due to hydration gradient or osmotic force in the skin.<sup>30, 31, 32</sup>

These ultradeformable drug carriers trespass the intact skin spontaneously, probably under the influence of the naturally occurring, transcutaneous hydration gradient. The added surfactants acts as edge activators and control the flexibility of the vesicle. The transferosomes are considered as unique among drug carrier systems because it can accommodate hydrophilic, lipophilic as well as amphiphilic drugs.<sup>30, 31</sup>

### **1.9.3 Niosomes:-**

Niosomes are vesicular drug delivery system encapsulating the medicament. The vesicles are composed of non-ionic surface active agents of the alkyl or dialkyl polyglycerol ether class and cholesterol as a vesicle rigidizer. Structurally, niosomes are of a bilayer structure similar to liposomes, however, the non-ionic surface active agents forms the bilayer structure in the case of niosomes. Usually, surface active agents when dispersed in water produce micellar structures however some of the surfactants produce bilayer vesicles niosomes. Niosomes may be unilamellar or multilamellar depending on the method of preparation. The niosomes are classified on the basis of the number of bilayer such as multilamellar vesicles, small unilamellar vesicles etc. or depending on the method of preparation such as reverse phase evaporation, dried reconstituted vesicles etc. Niosomes present a structure similar to liposome and hence they can represent alternative vesicular systems with respect to liposomes, due to the niosome ability to encapsulate different type of drugs within their multi environmental structure.

The technology utilized in niosomes is still greatly in its infancy, and already it is showing promise in the fields of cancer and infectious disease treatments.

Niosomes are vesicles of lamellar structures formed by mixture of nonionic surfactant with subsequent hydration in aqueous media. Non-ionic surfactant acts as a penetration enhancer and hence can overcome the barrier of stratum corneum.<sup>32</sup>

Niosomes are reported to entrap both hydrophilic and lipophilic drugs, either in aqueous core or in the membrane made of lipid materials. It is reported to attain better stability than liposome. Proniosomes are dry carriers coated with surfactants, which can be rehydrated by brief agitation in hot water. These are considered superior drug delivery system because of low cost, greater stability, non-toxic, biocompatible, biodegradable and non-immunogenic, as it is nonionic in nature.<sup>31, 32</sup>

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## CHAPTER- II

### Literature Review

#### 2.1 Background:-

Nonsteroidal anti-inflammatory drugs and corticosteroids are most widely used and effective Drugs for treatment of Arthritis. Nonsteroidal anti-inflammatory drugs (NSAIDs) that act by inhibiting cyclooxygenase and the formation of prostaglandins, are known to cause GI toxicity, leading to the formation of peptic ulcers, unwanted antiplatelet effects (nonselective inhibitors of cyclooxygenase), cardiotoxicity, renal toxicity and anaphylactic reactions in selected patients.

Most of the current therapies for RA do not achieve target specificity and to reach effective drug concentrations in affected joint tissues, high dose of drug must be administered, which may lead to significant adverse systemic side effects. Reduction in drug doses may exhibit lesser toxicity but may lead to decreased therapeutic efficacy. To overcome this problem, approaches that can specifically target the therapeutic agent to affected joints offer unique promise. <sup>1, 2, 4</sup>

Transdermal delivery of drugs has many advantages such as avoidance of hepatic first-pass metabolism., improved patient compliance and ease of access, provide a means to quickly terminate dosing sustained therapeutic drug levels, possible self - administration, non-invasive (no needles or injections needed),avoids food related Interaction, reduction of doses as compared to oral dosage forms and intravenous therapy. Topical route allows drug to diffuse out of its vehicle onto the surface tissues of skin.

In fact ease of applicability makes this route more comfortable for the patient which results in better patient compliance. <sup>8,9,10</sup>

There is no significant obstacle to penetration, once the drug permeates through stratum corneum of epidermis of skin. Topical NSAIDs have been reported to have reduced incidences of systemic side effects like gastric bleeding and peptic ulcer. The side effects of aceclofenac and indomethacin, such nephrotoxicity and neurotoxicity, limit its use as a

drug for arthritis. So far most of the analgesics and anti-inflammatory drugs used for the treatment of arthritis are administered mainly by transdermal route.<sup>8,10,11</sup>

The feasibility of topical route over parenteral route in treatment of arthritis has been evaluated in several studies.

## 2.2 Skin Permeation Enhancement/Optimization techniques:-

The skin permeation is a major task for the transdermal drug delivery system to achieve efficacy of formulation. Various approaches have been tried earlier to cross the skin barrier layers and to enhance permeation of drugs.<sup>9,10,11,12</sup>

**Table 2.1 Skin permeation enhancement techniques**

Drug/Vehicle based	Stratum corneum modification
↓	↓
Drug selection	Hydration
Pro drugs and ion pairs	Lipid fluidization
Drug-vehicle interaction	Bypass/removal
Chemical potential of drug	Electrical method
Eutectic system	
Complexes	
Liposomes	
Vesicles and particles	

Another approaches investigated are replacement of surfactant with skin penetration enhancers such as oleic acid and limonene as the edge activators. In addition, formulation with low percentage of alcohol, reduces the liposome size and increase the drug release and skin flux. Many enhancers, such as azone, DMSO, alcohols, fatty acids and terpenes, have been shown to increase permeability by disordering or 'fluidising' the lipid structure of the stratum corneum.<sup>19,20,21,22</sup>



### 2.3 Novel treatment strategies of arthritis:-

Several advancements have been made in recent past to address the issue of side effects in case of chronic therapy in arthritis. Formulation approaches like liposome, PEG liposome, polymeric micelles, solid lipid nanoparticles, polymeric vesicles etc. have been explored to achieve limited success. Liposomes still faces major deficiencies including lack of control over drug release rate, insufficient loading of drugs for which pH and ion gradients do not apply and lack of means to override biological barriers. Surface modification of liposomes by the inclusion of hydrophilic components (e.g., carbohydrates, glycolipids or polymers) to form long-circulating liposomes cause changes in the pharmacokinetic pattern seen for unmodified (classical) liposomes. Problem associated with polymeric carriers is that they are less biocompatible and more toxic as compared to liposomes and selection of polymers and their use for drug delivery must be carried out with caution.<sup>26, 27, 28, 29, 31</sup>

Topical methotrexate gel prepared with poloxamer 407 polymer have been observed to produce sustained and higher drug levels in muscle tissues beneath the site of administration.<sup>35</sup>

For the purpose to enhance the skin permeability of ketoprofen, various topical formulations have been formulated; one such formulation includes topical Oleo hydrogel ketoprofen preparation with enhanced skin permeability.<sup>36</sup>

Various approaches for targeted drug delivery have been widely used in previous studies. In inflammatory diseases several circumstances are known to activate the cellular immune response. An increased presence of immune-related cells like macrophages is common in the inflamed area.

In general, macrophages which are produced by the spleen leading to an inflammatory response are the primary target cells in this drug targeting approach. Although selective targeting to those immune-related cells to organs different from liver and spleen is challenging however, essential for the success of a potential anti-inflammatory therapy. The nanoscaled drug carriers improve selective delivery of drugs to the site of action, so-called drug targeting which can be either passive or active targeting. Passive targeting can be achieved without further integration of a specific targeting moiety on the particle surface. It has been shown that microspheres and nanoparticles can be efficiently taken up by macrophages, and mainly by phagocytosis. Thus, particle uptake into those

immune related cells or the disruption of the epithelium could allow the selective accumulation of the nanocarrier based drug delivery system in the desired area.<sup>2, 4, 7, 26, 27</sup>

Phagocytic cells such as macrophages and neutrophils play important role in induction and maintenance of inflammation. Strategies to optimize the drug delivery within a localized arthritis tissue and the uptake of anti-inflammatory drugs to these cells need to be quantified.

Therefore, passive targeting can be achieved in inflammatory diseases due to immune response. To reach efficient uptake into those immune-related cells, particle properties like size and surface charge play a key role.<sup>7, 26, 27</sup>

So far most of the analgesics and anti-inflammatory drugs used for the treatment of RA, are administered mainly by transdermal route. Currently anti inflammatory drugs are mainly delivered by transdermal iontophoresis. New drug application (NDA) of Alza corporation for iontophoretic fentanyl containing transdermal analgesic have been approved by US FDA.<sup>37</sup> Iontophoresis is a special method of applying drug to and pushing it through the skin to reach the blood vessels and surrounding deeper tissues by electric transmission. A significant amount of Piroxicam was retained in the skin after transdermal iontophoresis from piroxicam.<sup>38</sup>

In order to increase penetration and a prolonged release, lipid nano/submicron emulsion can be used as a vehicle for transdermal delivery of drugs.

The success of transdermal delivery depends on the ability of the drug to permeate the skin in sufficient quantities to achieve its desired therapeutic effect.

Stratum corneum layer of skin is considered as the major barrier for several compounds which need to permeate through the skin. Various approaches have been tried in earlier studies to cross this skin barrier. A promising approach for increasing the skin permeation of drugs can be the use of vesicular systems, such as liposomes, transferosomes and niosomes.<sup>30, 31, 32, 33</sup>

## **2.4 Review of literature on transdermal drug delivery system of nonsteroidal anti-inflammatory drugs:-**

**1. Sheikh A. et al in year 2011**, formulated and characterized aceclofenac gel containing linseed oil and ginger oleoresin and concluded that aceclofenac gel was prepared using different gelling agent like carbopol, HPMC K4M, sodium CMC. It was found that gel with carbopol 974P showed better physical properties and better permeability as compared to other gel base in gel formulation. The evaluation parameters were found to be in range when compared with marketed preparation NSAIDs Gel.

After 3 months of stability studies, the drug content of the formulation was found to be in limit (98.60%).<sup>40</sup>

**2. Karade P G. et al in year 2012**, formulated and evaluated celecoxib gel and concluded that the gel prepared with different gelling agent (Carbopol, Sodium Carboxy methyl cellulose, Sodium Alginate) showed good physical characteristics. The formulation containing 6.25% w/w sodium alginate and 2% DMSO as penetration enhancer was found to be suitable for topical application based on in vitro evaluation.<sup>41</sup>

**3. Abrar B. et al in year 2011**, reviewed on formulation and in vitro evaluation of NSAID's gel and concluded that the topical gels gives sustained delivery of drug onto the skin, so they are interesting promises to improve skin absorption of non steroidal anti-inflammatory drugs and to prevent side effects associated. The gel preparation is excellent in the percutaneous absorption of diclofenac or its salts and gives good properties and medical effect.<sup>42</sup>

**4. Rasool B. et al in year 2010**, developed and evaluated Ibuprofen transdermal gel and concluded that the apparent viscosity of the test formulations was comparable to that of the reference standard, Ibutop®. The inclusion of propanol increased the apparent viscosity of the test gels. The use of menthol, glycerol and propylene glycol (PG) as permeation enhancers significantly increased drug release rate constants. Ibuprofen solubility is enhanced by the addition of menthol. The in vivo study of anti inflammatory action by carageenan induced paw method showed that the marketed preparation (Ibutop) as well as formulation containing 5% menthol as penetration enhancer and 20%

propylene glycol as viscosity enhancing agent doesn't show anti-inflammatory action, this is due to requirement of higher dose of ibuprofen. But this formulation showed analgesic activity higher than that of marketed formulation.<sup>43</sup>

**5. Das M K. et al in year 2007**, formulated and performed ex vivo evaluation of topical rofecoxib gel and concluded that gel showed good homogeneity and spreadability with good physical appearance using hydroxypropylmethylcellulose (HPMC), sodium alginate and carbopol 940 as gelling agent. The gel formulation consisting of 4% w/w sodium alginate-Carbopol 940 at 3:1 ratio was found to be suitable for topical application based on *in vitro* evaluation and ex-vivo permeation studies. The anti-inflammatory activity of the rofecoxib gel formulation was evaluated using the rat hind paw edema model.<sup>44</sup>

**6. Bacchav Y G. et al in year 2010**, formulated meloxicam gel for topical application and evaluated in vitro and in vivo and showed that the draize's test for skin irritation for meloxicam gel showed zero score indicating its safety and acceptability for topical application. Meloxicam gel also exhibited two fold higher anti-inflammatory activity in carageenan induced paw method.<sup>45</sup>

**7. Tanwar et al in year 2012** prepared topical gel formulation of diclofenac sodium using different gelling agent carbopol, Na CMC, HPMC (K4M) and sodium alginate in different concentration. Studies showed that drug release was decreased with increase in gelling agent concentration because as the polymer concentration increases, viscosity also increases.<sup>46</sup>

**8. Nigam N. et al in year 2012** modulated anti-inflammatory activity of NSAIDS in normal rats treated with antihistaminic and concluded that the anti-inflammatory action of aspirin, ibuprofen, piroxicam was studied alone as well as along with anti-histaminic shows anti-inflammatory effects. Anti-inflammatory activity was determined by carageenan induced inflammation paw method.<sup>47</sup>

**9. Khullar et al in year 2013** formulated emulgel of mefenamic acid, a NSAID, using carbopol 940 as a gelling agent. Mentha oil and clove oil were used as penetration

enhancers. The emulsion was prepared and it was incorporated in gel base. The formulations showed comparable analgesic and anti-inflammatory activity when they compared with marketed diclofenac sodium gel.<sup>48</sup>

**10.** In a study by **Kumar et al in year 2013**, topical gel formulations of aceclofenac were prepared using these polymers such as Carbopol-934, Carbopol-940, HPMC, Poloxamer 407 in different concentration for the treatment of rheumatoid arthritis. The release rate of the gel was found to obey Higuchi model. The percentage of drug release was found to be highest from the gel prepared using Poloxamer-407 followed by Carbopol- 940 & Carbopol-934.<sup>49</sup>

**11.** In a study by **Nemat et al in year 2015** found that, Copper- Indomethacin topical gel has potent anti-inflammatory activity against joint inflammation in the monosodium iodoacetate treated rat model of osteoarthritis at doses of 0.25, 0.5, and 1 g/kg. The lowest studied dose was also satisfactorily complying to both safety and efficacy parameters.<sup>50</sup>

**12.** In a study by **Raju et al in year 2015**, the transemulgel was prepared from aqueous Aloe vera gel and aceclofenac emulsion. The formulated aceclofenac-Aloe vera transemulgel and aceclofenac marketed gel were subjected to pharmacodynamic studies in albino rats of Wistar strain employing carrageenan induced left hind paw edema method to analyse the anti-inflammatory effect. The prepared aceclofenac Aloe vera transemulgel was found to provide better in-vitro drug release as compared to the aceclofenac marketed gel formulation. Anti-inflammatory activity in treated rats also showed the significant paw volume reduction at  $p < 0.05$  compared with that of control.<sup>51</sup>

**13.** **Shakeel et al in year 2007**, investigated the potential of a nanoemulsion formulation for transdermal delivery of aceclofenac. Several oil-in-water nanoemulsions were prepared in the study by the spontaneous emulsification method. As outcomes of the study, permeability parameters such as steady-state flux ( $J(ss)$ ), permeability coefficient ( $K(p)$ ), and enhancement ratio ( $E(r)$ ) were analyzed for optimized nanoemulsion formulation containing 2% w/w of aceclofenac which showed promising results. The

anti-inflammatory effects of nanoemulsion gel formulation showed a significant increase ( $P < .05$ ) in percent inhibition of inflammation after 24 hours when compared with aceclofenac conventional gel as determined by carrageenan-induced paw edema in rat.<sup>52</sup>

**14.** In a study by **DasGupta et al in year 2013**, the oil-in-water nanoemulsions were prepared by screening the excipients from the nanoemulsion region of pseudoternary phase diagram. The nanoemulsion formulations that passed thermodynamic stability tests were characterized for viscosity, droplet size, transmission electron microscopy, refractive index and in vitro skin permeation. The in vitro skin permeation profile of optimized nanoemulsion was compared with marketed gel formulation. The significant ( $p < 0.001$ ) increase in in-vitro permeability and in-vivo anti-inflammatory efficacy of the formulation has also been observed in comparison with the marketed gel formulation of aceclofenac.<sup>53</sup>

## 2.5 Novel drug carrier systems for arthritis:-

### 2.5.1 Liposomes:

Liposomes are spherical vesicles made of phospholipid bilayer comparable to mammalian cell membrane. Liposomes contain an aqueous compartment which can carry molecules that are protected from the external environment. The different types of liposomes include small unilamellar vesicles made of a single bilayer, large unilamellar vesicles and multilamellar vesicles that contain several bilayers in a concentric manner.

Liposomes due to their biphasic characteristic and variability in design and composition, offer a dynamic and adaptable technique for enhancing drug solubility.

The systemic use of liposomes has drawbacks such as rupturing and rapid clearance from the blood, thereby release of drugs at undesirable sites. Liposomes still face major deficiencies including: lack of control over drug release rate; sufficient loading of drugs for which pH and ion gradients do not apply and lack of means to override biological barriers. Easy oxidation of phosphatidylcholine, the main membrane component, is the limitation of the introduction of liposomes to the medicinal practice on a large scale. Unsatisfying chemical stability of lecithin has an adverse influence on drug and liposome stability. Liposomes may decompose during storage leading to leakage of the encapsulated drug.<sup>54,55</sup>

There are several advantages of using liposomes in transdermal therapy. They may adsorb and fuse with skin surface, the collapse of Formulation on tissues increases the driving force for permeation of liberated drug molecules and hence increase penetration. They provide a reservoir of drug from which the slow release of drug allow the drug content within arthritis tissues to increase. Through transdermal route, a lower systemic blood levels is reached as the drug is localized within skin membrane. Liposomes include easy encapsulation of hydrophilic drugs into their core compartment and hydrophobic drugs into their lipid bilayer, excellent biocompatibility, ability to penetrate effectively into cell membranes, delivery of drugs into the cell compartments and diversity in modifying the surface properties by altering or introducing new components into the lipid bilayer.<sup>54,55,56</sup>

Methods of forming liposomes include dispersing phospholipids in aqueous medium, sonication, high pressure extrusion, detergent dialysis etc.<sup>55</sup>

❖ **Methods of preparation of liposomes:-**

• **Thin film hydration method:-**

The required quantity of phospholipid is dissolved in chloroform-methanol mixture in a round bottom flask. The flask is connected to vacuum rotary evaporator in order to evaporate organic solvent under vacuum until total evaporation and formation of thin film forms on the sides of the flask. The flask is connected overnight to a vacuum pump for removal of every trace of organic solvent. The film is hydrated with phosphate buffer in the flask with vortex agitation. For lipids with high  $T^{\circ}\text{C}$  such as DPPC, DSPC, medium should be preheated above lipid  $T^{\circ}\text{C}$ . The prepared MLV Suspension is subjected to probe sonication until vesicle dispersion become completely transparent. Following sonication, the SUV suspension is left for two hours at a temp higher than  $T^{\circ}\text{C}$  to anneal any structural defects of vesicles.<sup>54,55</sup>

• **Solvent Injection Methods:-**

A solution of lipids dissolved in diethyl ether or ether/methanol mixture is slowly injected to an aqueous solution of the material to be encapsulated at 55-65 $^{\circ}\text{C}$  or under reduced pressure. The subsequent removal of ether under vacuum leads to the formation of liposomes.<sup>55</sup>



**❖ Review of literature on liposomes of nonsteroidal anti-inflammatory drugs:-**

1. A study conducted by **Lenard et al in year 2012**, says that for transdermal absorption of NSAIDs at the localized site of action, liposomes may be a useful tool. With the use of Fourier Transform infra-red, Nuclear Magnetic Resonance and Surface Plasma Resonance, it has been demonstrated that NSAIDs have a strong affinity to form ionic and hydrophobic associations with zwitter ionic phospholipids and specifically phosphotidylcholines and this association is pH dependent usually at pH 3.5 but there is no significant change at neutral pH. Therefore pH dependent partition of potent anti-inflammatory drugs into phospholipids may result in change in hydrophobicity, fluidity, permeability, biochemical properties and stability.<sup>56</sup>

2. **Caldwell et al in year 2004**, formulated a liposomal suspension of diclofenac sodium. A single topical application of diclofenac liposomal suspension has shown concentrations of diclofenac in transudate within 6 hours and significantly attenuated carrageenan-induced local production of prostaglandin . Results of this study suggest that DLS is readily absorbed transdermally and may be efficacious for reducing subcutaneous inflammation.<sup>57</sup>

3. In another study by **Nishihata by year 1987**, diclofenac sodium loaded liposomes were prepared by thin film hydration technique using soya lecithin, cholesterol followed by sonication and then incorporation into 1% carbopol gel. The particle size, polydispersity index and zeta potential of liposomes were found to be 230 nm, 0.247 and -41 respectively and the entrapment efficiency was found to be 62%.The cumulative amount of drug permeated in 24 hour form the liposomal gel formulation was found to be 1176.7 µg/cm.<sup>58</sup>

4. From the drug entrapment efficiency study of ketoprofen liposomal gel formulation conducted by **Mansoori et al in year 2012**, maximum drug encapsulation of 97.51% was observed in Formulation, in which lipid and cholesterol were used in ratio of 1:2. Therefore it can be interpreted that, in liposome preparation, cholesterol was found to acts as vesicle rigidiser and provided stability and rigidity to liposome. The marketed gel

of Ketoprofen released approximately 92% of drug within 24 hour, whereas the liposomal formulations showed 87% drug release respectively in 24 hour. Liposomal formulations showed sustained drug release compared to normal gel, also an increase in release rate was observed after 12 hour.<sup>59</sup>

**5. Puglia et al in year 2004** attempted to prepare LUV dispersions containing indomethacin by extrusion method using dipalmitoyl-L-alpha-phosphatidylcholine and cholesterol and observed a high percentage of entrapped drug (approximately 84%). Furthermore, in-vivo findings revealed that the anti-inflammatory effect was more prolonged when indomethacin was delivered from a liposomal gel formulation rather than from a gel formulation without liposomes. In particular, the indomethacin-loaded gel formulation LUV-A showed a sustained release, possibly related to an interaction between LUV lipids and stratum corneum lipid structure. The anti-inflammatory effect was also found to be more prolonged when indomethacin was delivered from a liposomal gel formulation rather than from a gel formulation without liposomes. Therefore the selection of lipids for the formulation can be considered critical as it can certainly effect the drug permeation and duration of anti-inflammatory effects.<sup>60</sup>

**6.** In a study by **Srinath et al in year 2000**, a series of liposomal formulations of indomethacin were prepared using various phospholipids. The effects of method of preparation, lipid composition, charge, and cholesterol (CH) on encapsulation of indomethacin in liposomes were investigated. Pharmacodynamic evaluation of the liposomes was performed by carrageenan-induced rat paw edema (acute) and adjuvant arthritis (chronic) models. The anti-inflammatory activity was increased from the first to fifth hour. Liposomes showed the highest percentage inhibition of edema. The anti-inflammatory activity of liposomal indomethacin was significantly higher than that of free indomethacin.<sup>61</sup>

**7.** Piroxicam liposomes were prepared by **Canto et al in year 1999** by thin film hydration technique using Phospholipids and cholesterol. Liposomes were characterized by electron transmission microscopy, and the mean structure diameter was found to be 278 nm. The encapsulation efficiency obtained was 12.73%. The topical anti-inflammatory effect was evaluated in vivo by the cotton pellet granuloma method.

Inhibition of inflammation by free piroxicam and piroxicam encapsulated in liposomes gel was observed to be 21.1%, and 47.4%, respectively. These results showed that the encapsulation of piroxicam produced an increase of topical anti-inflammatory effect. In addition it was also observed that, anti-inflammatory effect can be achieved using lower drug concentrations when formulated as liposomal gel.<sup>62</sup>

**8.** In a study by **Wasankar et al in year 2012**, liposomal gel of Dex-ibuprofen was prepared by rotary evaporation followed by sonication using phosphatidylcholine and cholesterol .Particle size of 5.40  $\mu\text{m}$  and entrapment efficiency of 61% was achieved. The Formulation showed sustained drug delivery for 12 hours. In the study, phosphatidylcholine and cholesterol ratios were varied and it was observed that, ratio of phosphatidylcholine and cholesterol significantly effect the entrapment efficiency of drug in liposomes.<sup>63</sup>

**9.** **Venkataharsha et al in year 2015**, formulated naproxen and nimesulide liposomal formulation for incorporation in *Aloe vera* trans-emulgel. The formulated naproxen and nimesulide liposomal formulation using *A. vera* trans-emul gel were evaluated for in-vitro studies such as drug release, permeation study, drug content and entrapment efficiency. The formulations were found to be stable and prepared gel base was found effective with high drug release and drug content as compared with commercial formulation .The in-vivo anti-inflammatory action measured by Paw edema method in Wistar rats induced by carrageenan was also observed to be significantly improved by liposomal formulations.<sup>64</sup>

**10.** In a study by **Barbara et al in year 2005**, Liposomes loaded with ketorolac tromethamine salt were prepared by using a thin layer evaporation method and the physical properties of liposomes were studied by using atomic force microscopy (AFM) and transmission electron microscopy (TEM). The drug content was found to be dependent on the lipid composition used in the formulation and, in particular, vesicles containing both cationic lipids and phosphatidylcholine were found to have higher entrapped efficiency than liposomes with phosphatidylcholine alone. The cationic liposomes observed to be useful as carriers for ketorolac tromethamine to control its release.<sup>65</sup>

### **2.5.2 Transfersomes:-**

These are ultradeformable vesicle, elastic in nature which can squeeze itself through a pore which is many times smaller (1/10th) than its size owing to its elasticity. These are applied in a non-occluded method to the skin and have been shown to permeate through the stratum corneum lipid lamellar regions as a result of the hydration or osmotic force in the skin. Transfersomes are made up of a phospholipid component along with a surfactant mixture (Sodium Cholate, Spans and Tweens).<sup>66, 67, 68</sup>

The ratio and total amount of surfactants which acts as edge activator controls the flexibility of the vesicle. The unique property of this type of drug carrier system lies in the fact that it can accommodate hydrophilic, lipophilic as well as amphiphilic drugs. These ultradeformable drug carriers trespass the intact skin spontaneously, probably under the influence of the naturally occurring, transcutaneous hydration gradient. The 'moisture seeking' (hydrotaxis) of transfersomes permits the carrier to bring more than 50% of the epicutaneously administered drug across the skin barrier.<sup>66, 67, 68, 69</sup>

Nagasamy et al in year 2014, reviewed various formulations of transfersome and concluded that transfersome can pass through even tiny pores (100nm) because of their ultra deformable nature. It can carry sufficient amount of drug per unit time across the skin. It can hold the larger molecule like peptide and drugs having poor penetration and modify them to produce faster and targeted action.<sup>70</sup>

#### **❖ General method of preparation of transfersomes:-**

##### **• Thin film hydration method:-**

The mixture of vesicles forming ingredients, phospholipids and surfactant are dissolved in volatile organic solvent (chloroform-methanol mixture) then, organic solvent was evaporated above the lipid transition temperature using rotary evaporator. Final traces of solvent were removed under vacuum for overnight.

The deposited lipid films were hydrated with buffer by rotation at the corresponding temperature. The resulting vesicles were swollen for 2 hr at room temperature. To prepare small vesicles, resulting LMVs are sonicated using a probe sonicator.<sup>67, 68</sup>

- **Ether injection method:**

The ether injection method is usually based on slow injection of transferosome ingredients in diethyl ether through a 14-gauge needle at the rate of approximately 0.25 ml/min into a preheated aqueous phase maintained at 60°C.

The probable reason behind the formation of larger unilamellar vesicles is that the slow vaporization of solvent results in an ether gradient extending towards the interface of aqueous-non aqueous interface and thus responsible for the formation of the bilayer structure. The disadvantages of this method are that a small amount of ether is frequently present in the vesicles suspension and is difficult to remove.<sup>67, 68, 69, 70</sup>

- ❖ **Literature review on transferosomes:-**

Agents with lesser molecular weights are usually delivered through transdermal route with the help of skin permeation enhancers that increase the drug diffusion and partitioning. Use of drug carriers liposomes and niosomes were not very much successful till date, due to the inability of these carriers to pass through the narrow intercellular passages (virtual pores of approximately 30 nm) of the outer skin layers.

A solution to this problem is the introduction of more deformable vesicles, transferosomes. These novel drug-carriers are driven across the skin by the naturally occurring transepidermal hydration gradient and transport efficiently the therapeutic agents into the body.<sup>66, 67, 68, 71</sup> Transferosomes were successfully used in animals and humans and clinically proven to be safe and efficacious. The theoretical rationale for selection of such drug carrier systems are described here in this section with the corresponding experimental examples.

1. It is evident from the studies carried out by **Gregor et al in year 2001** where transferosomes of diclofenac Na were prepared using soya phosphatidylcholine by

suspending lipids in aqueous phase containing drug and thereafter sonication, size achieved was in the range of 100-200 nm diclofenac association with ultradeformable carriers have a longer effect and reach 10-times higher concentrations in the tissues under the skin in comparison with the drug from a commercial hydrogel. In rats, a single epicutaneous application of 2 mg of diclofenac per kg body weight in highly deformable carriers produced at least 4 times higher drug concentration in the treated muscles than a drug-loaded hydrogel. These Transfersomes are proposed to cross the skin spontaneously under the influence of transepidermal water activity gradient.<sup>72</sup>

2. In another study on Ibuprofen transfersomes by **Irfan et al in year 2001**, the best formulations were observed with the use of span 80 and tween 80 where vesicle size was found to be 962 nm and 2250 nm respectively, and zeta potential (negatively charged) for span 80 and tween 80 was found to be -16.1 and -17.5 respectively. The %EE of ibuprofen in the vesicles was 47.8 and the elasticity of both increases with increase in surfactant conc. and were found to be 34.4 and 26.5. In vitro skin permeation studies were carried by human cadaver skin using Franz diffusion cell, and drug release after 24 hrs and flux were found to be 2.5824 and 1.9672  $\mu\text{g}/\text{cm}^2/\text{hr}$  respectively. Fourier Transform Infrared Spectroscopy (FT-IR) and Differential Scanning Calorimetry (DSC) analysis indicated that the application of transfersomes significantly disrupted the stratum corneum lipid.<sup>73</sup>

3. In a review conducted by **Chandra et al in year 2014**, on vesicular drug delivery it is described that, the various vesicular system provides flexibility for drug carrier thus overcoming various solubility and bioavailability problems.<sup>74</sup>

4. **Sarwa et al, in a study in year 2015**, formulated and evaluated the potential of capsaicin loaded transfersome in arthritic rats. Capsaicin transfersosomal formulation showed better anti-inflammatory activity as compared with conventional gel. The reason suggested were good entrapment efficiency & high skin permeability.<sup>75</sup>

5. **Shaji et al in year 2014**, prepared transfersosomal formulation for enhanced transdermal delivery of a COX-2 inhibitor. Piroxicam transfersome prepared, evaluated and compared with conventional gel and drug solution. Advantages of being

transferosomal gel had a higher cumulative drug permeation, flux and had a better anti-inflammatory activity.<sup>76</sup>

**6. Gupta et al in year 2012**, formulated and evaluated sertraline loaded transferosome and concluded that the transferosomal gel showed a higher cumulative drug permeation and flux and had a lower lag time compared to drug solution and drug gel. The modified forced swim test in mice revealed that the formulation had better antidepressant activity as compared to the control gel.<sup>77</sup>

**7. Mohammed et al in year 2012**, prepared and characterized the ibuprofen loaded transferosome and concluded that %entrapment efficiency and elasticity increased with increased in surfactant concentration. From the In-vitro permeation study, it was concluded that the transferosomal formulation had 5-6 time more transdermal flux and had a less lag time than liposome. Stability study showed that leakage was negligible at refrigerated storage condition.<sup>78</sup>

**8. Sureewan et al in year 2013**, developed meloxicam (MX)-loaded cationic transfersomes as skin delivery carriers and investigated the influence of formulation factors such as cholesterol and cationic surfactants on the physicochemical properties of transfersomes such as particle size, size distribution, droplet surface charge and morphology, entrapment efficiency, stability of formulations and *in-vitro* skin permeation of MX. Cationic transfersomes were found to provide greater MX skin permeation than conventional liposomes and MX suspensions. The results indicated that the barrier function of the SC was affected by the physicochemical characteristics of the vesicle systems (size, charge, and %EE) and lipid composition (cholesterol and surfactant). A penetration-enhancing mechanism of transfersomes by vesicle adsorption and fusion with the Stratum Corneum, was observed. This study suggests that cationic transfersomes have the potential to be dermal delivery carriers of MX.<sup>79</sup>

**9. In a study by Duangjit et al in year 2014**, novel ultradeformable liposomes (mentosomes; MTS), deformable liposomes (transfersomes; TFS) and conventional liposomes (CLP) were compared in their potential for transdermal delivery of meloxicam (MX). MTS, TFS and CLP were investigated for size, size distribution, zeta potential,

elasticity, entrapment efficiency and stability. In-vitro skin permeation using hairless mice skin was evaluated using confocal laser scanning microscopy (CLSM). The results indicated that the difference in physicochemical characteristics of MTS, TFS and CLP affected the skin permeability. MTS and TFS showed higher flux of MX than CLP. CLSM image showed deformable vesicles mechanism for delivery of MX across the hairless mice skin. The study suggested that ultradeformable and deformable liposomes (MTS and TFS) had a potential to use as transdermal drug delivery carriers for MX.<sup>80</sup>



### 2.5.3 Niosomes:-

Niosomes are vesicles of microscopic lamellar structures formed by admixture of nonionic surfactant of the alkyl or dialkyl polyglycerol ether class and cholesterol with subsequent hydration in aqueous media. Non-ionic surfactant acts as a penetration enhancer and hence can overcome the barrier of stratum corneum.

Niosomes can entrap both hydrophilic and lipophilic drugs, either in aqueous layer or in vesicular membrane made of lipid materials. It is reported to attain better stability than liposomes. Proniosomes are dry formulations of surfactant-coated carrier, which when needed, rehydrated by brief agitation in hot water. These are considered superior drug delivery system because of low cost, greater stability, non-toxic, biocompatible, biodegradable and non-immunogenic, as it is nonionic in nature.<sup>81, 82</sup>

#### ❖ Literature review on niosomes:-

1. The non-ionic surfactant vesicles niosomes were first reported for cosmetic applications by L'Oreal. Niosomes were formulated and patented by the industry **L'Oreal in the year 1970s and 80s**. The benefit of using niosomes in cosmetic and skin care applications was their ability to enhance the stability of entrapped drugs and to improve the bioavailability of poorly absorbed ingredients along with enhanced skin penetration.<sup>81,82</sup>

2. The effect of type of surfactant on the characteristics of niosomes is significant as evident from the work of **Ibrahim et al in year 2005**, which showed that ketorolac niosomes formed from span 60 and tween 20 exhibited a very high encapsulation efficiency owing to almost complete inclusion of the highly lipophilic portion of the drug within the lipid bilayer of the niosomes. The niosomal formulation using span 60 showed an entrapment efficiency of 98.9% and a vesicle size of 6.6 micron whereas for those made using tween 20, the entrapment efficiency of 99% and vesicle size of 27.5 micron was observed.<sup>83</sup>

3. In another study by **Mansoroi et al in year 2004**, a novel elastic bilayer vesicle (niosomes) entrapping diclofenac diethyl ammonium was prepared for topical use. This eighteen bilayer vesicular formulations comprised of DPPC, tween 61 or span 60 mixed with cholesterol in solvent ethanol at 25% (v/v), and prepared by chloroform film method with sonication. Niosomes made using tween 61 gave no sedimentation, no layer separation, unchanged particle sizes of about 200 nm. The entrapment efficiency of the drug in the conventional and elastic tween 61 niosomes was found to be 65% and 93%, respectively and demonstrated the enhancement of transdermal absorption through rat skin as well as enhanced in vivo anti-inflammatory effect.<sup>84</sup>

4. In a study by **Solanki et al in year 2009**, the ketoprofen niosomal gel prepared by slurry method using span 40, cholesterol and malt dextrin (carrier) followed by probe sonication, shows 54.82% percentage drug entrapment and mean vesicle diameter of 4.92 micrometer. The cumulative amount of drug permeation of ketoprofen from niosomal gel was found to be  $403.65 \mu\text{gcm}^{-2}$  and from plain gel was  $346.48 \mu\text{gcm}^{-2}$ . The steady state transdermal flux from niosomal gel was found to be significantly higher  $50.81 \mu\text{gcm}^{-2}\text{h}^{-1}$  than from the plain gel i.e.  $38.12 \mu\text{gcm}^{-2}\text{h}^{-1}$ . The developed niosomal gel formulation demonstrated significant enhancement of permeation as compared to plain drug gel.<sup>85</sup>

5. Piroxicam niosomes prepared with coacervation method in a study conducted by **Chandra et al in year 2008**, using various grades of non-ionic surfactants spans, cholesterol & lecithin, it was observed that, span 40 and span 60 showed better percentage drug entrapment of 90.4 and 94.8% respectively. Niosomes prepared using span 60 were smaller in size, demonstrated higher entrapment efficiency and higher surface area as compared to that of span 40. Maximum flux achieved was  $35.61 \text{ g/cm}^2/\text{h}$  from the niosomes, and thus an enhancement of 7.39 times drug permeation was achieved for transdermal system based on proniosomal gel as compared to control gel.<sup>86</sup>

6. In a study by **Alam et al in year 2010**, celecoxib proniosomes were prepared using surfactant (Span 40 and Span 60), alcohol (ethanol or isopropyl alcohol) and CXB (100 mg) solution, in a ratio 5:5:4 w/w/w. The gel formulation was made using hydroxypropyl methyl cellulose (HPMC 4% w/v in ethanol) with entrapment efficiency 93.8% and mean

size of 317 to 449 nm. The formulation produced 95% and 92% inhibition after 12 h and 24 h, respectively. Based on the entrapment of the drug, Span 40 and Span 60 were selected as non-ionic surfactants. The non-ionic surfactants span 40 and span 60 produced less leaky niosomes and have highest phase transition temperature. Soya lecithin is preferred over egg lecithin because the former gives vesicles of larger size, possibly due to differences in the intrinsic composition of soya and egg derived lecithin. Preparations with a white semi-solid appearance were obtained with span and cholesterol while incorporation of lecithin results in a gel-like appearance. The types of alcohol affect the size of niosomal vesicles as well; ethanol gave the largest and isopropanol gave the smallest. The larger size with ethanol may be due to the slower phase separation because of its greater solubility in water. The smaller size with isopropanol may be due to its branched chain. In proniosomal formulation the entire drug may be intercalated within the bilayers as opposed to the aqueous spaces in the gel. This result was consistent with the entrapment efficiency of, ketorolac and oestradiol in Span 40 and 60 proniosomes. The cholesterol content contributed to an increase in the hydrophobicity, with subsequent reduction of vesicle size thereby also reducing the entrapment efficiency.<sup>87</sup>

7. Celecoxib loaded niosomes were formulated and characterized by in-vitro, ex-vivo and in- vivo studies by **Kaur et al in year 2007**, the niosomal gel was found to provide 6.5 times higher drug deposition in skin as compared to conventional gel of the drug. The ratio of muscle to plasma concentration was found to be 2.16 µg/g and 0.34µg /ml and also, there was significant reduction of rat paw edema observed as compared to conventional gel indicating better skin penetration and deposition of celecoxib from niosomes formulation. The results demonstrated that niosomal gel formulation possess great potential for enhanced skin accumulation, prolonging drug release and improving the site specificity of celecoxib.<sup>88</sup>

## 2.6 Evidences of capability of transdermal route to replace oral route for NSAIDs:-

In a pharmacokinetic study, it was observed that, 25 mg of dose of diclofenac sodium ensure good analgesic effect which corresponds to therapeutic concentration in muscles of approx 0.5  $\mu\text{g/g}$ . Oral dose of 9 mg/kg body wt crates serum conc. of 1  $\mu\text{g/ml}$ .

Diclofenac Sodium in transferosomal gel in dose of 0.25 mg/kg body wt achieve muscle concentration of approx 0.5  $\mu\text{g/g}$  with a Transdermal flux of 10  $\mu\text{g/h/cm}^2$ ,so crosses the skin readily. Oral dose of 2.25mg/kg body wt of diclofenac sodium creates serum concentration of 0.37  $\mu\text{g/ml}$  and joint concentration of 3 $\mu\text{g/g}$  and synovial fluid conc. of approx 0.8  $\mu\text{g/ml}$ .

Diclofenac Sodium in emulgel in a dose 1.25mg/kg body wt. creates the drug level of 0.5  $\mu\text{g/g}$  & Transdermal flux of 0.36  $\mu\text{g/h/cm}^2$ . Dose of 2.25mg/kg body wt creates serum conc. of 0.03  $\mu\text{g/ml}$ .<sup>89</sup>

From tablet, a  $t_{\text{max}}$  of 2 hrs,  $C_{\text{max}}$  of 10.2  $\mu\text{g/ml}$  and  $\text{AUC}_{0 \rightarrow t}$  of 20.8  $\mu\text{g.h/ml}$  was observed whereas, from nanoemulsion gel, a  $t_{\text{max}}$  of 6 hrs,  $C_{\text{max}}$  of 8.8 ( $\mu\text{g/ml}$ ) and  $\text{AUC}_{0 \rightarrow t}$  of 54.2  $\mu\text{g.h/ml}$  was observed in a study for aceclofenac.<sup>90</sup>

These studies on nonsteroidal anti-inflammatory drugs clearly indicate the potential of novel transdermal gels to replace the oral therapy in arthritis.

## **2.7 Patent search on drug carriers based transdermal drug delivery of NSAIDs for arthritis:-**

The topical Compositions of NSAID,s have been prepared earlier such as gels, lotion, cream, Patch for active ingredients such as naproxen, ibuprofen, piroxicam, diclofenac, but still topical formulations suffer from the drawbacks of poor permeability through skin mainly through the stratum corneum layer. Therefore there is a need to improve the efficacy of topical formulation by increasing the permeability and by achieving a pronged drug release from the formulation.

For example, in the invention in Patent US 6368618 B1 describes a novel topical formulation for delivery of nonsteroidal anti-inflammatory drugs (NSAIDs) that is characterized by enhanced transdermal absorption and efficacy. A two phase liquid composition has aqueous and oil phases, the oil phase having a relatively high concentration of the NSAID to enhance transdermal absorption and efficacy when incorporated into the topical anti-inflammatory formulation. The two phase liquid composition preferably contains, in addition to an NSAID, at least one melting point depressing agent. A preferred topical anti-inflammatory composition includes S (+)-ibuprofen, thymol, and ethyl alcohol or isopropyl alcohol. In this known prior art, the formulation is a cream and large amount of pharmaceutical oil in cream may retard the penetration rate of drug due to increased diffusion distance of drug molecule through oil phase before reaching the skin surface.<sup>91</sup>

The invention in US 7473432 B2 describes novel formulations of nonsteroidal anti-inflammatory drugs (NSAIDs) based on complex aggregates with at least three amphipathic components suspended in a suitable, e.g. pharmaceutically acceptable, polar liquid medium. A suitably ionized NSAID is one of the two, amongst said three, components that tends to destabilize lipid membranes, the other system component with such activity being typically a surfactant. In contrast, the remaining amongst said at least three amphipathic components typically forms a stable lipid membrane on its own. An essential characteristics of the resulting, relatively large, aggregates is an improved ability to penetrate pores, in a semi-permeable barrier, at least 30%, and often much smaller than the average diameter of the complex aggregate. This said aggregates is used

to mediate NSAID transport through semi-permeable barriers including mammalian skin. As a result of the skin penetration by NSAID loaded large aggregates, the drug delivered transcutaneously with such carriers gets deeper into the tissue than the corresponding NSAID from a solution on the skin surface. This is believed to be due to the special ability of suitable large carriers to bypass the local sink of blood capillaries at the epidermal-dermal junction in the skin. The carrier-mediated delivery of locally applied NSAIDs thus allows therapy of deep tissues under the drug administration site, which is medically highly desirable.

In this known prior art, the NSAID,s selected for preparation are diclofenac, ibuprofen and ketoprofen, the described approach of carrier mediated delivery has not been tried for Other NSAIDs such as aceclofenac, indomethacin etc. Also the preparation is a non occlusive patch which may be less patient compliant.

Although the above described systems of cream, non occlusive patch etc. have assisted in solving problems of poor skin permeability and lesser efficacy through transdermal administration of NSAIDs, there is still a scope of improvement in the drug permeation characteristics and efficacy and to provide a patient compliant dosage form for arthritis by formulating a transdermal gel containing drug loaded carrier systems.<sup>92</sup>

The invention in European patent bearing no EP 1551370 B1 describes a pharmaceutical preparation prepared which comprises of a bilayer membrane vesicles suspended in a liquid medium. The components were bilayer forming lipid, an amphipathic analgesic drug and a surfactant capable of self-aggregation in the suspension medium. Surfactant selected were preferably nonionic such as polyethylene glycol sorbitan long fatty chain ester, a polyethylene glycol-long fatty chain ester or ether and a polyhydroxy ethylene long fatty chain ester.<sup>93</sup>

In a study in the Australian patent bearing no AU 2005/281351A1, liposome was prepared consisting of a combination of a methyl prednisolone, phospholipid, and cholesterol. Phospholipids were a combination of hydrogenated soybean phosphatidylcholine (HSPC), polyethylene glycol coated distearoyl phosphatidyl ethanolamine (PEG-DSPE) and cholesterol. HSPC/Cholesterol/PEG-DSPE-2000 were used at mole ratio of 55:40:5 In the work, methyl prednisolone derivative encapsulated in

a liposome was essentially retained in said liposome for 6 months, liposomes were uniformly sized to a selected size range between 70-100 nm, preferably about 80 nm.<sup>94</sup>

According to a patent bearing no- US 2008/0003276 A1, the lipids mixture forming the liposome can be selected to achieve a specified degree of fluidity or rigidity, to control the stability of the liposome in serum and to control the rate of release of the entrapped agent in the liposome.

Charge-inducing lipids, such as phosphatidylglycerol can be incorporated into the liposome bilayer to decrease vesicle-vesicle fusion and to increase interaction with cells, while cholesterol and sphingomyelin can be included in formulations in order to decrease permeability and leakage of encapsulated drugs. At neutral pH buffers can decrease hydrolysis. Addition of an antioxidant, such as sodium ascorbate can decrease oxidation.<sup>95</sup>

Ketoprofen transferosome formulation has got approval for marketing by the Swiss regulatory agency (Swiss Medic) in year 2007, under the trademark Diractin of IDEA AG (Munich) . U.S. Patent.bearing no. 6,165,500 (Idea AG) says about the formulation of an adaptable bilayer vesicle which consist of a phospholipids combined with edge activators of category alcohols and surfactants such as cholates or polyoxyethylene ethers. These ultra deformable particles were termed as transferosomes and found to be suitable for delivery of both hydrophilic and lipophilic active pharmaceutical agents through the skin pores. Transferosomes in the size range of 200 to 600 nm visually appear as milky emulsions. For dermal delivery of drugs, a sizes range of 100 to 200 nm was found to be satisfactory.<sup>96</sup>

A preferred formulation according to the invention was that comprising phosphatidylcholine (PC) such as egg PC (EPC) or hydrogenated soy PC (HSPC) as a the liposome forming lipid. Charge-inducing lipids, such as phosphatidylglycerol can be incorporated into the liposome bilayer to decrease vesicle-vesicle fusion and to increase interaction with cells, while cholesterol and sphingomyelin included in formulations decrease permeability and leakage of encapsulated drugs. Buffers at neutral pH can decrease hydrolysis. Addition of an antioxidant, such as sodium ascorbate can decrease oxidation of liposomes. The lipids containing high content of phosphatidylcholine &

lyso-phosphatidylcholine (lyso- PC) can permeate into the stratum corneum layer of skin, probably due the role of lyso-PC as an edge activator and enhancing the elasticity of the vesicles.<sup>97</sup>

The invention described in European patent bearing no EP 1551370 B1 says that surfactant selected should be preferably nonionic such as polyethylene glycol sorbitan long fatty chain ester, a polyethylene glycol long fatty chain ester or ether and a polyhydroxy ethylene long fatty chain ester.<sup>98</sup>

The US patent no US 8,865,206 B1 says about a surface modified multilayered nanostructures for dermal delivery. Dermal delivery is best suited for the various skin diseases or disorders. However, the stratum corneum limits the permeation of number of suitable pharmaceutical agents for dermal delivery. Certain embodiments of the present invention include surface modified multilayered nanostructures. The modification was completed by using fatty acids enabling delivery of a significant amount of one or more pharmaceutical agent(s) into deeper layers of the epidermis and dermis to treat skin diseases or disorders. Each active pharmaceutical agent can be encapsulated into the separate layers of the nanostructures. The nanostructures are composed of lipids, polymers, emulsifying agents and surfactants.<sup>99</sup>

The US patent no- US 9,492,385 B2 in year 2016 says about temperature sensitive liposomal formulations. The surface active agent is contained in the bilayer membrane in an amount sufficient to increase the percentage of active agent released at the phase transition temperature of the lipid bilayer compared to that which would occur in the absence of the surface active agent. The surface active agent is present in the lipid bilayer membrane such that the membrane is stable in the gel-phase, i.e., the presence of the surface active agent does not destabilize the membrane, particularly prior to the melting of the lipid bilayer.<sup>100</sup>

US 9,066,867 B2 in year describes a serum-stable mixture of lipids capable of encapsulating an active agent to form a liposome, said mixture comprising phosphatidylcholine and phosphatidylethanolamine in a ratio in the range of about 0.5 to about 8. The mixture may also include pH sensitive anionic and cationic amphiphiles, such that the mixture is amphoteric, being negatively charged or neutral at pH 7.4 and positively charged at pH 4. The drug/lipid ratio may be adjusted to target the liposomes to particular organs or other sites in the body.<sup>101</sup>



A Korean patent KR 20120124112 A describes a transferosome formulation comprising of Lyso-phospholipid and Surfactants. A transdermal formulation containing transferosome with lyso-phospholipid and surfactant has been developed to ensure excellent skin permeability and high drug accumulation. A transferosome composition contains 12% or more of lyso-phospholipid and surfactant. The surfactant selected in study are Tween 20, Tween 60, Brij 72, Brij 76, or Brij 78. The size of the transferosome is 50-200 nm.<sup>102</sup>

The US Patent no US 20090060990 A1 says about an invention comprising of NSAID formulations, based on highly adaptable aggregates, for improved transport through barriers and topical drug delivery. An essential characteristics of the resulting, relatively large, aggregates is an improved ability to penetrate pores, in a semi-permeable barrier, at least 30%, and often much smaller than the average diameter of the complex aggregate. This enables said aggregates to mediate NSAID transport through semi-permeable barriers including mammalian skin. As a result of the skin penetration by NSAID loaded large aggregates, the drug delivered transcutaneously with such carriers gets deeper into the tissue than the corresponding NSAID from a solution on the skin surface. This is believed to be due to the special ability of suitable large carriers to bypass the local sink of blood capillaries at the epidermal-dermal junction in the skin.<sup>103</sup>

The US Patent no- US 20020048596 A1 described an invention where the active substance in the form of minute droplets with a membrane-type sheath of layers of amphiphilic carrier substance, for the transport of an active substance into and through natural barriers and constrictions such as skin. it was recognized that all such carriers, which are sufficiently elastic in order to be able to penetrate through the constriction of the barrier, such as of the skin, are suitable for penetrating into and through permeability barriers.<sup>104</sup>

The US patent no US 7473432 B2 says about an invention of NSAID formulations, based on highly adaptable aggregates, for improved transport through barriers and topical drug delivery. The aggregates are composed of suitably ionised NSAID, lipid membranes and surfactants. An essential characteristics of the resulting, relatively large, aggregates is an improved ability to penetrate pores, in a semi-permeable barrier, at least 30%, and often much smaller than the average diameter of the complex aggregate. This enables said aggregates to mediate NSAID transport through semi-permeable barriers including mammalian skin. As a result of the skin penetration by NSAID loaded large aggregates, the drug delivered transcutaneously with such carriers gets deeper into the tissue than the

corresponding NSAID from a solution on the skin surface. This is believed to be due to the special ability of suitable large carriers to bypass the local sink of blood capillaries at the epidermal-dermal junction in the skin.<sup>105</sup>

A method of forming liposomes with a higher inside/lower outside pH gradient, comprising preparing a suspension of liposomes in an aqueous solution of a salt of a weak acid which, in protonated form is uncharged, and is capable of freely permeating the trans membrane barrier of liposomes, adjusting the external medium to produce a higher inside/lower outside concentration gradient of the weak acid salt, and allowing the weak acid to distribute itself between inner and outer liposome compartments, with the weak acid acting as an inside-to-outside proton shuttle, thereby generating a higher inside/lower outside pH gradient.

The compound loaded into the liposomes were selected from the group consisting of non-steroidal anti-inflammatory drugs such as ibuprofen and indomethacin.<sup>106</sup>

There is provided homogeneous pharmaceutical compositions for the treatment of inflammatory disorders comprising an anti-inflammatory and/or antihistaminic active ingredient, a polar lipid liposome and a pharmaceutically-acceptable aqueous carrier. Liposomes have also been employed to encapsulate various drug compounds including aceclofenac and indomethacin for delivery via the nasal route, in order to improve bioavailability or as an adjuvant. Liposomes may be prepared by various methods using solvents, reduced pressure, two-phase systems, freeze drying, sonication etc.<sup>107</sup>

Niosomes were first of all mentioned in the U.S. Patent bearing no.4,217,344 of the inventor industry L'Oreal. The invention described the formulation of niosome vesicles of size range 100 to 1000 nm in diameter. In the study, the mixtures of oleth-10 and oleth-2 together with glycerol were used which produced milky dispersions of vesicles. In Subsequent findings, it was observed that, the easily available surfactants sorbitan fatty acid esters with an HLB range of 4-8 were found to be compatible with niosome vesicle formation. These biodegradable, cheap and non-toxic surfactants were found to have extensive applications in the cosmetic and pharmaceutical fields.<sup>108</sup>

## 2.8 Profile of drug candidates selected –Aceclofenac and Indomethacin and selected excipients:-

The properties of the drug candidates selected and the Excipients of the formulation were studied in official pharmacopeias and reference books for checking their suitability for the formulation. The purpose was to know the identification characters and physicochemical properties of the ingredients based on literature review.<sup>109, 110, 111, 112, 113, 114, 115, 116</sup>

### 2.8.1 General information- Aceclofenac

- **Generic Name:** Aceclofenac
- **Chemical IUPAC Name:** 2,6-dichlorophenyl)amino] phenylacetoxycetic acid
- **Trade names of transdermal formulations:-** Hifenac gel, Accept gel
- **CAS Registry Number:** 89796-99-6
- **Molecular Formula:** C<sub>16</sub>H<sub>13</sub>Cl<sub>2</sub>NO<sub>4</sub>
- **Structural formula:**

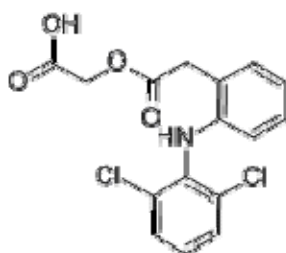


Figure 2.1 Structure of aceclofenac

- **BCS Classification:** Class II drug
- **Molecular Weight:** 354.2
- **Physical Form:** A white or almost white, crystalline powder.
- **Melting Point:** 149-153°C

- **Solubility:** Practically insoluble in water, as the pH increased after 6, there is an increase in drug solubility .freely soluble in acetone, soluble in alcohol.
- **Partition Coefficient (log P):** 8.0
- **Category:** NSAID
- **Mechanism of action:** Aceclofenac, a phenylacetic acid derivative, is a potent inhibitor of cyclooxygenase enzyme, involved in the production of prostaglandins, causing marked anti-inflammatory and analgesic properties.
- **Indication and usage:-** Ankylosing spondylitis, Rheumatoid arthritis, Osteoarthritis
- **Dosage and Administration:** Oral Adult: 100 mg bid, in the morning and in the evening, Topical dose- 1% to 1.5 %
- **Adverse reactions:**

Gastro intestinal disorders (e.g. dyspepsia, abdominal pain, nausea, vomiting, diarrhoea, flatulence, constipation, malaena, haematemesis, ulcerative stomatitis, gastritis), rash, ruber, urticaria, enuresis, headache, dizziness, paraesthesia, drowsiness; asthma, bronchospasm, dyspnoea, pruritus, purpura, angioedema. MI, stroke, acute coronary syndrome, oedema, HTN, cardiac failure; nephritis, nephritic syndrome, renal failure; abnormal liver function, hepatitis, jaundice; visual disturbance, optic neuritis; thrombocytopenia, neutropenia, agranulocytosis, aplastic and haemolytic anaemias. Rarely, pancreatitis, exfoliative and bullous dermatoses (e.g. epidermal necrolysis, erythema multiforme, Steven Johnson syndrome, toxic epidermal necrolysis).

Potentially Fatal: GI bleeding, ulceration or perforation.
- **Candidature for transdermal drug delivery:-**

Aceclofenac has moderate lipid and aqueous solubilities. Hence, the drug could be considered as a good candidate for transdermal delivery system, since it could be soluble in the sebum of the skin, and then readily penetrate into the lower skin layers to dissolve in the tissue fluids.

### 2.8.2 General informations: Indomethacin

- **Generic Name:** Indomethacin
- **Chemical IUPAC Name:** 1-(4-chlorobenzoyl)-5-methoxy-2-methylindol-3-yl acetic acid.
- **Proprietary Names:** VI-Gel, Elmetacin, M-CIN, Satogesic, Indobene gel
- **CAS Registry Number:** 53-86-1
- **Molecular Formula:** C<sub>19</sub>H<sub>16</sub>ClNO<sub>4</sub>
- **Structural formula:**

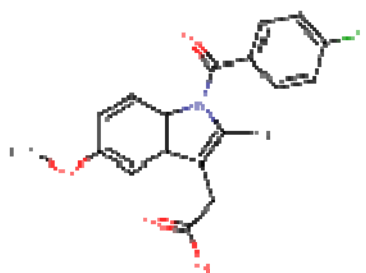


Figure 2.2 Structure of indomethacin

- **BCS Classification:** Class II drug
- **Molecular Weight:** 357.8
- **Physical Form:** A white to pale yellow, crystalline powder; odourless or almost odourless.
- **Melting Point:** 158°C
- **Solubility:** Practically insoluble in water, as the pH increased after 6, there is an increase in drug solubility .freely soluble in acetone, soluble in alcohol.
- **Partition Coefficient (logP):** 3.4
- **Category:** NSAID
- **Mechanism of action:** by inhibiting cyclooxygenase and the formation of prostaglandins.
- **Indication and usage:** Pain and inflammation associated with musculoskeletal and joint disorders.
- **Dosage and Administration:** Topical dose 1% as per USP 32 NF 27
- **Adverse reactions:** CHF, tachycardia, chest pain, arrhythmia, palpitations, HTN, pulmonary HTN, oedema, GI disturbances (e.g. nausea, vomiting, dyspepsia, indigestion, heartburn, epigastric pain),headache, frontal throbbing, apparent swelling of the temporal

vessels, tinnitus, ataxia, tremor, dizziness, insomnia, vertigo, lightheadedness, confusion, psychiatric disturbances; haemolytic anaemia, bone marrow depression, agranulocytosis, leucopenia, thrombocytopenic purpura; corneal deposits and retinal disturbances, acute interstitial nephritis, haematuria, proteinuria, pruritus, urticaria, rash, macular and morbilliform eruptions, apnoea and exacerbation of pulmonary infection.

Potentially Fatal: Anaphylactic reactions, Stevens-Johnson syndrome, toxic epidermal necrolysis, exfoliative dermatitis, aplastic anaemia, jaundice and fatal fulminant hepatitis, liver necrosis, hepatic failure, fulminant necrotising fasciitis, renal failure, MI, stroke, GI ulceration, perforation and hemorrhage.

- **Candidature for transdermal drug delivery:-** Low molecular weight, good permeability and shorter half-life of indomethacin made it a suitable drug candidate for the development of transdermal drug delivery system.

## 2.9 Excipients Profile:-

### 2.9.1 Carbopol 934

Carbopol-934 is a synthetic polymer of high molecular weight which is composed of acrylic acid cross-linked with polyalkenyl polyether. The average molecular weight is  $3 \times 10^6$  Daltons. It contains not less than 56% and not more than 68% of carboxylic acid (-COOH) groups.

- **Synonym:** Acritamer, Acrylic acid polymer, Carboxy vinyl polymer.
- **Non proprietary names:** BP carbomer, USP carbomer
- **Chemical name:** Carboxyl polymethylene
- **Empirical formula:**  $(C_3H_4O_2)_x (-C_3H_5\text{-sucrose})_y$
- **Structure:**

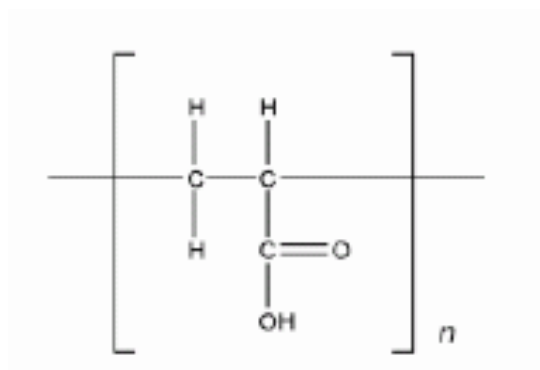


Figure 2.3 Structure of Carbopol 934

- **Category:** Bioadhesive, emulsifying, suspending & viscosity enhancing agent, tablet binder and release-modifying agent.
- **Description:** White, fluffy, acidic, hygroscopic powder with a slight characteristic odour.
- **Solubility:** After neutralization with alkali hydroxides or amines, soluble in water, in ethanol (95%) and in glycerol.
- **pH:** 2.5-3.0 (1% aqueous solution)
- **Glass transition temperature:** 100 °C -105°C
- **Melting point:** The compound starts decomposing at 260°C.
- **Specific gravity:** 1.41 gm/ml
- **Viscosity:** Carbopols dissolves in water to produce colloidal acidic solutions of low viscosity which on neutralization becomes highly viscous gels of viscosity approximately 29,400 to 39,400 cps at 25°C for 0.5% neutralized solution.

- **Concentration to be used:** For gelling agent 0.5-2%
- **Stability and storage:** Carbomers are considered as stable, though hygroscopic in nature and can withstand temperature of 104°C for up to 2 hours without affecting their thickening characteristics.
- **Applications:** It is used as thickening, emulsifying and gelling agent. It is used as a tablet binder and matrix forming agent in sustained-release formulations affording zero- to near zero-order release. It is used as the bioadhesive component in mucoadhesive ointments, gels and tablets.
- **Safety:** Carbomers are regarded as non toxic and non-irritant.

### 2.9.2 Propylene glycol:-

- **Chemical name:** 1, 2-Propanediol
- **Formula:** C<sub>3</sub>H<sub>8</sub>O<sub>2</sub>
- **Molecular Weight:** 76.09
- **Description:** It is a clear, colourless, viscous, practically odourless liquid with a sweet and slightly acid taste resembling that of glycerin.

#### Structural formula:

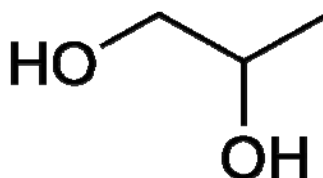


Figure 2.4 Structure of Propylene glycol

- **Boiling point:** 188 °C
- **Density:** 1.038 g / cm<sup>3</sup> at 20°C
- **Melting point:** -59 °C
- **Solubility:** Miscible with acetone, chloroform, ethanol (95%), glycerin and water, soluble at 1 in 6 parts of ether, not miscible with light mineral oil or fixed oils, but will dissolve in some essential oils.



- **Stability:** Stable in a well-closed container at cool temperature. It is chemically stable when mixed with ethanol (95%), glycerin, or aqueous solutions.
- **Applications:** In parenteral and non parenteral pharmaceutical formulations, widely used as a solvent and preservative. Commonly used as a plasticizer and as a carrier for emulsifiers.
- **Incompatibilities:** Incompatible with potassium permanganate.
- **Safety:** Regarded as a relatively nontoxic material and minimally irritant in topical preparations. It is also used in foods and cosmetics. Pain or irritation may cause due to parenteral administration when it is used in high concentration

### 2.9.3 Cholesterol:-

- **Official in:-** BP, JP, USP-NF
- **Synonyms:-** Cholesterin; cholesterolum.
- **Chemical Name and CAS Registry Number:-** Cholest-5-en-3b-ol [57-88-5]
- **Structure:-**

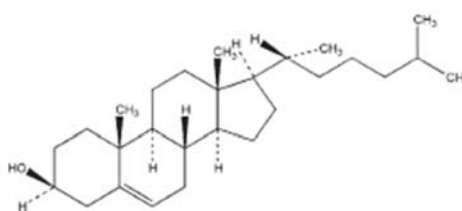


Figure 2.5 Structure of Cholesterol

- **Category:-** Emollient; emulsifying agent.
- **Physical form:-** Cholesterol is found as white or faint yellow, almost odorless, pearly granules, needles or powder.
- **Density:-** 1.052 g/cm<sup>3</sup> for anhydrous form.
- **Melting point:-** 147–150°C
- **Safety:-** Nontoxic and non-irritant, has exhibited experimental teratogenic and reproductive effects.
- **Regulatory status:-** Included in the FDA Inactive Ingredients database as dosage forms of injections, ophthalmic, topical, and vaginal preparations. It is also included in the category of non parenteral medicines licensed in the UK.

### 2.9.4 Sorbitan ester (span)

- **Synonym :-** SPAN 60, FEMA 3028, ARLACEL 60, SPAN(TM) 60, Barchlor 16S
- **Structure:-**

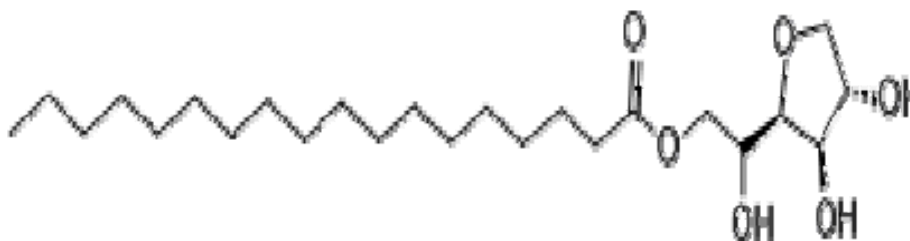


Figure 2.6 Structure of span 60

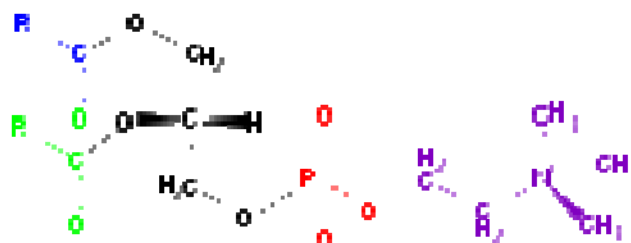
- **CAS Registry Number:-** 1338-41-6
- **Molecular Weight:-** C<sub>24</sub>H<sub>46</sub>O<sub>6</sub>
- **Appearance:-** Cream to amber color liquid or solid
- **Functional category:-** Emulsifying agent, nonionic surfactant, wetting agent, surface edge activator
- **Water solubility:-** Insoluble
- **Applications:-** Sorbitan esters have wide applications in cosmetics, food products, and pharmaceutical formulations as lipophilic non-ionic surfactants. They are basically used in pharmaceutical formulations as emulsifying agents in the formulation of creams, emulsions, and ointments for topical application. Sorbitan esters can be used to produce stable water-in oil emulsions and microemulsions.
- **Storage conditions:-** Sorbitan esters are stable in weakly acidic and weakly basic solvents. They must be stored in a well closed container in a cool and dry place.
- **Regulatory Status:-** Sorbitan esters are accepted as food additive in the FDA database and can be used in the inhalations, IM injections, ophthalmic and topical dosage forms.

### 2.9.5 Phospholipids:-

Phospholipids are a category of lipids that forms a major component of cell membranes. They have the ability to form lipid bilayers because of their amphiphilic character. The structure of the phospholipid molecule usually contains two hydrophobic fatty acid tails and a hydrophilic head molecule consisting of a phosphate group. These two components

are joined together by a glycerol molecule. The phosphate groups can be modified by attachment with simple organic molecules such as choline.

- **Structure of Phosphatidylcholine :-**



**Figure 2.7 Basic Structure of Phosphatidylcholine**

The phospholipid head consists of a negatively charged phosphate group and glycerol molecule, that is hydrophilic in nature. The phospholipid contains 2 fatty acid chains, which are hydrophobic in nature. When placed in water, phospholipids form a variety of structures depending on the specific properties of the phospholipid with tails typically aggregating to minimize interactions with water molecules.

- **Application:** - Phospholipids can be used to prepare liposomes and other nano carrier based formulations of oral, topical and parenteral drugs for the purpose of improving bio-availability, reducing toxicity and increasing drug permeation. Liposomes are composed of phospholipids enriched with phosphatidylcholine.

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## CHAPTER III

### Aim, Objective and Rationale

**3.1 Aim:-** Novel carrier systems for targeted drug delivery in the treatment of arthritis.

**3.2 Hypothesis:-**

1. Nanocarriers such as liposomes and transferosomes will enhance the localization of NSAIDs to the target site and will provide sustained drug release in Arthritis.
2. Drug Carriers incorporated in gel bases will achieve sustained release and greater localization of drug in targeted arthritis tissues through transdermal route.

**3.3 Objectives:-**

1. To develop a drug delivery system in drug carriers liposomes and transferosomes with lesser vesicle size as well as more drug loading capacity.
2. To incorporate drug carriers into transdermal gels and to evaluate the formulation and optimization to achieve an effective concentration of drug at inflamed target sites with a prolonged period of activity.

**3.4 Rationale:-**

1. The non-steroidal anti-inflammatory drugs in transdermal gel can eliminate gastrointestinal side effects and systemic toxicity associated with treatment.

2. NSAID's such as aceclofenac and indomethacin have moderate lipid and aqueous solubilities, therefore good candidates for transdermal delivery system, since it could be soluble in the sebum of the skin, and then readily penetrate into the lower skin layers to dissolve in the tissue fluids.
3. Through transdermal route due to avoidance of hepatic first pass metabolism, bioavailability may be increased. The pharmacokinetic profile through transdermal route has the potential to replace oral therapy for arthritis.
4. The selected carrier systems as transdermal delivery may give a sustained drug release and more drug absorption.
5. Lipid rich vesicles are hypothesized to carry significant quantity of drugs across the skin thus, enhancing the systemic absorption of drugs. Phospholipids have the ability to penetrate and to disturb the structure of stratum corneum lipid bilayers.
6. NSAIDs in transdermal drug delivery can avoid many side effects such as abdominal pain, peptic ulcer and liver toxicity associated with its oral therapy.
7. Due to the short half-life, they requires frequent dosing that can be solved by transdermal drug delivery which can provide a sustained drug release.
8. Drug carriers can provide better permeation through skin and a reservoir for sustained drug release for control of inflammation for a prolonged period.
9. In carrier systems, skin irritation is supposed to be less as compared to conventional transdermal systems.

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# CHAPTER –IV

## Experimental Work

### 4.1 Requirements for research work:-

#### 4.1.1 Equipments:-

**Table 4A.1 Equipments used in present investigation**

S.No	Equipment	Make
1.	Weighing balance	Shimadzu Pvt. Ltd (AX 200)
2.	Rotary vacuum evaporator	Supertechno associates, Mumbai
3.	Probe sonicator	Vibra cell-Sonics(Inkarp)
4.	Cooling centrifuge	Remi Electrotechnic Ltd.(CM-112 PLUS)
5.	UV spectrophotometer	Shimadzu pvt. ltd., Japan
6.	High performance liquid chromatography	Agilent Technologies.1220 LC Infinity with DAD and autosampler, C <sub>18</sub> column (100 mm × 4.6 mm, 3.5 μ)
7.	FTIR	Analytical Techno Pvt. Ltd (WQF 520)
8.	pH meter	Welltronics pvt.ltd., Ahmedabad
9.	Franz diffusion cell(Modified)	Durga Scientific corporation, Ahmedabad
10.	Dialysis membrane (pore size 2.4 nm, molecular wt. cutoff, 12000-14000)	Himedia
11.	Magnetic stirrer	Remi lab instruments,India
12.	Zeta sizer	Malvern Instruments Ltd. Malvern, UK(MAL100206)
13.	Gas chromatography	Auto System XL, Perkin Elmer
14.	Viscometer	Brookfield LVDV II
15.	Differential scanning calorimetry	Perkin Elmer Pyris1 DSC
16.	Transmission electron microscopy	Model: Tecnai 20, Make: Philips, Holland
17.	Trinocular microscope	Carl Zeiss

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4.1.2 Materials:-

Table 4A.2 Chemicals and reagents used in the work (AR grade)

S.no	Chemicals	Supplied by
1.	1-2 disteroyl-sn- glycerol-3-phosphoethanolamine ,Na	Gift sample by Lipoid ,Germany
2.	1-2 disteroyl-sn- glycerol-3-phosphate monosodium salt	Gift sample by Lipoid, Germany
3.	hydrogenated Phosphotidylcholine,	Gift sample by Lipoid, Germany
4.	1, 2-dioloxy-3-trimethyl ammonium Propane chloride,	Gift sample by Lipoid, Germany
5.	Leciva S-35, Leciva S-50 D, Lipova E-120	Gift sample by VAV Life sciences, Mumbai
6.	Methanol(HPLC grade)	Gujarat Chemicals, Gujarat
7.	Acetonitrile(HPLC grade)	Gujarat Chemicals, Gujarat
8.	Water(HPLC grade)	Gujarat Chemicals, Gujarat
9.	Chloroform	Gujarat Chemicals, Gujarat
10.	Egg Phosphotidylcholine	Chemdyes corporation, Gujarat
11.	Soya Phosphotidylcholine	Chemdyes corporation, Gujarat
12.	Carageenan	Chemdyes corporation, Gujarat
13.	Indomethacin	Chemdyes corporation, Gujarat
14.	PLGA	Chemdyes corporation, Gujarat
15.	Cholesterol	Chemdyes corporation, Gujarat
16.	Dimethyl Acetamide	Chemdyes corporation, Gujarat
17.	N-methyl-2-pyrrolidone	Chemdyes corporation, Gujarat
18.	Propylene Glycol	Chemdyescorporatio, Gujarat
19.	PEG 4000	Balaji drugs, Gujarat
20.	Polyacrylamide	Balaji drugs, Gujarat
21.	Polyvinylpyrrolidone	Balaji drugs, Gujarat
22.	Polyvinyl Alcohol	Balaji drugs, Gujarat
23.	Pluronic F-68	Balaji drugs, Gujarat

S.no	Chemicals	Supplied by
24.	HPMC	Balaji drugs, Gujarat
25.	Ethyl Cellulose	Balaji drugs, Gujarat
26.	Sodium Dodecyl Sulfate	Balaji drugs, Gujarat
27.	Ethanol	Balaji drugs, Gujarat
28.	Methanol	Balaji drugs, Gujarat
29.	Chloroform	Balaji drugs, Gujarat
30.	Benzene	Balaji drugs, Gujarat
31.	n-decane	Balaji drugs, Gujarat
32.	Glutaraldehyde	Balaji drugs, Gujarat
33.	Sterylamine	Balaji drugs, Gujarat
34.	Diethyl Acetate	Qualikems Ltd, Gujarat
35.	Cetyl Alcohol	Qualikems Ltd, Gujarat
36.	n-butanol	Qualikems Ltd, Gujarat
37.	Oleic Acid	Qualikems Ltd, Gujarat
38.	Carbomer-934,940	Qualikems Ltd, Gujarat
39.	Soya Phosphotidylcholine	Qualikems Ltd, Gujarat
40.	Cholesterol	Qualikems Ltd, Gujarat
41.	Acceclofenac	Qualikems Ltd, Gujarat
42.	Triethanolamine	Gujarat Chemicals Gujarat
43.	Liquid Paraffin	Gujarat Chemicals Gujarat
44.	Pot.Dihydrogen Phosphate	Gujarat Chemicals Gujarat
45.	Disod. Hydrogen Phosphate	Gujarat Chemicals Gujarat
46.	Disodium Citrate	Gujarat Chemicals,Gujarat



## 4.2 Plan of work:-

- Screening of formulation components and process parameters for formulation of drug carriers liposomes and transferosomes of nonsteroidal anti-inflammatory drugs using factorial design.
- Preformulation studies and drug- excipients compatibility studies.
- Formulation of drug carriers by using screened phospholipids, cholesterol and surfactants by an optimized method of thin film rehydration using experimental design.
- Optimization of carrier composition depending on characteristics of drug carriers and establishment of quality control parameters such as particle size by suitable equipments using zeta sizer and transmission electron microscopy, surface charge (zeta potential) and drug entrapment.
- Incorporation of drug carriers into transdermal gel formulation and characterization by in-vitro release and membrane diffusion studies.
- Formulation optimization by statistical analysis of responses.
- Ex-vivo permeation studies of optimized formulation to determine drug permeation through rat skin.
- Pharmacokinetic studies for determination of drug concentration in plasma,  $t_{max}$ ,  $C_{max}$  and AUC after transdermal administration of formulation in rats.
- Skin permeation studies through radioactive labeling experiments.
- Anti-inflammatory studies by rat paw edema method using plethysmometer and comparison with marketed preparation.
- Analgesic activity by latency period method in Eddy's hot plate in rats and comparison with marketed preparation.
- Statistical analysis and establishment of statistical significant improvement in efficacy of the prepared formulation as compared to conventional formulation
- Stability studies as per ICH Guidelines.

#### **4A. Drug carriers based transdermal gel of aceclofenac:-**

##### **4A.1 Preformulation studies:**

- Identification of drug was carried out by Differential Scanning Calorimetry, Fourier Transform Infra-Red and UV-Visible spectrophotometry.

**4A.1.1 DSC studies:-** DSC studies were carried out at a temperature range of 25 °C to 165 °C at 10 °C increments of temperature as per the methods mentioned in literature.<sup>111,112</sup>

**4A.1.2 FTIR studies:-** The FTIR of drug sample was carried out by FTIR Shimadzu Corporation- 8400S DRS. The sampling technique involved KBr pellet with resolution of 6 and scanning for 12-24 times.<sup>110,111,112</sup> IR scan of drug aceclofenac was taken after keeping at 40 °C and 75% RH for 3 months. The IR spectrum of the plain drug was compared with reference IR Spectrum.

##### **4A.1.3 UV Scan for determining $\lambda_{\text{max}}$ of aceclofenac:-**

The aceclofenac drug concentration of 10 µg/ml in methanol was scanned between wavelengths of 200 nm to 400 nm using UV-Visible spectrophotometer Shimadzu Corporation, Japan (UV-1800) for determining  $\lambda_{\text{max}}$  of aceclofenac.

## **4A.2 Analytical method for estimation of aceclofenac by UV–Visible Spectrophotometry:-**

The method of analysis was carried out as per the reported method in literatures.<sup>109, 110, 112, 113</sup>

### **4A.2.1 Standard plot of aceclofenac in media methanol:-**

A standard plot was prepared using a concentration range 5 to 30 µg /ml. Methanol was used as blank solution and absorbance was measured for each solution at  $\lambda_{\max}$  of 278 nm using Shimadzu Corporation, Japan (UV-1800), and the graph was plotted for absorbance versus concentration of aceclofenac. All the readings were taken in triplicate to minimize error and standard deviation was determined.

### **4A.2.2 UV scan of aceclofenac in media phosphate buffer saline pH 7.4 :-**

The aceclofenac drug concentration of 10 µg/ml in phosphate buffer saline pH 7.4 was scanned between wavelengths of 200 nm to 400 nm using UV-Visible spectrophotometer Shimadzu Corporation, Japan (UV-1800).

### **4A.2.3 Standard calibration curve of aceclofenac in phosphate buffer saline pH 7.4**

Standard plot was prepared using solutions of concentration range 5 to 30 µg /ml. Phosphate buffer saline pH 7.4 was used as blank solution and absorbance was measured for each solution at  $\lambda_{\max}$  of 273 nm using Shimadzu Corporation, Japan (UV-1800).The graph was plotted for absorbance versus concentration of aceclofenac. All the readings were taken in triplicate to minimize error and standard deviation was determined.

### **4A.2.4 Linearity, precision and accuracy of analytical method**

#### **❖ Linearity and range**

The linearity was determined by analyzing 6 independent levels of standard curve in the concentration range of 5-30 µg/ml. Absorbance of each solution against media

was recorded at  $\lambda_{\max}$ . The curve of absorbance vs. concentration was plotted and correlation co-efficient and regression line equation for drug were determined.

❖ **Precision:**

Intraday precision was determined by analyzing drug in concentration range of 5-30  $\mu\text{g/ml}$  at three different time points of the same day and inter day precision was determined by analyzing aceclofenac at three different time points on different days and % RSD was calculated.

❖ **Accuracy of Analytical method:-**

The prepared samples were spiked and % recovery was calculated to confirm the accuracy of analytical method.

### 4A.3. Process variable optimization:-

The process of rotary vacuum evaporation was varied by varying the process variables to investigate their effect of characteristics of carrier systems. The method of rotary vacuum evaporation was performed as per the methods mentioned in literatures.<sup>54, 55</sup>

Process variables in Rotary vacuum evaporation and probe sonication were optimized.

**4A.3.1 Rotary vacuum evaporation process variables:** - The process variables, temperature, RPM and time of operation were varied based on 3 factors, 3 level general factorial design and based on the quality of film produced, the process was optimized.

**Table 4A.3 Process variables in rotary vacuum evaporation**

Parameter	Temperature	RPM	Variables
Level	High (70° C)	High (90)	High (20)
	Medium (60° C)	Medium (80)	Medium (15)
	Low (50° C)	Low (60)	Low (10)



**Figure 4A.1 Rotary vacuum evaporator**

The process parameters such as temperature, rotations per minute and time were varied in thin film hydration method and it was observed that at 50°C temperature and 90 rpm, when film formation was allowed for 20 minutes in rotary vacuum evaporator, a thin film formed which was uniform and translucent in appearance. Also there was no change observed in appearance of film when, temperature was varied from 50°C to 60°C and rpm was varied from 80 to 90 rpm. It was also observed that, at 50°C temperature and 60 rpm, when film formation was allowed for 10 minutes in rotary vacuum evaporator, a dry thin film could not be formed and also no change was observed, when rpm was changed from 60 rpm to 80 rpm.

The thin film which was formed at 50°C temperature and 90 rpm for 20 minutes was hydrated with media phosphate buffer saline pH 7.4 and then prepared liposomes were reduced in size by probe sonication.

#### **4A.3.2 Probe sonication process variables:-**

Sonication was performed to downsize the vesicles as per the methods mentioned in literature.<sup>51,52</sup> The probe sonicator was operated using 13 mm probe at amplitude of 60%. The effect of sonication cycles on the transparency of vesicular dispersion and average size of vesicles was studied. % transmittance was measured using UV spectrophotometer and size was measured using trinocular microscope (Carl Zeiss)



**Figure 4A.2 Probe sonicator**

**Table4A.4 Process optimization of probe sonication**

Probe	Amplitude	Time and Pulse	Temperature
13 mm standard	60 %	2 minutes 2 sec on,2 sec off	4° C

#### **4A.4 Preliminary studies on formulation for screening of excipients:-**

##### **4A.4.1 Selection of excipients by formulation of trial batches of carrier system**

The preliminary trial batches of liposomes and transferosomes were prepared using selected process parameters to check the feasibility of preparation of liposomes by using the available excipients that were also reported in earlier studies.

The phospholipids subjected to screening were –

1. 1-2 disteroyl-sn- glycerol-3-phosphoethanolamine Na salt,
  2. 1-2 disteroyl-sn- glycerol-3-phosphate monosodium salt
  3. Hydrogenated Phosphatidylcholine,
  4. 1, 2-dioloxy-3-trimethyl ammonium Propane chloride,
  5. Leciva S-35, Leciva S-50 D, Lipova E-120.
- Other excipients utilized were cholesterol and the solvents used were chloroform, methanol and phosphate buffer saline pH 7.4

##### **4A.4.2 Process of synthesis of liposome as drug carriers by film hydration method:-**

- The excipients Phospholipids (0.067 mMol to 0.133 mMol) & cholesterol(0.064 to 0.129 mMol) were solubilized in 10 ml chloroform-methanol (9:1) mixture. Drug: Lipid molar ratio (2.12 to 4.20 mMol).The quantities were converted into mg for formulation. Hydration volume was kept as 20 ml.

- Thin film formation was done by vacuum rotary evaporator at temperature of 50°C, 90 rpm for 20 minutes. Thin film was evaporated under vacuum for removal of even trace amount of organic solvent and then kept in desiccators overnight for removal of even trace amount of organic solvent.
- Dried thin film was hydrated by 20 ml phosphate buffer saline pH 7.4 containing 100 mg of drug to prepare drug loaded multilamellar vesicles.
- The vesicular dispersion was sonicated by probe sonicator (Vibra cell ,Sonics) for 5 cycles of 2 minutes at pulse of 2 s using standard 13 mm probe at amplitude of 60% to obtain small unilamellar vesicles form large multilamellar vesicles. The vesicles were observed under Carl Zeiss trinocular microscope at 40 X and 100 X magnification.

#### **4A.4.3 . Study of influence of quantities of excipients on quality of liposomes by 3<sup>2</sup> full factorial design:-**

The selected excipients in the preliminary studies were put in 2 factors, 2 levels factorial design and screened on the basis of outcomes of size and entrapment efficiency.

<b>Factors-</b>	<b>Responses-</b>
Quantity of Phospholipids	Size
Quantity of cholesterol	Entrapment efficiency

#### **4A.4.4 Preparation of trial batches of liposomes based on factorial design:-**

The quantity of phospholipid was varied as 0.067 mMol to 0.133 mMol (50 -100 mg) & cholesterol as 0.064 to 0.129 mMol (25-50 mg) for preparation of factorial batches of liposomes. The quantities were converted into mg. The batches were named as L1 to L4. The drug to lipid molar ratio was taken as 2.12 to 4.20 mMol and the hydration volume was kept as 20 ml. The quantities of drug and excipients were converted into milligrams for preparation of batches.



**Table 4A.5 Factorial design for factors screening in liposome preparation**

<b>Formulation</b>	<b>Quantity of phospholipid (mg)</b>	<b>Quantity of cholesterol (mg)</b>
L1	100	50
L2	100	25
L3	50	50
L4	50	25

**4A.4.5 Evaluation of carrier systems**-Evaluation was carried out by following process as per the methods reviewed in literatures. <sup>51,52,56,57</sup>

The methods are described as follows:-

- **Particle shape determination of drug carriers:**

Shape was determined using trinocular microscope (Carl Zeiss) at BIP, Baroda.

- **Particle size, Zeta potential and polydispersity index determination of drug carriers:**

Particle size of drug carriers was determined using trinocular microscope (Carl Zeiss) and Malvern sizer (Malvern Instruments Ltd., UK, MAL100206).

- **Drug entrapment efficiency:-**

Drug loaded vesicles dispersed in media phosphate buffer saline pH 7.4 were centrifuged at 15000rpm for 15 minutes at 4° C on Remi lab centrifuge. The supernatant was collected and again centrifuged at 15000 rpm for 15 minutes and the drug content of supernatant was analyzed after 1/100 dilution to determine untrapped drug content. The entrapped drug content in the drug carriers were determined by subtracting the value of untrapped drug from the total drug added for the preparation.

#### **4A.4.6 Analysis of factorial batches of liposomes of aceclofenac:-**

The factorial batches were analyzed by pareto chart, main effect plot, interaction plot and cube plot using statistical software Minitab 16 to determine the effect and influence of factors on responses of % drug entrapment and size of liposomes.

#### **4A.4.7 Process of Synthesis of trial batch of transferosomes as Drug carriers by thin film hydration Method:-**

The transferosomes were prepared as per the methods described in literatures with modifications based on requirements of formulation.<sup>75, 76,77,78,79</sup>

- Phospholipid (0.067 mMol to 0.133 mMol), surfactant (0.06 to 0.12 mMol) & cholesterol (0.064 to 0.129 mMol) were solubilized in 10 ml of chloroform-methanol (9:1) mixture. Thin film formation was done by rotary vacuum evaporator at temperature of 50°C, 90 rpm for 20 minutes.
- Thin film was evaporated under vacuum for removal of even trace amount of organic solvent and then kept in desiccators overnight for removal of even trace amount of organic solvent.
- Dried thin film was hydrated by 20 ml phosphate buffer pH 7.4 containing 100 mg of drug and 100 mg surfactant to prepare drug loaded multilamellar vesicles. Drug: Lipid molar ratio used was 2.12-4.20.
- The vesicular dispersion was sonicated by probe sonicator (Vibra cell -Sonics) for 2 cycles of 2 minutes at pulse of 2 sec using standard 13 mm probe at amplitude of 60% to obtain small unilamellar vesicles form large multilamellar vesicles.
- The vesicles were observed under Carl Zeiss trinocular Microscope at 40 X and 100X magnification.

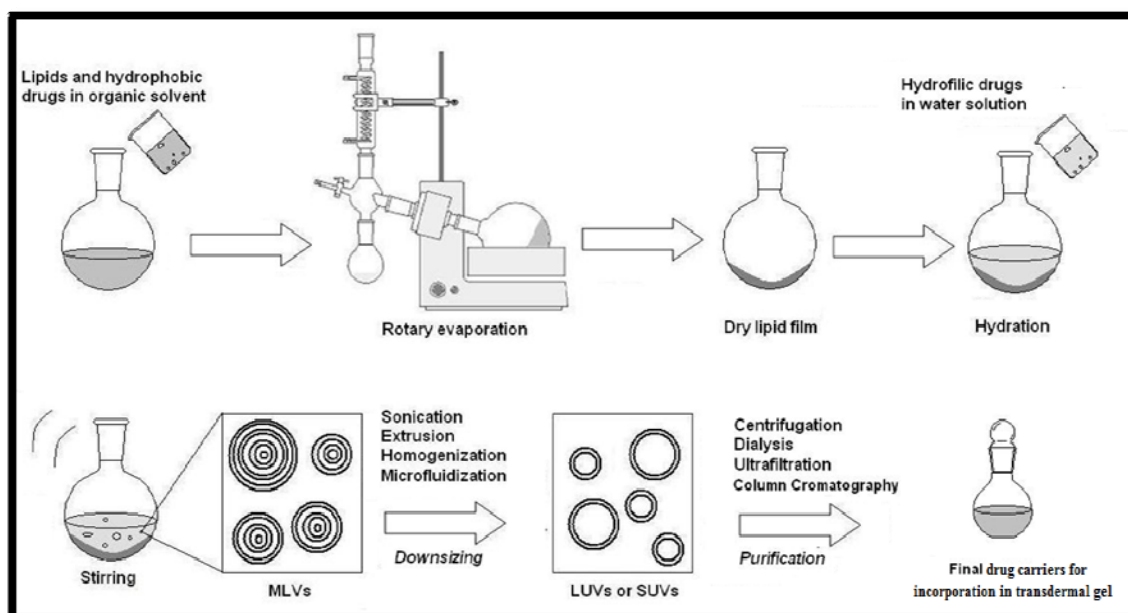


Figure 4A.3 Schematic diagram of preparation of vesicles by thin film hydration method

#### 4A.4.8 Selection of surfactants for preparation of transferosomes:-

The surfactant was selected based on preliminary evaluation of trial batches of transferosomes. The phospholipid, 1, 2-disteroyl-sn-glycero-3-Phospho-ethanolamine, Na salt selected in the earlier studies on liposomes was used for preparation of transferosomes also.

#### 4A.4.9 Preparation of trial batches for selection of surfactants:-

The quantity of surfactants was varied as 50 mg and 100 mg and cholesterol were varied as 25 mg and 50 mg for preparation of factorial batches of transferosomes. Transferosomes were prepared using the surfactants span 40, span 60, span 20 and sodium cholate which are reported earlier for transferosome preparation.<sup>75, 76,77,78,79</sup> The quantity of phospholipid was kept 100 mg for all the trial batches and the batches were named as T1 to T16. Selection was performed based on evaluation data of % drug entrapment and size of transferosomes formed.

Table 4A.6 Formulation of transferosomes using different surfactants

Formulation	Drug(mg)	Phospholipid (mg)	Span40(mg)	Cholesterol(mg)
T1	100	100	50	25
T2	100	100	100	50
T3	100	100	100	25
T4	100	100	50	50

Formulation	Drug(mg)	Phospholipid (mg)	Span60(mg)	Cholesterol(mg)
T5	100	100	100	50
T6	100	100	100	25
T7	100	100	50	25
T8	100	100	50	50

Formulation	Drug(mg)	Phospholipid (mg)	Span20(mg)	Cholesterol(mg)
T9	100	100	50	50
T10	100	100	100	25
T11	100	100	50	25
T12	100	100	100	50

Formulation	Drug(mg)	Phospholipid (mg)	Na.Cholate(mg)	Cholesterol(mg)
T13	100	100	100	25
T14	100	100	50	50
T15	100	100	50	25
T16	100	100	100	50

#### 4A.4.10 Drug release study of transferosome prepared using span 40 and span 60:-

As the drug entrapment and size were found to be nearly same for the transferosomes prepared using span 40 and span 60, the transferosomes were incorporated into gel and further evaluated for % drug release for 6 hrs.

**4A.4.11. Study of influence of quantities of excipients on quality of transferosomes by 3<sup>2</sup> full factorial design:-**

The selected excipients in the preliminary studies were put in 3 factors, 2 levels factorial design and the influence of the levels of factors on the outcomes of responses of size and entrapment efficiency was studied.

**Factors-**

Quantity of Phospholipids  
 Quantity of surfactant  
 Quantity of cholesterol

**Responses-**

Size  
 Entrapment efficiency

**4A.4.12 Preparation and evaluation of trial batches of transferosomes based on factorial design:-**

For preparation of factorial design bathes of transferosomes, the quantity of phospholipid was varied as 50 mg and 100 mg (0.067-0.133 mMol), the quantity of selected surfactant span 60 was varied as 25 mg and 50 mg (0.06-0.12 mMol) and quantity of cholesterol was varied as 25 mg and 50 mg(0.064-0.129 mMol). Batches were named as T1 to T8 and evaluated for size and entrapment efficiency.

**Table 4A.7 Factorial design for factors screening in transferosome preparation**

<b>Formulation</b>	<b>Quantity of phospholipid (mg)</b>	<b>Quantity of surfactant (mg)</b>	<b>Quantity of cholesterol (mg)</b>
T1	50	50	25
T2	50	25	25
T3	100	25	25
T4	100	50	25
T5	50	50	50
T6	100	25	50
T7	50	25	50
T8	100	50	50

#### **4A.5 Drug-excipient compatibility studies for formulation components of aceclofenac:-**

The screened phospholipid-1, 2-disteroyl-sn-glycero-3-Phospho-ethanolamine, Na salt and surfactant span 60 were subjected to compatibility studies with drug aceclofenac to find out any interaction with drug. The interaction of cholesterol and carbopol gel base with drug, phospholipid and surfactant mixture was also studied.

##### **4A.5.1 FTIR analysis of pure drug and mixture of drug and excipients for studying drug-excipient compatibility:-**

FTIR analysis was performed for studying drug-excipient compatibility among the drug and the screened excipients.

A. IR Scan of aceclofenac

B. IR scan of mixture of aceclofenac and phospholipid in ratio of 1:1.

C. IR scan of mixture of aceclofenac, phospholipid and cholesterol in ratio of 1:1:1.

D. IR scan of mixture of aceclofenac, phospholipids, cholesterol and surfactant in ratio of 1:1:1:1.

E. IR scan of mixture of aceclofenac, phospholipids, cholesterol, surfactant and gel base carbopol934 in ratio of 1:1:1:1:1.

##### **4A.5.2 DSC analysis of pure drug and mixture of drug and excipients for studying drug- excipient compatibility:-**

For drug excipient interaction studies, DSC analysis was performed at sophisticated instrumentation centre for applied research and testing (SICART), Gujarat as per the methods mentioned in literature.<sup>111, 112</sup> DSC analysis was performed for studying drug-excipient interaction in a temperature range of 25°C to 160°C at an increment of 10°C.

❖ **Preparation of transdermal gel:-** A 1% carbopol gel was prepared as per standard methods by dispersing carbopol 934 in distilled water as a base for incorporation of drug loaded carriers for transdermal delivery. The pH was adjusted to 7 using triethanolamine. <sup>12,15,17,19,20,118</sup>

#### **4A.6 Formulation batches of liposomes incorporated gel based on experimental design (Central composite design):-**

The experimental batches based on central composite design were further prepared by varying the level of screened phospholipid and cholesterol as 50 to 100 mg (0.067 mMol to 0.133 mMol) and 25 to 50 mg (0.064 to 0.129 mMol) respectively. The Drug: Lipid molar ratio taken was 2.12 to 4.20 mMol and the hydration volume kept was 20 ml.

**Table 4A.8 Liposome batches based on central composite design**

<b>Formulation</b>	<b>Quantity of Phospholipid (mg)</b>	<b>Quantity of Cholesterol(mg)</b>
M1	75.00	19.82
M2	75.00	37.50
M3	100.00	25.00
M4	75.00	37.50
M5	39.64	37.50
M6	50.00	25.00
M7	50.00	50.00
M8	75.00	37.50
M9	75.00	37.50
M10	75.00	37.50
M11	75.00	55.17
M12	100.00	50.00
M13	110.35	37.50

➤ The responses measured for batches were % drug entrapment and in-vitro permeation flux and the effect of factors on the responses were studied.

#### 4A.7 Formulation batches of transferosomes incorporated gel based on experimental design (Box Behnken design):-

From the analysis of factorial design studies, it was evident that, the quantities of phospholipid, surfactants and cholesterol have effect on the size and drug entrapment of drug carriers, the experimental batches based on Box Behnken design were further prepared by varying the level of phospholipid, surfactants and cholesterol as 50 to 100 mg (0.067- 0.133 mMol), 25 to 50 mg (0.06-0.120 mMol) and 25 to 50 mg (0.064- 0.129 mMol) respectively. The responses measured for batches were %drug entrapment and in-vitro permeation flux and the effect of factors on the responses were studied.

**Table4A.9 Formulation batches of transferosomes incorporated gel**

<b>Formulation</b>	<b>Quantity of Phospholipid (mg)</b>	<b>Quantity of Surfactant(mg)</b>	<b>Quantity of Cholesterol (mg)</b>
F1	100	37.5	25.0
F2	50	37.5	50.0
F3	75	25.0	50.0
F4	75	37.5	37.5
F5	100	50.0	37.5
F6	50	37.5	25.0
F7	75	25.0	25.0
F8	75	37.5	37.5
F9	75	50.0	50.0
F10	50	25.0	37.5
F11	100	37.5	50.0
F12	75	50.0	25.0
F13	75	37.5	37.5
F14	100	25.0	37.5
F15	50	50.0	37.5



#### **4A.8 Evaluation of liposomal gel and transferosomal gel based on gel characteristics:-**

The drug loaded liposomes and transferosomes incorporated gels along with plain drug gel were evaluated for pH, spreadability, gel strength, extrudability and drug release and permeation studies based on the methods in literatures.<sup>10,11,12,15,17,19,20,118</sup>

##### **4A.8.1 Determination of pH:-**

The pH of gels was checked by using a digital pH meter at room temperature. Initially, the pH meter was calibrated using standard buffers of pH 7 and then 10 gm of gel was weighed and dispersed in 25 ml of distilled water and then electrode of pH meter was dipped in the dispersion and the pH was noted.

##### **4A.8.2 Spreadability:-**

A quantity of 0.5 g gel was placed within a circle of 1 cm diameter on a premarked glass slide over which a second glass slide was placed. A weight of 2 g was allowed to rest on the upper glass slide for 1min. The increase in the diameter due to spreading of the gel was noted. Spreadability was calculated by using the following formula:

$$S = M.L / T$$

Where, S = Spreadability, M = weight attached to upper slide, L = length of spread, T = time taken.

##### **4A.8.3 Gel strength:-**

The apparatus for measuring gel strength consisted of a plunger having a pan to hold weights at one end whereas the other end was immersed into the gel. Formulated gels were placed in glass bottle where marking was done at 1cm below the filling mark. The weight required for the plunger to sink to a depth of 1 cm through the prepared gel was measured for each formulation.

#### 4A.8.4 Extrudability:-

The prepared gel was filled in tube and sealed. 3 Markings were made at intervals of 1.5 cm from bottom of tube. The tube was pressed at marking using Pfizer hardness tester with a pressure of  $1 \text{ kg/cm}^2$ , the weight of gel expelled in form of continuous ribbon was measured for each formulation and uniformity of release of gel from the tube was determined.



Figure 4A.4 Extrudability studies using Pfizer hardness tester

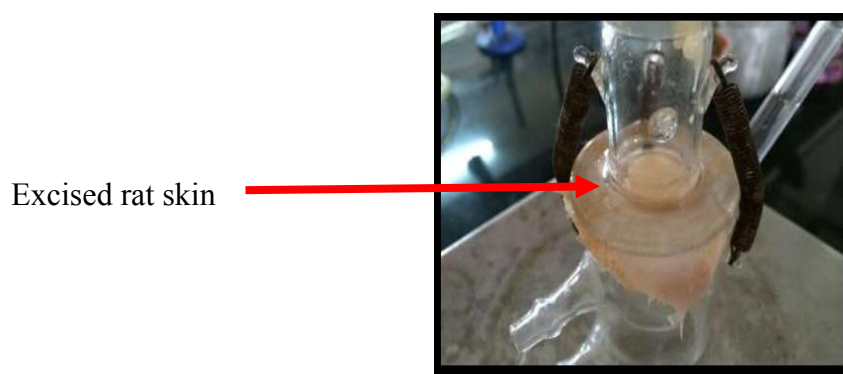
#### 4A.8.5 Rheological studies:-

The viscosity of gels was determined by using Brookfield helipath (LVDV 2) viscometer. The gel was placed in the sample holder and spindle no-96 was attached to viscometer. The spindle was lowered perpendicularly into the sample and then it was allowed to rotate at a constant optimum speed at room temperature to achieve a torque of minimum 10%. The readings of viscosity of the formulation were measured at different rpm.

#### 4A.8.6 Release studies of drug carriers based transdermal gel :-

In-vitro and ex- vivo permeation studies: (IAEC Approval no. PhD/13-14/23 dated. 14<sup>th</sup> December 2013). In-vitro drug release studies was performed for all the experimental design batches using modified Franz diffusion cell and dialysis membrane (Himedia, pore size 2.4 nm, molecular wt. cutoff , 12000-14000). The ex- vivo study was performed for optimized batch of drug carriers incorporated gel. For ex-vivo permeation studies, in place of dialysis membrane the excised skin of dorsal side of 5-6 weeks old rat was mounted on donor compartment after removing hair and

then clamped between the donor and the receptor chamber of modified diffusion cells with the stratum corneum facing the donor chamber. Then, 0.2 g of gel containing aceclofenac was applied on the excised skin part in the donor chamber. The receptor compartment was filled with 20 ml of phosphate buffer saline pH 7.4 as diffusion media for release of drug in receptor compartment. The receptor medium was maintained at a temperature of  $37 \pm 0.5^\circ \text{C}$  and stirred at 600 rpm throughout the experiment. Aliquots of 5 ml were sampled from the receptor compartment at time interval of 1 hr and then immediately replaced with the same volume of pure medium. Aliquots withdrawn were analyzed by UV spectroscopy method and total amount of drug released at each time interval was determined. The cumulative amount of drug released across the rat skin was determined as a function of time.



**Figure 4A.5 Drug permeation study**

In-vitro permeation studies were carried out for all the experimental batches of liposomal gel and the cumulative drug release as well as the permeation flux was determined. The permeation flux for experimental batches of liposomal gel, transferosomal gel and plain drug gel were determined. Permeation flux is the slope of percentage drug release v/s time. It is expressed as  $\mu\text{g}\cdot\text{cm}^{-2}/\text{hr}^{-1}$

Studies were carried out for all the experimental batches of transferosomal gel also and the cumulative drug release as well as the permeation flux was determined.

#### **4A.9 Statistical analysis and optimization of liposomal and transferosomal gel formulation:-**

The analysis of liposomal gel and transferosomal gel design batches was performed by response surface methodology method. The evaluation responses of batches were analyzed by contour plots and surface plots to observe the design space which can give suitable quantities of excipients for maximum responses. To achieve the maximum possible desired response, a target value of the responses was set in the optimization plot, in such a way that it was within the limits of responses already obtained from the design batches. The formulations factors and levels obtained from the optimization plot was taken as the optimization formula which could produce the desired target responses.

##### **4A.9.1 Development of optimized formulation of transferosomal and liposomal gel:-**

The novel transdermal gel has been developed containing drug loaded liposomes and transferosomes based on the optimized formula and process. The optimized batch was further evaluated to check the ability to achieve the target responses. The developed formulations were also compared with the conventional transdermal gel formulations.

##### **4A.9.2 Size and Morphology determination of optimized batch of drug carriers:-**

Studies were performed by Malvern zeta sizer and transmission electron microscopy for determining size, zeta potential and morphology of liposomal and transferosomal dispersion.<sup>76,77</sup>

Surface morphology of optimized formulation of transferosomes and liposomes was determined using Transmission electron microscopy (Tecnai 20, Make: Philips, Holland) at SICART, Gujarat. For analysis 2 drops of sample was placed on carbon support film, dried and then 1% solution of Urenyl acetate was used for negative staining.



**Figure 4A.6 Transmission electron microscope**

#### **4A.9.3 Determination of amount of aceclofenac permeated and absorbed in rat skin by after diffusion studies:-**

The permeation of drug through skin was carried out as per the methods reviewed in literatures.<sup>72, 73,76,77,78</sup> After completion of ex-vivo diffusion and permeation studies for a time duration of six hours, the drug diffused in acceptor compartment was estimated using UV-Visible spectroscopic method. The gel applied on the excised skin in the donor compartment was collected back in a beaker and 10 ml methanol was added to it. The mixture was sonicated for 1 hr and kept overnight. Later methanol was separated from gel by simple filtration and then, volume was made up to 100 ml with phosphate buffer saline pH 7.4. The absorbance was measured at  $\lambda_{\max}$  of 273 nm after dilutions done with phosphate buffer saline pH 7.4 and the aceclofenac content was determined.

Amount of aceclofenac absorbed in rat skin was calculated by homogenizing the skin tissue in methanol. Later on, methanol was separated from gel by simple filtration and volume was made up to 100 ml with Phosphate buffer saline pH 7.4. The dilutions were done with phosphate buffer saline pH 7.4 and aceclofenac absorbed in the skin was determined by measuring the absorbance by UV spectrophotometric method at  $\lambda_{\max}$  of 273 nm. After the determination of percentage drug in acceptor compartment (% drug release at end of 6 hours), percentage drug remaining in donor compartment

and drug retained in skin, loss of drug was calculated by subtracting the sum of above mentioned values from 100.

Loss of drug =  $100 - (\% \text{ drug in acceptor compartment} + \% \text{ drug in skin} + \% \text{ drug remaining in donor compartment})$

The percentage drug diffused into in acceptor compartment, percentage drug absorbed in skin and percentage drug retained on skin in donor compartment was determined for optimized batch of liposomal gel, transferosomal gel and plain gel.

#### **4A.9.4 Pharmacokinetics studies and comparative pharmacokinetic profiles of transferosomal gel, liposomal gel and plain drug gel:-**

For Pharmacokinetic studies, HPLC method was used for the analysis of aceclofenac in plasma and preparation of standard curve of drug in plasma after referring literatures.<sup>119, 120, 121</sup>

A reported HPLC method has been used with some modifications for estimation of aceclofenac in plasma samples. Mobile phase used was 30 volume of water and 70 volume of acetonitrile. The Injection volume was 10 $\mu$ l and flow rate of 1 ml/min was kept. The  $\lambda_{\text{max}}$ -of 273 nm was set for measurements. The column used was C<sub>18</sub> column (100 mm  $\times$  4.6 mm, 3.5  $\mu$ ).

The blood samples withdrawn from the tail vein of rats were collected in stoppered tubes containing EDTA as anticoagulant and immediately centrifuged at 5000 rpm for 25 min. The separated plasma samples were stored at -21 $^{\circ}$  C until analyzed. The HPLC chromatogram of blank plasma sample was taken followed by standard concentration of drug in Plasma for preparation of standard curve.

For analysis, in 0.1ml of plasma, 25  $\mu$ l of internal standard solution and 20  $\mu$ l of standard aceclofenac solution was added. Then 200  $\mu$ l of acetonitrile was added to the mixture and mixed for a minute, and then volume was made up to 1 ml with acetonitrile. The resulting solution was vortexed for 60 s and centrifuged at 10,000 rpm for 10 min. The supernatant layer was separated and analyzed for aceclofenac content using a sensitive high performance liquid chromatographic method.

#### **4A.9.5 Plasma profile of drug administered through drug carriers liposomes and transferosomes incorporated transdermal gel:-**

Approval to carry out pharmacokinetic studies was obtained from the Institutional Animal Ethics Committee. Approval no- AEP. PhD/13-14/23 .These studies were performed on optimized liposomal gel (M3), transferosomal gel (F3) and plain gel. Albino rats were kept under standard laboratory conditions (temperature  $25 \pm 2^\circ\text{C}$  and relative humidity of  $55 \pm 5\%$ ). The rats were kept in cages (6/cage) and feeded with standard laboratory diet. About  $15 \text{ cm}^2$  of skin was shaved on the abdominal side of rats in each group. They were fasted for the period of 12 hr for observations of any unwanted effects. The rats were divided into 3 groups, each containing 3 rats. Group I received M3 transdermally, group II received F3 transdermally and group III received plain gel.

The rats were anaesthetized using light ether anesthesia and blood samples (0.5 ml) were withdrawn from the tail vein of rat at 0 (pre-dose), 30 minutes, 1, 2, 3, 4,6 and 8 h and kept in micro centrifuge tubes in which 6 mg of EDTA was added as an anticoagulant. The blood collected was mixed with the EDTA properly and centrifuged at 5000 rpm for 25 min for separation of plasma. The separated plasma was stored at  $-21^\circ\text{C}$  until drug analysis was carried out using high performance liquid chromatographic (HPLC) method.

For analysis, in 0.1ml of plasma sample, 25  $\mu\text{l}$  of internal standard solution was added. Then 200  $\mu\text{l}$  of acetonitrile was added to the mixture and mixed for a minute, and then volume was made up to 1 ml with acetonitrile. The resulting solution was vortexed for 60 s and centrifuged at 10,000 rpm for 10 min. The supernatant layer was separated and analyzed for aceclofenac content at the set parameters of high performance liquid chromatographic method.

#### **4A.10 Residual solvent analysis for transferosomes and liposomes:-**

As chloroform and methanol were used as solvent in the thin film hydration method during preparation of transferosomes and liposomes, the removal of complete organic solvent in the process was confirmed<sup>122</sup>. The solvents methanol and chloroform come under solvent of Class II, their amounts must be under limits of 3000 ppm and 60 ppm respectively in the formulation according to ICH Guideline Q3C\_R5.

Therefore formulations were analyzed by Gas Chromatography (Auto System XL, Perkin Elmer) at Sophisticated Instrumentation Centre for Applied Research & Testing, Anand, Gujarat.

#### **4A.11 Radioactive tagging experiment for skin permeation studies:-**

##### **4A.11.1 Steps of radiolabelling of formulation:-**

The radiolabelling was performed at Institute of Nuclear Medicine and Allied Sciences, DRDO, New Delhi as per the standard procedure.<sup>123, 124</sup>

- Aceclofenac transferosomal dispersion 1ml (conc.3.5 mg/ml) was mixed with 0.1 ml SnCl<sub>2</sub> (1 mg/ml in 10% Glacial acetic acid).Then, 1 ml <sup>99m</sup>Tc-NaTcO<sub>4</sub> (2 mci/ml) was added and mixed.
- The radiolabeling of Compound was performed as per standard protocol of INMAS and radiolabeling efficiency was determined at various pH, temperature and incubation time by instant thin layer chromatography.The maximum radiolabeling was observed at pH 7,temperature of 30°C and incubation time of 30 minutes. pH was adjusted to 7 using NaHCO<sub>3</sub> solution (1%)
- Then the mixture was incubated for 30 minutes at 30°C to produce labeled <sup>99m</sup>Tc-aceclofenac transferosomes.

The chemical form of <sup>99m</sup>Tc available from the Moly generator is sodium pertechnetate (<sup>99m</sup>Tc-NaTcO<sub>4</sub>) which is nonreactive. In <sup>99m</sup>Tc-labeling, prior reduction of <sup>99m</sup>Tc- NaTcO<sub>4</sub> from the 7<sup>+</sup> state to a lower oxidation state is required using reducing agent SnCl<sub>2</sub>.

##### **4A.11.2 In-vitro saline stability of radiolabelled drug:-**

- ❖ In vitro saline stability of radiolabelled compound was determined by instant thin layer. Study was performed by mixing 100µl of <sup>99m</sup>Tc- Compound with 900µl of saline, vortex it for proper mixing.
- ❖ Mixture was incubated at 37°C Small aliquots were withdrawn at 0.25, 0.5, 1, 2,4,6,18,24 hrs. and radiochemical purity of <sup>99m</sup>Tc-Compound was evaluated by standard ITLC method using acetone as mobile phase.
- ❖ The developed strips were cut in 7:3 ratios and radioactivity in each part was measured to calculate the % labeled compound.



- ❖ Any decrease in percentage of radio labeled complex was taken as degradation.

### 4A.11.3 Procedure of imaging:-

The imaging was performed as per the standard methods of INMAS, New Delhi and described as follows:-

- Symbia 2T dual Head SPECT-CT machine (Siemens) was used for scintigraphy.
- 25 cm<sup>2</sup> round area was shaved and cleaned with normal saline prior to application of the formulations.
- Application area of 2 cm<sup>2</sup> was marked by a marker pen and transferosomal gel was applied.
- Scintigraphic images of pre-wash area and post-wash area were taken at the end of 1 hour, 2 hour and 3 hour after the gel application on individual rabbits.
- Washing at every time point was done with cotton wet with saline from periphery to centre to avoid contamination.

### 4A.12 Anti- inflammatory studies of optimized formulations by rat paw edema method:-

- The anti-inflammatory activity was carried out by carrageenan induced paw edema method to compare the activity of liposomal gel, transferosomal gel and marketed gel of aceclofenac using Plethysmometer.<sup>125,126,128</sup>
- The albino rats weighing between 250 to 300 gms. were used for the study.
- The rats were fed with standard food and water. Food was withdrawn 12 hours before and during the experimental studies.
- The animals were divided into four groups having two animals in each group.
- First group served as normal control receiving gel base without drug, second group received liposomal aceclofenac gel, third group received transferosomal aceclofenac gel and fourth group received marketed aceclofenac gel at the right hind paw.
- After 30 minutes of topical application of formulations on the right hind paw of rats, 0.1 ml of 1%w/v carrageenan (in 0.9% saline solution) was injected in the subplantar region of right hind paw of rats. The initial paw volume just after injection and subsequent readings up to 6 hours and then at 24 hrs were measured.

**Table 4A.10 Antiedema activity determination by rat paw edema method using plethysmometer**

Rat Group	Applied Formulation
I	Control
II	Aceclofenac liposomal gel
III	Aceclofenac transferosomal gel
IV	Aceclofenac marketed gel

❖ The % inhibition of edema was calculated for each group using the following equation:-

$$\% \text{ inhibition of edema} = 1 - [(a-x / b-y)] \times 100$$

a= mean paw volume of treated animal after carageenan injection

x= mean paw volume of treated animal before carageenan injection

b= mean paw volume of control animal after carageenan injection

y= mean paw volume of control animal before carageenan injection

#### **4A.13 Analgesic activity of optimized formulations by hot plate method in rats:-**

Analgesic activity of transdermal gel formulations was carried out by latency period measurements using Eddy's hot plate as per the methods described in literature.<sup>127, 128</sup>

- Albino rats weighing between 250-300 gms. were used for evaluation of analgesic activity, in each group two albino rats were kept. Albino rats were divided into four different groups each containing two animals, the animals were marked individually. Food was withdrawn 12 hours prior to drug administration till completion of experiment. The animals were weighed and numbered appropriately. First group did not receive anything, second group received liposomal gel, third group received transferosomal gel and fourth group received marketed gel on hind paws.

- After 60 minutes, the animals placed on the hot plate at temperature of  $55^{\circ}\text{C} \pm 5^{\circ}\text{C}$  and the observations were recorded and at the time interval of 30, 60 and 90,120,150 and 180 minutes. A cut off time of 30 s was followed to avoid any thermal injury to the paws. The time of latency was determined as the time period between the zero point, when the animal is placed on the hot plate surface, and the time when the animal jumps off to avoid thermal pain.
- The statistical analysis was performed to confirm the significant improvement of analgesic activity of drug carriers based gel as compared to plain drug gel at 95% confidence interval.



Figure 4A.7 Eddy's hot plate

### 4A.14 Stability Studies of optimized formulation:-

Stability studies were performed as per ICH guidelines at accelerated condition of temperature of  $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$  and humidity of  $60\% \text{ RH} \pm 5\% \text{ RH}$  for six months and evaluated for- size, zeta Potential ,entrapment efficiency, gel characteristics and In-vitro permeation.<sup>129</sup> The data were analyzed statistically at 95% confidence interval for finding out any significant change in properties.

## **4B Drug carriers (transferosomes) based transdermal gel of indomethacin:-**

### **4B.1 Preformulation studies:-**

Identification of drug was carried out by Fourier Transform Infra-Red and UV-Visible spectrophotometry as per the methods described in literature.<sup>109, 110, 111, 130, 132</sup>

**4B.1.1 FTIR studies:-** The FTIR of drug sample was carried out by FTIR Shimadzu Corp.(8400S DRS) .The Sampling technique involved KBr pellets with resolution of 6 and scanning for 12-24 times. IR scan of drug indomethacin was taken after keeping at temperature of 40° C and humidity of 75% RH for 3 months. The IR spectra of the plain drug were compared with reference IR Spectra.

### **4B.1.2 UV scan of indomethacin in methanol :-**

The indomethacin drug concentration of 10 µg/ml in methanol was scanned between wavelengths of 200 nm to 400 nm using UV-visible spectrophotometer Shimadzu Corporation, Japan (UV-1800) for determining  $\lambda_{\text{max}}$  of indomethacin.

### **4B.1.3 Drug-excipient compatibility studies:-**

The screened phospholipid -1, 2-disteroyl -sn- glycerol-3-Phospho-ethanolamine, Na salt and surfactant span 60 were subjected to compatibility studies with drug indomethacin to find out any interaction with drug. The interaction of cholesterol and carbopol gel base with drug, phospholipid and surfactant mixture was also studied.

FTIR analysis was performed for studying drug-excipient compatibility using the following physical mixtures:-

A. IR scan of indomethacin

B. IR scan of mixture of indomethacin, phospholipids, cholesterol and surfactant in ratio of 1:1:1:1.

C. IR scan of mixture of indomethacin, phospholipids, cholesterol, surfactant and gel base carbopol 934 in ratio of 1:1:1:1:1.

## **4B.2. Analytical method for estimation of indomethacin by UV-Visible Spectrophotometry:-**

The method of analysis was carried out as per the reported method in literature.<sup>109, 110, 111, 130, 132.</sup>

### **4B.2.1 Standard calibration curve of indomethacin in methanol:-**

Standard plot was prepared using a concentration range 2 to 16 µg /ml.

Methanol was used as blank solution and absorbance was measured for each solution at  $\lambda_{\max}$  of 239 nm using Shimadzu Corporation, Japan (UV-1800), and the graph was plotted for absorbance versus concentration of indomethacin. All the readings were taken in triplicate to minimize error and standard deviation was determined.

### **4B.2.2 UV Scan for determining $\lambda_{\max}$ of indomethacin in Phosphate buffer pH 7.4:-**

The indomethacin drug concentration of 10 µg/ml in phosphate buffer pH 7.4 was scanned between 200 nm to 400 nm using UV-Visible spectrophotometer Shimadzu Corporation, Japan (UV-1800) for determining  $\lambda_{\max}$  of indomethacin.

### **4B.2.3 Standard plot of indomethacin in media Phosphate buffer pH 7.4 :-**

Standard plot was prepared using a concentration range 3 to 30 µg /ml. Phosphate buffer pH 7.4 was used as blank solution and absorbance was measured for each solution at  $\lambda_{\max}$  of 320 nm using Shimadzu Corporation, Japan (UV-1800), and the graph was plotted for absorbance versus concentration of indomethacin. All the readings were taken in triplicate to minimize error and standard deviation was determined.

### **4B.2.4 Linearity, precision and accuracy of analytical method:**

#### **❖ Linearity and range**

The linearity was determined by analyzing 6 independent levels of standard curve in the range of 3-30 µg/ml. Absorbance of each solution against media was recorded at  $\lambda_{\max}$ . Curve of absorbance vs. concentration was plotted and correlation co-efficient and regression line equation for drug were determined.

**❖ Precision:**

Intra-day precision was determined by analyzing drug (3-30 µg/ml) at three different time points of the same day and inter day precision was determined by analyzing aceclofenac at three different time points on different days and %RSD was calculated.

**❖ Accuracy of Analytical method:-**

The prepared samples were spiked and % recovery was calculated to confirm the accuracy of analytical method.

**4B.3 Process of preparation of transferosomes as drug carriers by thin film hydration method based on experimental design:-**

<b>Factors:-</b>	<b>Responses:-</b>
Quantity of Phospholipids	Size
Quantity of cholesterol	Entrapment efficiency
Quantity of surfactant	

The quantity of phospholipid was varied as 0.067 mMol to 0.133 mMol (50 -100 mg), surfactant 0.06-0.12 mMol (25-50 mg) and cholesterol as 0.064 to 0.129 mMol (25-50 mg) for preparation of experimental design batches of transferosomes. The quantity of drug indomethacin was taken as 0.279 mMol (100 mg) .The prepared batched were evaluated for size and entrapment efficiency.

#### 4B.3.1 Steps of preparation of transferosomes of indomethacin:-

- Phospholipid (0.067 mMol-0.133 mMol), surfactant (0.06 - 0.12 mMol) & cholesterol (0.064 - 0.129 mMol) were solubilized in 10 ml chloroform-methanol (9:1) mixture. Drug: Lipid molar ratio=2.12mMol - 4.20 mMol).
- Thin film formation was done by vacuum rotary evaporator at temperature of 50°C, 90 rpm for 20 minutes.
- Thin film was evaporated under vacuum for removal of even trace amount of organic solvent and then kept in desiccators overnight for removal of even trace amount of organic solvent.
- Dried thin film was hydrated by 20 ml phosphate buffer pH 7.4 containing 100 mg of drug and 100 mg surfactant to prepare drug loaded multilamellar vesicles
- The vesicular dispersion was sonicated by probe sonicator ( Vibra cell -Sonics) for 2 cycles of 2 minutes at pulse of 2 sec using standard 13 mm probe at amplitude of 60% to obtain small unilamellar vesicles form large multilamellar vesicles.
- The vesicles were observed under trinocular Microscope at 40 X and 100 X magnification.

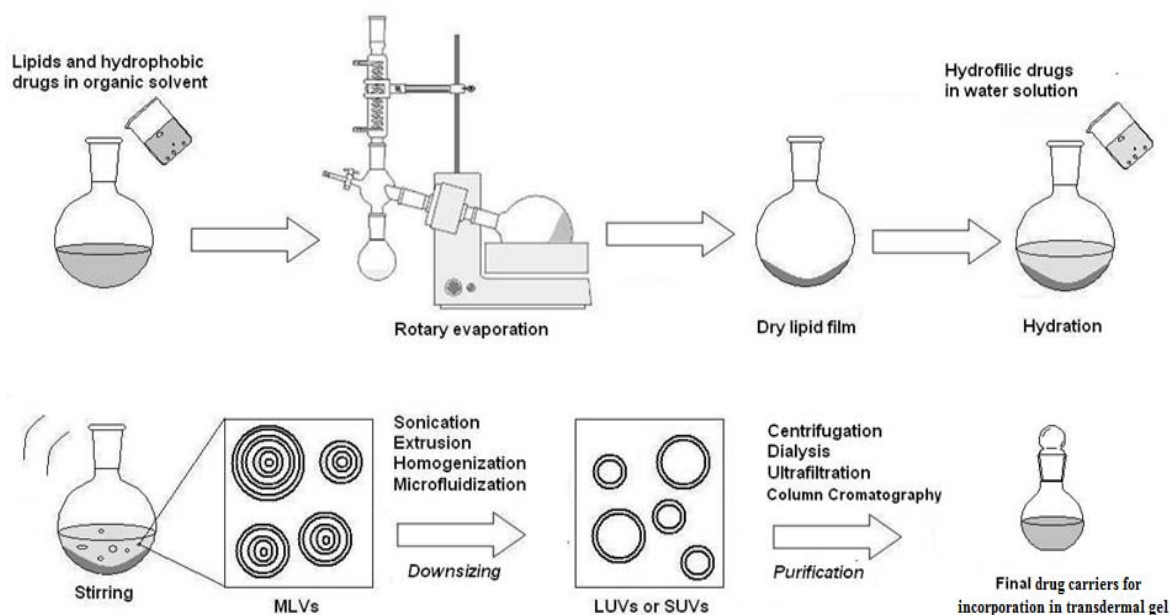


Figure4B.1 Schematic diagram of preparation of vesicles of indomethacin by thin film hydration

The residue at bottom was soaked in methanol for 30 minutes and then agitated, and analyzed for the entrapped drug content.

#### **4B.3.2 Formulation batches of transferosomes incorporated gel based on Experimental design (Box Behnken):-**

From the analysis of factorial design studies, it was evident that ,the quantities of phospholipid ,surfactants and cholesterol have effect on the size and drug entrapment of drug carriers, the experimental batches based on Box Behnken design were further prepared.

The quantity of phospholipid was varied as 50 mg to 100 mg (0.067-0.133 mMol), the quantity of surfactant varied as 25 mg to 50 mg(0.06 mMol-0.12 mMol) and quantity of cholesterol was varied as 25 mg to 50 mg (0.064-0.129mMol) for the preparation of factorial batches .Quantity of indomethacin was 100 mg (0.279 mMol).

**Table 4B.11 Formulation batches of transferosomes incorporated gel**

<b>Formulation</b>	<b>Quantity of Phospholipid(mg)</b>	<b>Quantity of surfactant(mg)</b>	<b>Quantity of Cholesterol(mg)</b>
F1	75	50	25
F2	75	25	25
F3	50	50	37.5
<b>F4</b>	<b>100</b>	<b>37.5</b>	<b>50</b>
<b>F5</b>	<b>100</b>	<b>25</b>	<b>37.5</b>
F6	75	25	50
F7	50	37.5	25
F8	75	37.5	37.5
F9	75	37.5	37.5
F10	100	50	37.5
F11	50	25	37.5
F12	50	37.5	50
F13	75	37.5	37.5
F14	75	50	50
F15	100	37.5	25



#### **4B.4 Evaluation of formulation:-**

**4B.4.1 Evaluation of transferosomes formulation:-**The evaluation of transferosomes were performed based on the methods reviewed in literatures.<sup>133, 139,140,141</sup>

❖ **Particle shape determination of drug carriers:-**

Shape was determined using trinocular microscope (Carl Zeiss) at BIP, Baroda

❖ **Particle size, Zeta potential and polydispersity index determination of drug carriers:-**Drug carriers particle size was determined using trinocular microscope (Carl Zeiss) and Malvern sizer (Malvern Instruments Ltd., UK, MAL100206)

❖ **Drug entrapment efficiency:-**

Drug entrapment efficiency was determined as per the methods given in literatures.<sup>134, 140, 141,142,148</sup>

Drug loaded Vesicles in phosphate buffer saline pH 7.4, were centrifuged at 15000rpm for 15 minutes at 4° C on Remi Lab centrifuge. The supernatant was collected and again centrifuged at 15000 rpm for 15 minutes and the drug content of supernatant was analyzed after 1/100 dilution to determine untrapped drug content. The entrapped drug was determined by subtracting the untrapped drug from the total drug added.

#### **4B.4.2 Evaluation of transferosomal gel based on gel characteristics and release studies:-**

The drug loaded transferosomes incorporated gels along with plain drug gel were evaluated for Refractive index, pH, spreadability, gel strength and extrudability.<sup>140, 141,142,148,150</sup>

❖ **Refractive index:-**

Refractive index was measured using Abbe refractrometer, dolphin at BIP, Baroda

❖ **Determination of pH:**

The pH of gels was checked by using a digital pH meter at room temperature. Initially, the pH meter was calibrated using standard buffers of pH 7 and then 10 gm of gel was weighed and dispersed in 25 ml of distilled water and then electrode of pH meter was dipped in the dispersion and the pH was noted.

❖ **Spreadability:-**

A quantity of 0.5 g gel was placed within a circle of 1cm diameter on a premarked glass slide over which a second glass slide was placed. A weight of 2 g was allowed to rest on the upper glass slide for 1min.

The increase in the diameter due to spreading of the gel was noted. Spreadability was then calculated by using the formula:

$S = M.L / T$  where, S = Spreadability, M = weight attached to upper slide, L = length of spread, T = time taken.

❖ **Gel strength:-**

The apparatus for measuring gel strength consist of plunger having pan to hold weights at one end and the other end was immersed into gel. Formulated gels were placed in glass bottle where marking was done 1cm below the filling mark. The weight required for the plunger to sink to a depth of 1 cm through the prepared gel was measured for each formulation.

❖ **Extrudability:**

Prepared gel was filled in tube and sealed. 3 Markings were done at interval of 1.5 cm from bottom of tube. The tube was pressed at marking using Pfizer hardness tester with  $1 \text{ kg/cm}^2$ , the weight of gel in continuous ribbon expelled is measured for each formulation.



Figure 4B.2 Extrudability studies using Pfizer hardness tester

### ❖ **Rheological studies:-**

The viscosity of gels was determined by using Brookfield helipath (LVDV) viscometer. The gel was placed in the sample holder and no- 96 spindle was lowered perpendicularly into the sample. The spindle was attached to viscometer and then it was allowed to rotate at a constant optimum speed at room temperature. The readings of viscosity of the formulation were measured at different rpm.

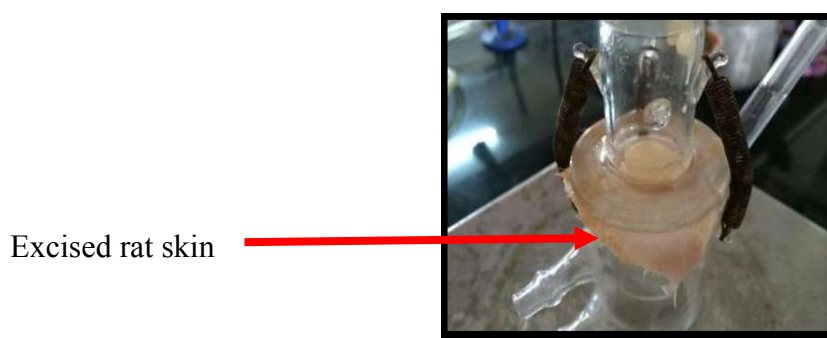
### ❖ **Release studies of transdermal formulations of indomethacin:-**

In-vitro and ex-vivo diffusion studies: (IAEC Approval no. PhD/13-14/23 dated. 14<sup>th</sup> December 2013). In-vitro diffusion studies was performed using modified Franz diffusion cell and dialysis membrane (HIMEDIA, pore size 2.4 nm, molecular wt. cutoff,12000-14000).The ex-vivo studies were performed using excised rat skin samples.<sup>10,15,143,146,147,150,151</sup> The in-vitro studies were carried out for all the experimental design batches whereas ex-vivo study was performed for optimized batch. For ex-vivo study, the excised skin of dorsal side of 5-6 weeks old rat was mounted on donor compartment after removing hair. It was then clamped between the donor and the receptor chamber of modified diffusion cells with the stratum corneum facing the donor chamber. Then, 0.2 g of gel containing indomethacin was applied on the skin in donor chamber. The receptor chamber was filled with 20 ml of with phosphate buffer saline pH 7.4 as diffusion media in receptor compartment.

The receptor medium was maintained at a temperature of  $37 \pm 0.5^\circ \text{C}$  and stirred at 600 rpm throughout the experiment. Aliquots of 5 ml were sampled from the receptor compartment at time interval of 1 hr and then immediately replaced with the same volume of pure medium. Aliquots withdrawn at specific time intervals were analyzed by UV spectroscopy method. Cumulative corrections were made to obtain the total amount of drug released at each time interval. The cumulative amount of drug released across the excised rat skin was determined as a function of time.

❖ **Permeation flux of transdermal formulation:-**

The permeation flux for experimental batches of transferosomal gel and plain drug gel were determined as per the studies reported in literatures.<sup>10,15,143,145,146,147,148</sup> Permeation flux is the slope of percentage drug release v/s time. It is expressed as  $\mu\text{gcm}^{-2}/\text{hr}^{-1}$ . Studies were carried out for all the experimental batches of transferosomes and the cumulative drug release as well as the permeation flux was determined.



**Figure 4B.3Ex-vivo permeation study**

**4B.5 Statistical analysis and optimization of formulation:-**

**4B.5.1 Analysis of transferosomal gel design batches by response surface methodology:-**

The analysis of transferosomal gel design batches was performed by response surface methodology method. The evaluation responses of batches were analyzed by contour plots and surface plots to observe the design space which can give suitable quantities of excipients for maximum responses. To achieve the maximum possible desired response, a target value of the responses was set in the optimization plot, in such a way that it was within the limits of responses already obtained from the design batches. The formulations factors and levels obtained from the optimization plot were taken as the optimization formula which could produce the desired target responses.

#### **4B.5.2 Development of optimized formulation of transferosomal gel:-**

The novel transdermal gel has been developed containing drug loaded transferosomes based on the optimized formula and process. The optimized batch was further evaluated to check the ability to achieve the target responses. The developed formulations were also compared with the conventional transdermal gel formulations.

The transferosomal gel showed better % drug entrapment and ex-vivo Permeation flux as compared to plain drug gel.

#### **4B.5.3 Size and morphology determination of optimized batch of drug carriers:-**

Studies were performed by Malvern zeta sizer and transmission electron microscopy for determining size, zeta potential and morphology of transferosomal dispersion.<sup>140,141,143</sup>

Surface morphology of optimized formulation of transferosomes and liposomes was determined using Transmission electron microscopy at SICART, Gujarat.



**Figure 4B.4 Transmission electron microscopy of transferosome of indomethacin**

#### **4B.5.4 Determination of amount of indomethacin permeated and absorbed in rat skin by after diffusion studies:-**

After completion of ex-vivo diffusion study of six hours, the drug diffused in acceptor compartment was estimated using UV spectroscopic method.<sup>8,9,29,30,33,36</sup> The gel in the donor compartment was collected, to it 10 ml methanol of was added, was sonicated

for 1 hr and kept overnight. Later methanol was separated from gel by simple filtration and volume was made up to 100 ml with phosphate buffer pH 7.4 and later dilution was done with Phosphate buffer pH 7.4. Indomethacin content was measured by UV spectroscopic method at  $\lambda_{\max}$  of 320 nm.

Amount of indomethacin absorbed in rat skin was calculated by homogenizing skin tissue in methanol. Later methanol was separated from gel by simple filtration and volume was made up to 100 ml with phosphate buffer saline pH 7.4 and later dilution was done with phosphate buffer pH 7.4 and indomethacin content was measured by UV spectroscopic method at  $\lambda_{\max}$  of 320 nm.

After the determination of percentage drug in acceptor compartment (% drug release at end of 6 hours) and percentage drug remaining in donor compartment and drug retained in skin, loss of drug was calculated by subtracting the sum of above mentioned values from 100.

Loss of drug =  $100 - (\% \text{ drug in acceptor compartment} + \% \text{ drug in skin} + \% \text{ drug remaining in donor compartment})$ .

The percentage drug diffused into in acceptor compartment, percentage drug absorbed in skin and percentage drug retained on skin in donor compartment was determined for optimized batch of transferosomal gel and plain drug gel.

### **4B.6 Anti-inflammatory studies of optimized formulations by rat paw edema method:-**

- The anti-inflammatory activity was carried out by carrageenan induced paw edema method to compare the activity of transferosomal gel and plain drug gel using plethysmometer.<sup>125,126,128</sup>
- The albino rats weighing between 250-300 gms. were used.
- The rats were fed with standard food and water. Food was withdrawn 12 hours before and during the experimental studies.
- The animals were divided into four groups having two animals in each group. First group served as normal control receiving gel base without drug

Second group received transdermal indomethacin gel and third group received plain drug gel at the right hind paw.

- After 30 minutes of topical application of formulations on the right hind paw of rats, 0.1 ml of 1%w/v carageenan (in 0.9% saline solution) was injected in the subplantar region of right hind paw of rats. The initial paw volume just after injection and subsequent readings upto 6 hours and then at 24 hrs were measured.

**The % inhibition of edema was calculated for each group using the following equation-**

$$\% \text{ inhibition of edema} = 1 - [(a-x) / (b-y)] \times 100$$

a= mean paw volume of treated animal after carageenan injection

x= mean paw volume of treated animal before carageenan injection

b= mean paw volume of control animal after carageenan injection

y= mean paw volume of control animal before carageenan injection

**Table 4B.12 Antiedema activity determination by rat paw edema method using plethysmometer**

Rat Group	Applied Formulation
I	Control
II	Indomethacin transdermal gel
III	Indomethacin plain drug gel

#### **4B.7 Analgesic activity of optimized formulations by latency period method:-**

The analgesic activity of transdermal gel formulations was determined by latency period method using Eddy's hot plate as per the methods in literature.<sup>127, 128</sup>

- Albino rats weighing 250-300 gms. were used for evaluation of analgesic activity; in each group two albino rats were kept.
- Albino rats were divided into three different groups each containing two animals, the animals were marked individually. Food was withdrawn 12 hours prior to drug

administration till completion of experiment. The animals were weighed and numbered appropriately. First group did not receive anything, second group received indomethacin transferosomal gel and third group received indomethacin plain drug gel on hind paws.

- After 60 minutes, the animals placed on the hot plate at temperature of  $55^{\circ}\text{C} \pm 5^{\circ}\text{C}$  and the observations were recorded and at the time interval of 30, 60, 90, 120, 150 and 180 minutes.
- A cut off time of 30 s was followed to avoid any thermal injury to the paws.
- The time of latency was determined as the time period between the zero point, when the animal is placed on the hot plate surface, and the time when the animal jumps off to avoid thermal pain.
- The statistical analysis was performed to confirm the significant improvement of analgesic activity as compared to plain drug gel at 95% confidence interval.

### ❖ Analgesic activity by hot plate method in rats:



Figure4B.5 Eddy's hot plate analgesic activity determination of transferosomal gel of indomethacin

### 4B.8 Stability Studies of optimized formulations:-

Stability studies were performed as per ICH guidelines at accelerated condition of temperature of  $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$  and humidity of  $60\% \text{RH} \pm 5\% \text{RH}$  for six months and



evaluated for size, zeta Potential ,entrapment efficiency, gel characteristics and in-vitro permeation.<sup>129</sup> The data were analyzed statistically at 95% confidence interval for finding out any significant change in properties. Stability studies were performed for transferosomal gel of drugs indomethacin.

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# CHAPTER-V

## Results and Discussion

### 5A Drug carriers, transferosome and liposome based transdermal gel of aceclofenac:-

#### 5A.1 Preformulation studies:-

Identification of drug was carried out by Differential scanning calorimetry, Fourier Transform Infra Red and UV spectrophotometric methods.

**5A.1.1 DSC studies:-**DSC studies were carried out at a temperature range of 25 °C to 165 °C at 10 °C increments of temperature.

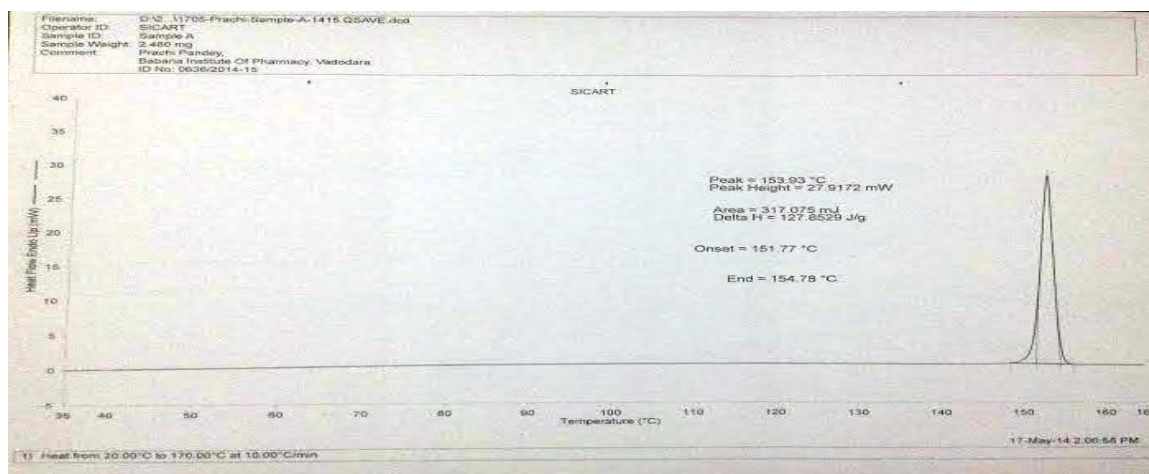
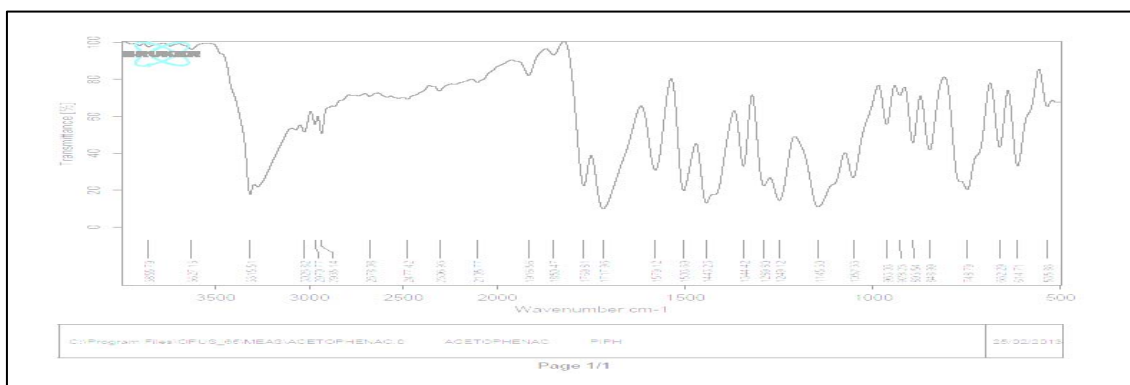


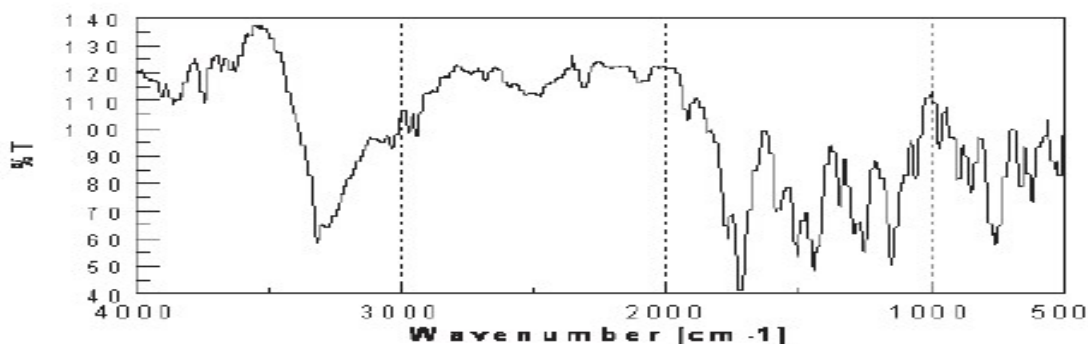
Figure 5A.1 DSC thermogram of aceclofenac

- DSC thermogram indicates the M.P of 154 °C which fairly matches with reported M.P of aceclofenac, thus confirming the identity and purity of aceclofenac.

**5A.1.2 FTIR studies:** - FTIR of pure drug aceclofenac was performed to confirm the identity.



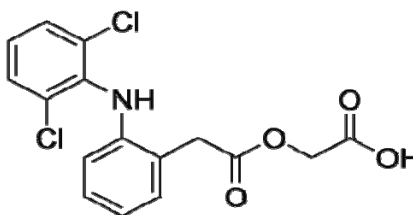
**Figure 5A.2 FTIR spectra of aceclofenac**



**Figure.5A.3 Standard FTIR spectra of aceclofenac**

**Source:-** Gupta V, Barupal AK, Ramteke S ,2008,Formulation Development and in vitro Characterization of Proliposomes for Topical Delivery of Aceclofenac, Indian J Pharm Sci , 70(6),pp: 768–775

**FTIR Interpretation:-**



**Figure 5A.4 Chemical structure of aceclofenac**

**Table 5A.1 Interpretation of FTIR spectra of aceclofenac**

Functional groups	Absorption (cm <sup>-1</sup> )	Range (cm <sup>-1</sup> )
C=O stretching	1736.41	1540–1870
OH stretching	2925.50	2500–3100
NH stretching	3359.18	3300–3500
C-Cl	668.00	550–850

**Interpretation:-** The FTIR spectra of test sample of drug aceclofenac fairly matches with that of reference spectra particularly in fingerprint region thereby confirming the identity of aceclofenac.

### 5A.1.3 UV Scan for determining $\lambda_{\max}$ of aceclofenac in methanol:-

The aceclofenac drug concentration of 10  $\mu\text{g/ml}$  in methanol was scanned between wavelengths 200 nm to 400 nm using UV-Visible spectrophotometer (Model-Shimadzu Corporation, Japan, UV-1800).

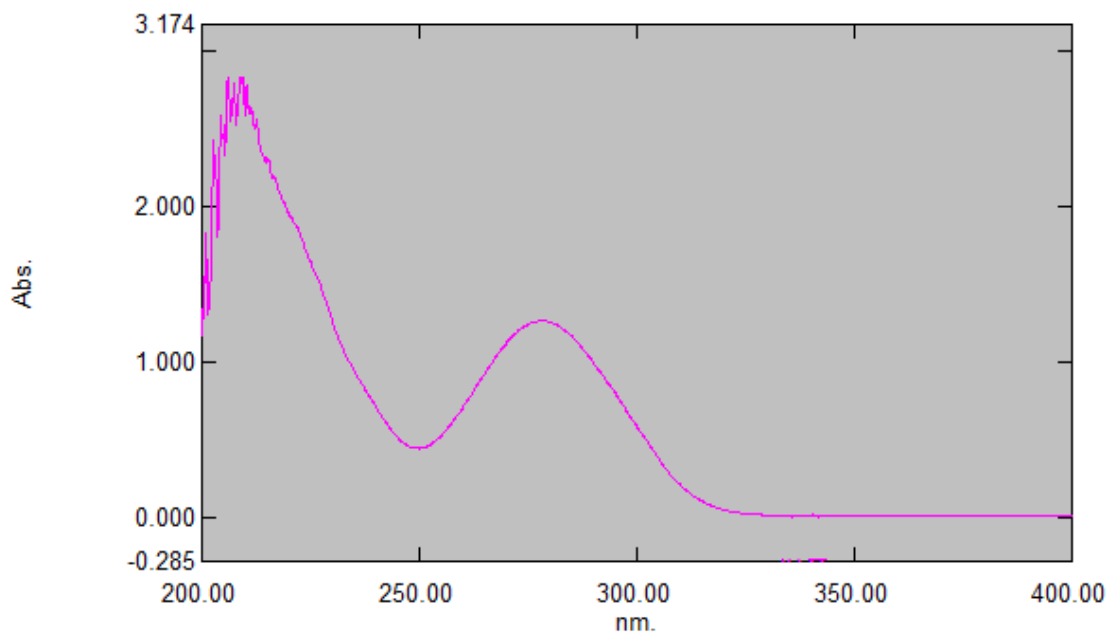


Figure 5A.5 UV scan of aceclofenac in methanol

**Interpretation:-** From the UV scan of aceclofenac, maximum absorbance was observed at 278 nm in media methanol using different concentrations of drug. The reported  $\lambda_{\max}$  is 277 nm, hence it can be considered as an evidence for identification of aceclofenac.

### 5A.2 Analytical method for estimation of aceclofenac by UV Spectrophotometer:-

The method of analysis was carried out as per the reported method in literature.<sup>30, 31, 32,</sup>

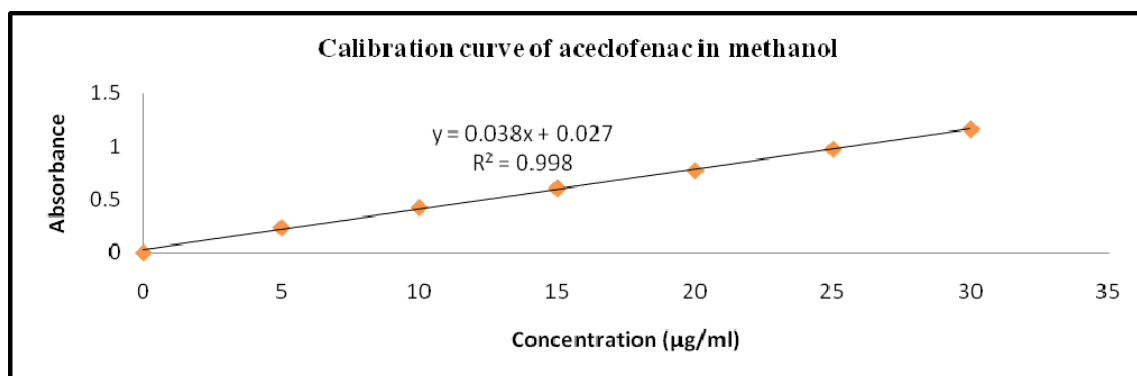
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**5A.2.1 Standard plot of aceclofenac in media methanol:-**

The standard plot of aceclofenac was prepared in methanol using a concentration range 5 to 30 µg /ml. Methanol was used as blank solution and absorbance was measured for each solution at  $\lambda_{\text{max}}$  of 278 nm using UV-Visible spectrophotometer (Model-Shimadzu Corporation, Japan,UV-1800) and the graph was plotted for absorbance versus concentration of aceclofenac. All the readings were taken in triplicate to minimize error and standard deviation was determined.

**Table 5A.2 Calibration curve of aceclofenac in methanol**

Concentration (µg/ml)	Absorbance
0	0.00
5	0.236±0.001
10	0.424± 0.0005
15	0.607± 0.0011
20	0.774± 0.001
25	0.975± 0.0005
30	1.165± 0.0015

**Figure 5A.6 Calibration curve of aceclofenac in methanol**

- Good linearity was observed in the concentration range of 5 to 30 µg /ml as  $R^2$  value was found to be 0.998.

### 5A.2.2 UV scan of aceclofenac in media phosphate buffer saline pH 7.4:-

The aceclofenac drug concentration of 10  $\mu\text{g/ml}$  in phosphate buffer saline pH 7.4 was scanned between wavelengths 200 nm to 400 nm using UV-Visible spectrophotometer (Model-Shimadzu Corporation, Japan, UV-1800).

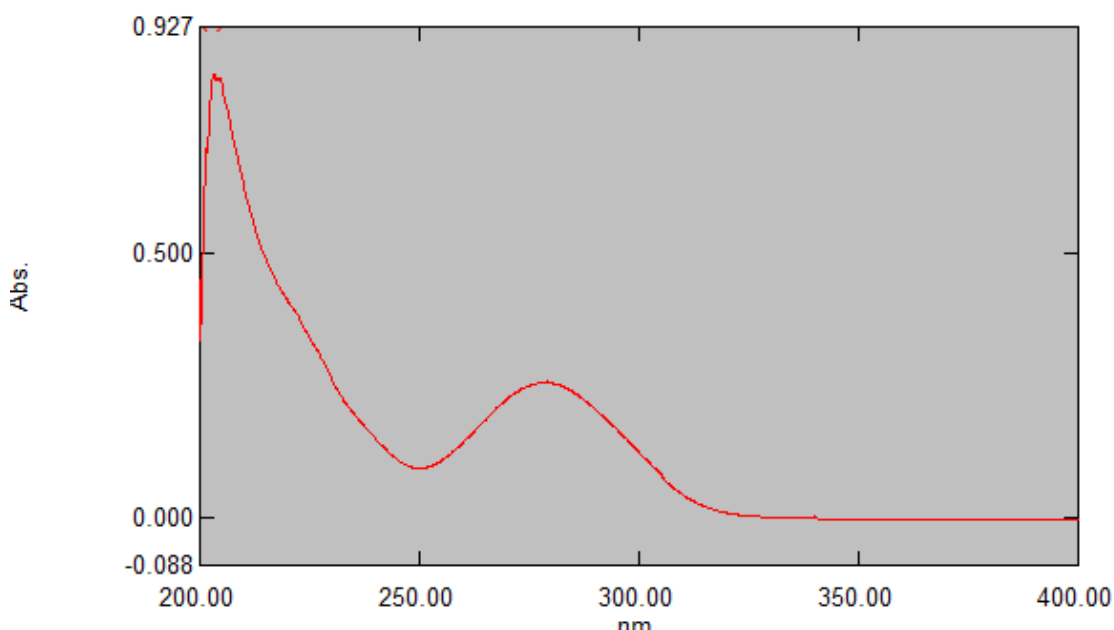


Figure 5A.7 UV scan of aceclofenac in phosphate buffer saline pH 7.4

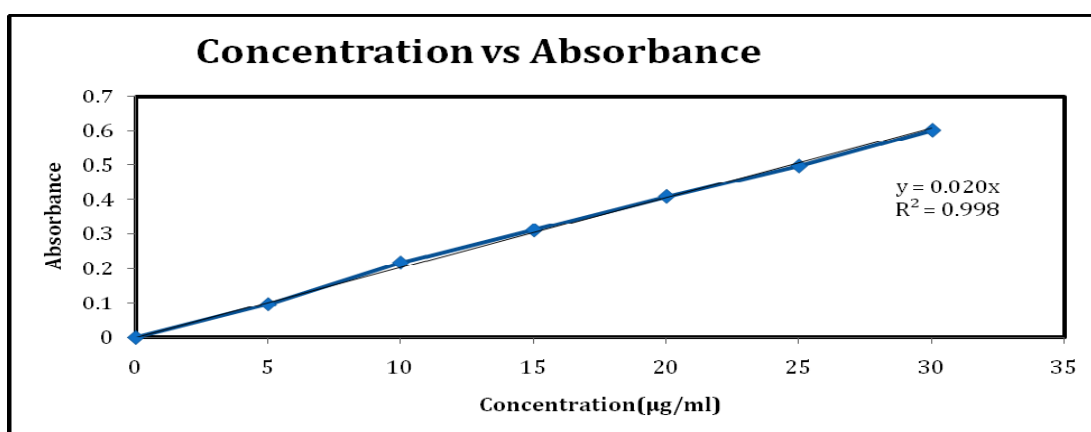
**Interpretation:** - From the UV scan of aceclofenac, maximum absorbance was observed at 273 nm in media phosphate buffer saline pH 7.4 at different concentration. The reported  $\lambda_{\text{max}}$  is 273 nm, hence it can be taken as working wavelength for UV spectroscopic analysis of aceclofenac.

### 5A.2.3 Standard calibration curve of aceclofenac in phosphate buffer saline pH 7.4:-

Calibration curve of drug aceclofenac was prepared using a concentration range 5 to 30  $\mu\text{g/ml}$  in media phosphate buffer saline pH 7.4.

**Table 5A.3 Calibration curve of aceclofenac in phosphate buffer saline pH 7.4**

Concentration ( $\mu\text{g/ml}$ )	Absorbance
0	0.00
5	0.096 $\pm$ 0.001
10	0.217 $\pm$ 0.0005
15	0.311 $\pm$ 0.001
20	0.409 $\pm$ 0.001
25	0.498 $\pm$ 0.001
30	0.602 $\pm$ 0.001

**Figure 5A.8 Standard calibration curve for aceclofenac in phosphate buffer saline pH 7.4**

**Interpretation:-** Linearity was observed in the concentration range of 5 to 30  $\mu\text{g/ml}$  as  $R^2$  value was found to be 0.998, readings were taken in triplicate and standard deviation was calculated which was found to be under limits.

#### 5A.2.4. Linearity, precision and accuracy of analytical method

##### ❖ Linearity and range

The linearity was determined by analyzing 6 independent levels of standard curve in the range of 5- 30  $\mu\text{g/ml}$ .

**Table 5A.4 Results of Linearity and range**

Solvent system	Range ( $\mu\text{g/ml}$ )	Coefficient of linearity found from graph
Phosphate buffer saline pH 7.4	5-30	0.998

**Interpretation:-**The values of coefficient of linearity were found to be nearer to 1 for all 3 stock solutions which indicates the linearity of the range selected.

❖ **Precision:**

Intra-day precision was determined by analyzing drug (5-30 µg/ml) at three different time points of the same day and inter day precision was determined by analyzing aceclofenac at three different time points on different days and % RSD was calculated.

**Table 5A.5 Interday precision of analysis of aceclofenac**

Conc. µg/ml	Absorbance at 273 nm			Avg. abs.	S.D	% RSD
	Stock 1	Stock 2	Stock 3			
5	0.096	0.097	0.097	0.097	0.0005	0.51
10	0.217	0.216	0.217	0.217	0.0005	0.23
15	0.311	0.312	0.313	0.312	0.001	0.32

**Table 5A.6 Intraday precision of analysis of aceclofenac**

Conc. µg/ml	Absorbance at 273 nm			Avg. Abs.	S.D	% RSD
	Stock 1	Stock 2	stock 3			
5	0.098	0.097	0.097	0.097	0.0005	0.51
10	0.218	0.216	0.217	0.217	0.001	0.46
15	0.313	0.311	0.312	0.312	0.001	0.32

**Interpretation:** - The % RSD (Relative Standard Deviation) is less than 1 for intraday precision and for interday precision suggests that the method is precise and shows reproducibility.



**5A.2.5. Accuracy of analytical method:-**

The prepared samples were spiked and % recovery was calculated to confirm the accuracy of analytical method.

**Table 5A.7 Accuracy of analysis of aceclofenac**

Solvent	Amount taken µg/ml	Amount added		Theoretical drug conc. µg/ml	Actual conc. µg/ml	% Recovery
		% of original amount	Conc. in µg/ml of original amount			
Phosphate buffer saline pH 7.4	15	25	3.75	18.75	18.49	98.61
	15	50	7.50	22.50	22.23	98.80
	15	75	11.25	26.25	26.02	99.12

**Interpretation** –The method showed % recovery of more than 98%, thus confirming the accuracy of method of determining aceclofenac in phosphate buffer saline pH 7.4 by UV spectrophotometer.

**5A.3 Process variable optimization:-**

The process of rotary vacuum evaporation and probe sonication were varied by varying the process variables to investigate their effect of characteristics of carrier systems. Process variables in rotary vacuum evaporation and probe sonication were optimized.

**5A.3.1 Rotary vacuum evaporation –**

The process variables, temperature, RPM and time of operation were varied based on 3 factors, 3 level general factorial design and based on the quality of film produced, the process was optimized.

**Table 5A.8 Process variables of rotary vacuum evaporation**

<b>Parameter</b>	<b>Temperature</b>	<b>RPM</b>	<b>Variables</b>
Level	High (70° C)	High (90)	High (20)
	Medium (60° C)	Medium (80)	Medium (15)
	Low (50° C)	Low (60)	Low (10)



**Figure 5A.9 Rotary vacuum evaporator**

Table 5A.9 Effect of process parameters

Process Parameters		Observation	
Temperature °C	RPM	Time (min)	Quality of film
<b>60</b>	<b>90</b>	<b>20</b>	<b>Thin film formed- uniform and translucent in appearance</b>
60	80	10	Dry thin film not formed
50	80	20	Thin film formed- uniform in appearance
60	90	15	Thin film formed- not uniform in appearance
70	80	15	Thin film formed- not uniform in appearance
<b>50</b>	<b>90</b>	<b>20</b>	<b>Thin film formed- uniform and translucent in appearance</b>
70	80	20	Thin film formed- uniform in appearance
60	80	20	Thin film formed- uniform in appearance
50	90	15	Thin film formed- not uniform in appearance
70	60	10	Dry thin film not formed
50	90	10	Dry thin film not formed
70	60	20	Thin film formed- uniform in appearance
70	90	20	Thin film formed- uniform in appearance
70	80	10	Dry thin film not formed
60	60	20	Thin film formed- uniform in appearance

**Interpretation-**

The process parameters such as temperature, rotations per minute and time were varied in thin film hydration method and it was observed that at 50° C temperature and 90 rpm, when film formation was allowed for 20 minutes in rotary vacuum evaporator, a thin film was formed which was uniform and translucent in appearance. Also there was no change observed in appearance of film when, temperature was varied from 50° C to 60° C and rpm was varied from 80 to 90 rpm.

It was also observed that, at 50° C temperature and 60 rpm, when film formation was allowed for 10 minutes in rotary vacuum evaporator, a dry thin film could not be formed and also no change was observed, when rpm was changed from 60 rpm to 80 rpm.

Then, hydration of thin film was performed with solvent Phosphate buffer saline pH 7.4 at 50 °C temperature and 90 rpm for 20 minutes. Similar studies of screening of process parameters were performed for transferosomes also using the same combinations of process conditions and it was observed that thin film formation was possible at 50 °C temperature and 90 rpm, when film formation was allowed for 20 minutes in rotary vacuum evaporator. The hydration of thin film of transferosomes was done using the similar method as applied for liposomes.

Then prepared liposomes and transferosomes were reduced in size by probe sonication.

### 5A.3.2 Probe sonication-

The probe sonicator was operated using 13 mm probe at an amplitude of 60%. The effect of sonication cycles on the transparency of vesicular dispersion and average size of vesicles was studied. The % transmittance was measured using UV spectrophotometer and size was measured using trinocular microscope.



Figure 5A.10 Probe sonicator

Table 5A.10 Process optimization of Probe Sonication

Probe	Amplitude	Time and Pulse	Temperature
13 mm standard	60 %	2 minutes 2 s on, 2 s off	4 ° C

**Table 5A.11 Effect of sonication cycles on vesicle size of liposomes**

Cycles	Observation	Mean vesicle Size
2	Vesicles dispersion was slightly Hazy	3.5 $\mu$
3	Vesicles dispersion was almost transparent	2.0 $\mu$
4	Vesicles dispersion was almost transparent	0.6 $\mu$
5	Vesicle dispersion was transparent, Process repeated thrice % Transmittance was 90% in every analysis.	0.4 $\mu$

**Table 4A.12 Effect of sonication cycles on vesicle size of transferosomes**

Cycles	Observation	Mean vesicle Size
1	Vesicles dispersion was slightly hazy	1.5 $\mu$
2	Vesicles dispersion was transparent. % Transmittance was 90% in every analysis.	0.5 $\mu$
3	Vesicles dispersion transparent but frothing was observed	0.5 $\mu$

**Interpretation:-**It was found that sonication for 2 cycles of 2 minutes produced vesicles dispersion which was slightly hazy and mean vesicle size was found to be 3.5  $\mu$ m whereas at 3 and 4 cycles for 2 minutes produced vesicles dispersion which was almost transparent with mean vesicle size of 2  $\mu$ m and 0.6  $\mu$ m. The sonication for 5 cycles of 2 minutes produced vesicle dispersion which was transparent and mean vesicle size observed was 0.4  $\mu$ . In case of transferosomes, sonication for 2 cycles of 2 minutes produced vesicle dispersion which was transparent and mean vesicle size observed was 0.5  $\mu$ . The process was repeated thrice and % transmittance was found to be 90% in every analysis. Therefore the method of 5 sonication cycle each of 2 minutes and 2 sonication cycle each of 2 minutes at amplitude of 60% using 13 mm

standard probe can produce transparent vesicular liposomal dispersion and transferosomal dispersion respectively in reproducible manner with uniformity in vesicle size. Therefore, the process parameters selected for further studies were:-

- **For rotary vacuum evaporation:** 50°C temperature, rotation at 90 rpm and time 20 minutes.
- **For probe sonication:** 5 sonication cycle each of 2 minutes at amplitude of 60% for liposomes & 2 sonication cycle each of 2 minutes at amplitude of 60% for transferosomes.

#### **5A.4 Preliminary studies for screening of excipients:-**

##### **5A.4.1 Selection of excipients by formulation of trial batches of carrier system:-**

The preliminary trial batches of liposomes were prepared using drug and phospholipid in ratio of 1:1. Preparation was carried out at selected process parameters.

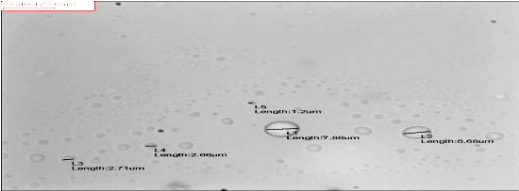
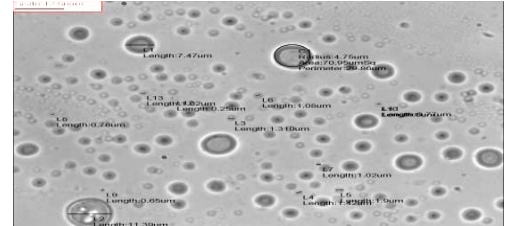

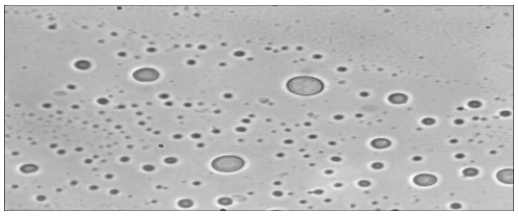

The phospholipids subjected to screening were:

1. 1-2 distearyl-sn- glycerol-3-phosphoethanolamine Na salt,
2. 1-2 distearyl-sn- glycerol-3-phosphate monosodium salt,
3. Hydrogenated Phosphatidylcholine,
4. 1, 2-diolsyl-3-trimethyl ammonium Propane chloride,
5. Lipova E-120.

Other excipient used in the preparation of liposomes was cholesterol as vesicle rigidizer and chloroform and methanol as solvents in rotary vacuum evaporation method.

❖ **Observation:**—The liposomes prepared were observed under trinocular microscope (Carl Zeiss) to determine the possibility of preparation by using the above mentioned phospholipids.

**Table 5A.13 Effect of Phospholipids on feasibility of production of vesicular systems**

Lipids	Observation	
Lipova-E-120	Homogenous dispersion , vesicles visible in trinocular microscope	
1,2-disteroyl -sn- glycerol -3-Phospho-ethanolamine, Na salt	Homogenous dispersion, vesicles visible in trinocular microscope, a prominent bilayer structure of vesicles was observed	
1,2-distroyl –sn-glycerol-3-Phosphate, monosodium salt	Homogenous dispersion, vesicles visible in trinocular microscope	
1,2-Dioleoyl-3-trimethyl ammonium Propane chloride	Homogenous dispersion, vesicles visible in trinocular microscope	
Hydrogenated Phosphatidylcholine	Not homogenous, slight lumps but vesicles visible in trinocular microscope	

**Interpretation-**

Using phospholipids named Leciva S-35 and Leciva S-50 D, it was observed that, on hydration of thin film produced, homogenous dispersion was not achieved and particles were visible. The phospholipid Lipova E-120 resulted in homogenous dispersion and no phase separation was observed, vesicles were found to be visible when seen through trinocular microscope.

Using 1,2-disteroyl-sn-glycerol-3-Phospho-ethanolamine,Na salt, 1,2-distroyl-sn-glycerol-3-Phosphate,monosodium salt, 1,2-Dioleoyl-3-trimethyl ammonium Propane

chloride, a homogenous dispersion was prepared on thin film hydration and , vesicles were found to be visible in trinocular microscope. The phospholipid 1,2-disteroyl-sn-glycero-3-Phospho-ethanolamine,Na salt was selected for further studies as prominent bilayer structure of vesicles was observed as well a uniform size distribution was found.

**5A.4.2 Preliminary studies on formulation of liposomes by 3<sup>2</sup> full factorial design:-**

The selected excipients in the preliminary studies were put in 2 factors, 2 levels factorial design and screened on the basis of outcomes of size and entrapment efficiency.

**Factors-**

Quantity of Phospholipids

Quantity of cholesterol

**Response-**

Size

Entrapment efficiency



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### 5A.4.3 Preparation of trial batches of liposomes based on factorial design:-

The quantity of phospholipids was varied as 50 mg and 100 mg (0.067- 0.133 mMol) and cholesterol were varied as 25 mg and 50 mg (0.064-0.129 mMol) for preparation of factorial batches of liposomes. The batches were named as L1 to L4.

**Table 5A.14 Factorial design for factors screening in liposome preparation**

<b>Formulation</b>	<b>Quantity of phospholipid (mg)</b>	<b>Quantity of cholesterol (mg)</b>	<b>Avg. Size (nm)</b>	<b>% Drug entrapment</b>
L1	100	50	398	43
L2	100	25	323	34
<b>L3</b>	<b>50</b>	<b>50</b>	<b>449</b>	<b>74</b>
L4	50	25	437	68

**Interpretation-** Highest drug entrapment was observed in the batch containing 50 mg of phospholipid and 50 mg of cholesterol but the size of vesicles was found to be higher than other formulations.

### 5A.4.4 Analysis of factorial batches –

The factorial batches were analyzed by pareto chart, main effect plot, interaction plot and cube plot to determine the effect and influence of factors on responses of % drug entrapment and size of liposomes.

## ❖ Pareto chart to identify the influence of factors on influence:-

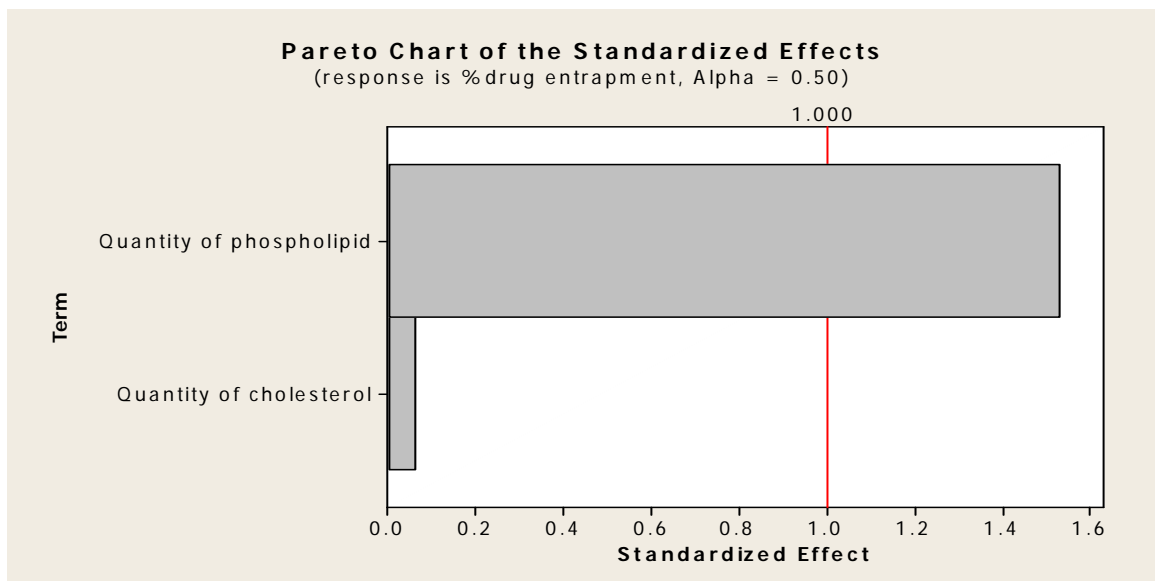
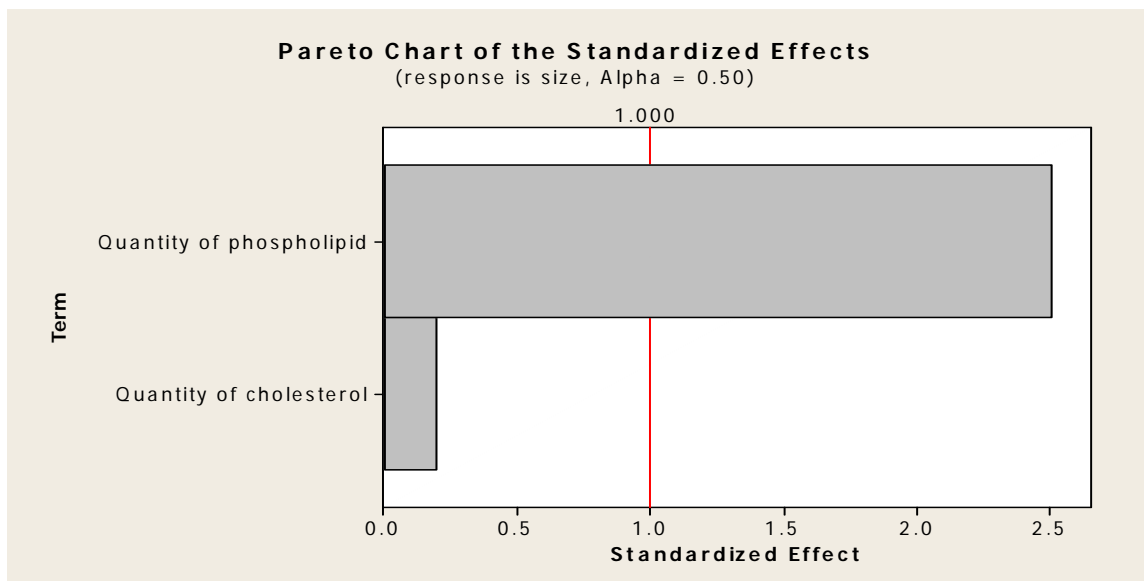
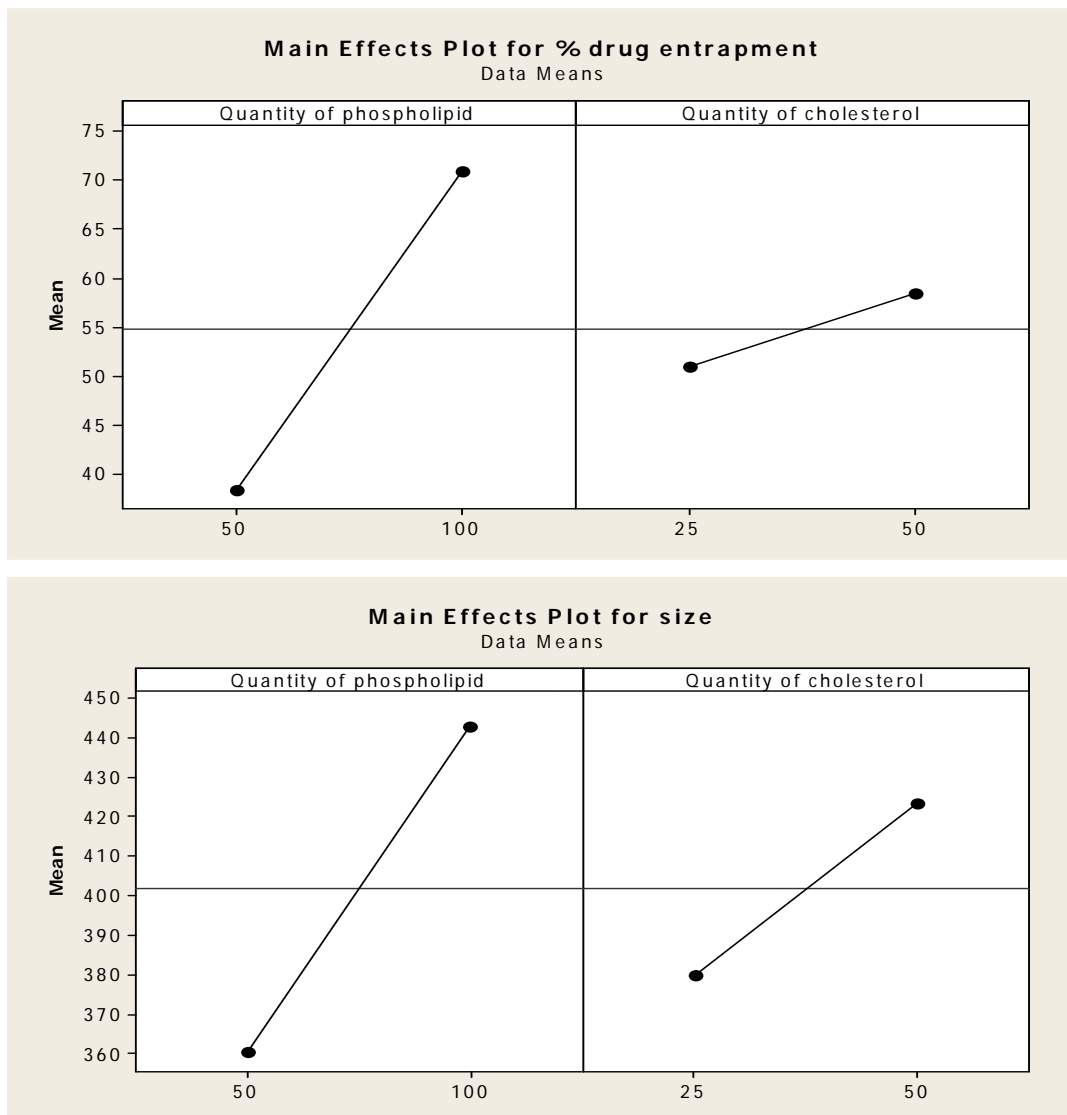


Figure 5A.11 Effect and influence of factors on responses

**Interpretation:-**

- Quantity of phospholipid has significant effect on size and % drug entrapment of liposomes as the quantity of phospholipids is crossing the line of significant influence on responses in the pareto chart.

❖ **Main effect plot to study the effect of factors and their levels on the responses:-**



**Figure.5A.12. Effect and influence of factors on responses**

**Interpretation:-**

- Higher quantity of phospholipid and surfactant results in higher drug entrapment as observed in main effect plot.
- Higher quantities of phospholipid and cholesterol results in larger size of liposomes as observed in the main effect plot.

## ❖ Interaction Plots of factors on the responses:-

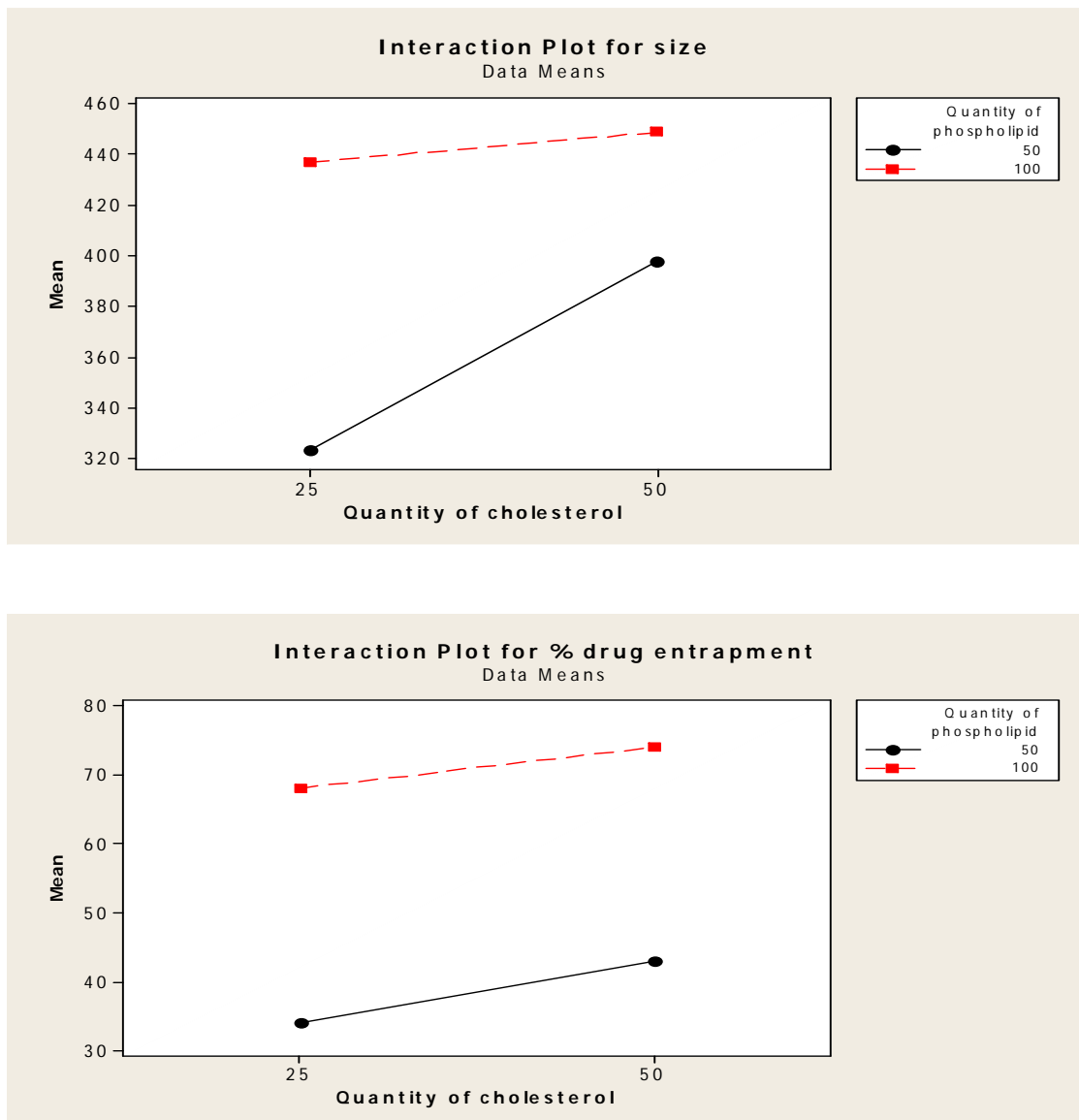
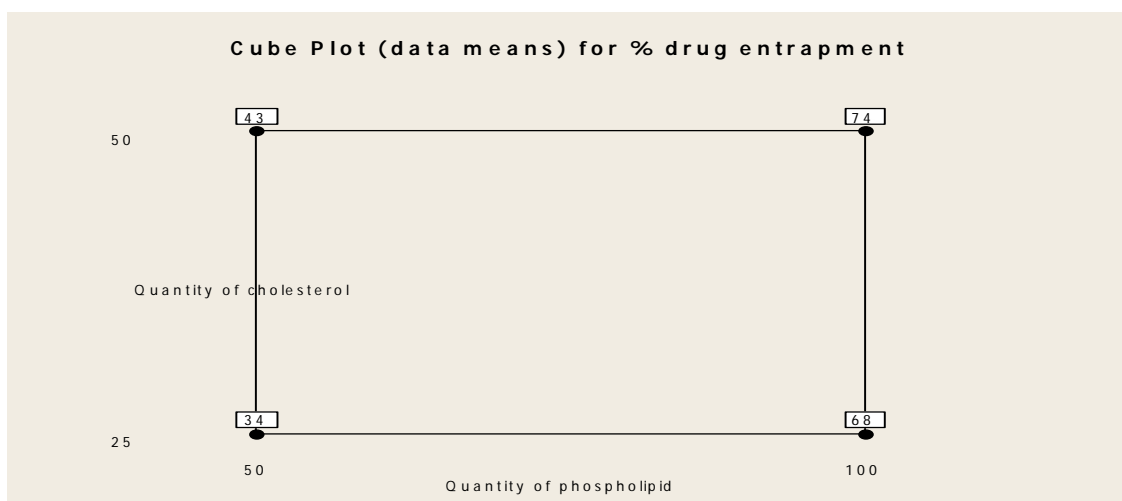
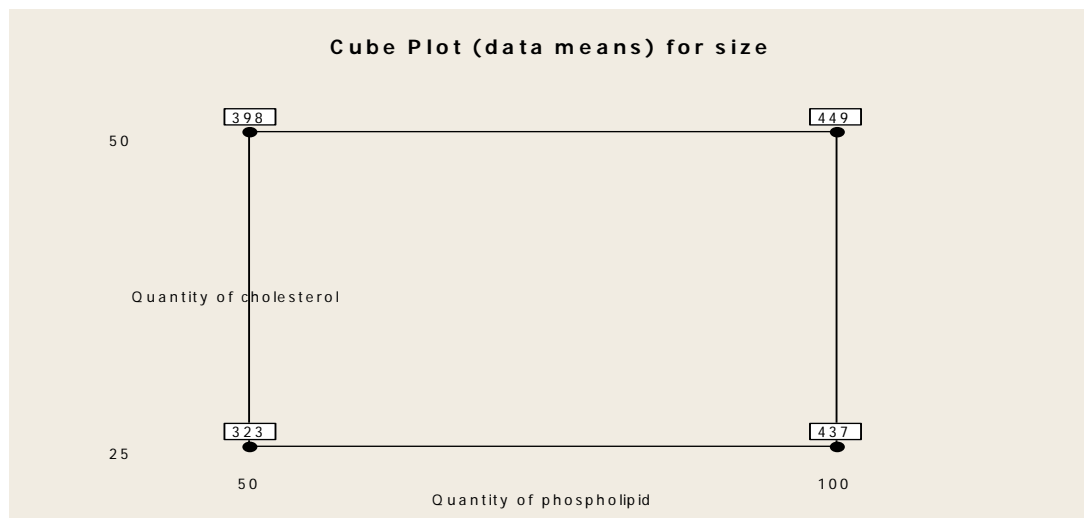


Figure.5A.13 Effect and influence of factors on responses

**Interpretation:-**

- Interaction plot indicate less interaction between quantities of phospholipid and cholesterol in affecting size of liposomes.
- Almost parallel lines indicate no interaction between quantities of phospholipid and cholesterol in affecting % drug entrapment of liposomes.

❖ **Cube plots to study effects of levels of factors on responses:-**



**Figure.5A.14. Effect and influence of factors on responses**

**Interpretation:-**

As observed from the cube plot,

- Quantity of phospholipid has positive influence on size (323 to 437).
- Quantity of cholesterol has positive influence on size (437 to 449).
- Quantity of phospholipid has positive influence on % drug entrapment (34 to 68).
- Quantity of cholesterol has greatest influence on % drug entrapment (68 to 74).

**5A.4.5 Shape and homogeneity of liposomes:-**The shape and homogeneity of factorial design batch L3 was studied by trinocular microscope (Carl Zeiss) at 40 X magnification.

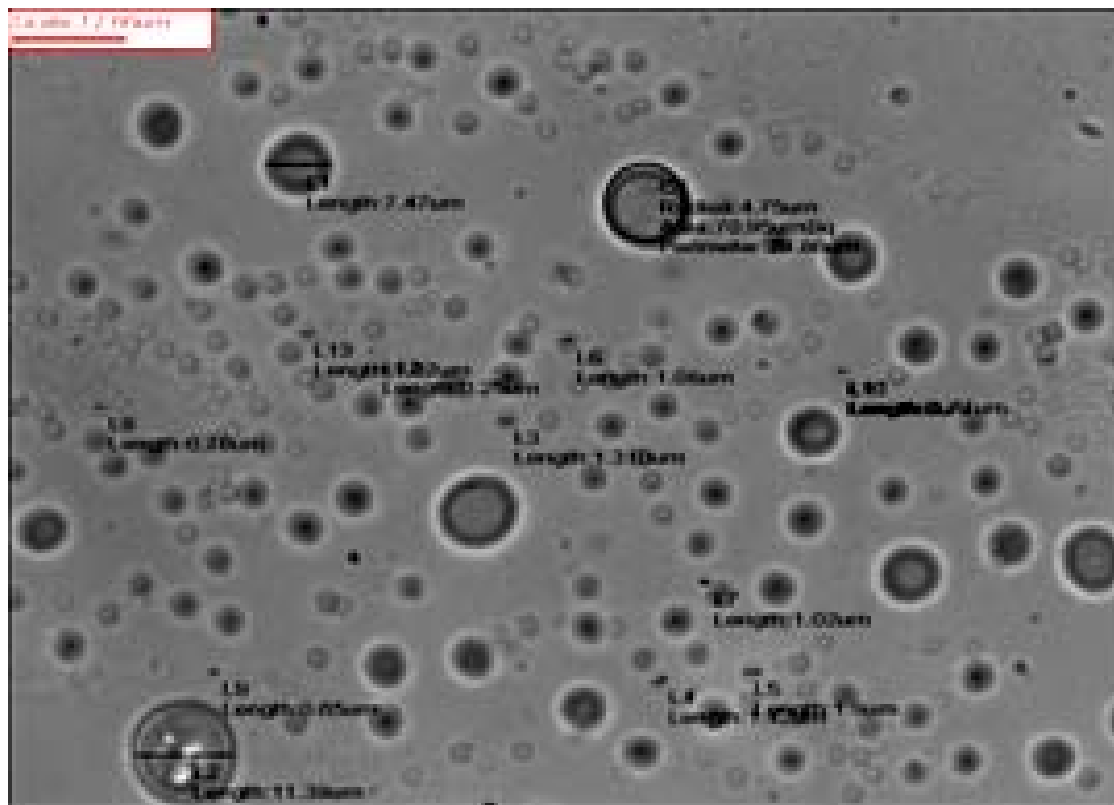


Figure 5A.15 Drug loaded liposomes

➤ The shape was almost spherical and the average vesicle size was found to be 0.5 to 1.0 microns.

**5A.4.6 Preliminary screening of surfactants for preparation of transferosomes:-**

The surfactant was selected based on preliminary evaluation of trial batches of transferosomes. The phospholipid, 1, 2-disteroyl-sn-glycero-3-phospho-ethanolamine, Na salt selected in the earlier studies on liposomes was used for preparation of transferosomes also.

**Table 5A.15 Selection of surfactant for transferosome preparation**

<b>Surfactant</b>	<b>Observation</b>
Sodium cholate	Transferosome dispersion was homogenous. On observation under trinocular microscope vesicles were visible.
Tween 80	Frothing observed in dispersion.
Span 40	Transferosome dispersion was homogenous. On observation under trinocular microscope, vesicles were visible.
Span 60	Transferosome dispersion was homogenous. On observation under trinocular microscope, vesicles were visible.
Span 20	Transferosome dispersion was homogenous. On observation under trinocular microscope, vesicles were visible.

- Surfactants span 40, span 60, sodium cholate and span 20 were found to produce transferosome vesicles. Tween 80 was not found suitable for preparing transferosomes as frothing was observed.

#### 5A.4.7 Screening of surfactant by formulation of transferosomes using different types and quantities of surfactants:-

The quantity of surfactants was varied as 50 mg and 100 mg and cholesterol were varied as 25 mg and 50 mg for preparation of factorial batches of transferosomes. The surfactants used for preparation of trial batches were span 40, span 60, span 20 and sodium cholate. The batches were named as T1 to T16. Quantity of drug taken was 100 mg and selection of surfactant was performed based on evaluation data of % drug entrapment and size.

**Table 5A.16 Formulation of transferosomes using different types and quantities of surfactants in combinations with cholesterol in varying quantities.**

Formulation	Drug (mg)	Phospholipid (mg)	Span 40 (mg)	Cholesterol (mg)	Avg. size (nm)	% Drug entrapment
T1	100	100	50	25	500	57.47
T2	100	100	100	50	450	61.69
T3	100	100	100	25	500	49.08
T4	100	100	50	50	400	42.63

Formulation	Drug (mg)	Phospholipid (mg)	Span 60 (mg)	Cholesterol (mg)	Avg. size (nm)	% Drug entrapment
T5	100	100	100	50	350	69.58
T6	100	100	100	25	300	52.64
T7	100	100	50	25	350	60.24
T8	100	100	50	50	400	65.45

Formulation	Drug (mg)	Phospholipid (mg)	Span 20 (mg)	Cholesterol (mg)	Avg. size (nm)	% Drug entrapment
T9	100	100	50	50	600	21.14
T10	100	100	100	25	750	29.34
T11	100	100	50	25	650	25.22
T12	100	100	100	50	700	28.12

Formulation	Drug (mg)	Phospholipid (mg)	Na. Cholate (mg)	Cholesterol (mg)	Avg. size (nm)	% Drug entrapment
T13	100	100	100	25	600	35.10
T14	100	100	50	50	500	30.33
T15	100	100	50	25	450	30.25
T16	100	100	100	50	500	38.43



**Interpretation:** - It was observed that transferosomes prepared from span 40 has lowest particle size and highest % drug entrapment as compared to sodium cholate. Transferosome prepared from span 60 has lowest particle size and highest % drug entrapment as compared to span 20. It was concluded that span 40 and span 60 gives better entrapment efficiency as well as particle size as compared to span 20 and sodium cholate. Both the surfactants span 40 and span 60 produced almost same entrapment efficiency in formulation, therefore diffusion study was carried out for further screening.

**5A.4.8 Drug release study of transferosome prepared using span 40 and span 60:-**

As the drug entrapment and size were found to be nearly same for the transferosomes prepared using span 40 and span 60, the transferosomes batches T1 to T8 were incorporated into gel and further evaluated for % drug release for 6 hrs.

**Table 5A.17 % Drug release of transferosomal gel formulations**

Formulation	Surfactant used	% Cumulative drug release
T1	span 40	17.25
T2	span 40	12.27
T3	span 40	19.57
T4	span 40	22.11
T5	span 60	85.63
T6	span 60	76.78
T7	span 60	71.65
T8	span 60	82.88

➤ From in-vitro % drug release studies, span 60 was found to give better drug release after 6 hrs as compared to span 40, therefore nonionic surfactant span 60 and cholesterol were selected as factors and their quantities varied in experimental design.

#### 5A.4.9 Formulation of transferosomes using screened factors by 3<sup>2</sup> full factorial design:-

The selected excipients in the preliminary studies were put in 3 factors, 2 levels factorial design and the influence of levels of factors on the responses of size and entrapment efficiency was studied.

<b>Factors-</b>	<b>Responses-</b>
Quantity of phospholipid	Size
Quantity of cholesterol	Entrapment efficiency
Quantity of surfactant	

#### 5A.4.10 Preparation of formulation batches of transferosomes based on factorial design:-

For preparation of transferosomes, the quantity of phospholipid was varied as 50 mg and 100 mg, the quantity of surfactant varied as 25 mg and 50 mg and quantity of cholesterol was varied as 25 mg and 50 mg for the preparation of factorial batches. The quantity of drug aceclofenac taken was 100 mg and batches were named as T1 to T8.

<b>Factors</b>	<b>Levels</b>
Quantity of Phospholipid(mg)	50 -100
Quantity of Surfactant (mg)	25-50
Quantity of Cholesterol (mg)	25-50

**Table 5A.18 Factorial design for factors screening in transferosome preparation**

<b>Formulation</b>	<b>Quantity of Phospholipid (mg)</b>	<b>Quantity of Surfactant (mg)</b>	<b>Quantity of Cholesterol (mg)</b>	<b>Size (nm) Avg.</b>	<b>%Drug Entrapment</b>
T1	50	50	25	200±20	34±1.4
T2	50	25	25	300±18	41±2.8
T3	100	25	25	800±35	52±3.9
T4	100	50	25	500±26	48±1.9
T5	50	50	50	300±15	38±2.1
<b>T6</b>	<b>100</b>	<b>25</b>	<b>50</b>	<b>900±27</b>	<b>58±4.3</b>
T7	50	25	50	400±45	46±3.7
T8	100	50	50	700±54	49±3.6

- Highest drug entrapment was observed in the batch containing 100 mg of phospholipid, 25 mg of surfactant and 50 mg of cholesterol but the size of vesicles was higher than other formulations.

#### 5A.4.11 Shape and homogeneity of transferosomes:-

The shape and homogeneity of transferosomes batch T6 was studied by trinocular microscope (Carl Zeiss) at 40 X magnification.

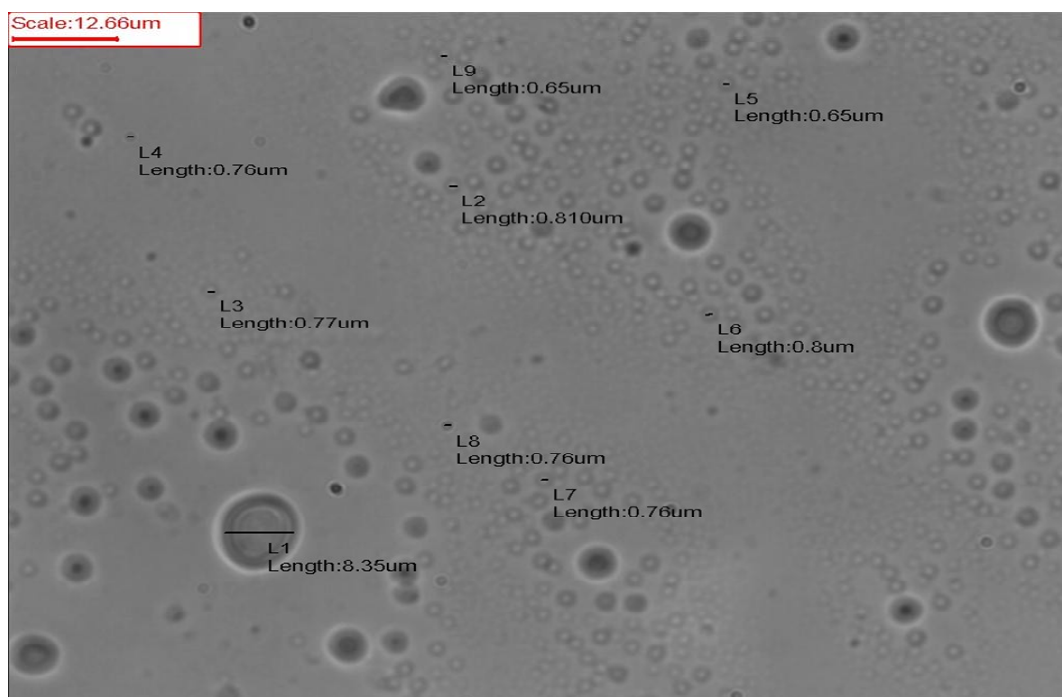


Figure 5A.16 Drug loaded transferosomes batch T6 containing both large MLV and SUV  
Size range- 0.5-1 μm

#### 5A.4.12 Interpretations of influence of factor on responses by analysis of factorial design:-

- ❖ Pareto chart was studied to identify the influence of levels of factors on the responses:-

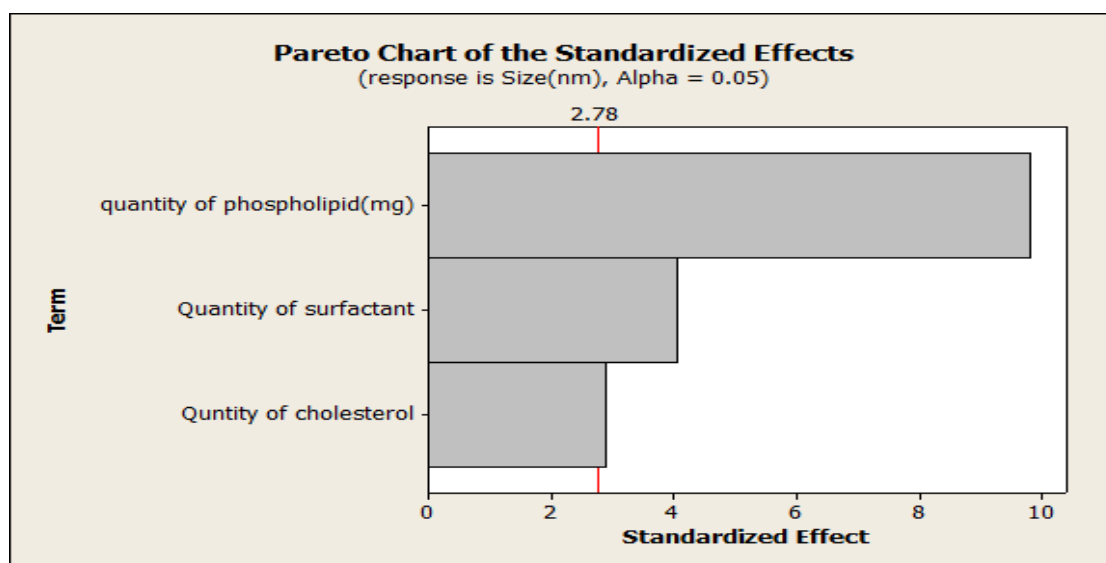
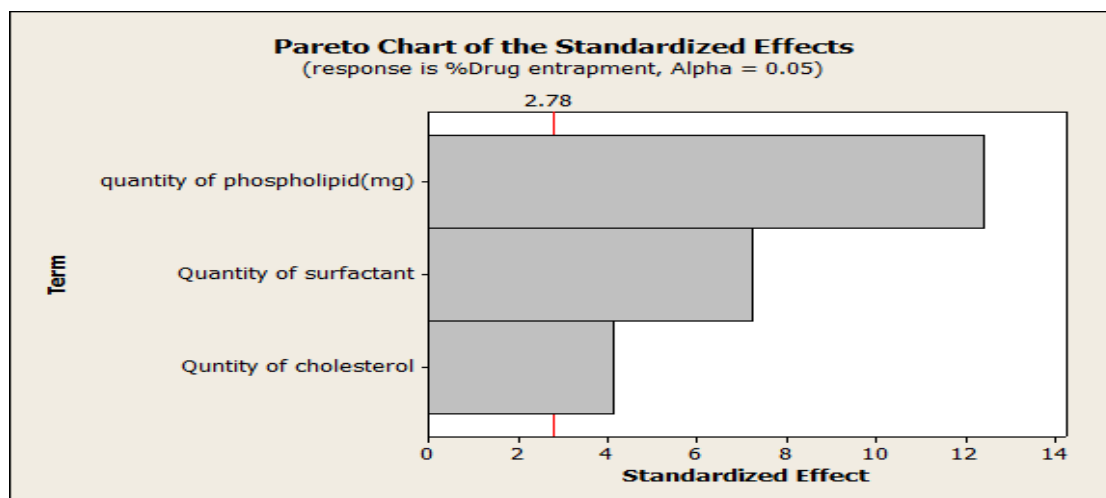


Figure 5A.17 The influence of factors on responses by pareto chart

- Quantity of phospholipids, surfactant and cholesterol significantly affect % drug entrapment as these factors are crossing the lines of significance for influencing the response of % drug entrapment.
- Quantity of phospholipids, surfactant and cholesterol significantly affect size as these factors are crossing the lines of significance for influencing the response of size.

## ❖ Main effect plot to study the effect of levels of factors on responses:-

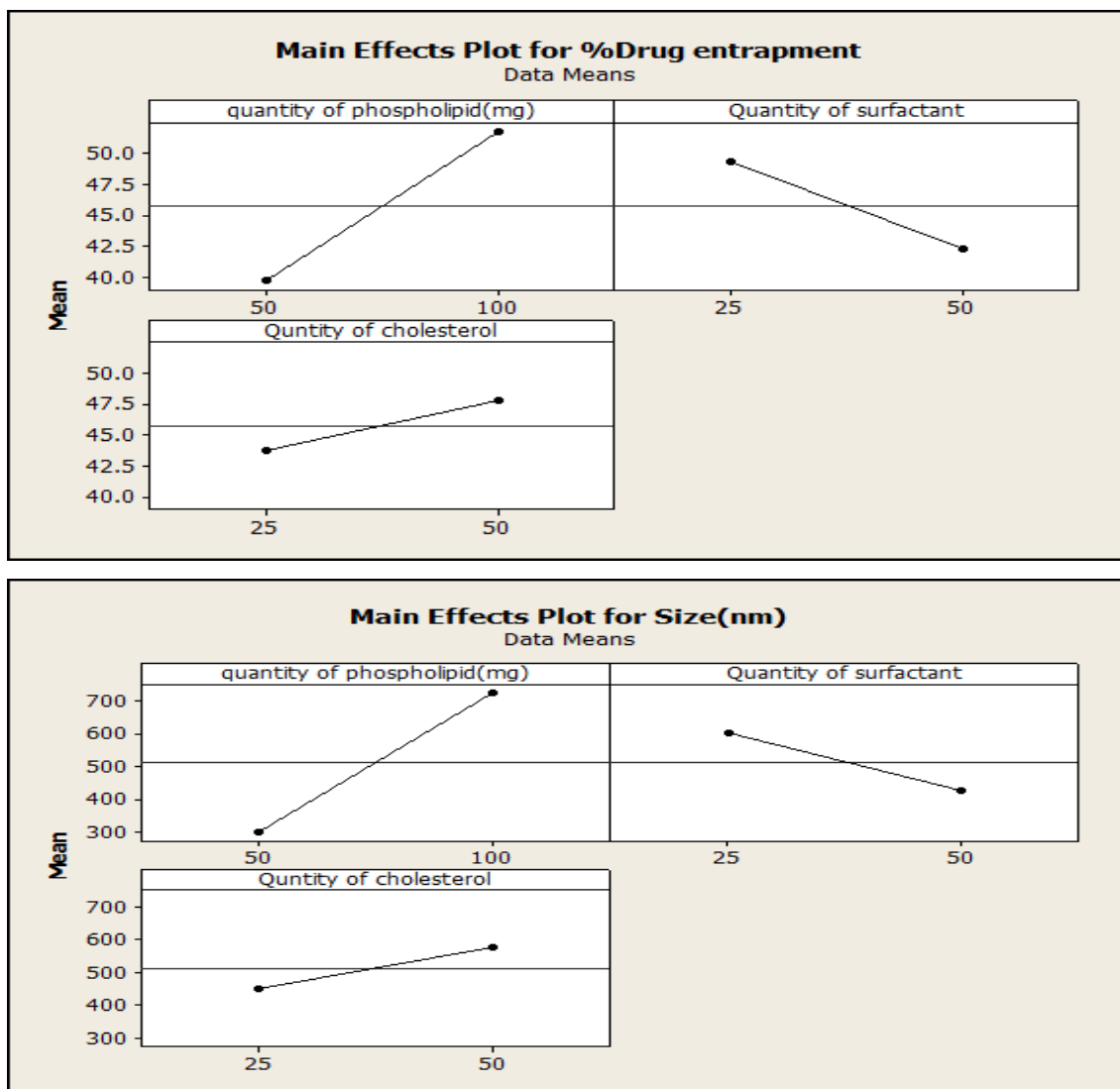


Figure.5A.18 Study of effect of factors on responses by main effect plot

- Higher quantity of lipid results in high entrapment
- Lower quantity of surfactant results in high drug entrapment
- High cholesterol results in high drug entrapment
- Lower quantity of lipid results in lower size.
- Higher quantity of surfactant results in lower size.
- Lower cholesterol results in lower size.

## ❖ Interaction plot for % drug entrapment and size:-

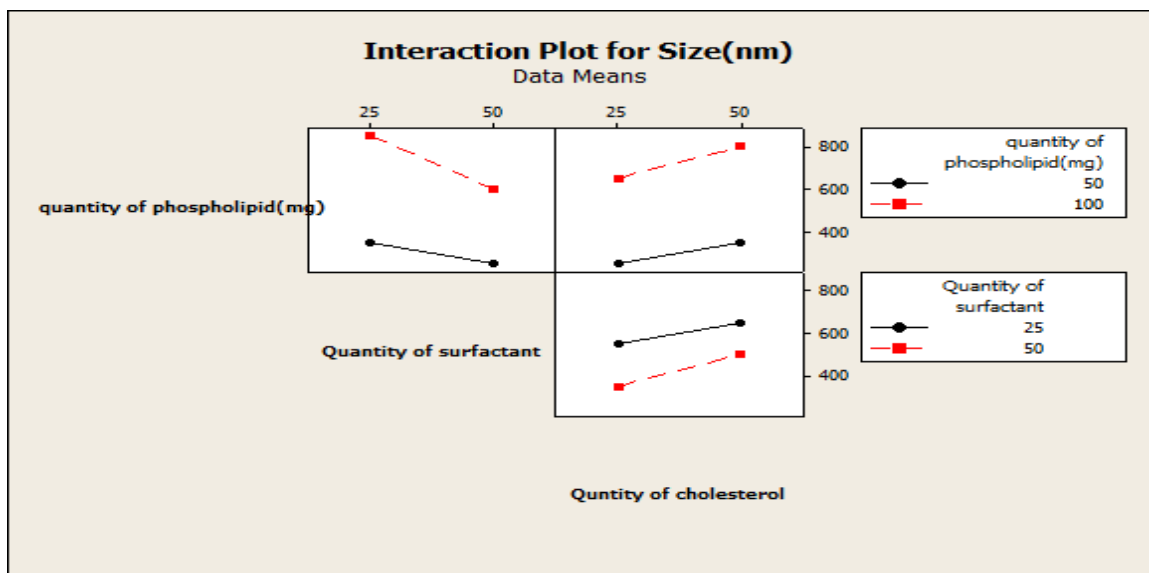
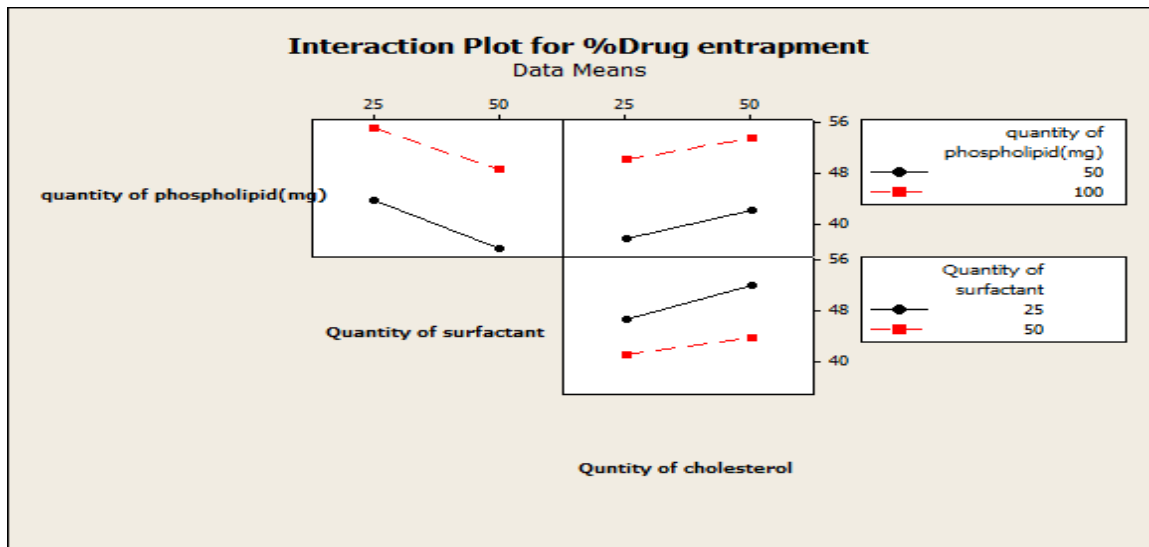


Figure 5A.19 Study of effect of interaction of factors on responses by interaction plots

- Almost parallel lines indicate no interaction among factors.
- Quantity of phospholipids and surfactants are affecting the % drug entrapment irrespective of cholesterol
- Almost parallel lines indicates no interaction among factors
- Quantity of phospholipids and surfactants are affecting the size irrespective of cholesterol

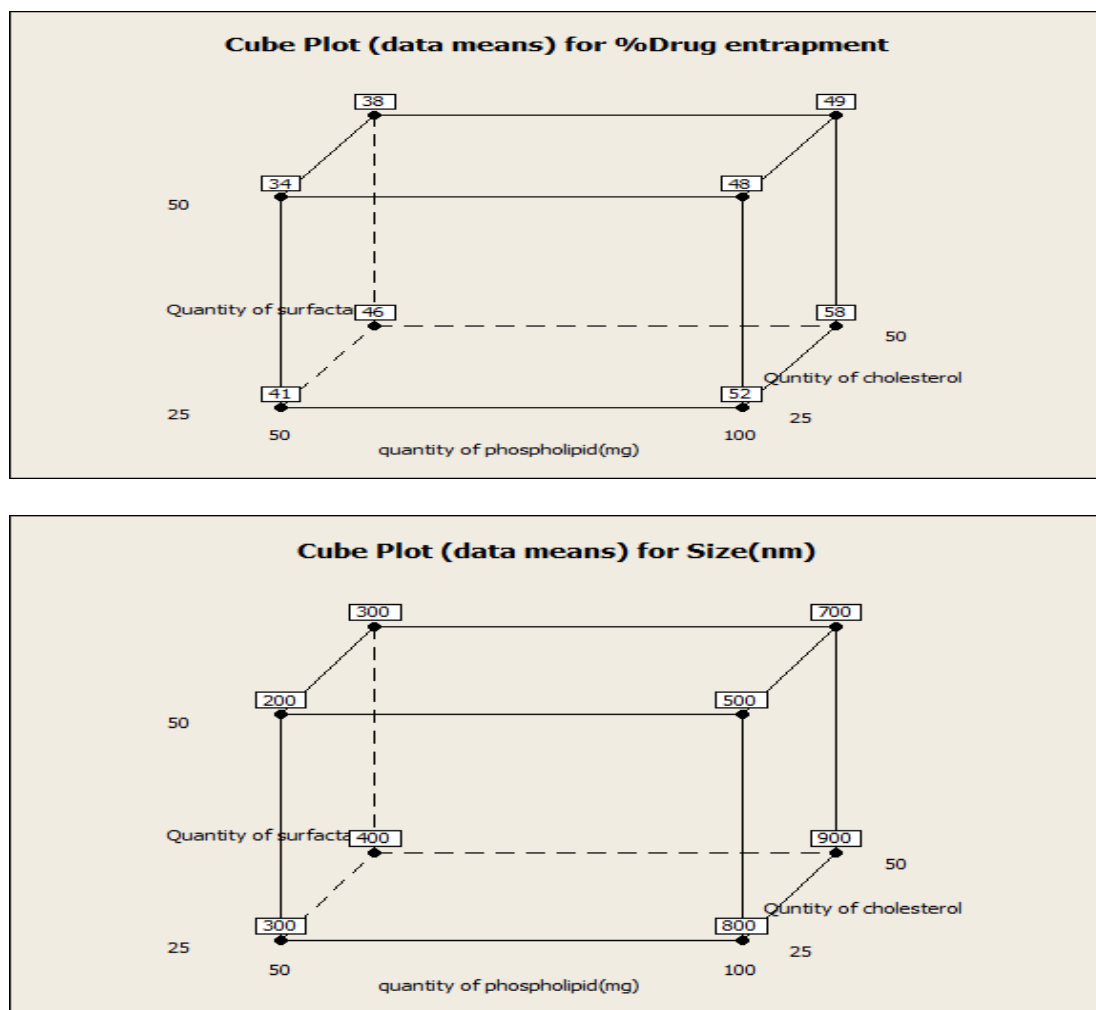
❖ **Cube plot (Data means) for % drug entrapment and size:-**

Figure 5A.20 Influence of factors on responses by cube plot

- ✓ Phospholipids has greatest positive influence on % drug entrapment (41 to 52)
  - ✓ Surfactant has negative influence (41 to 34)
  - ✓ Cholesterol has least positive significant effect (48 to 49)
  - ✓ Quantity of phospholipids has negative influence on size (300 to 800 nm)
  - ✓ Quantity of surfactant has positive influence on size (800 to 500 nm)
  - ✓ Cholesterol has least significant negative Effect (800 to 900 nm)
- From the analysis of factorial design studies, it became clear that, the quantities of phospholipid, surfactants and cholesterol have significant effect on the size and drug entrapment of drug carriers.

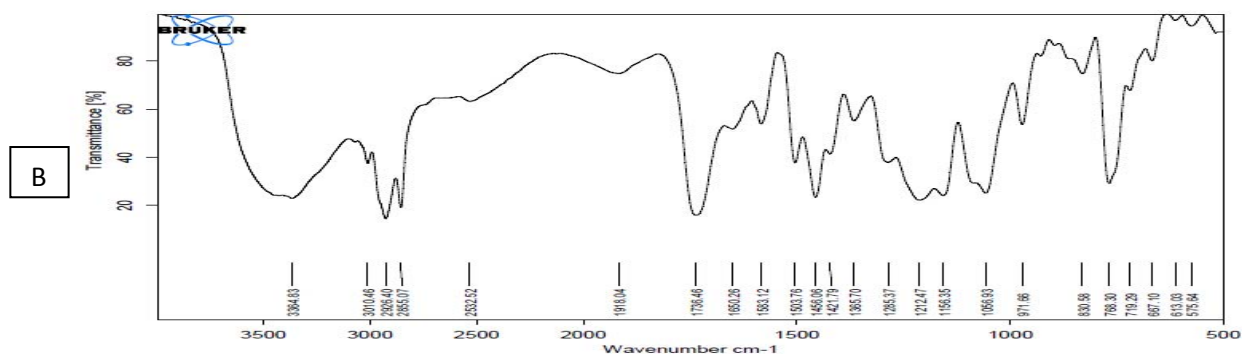
### 5A.5 Drug-excipient compatibility studies:-

The screened Phospholipid, 1, 2-disteroyl -sn- glycerol-3-Phospho-ethanolamine, Na salt and surfactant span 60 were subjected to compatibility studies with drug aceclofenac to find out any interaction with drug.

The interaction of cholesterol and carbopol gel base with mixture of drug aceclofenac, phospholipid and surfactant was also studied.

#### 5A.5.1 FTIR analysis of pure drug and mixture of drug and excipients for studying drug- excipient compatibility:-

FTIR analysis was performed for studying drug-excipient compatibility.





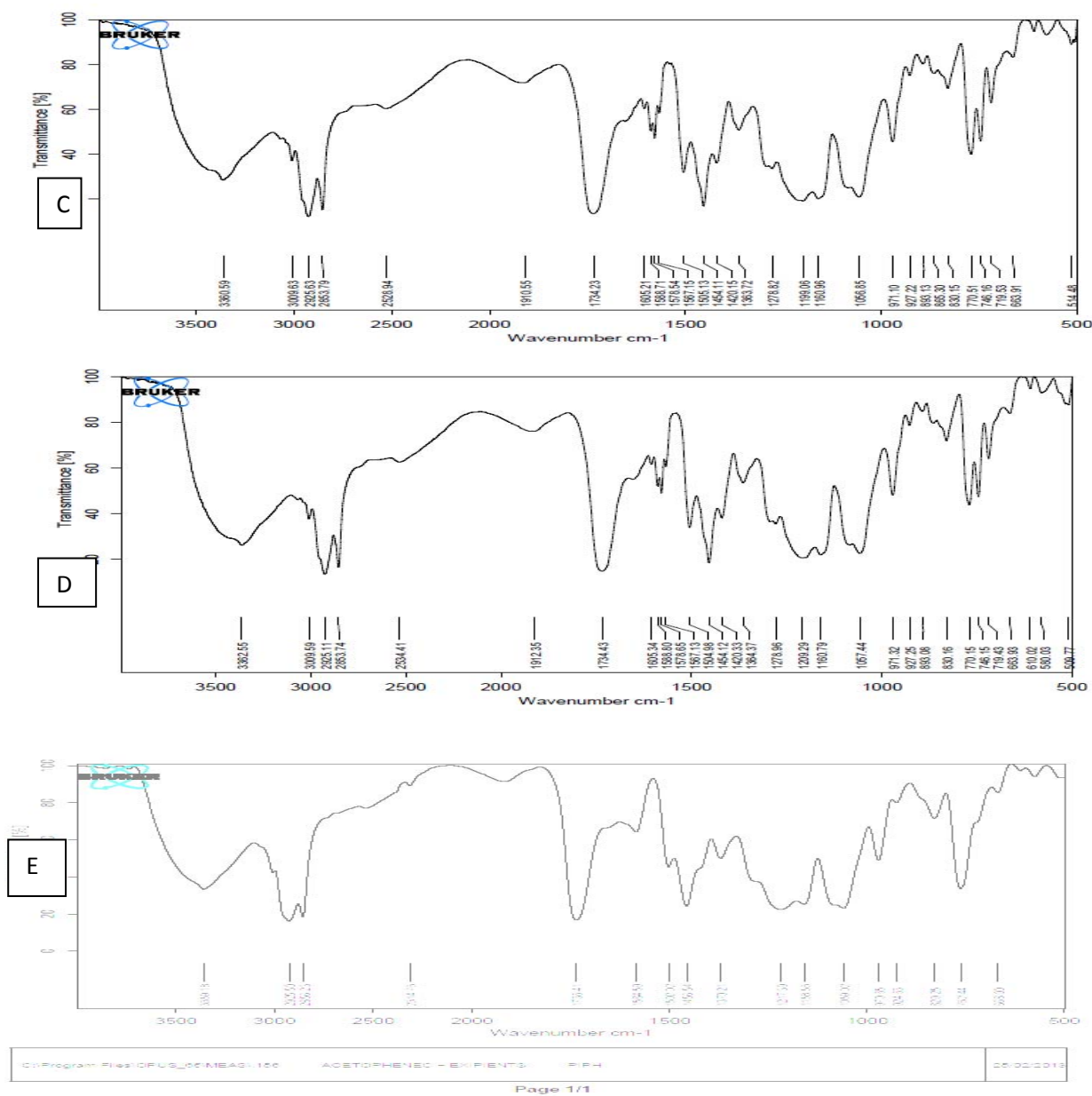


Figure 5A.21 FTIR Spectra of physical mixture of drug aceclofenac and excipients

- A. IR Scan of aceclofenac
- B. IR scan of mixture of aceclofenac and phospholipid in ratio of 1:1.
- C. IR scan of mixture of aceclofenac, phospholipids and cholesterol in ratio of 1:1:1.
- D. IR scan of mixture of aceclofenac, phospholipids, cholesterol and surfactant in ratio of 1:1:1:1.
- E. IR scan of mixture of aceclofenac, phospholipids, cholesterol, surfactant and gel base carbopol 934 in ratio of 1:1:1:1:1.

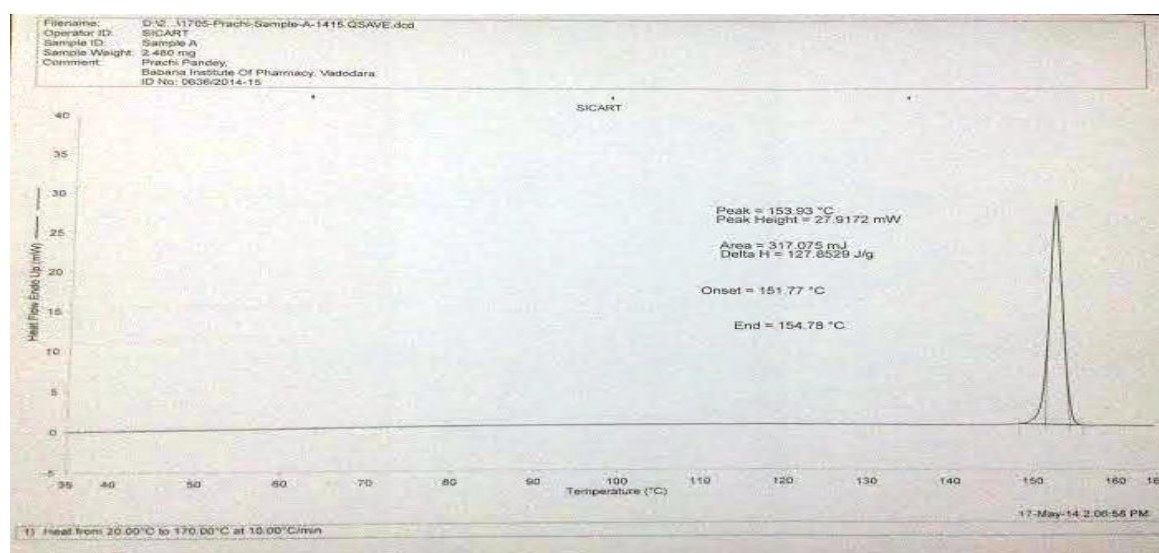
**Table 5A.19 Results of FTIR spectra for drug aceclofenac in physical mixture with excipients**

Compounds	FTIR Interpretation
Aceclofenac	Identity confirmed
Aceclofenac and phospholipids in ratio of 1:1	No interaction observed
Aceclofenac, phospholipids and cholesterol in ratio of 1:1:1.	No interaction observed
IR scan of mixture of aceclofenac, phospholipids, cholesterol and surfactant in ratio of 1:1:1:1.	No interaction observed
IR scan of mixture of aceclofenac, phospholipids, cholesterol, surfactant and gel base carbopol 934 in ratio of 1:1:1:1:1.	No interaction observed

➤ Aceclofenac retains its characteristic peaks in physical mixture with excipients, so it can be concluded that there is no interaction between drug and excipients.

### 5A.5.2 DSC analysis of pure drug and mixture of drug and excipients for studying drug- excipient compatibility:-

DSC analysis was performed for studying drug-excipient interaction in a temperature range of 25°C to 160°C at an increment of 10°C.

**Figure 5A.22 DSC thermogram of aceclofenac**

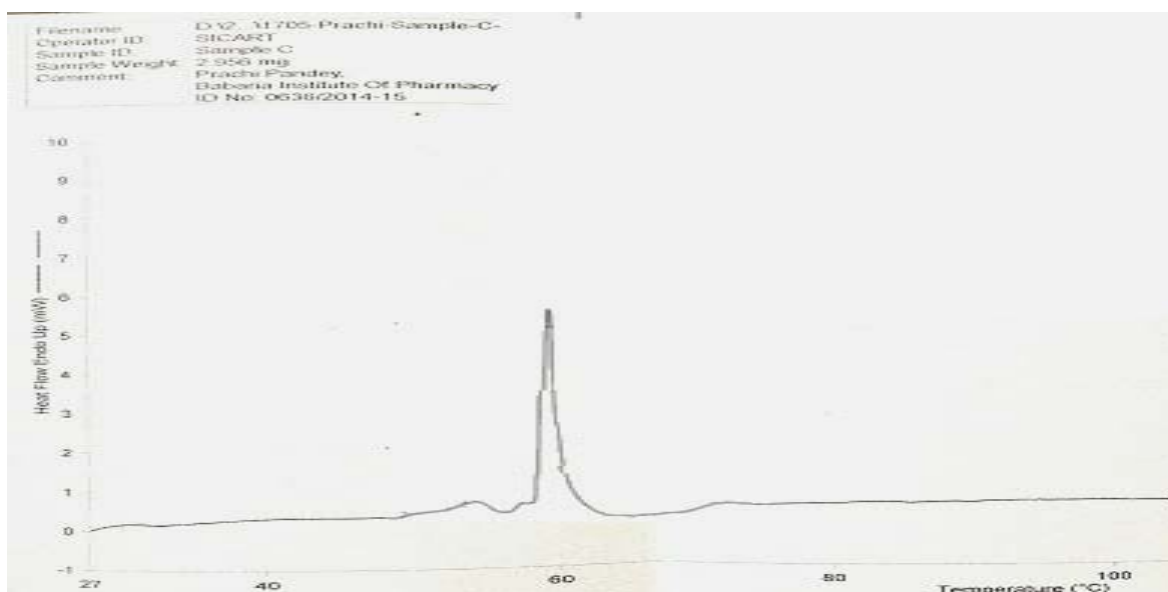


Figure 5A.23 DSC thermogram of phospholipid 1, 2-disteroyl –sn- glycero -3-Phospho-ethanolamine, Na salt

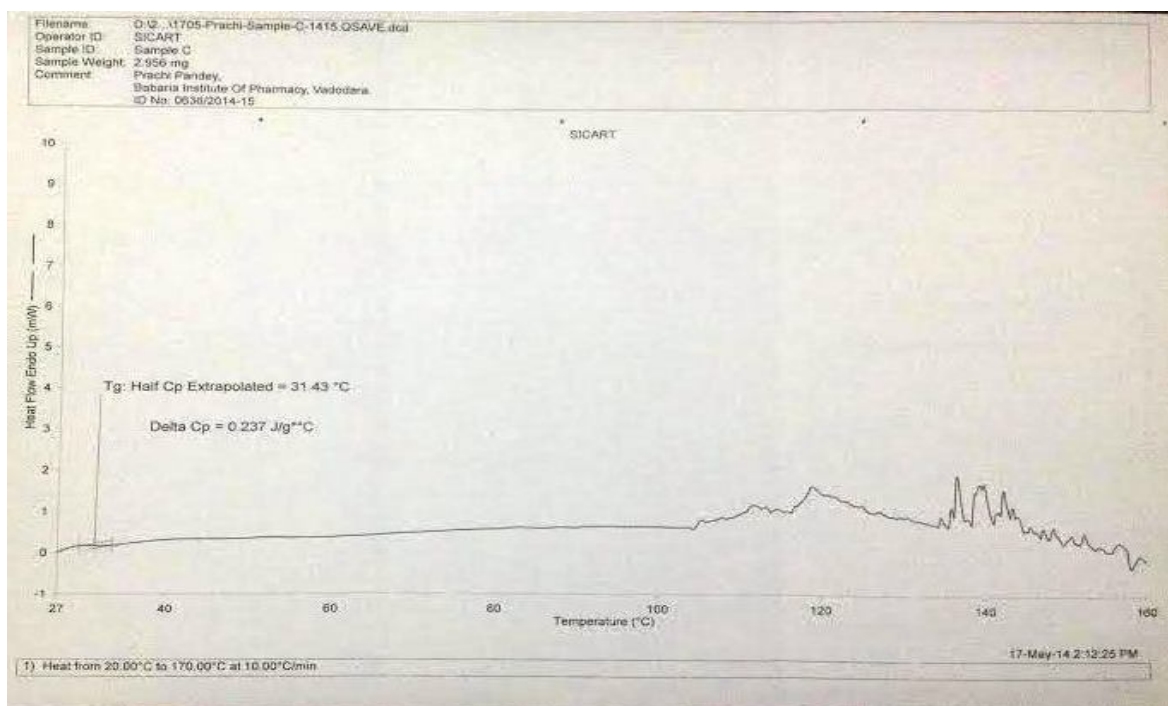
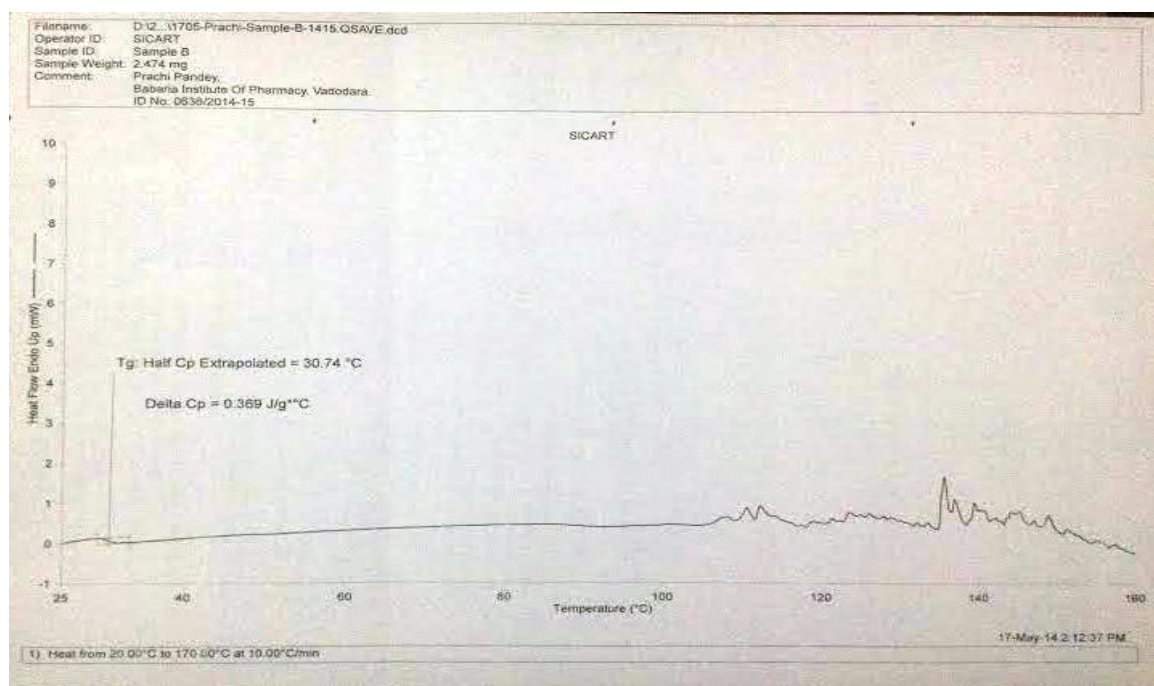


Figure 5A.24 DSC thermogram of mixture of aceclofenac and phospholipids 1, 2-disteroyl –sn- glycero -3-Phospho-ethanolamine, Na salt



**Figure 5A.25 DSC thermogram of mixture of aceclofenac and phospholipids 1,2-disteroyl -sn- glycerol-3-Phospho-ethanolamine,Na salt and surfactant span 40.**

### Interpretation-

Phospholipid showed two small peaks at 54°C and 72°C and a prominent peak at 58.45°C, while aceclofenac showed a sharp endothermic peak at 154.78°C. On the other hand mixture of aceclofenac and phospholipid (1:1) showed a broad peak (at 110°C) and a sharp peak at 135°C. Mixture of aceclofenac, phospholipid and surfactant (1:1:1) showed a broad peak at 120°C and a sharp peak at 137°C. There are clearly visible differences in the peaks between the mixture and individual components of the mixture. The thermogram of the mixture was not showing the peaks representing aceclofenac and phospholipids. Moreover, the phase transition temperature of the mixture (50°C) was found to be lowered than that of phospholipid alone showing interaction of phospholipid with drug. The DSC studies clearly indicate the conversion of crystalline drug to the amorphous form which could be attributed to complete dissolution of the drug in the molten lipid matrix.

## 5A.6 Formulation of drug carriers incorporated gel:-

### 5A.6.1 Preparation of transdermal gel:-

A 1% carbopol gel was prepared as per standard methods by dispersing carbopol 934 in distilled water as a base for incorporation of drug loaded carriers for transdermal delivery.

### 5A.6.2 Formulation batches of drug carriers (liposomes) incorporated gel based on experimental design (Central composite design):-

The experimental batches based on central composite design were further prepared by varying the level of phospholipids (0.067 mM to 0.133 mM) and cholesterol (0.064 to 0.129 mM). Drug: Lipid molar ratio= 2.12 mMol to 4.20 mMol .Hydration volume kept was 20 ml.

The responses measured for batches were % drug entrapment and in-vitro permeation flux and the effect of factors on the responses were studied.

**Table 5A.20 Liposome batches based on central composite design**

Formulation	Quantity of Phospholipid (mg)	Quantity of Cholesterol (mg)
M1	75.000	19.8223
M2	75.000	37.5000
M3	100.000	25.0000
M4	75.000	37.5000
M5	39.645	37.5000
M6	50.000	25.0000
M7	50.000	50.0000
M8	75.000	37.5000
M9	75.000	37.5000
M10	75.000	37.5000
M11	75.000	55.1777
M12	100.000	50.0000
M13	110.355	37.5000

### 5A.6.3 Formulation batches of transferosomes incorporated gel based on experimental design (Box Behnken):-

From the analysis of factorial design studies, it was evident that, the quantities of phospholipid, surfactants and cholesterol have effect on the size and drug entrapment of drug carriers, the experimental batches based on Box Behnken design were further prepared by varying the level of phospholipid, surfactants and cholesterol.

The responses measured for batches were % drug entrapment and in-vitro permeation flux and the effect of factors on the responses were studied.

**Table 5A.21 Formulation batches of transferosomes incorporated gel**

<b>Formulation</b>	<b>Quantity of Phospholipid (mg)</b>	<b>Quantity of surfactant (mg)</b>	<b>Quantity of Cholesterol (mg)</b>
F1	100	37.5	25.0
F2	50	37.5	50.0
F3	75	25.0	50.0
F4	75	37.5	37.5
F5	100	50.0	37.5
F6	50	37.5	25.0
F7	75	25.0	25.0
F8	75	37.5	37.5
F9	75	50.0	50.0
F10	50	25.0	37.5
F11	100	37.5	50.0
F12	75	50.0	25.0
F13	75	37.5	37.5
F14	100	25.0	37.5
F15	50	50.0	37.5

## 5A.7 Evaluation of liposomes and transferosomes incorporated gel:-

### 5A.7.1 Evaluation of liposomal gel on the basis of gel characteristics:-

Table 5A.22 Evaluation results of experimental batches of liposomes incorporated gel

Formulation	pH	Refractive index	Spread ability (gm.cm/sec)	Gel strength (gm)	Extrudability (gm)		
					Press 1	Press 2	Press 3
M1	6.7	1.42	1.4199	20.18	3.114	2.187	1.96
M2	6.2	1.39	1.3614	22.92	3.467	2.114	1.650
M3	6	1.38	1.3812	21.55	3.737	2.008	1.275
M4	6	1.44	1.3101	24.50	2.898	2.009	1.197
M5	6.1	1.36	1.3980	22.70	3.567	2.886	1.854
M6	5.9	1.45	1.3417	20.46	3.786	2.002	1.713
M7	6.3	1.48	1.2698	23.11	3.641	1.996	1.118
M8	6.2	1.39	1.4143	20.18	3.487	2.775	1.729
M9	6	1.43	1.3977	21.87	3.773	2.087	1.684
M10	6	1.39	1.2928	22.34	2.644	2.113	1.883
M11	6.7	1.42	1.3249	22.76	3.179	2.005	1.480
M12	6.6	1.41	1.3287	22.09	3.583	2.505	1.860
M13	6.7	1.38	1.3345	22.48	3.021	2.819	1.489
Plain drug gel	6	1.34	1.3595	17.23	2.880	1.996	1.234

**Interpretation:** From above table, based on evaluation of pH, refractive index, spreadability, gel strength and extrudability of all the formulations, it was observed that drug loaded liposomes incorporated in gel are better than plain drug gel.

## 5A.7.2 Evaluation of transferosomal gel on the basis of gel characteristics:-

Table 5A.23 Evaluation and characterization of transferosomes incorporated gel

Formulation	pH	Refractive index	Spread ability (gm cm/sec)	Gel strength (gm)	Extrudability (gm)		
					Press 1	Press 2	Press 3
F1	6	1.38	1.4285	21.48	3.208	2.133	1.118
F2	6.5	1.33	1.3990	23.02	3.323	2.024	1.236
F3	6.7	1.36	1.3428	22.62	3.542	2.046	1.289
F4	6	1.40	1.3595	25.72	2.988	2.012	1.208
F5	5.9	1.37	1.431	25.12	3.181	2.068	1.970
F6	6.2	1.38	1.4516	22.32	3.889	2.018	1.411
F7	6.5	1.41	1.2962	22.32	3.245	2.049	1.218
F8	6.3	1.33	1.4603	22.20	3.595	2.112	1.524
F9	6.2	1.39	1.4245	20.32	3.986	2.017	1.398
F10	6	1.35	1.3428	21.54	2.828	2.049	1.778
F11	6.7	1.38	1.2962	22.43	3.234	2.133	1.890
F12	6.8	1.38	1.3990	24.16	3.653	2.477	1.769
F13	6.6	1.34	1.4603	23.42	3.325	2.512	1.363
F14	6.5	1.36	1.3595	22.89	3.188	2.768	1.455
F15	6.5	1.37	1.4245	23.54	3.743	2.578	1.689
Plain drug gel	6	1.34	1.3595	17.23	2.880	1.996	1.234

**Interpretation:-** From above table, based on evaluation of pH, refractive index, spreadability, gel strength and extrudability of all the formulations, it was observed that drug loaded transferosome incorporated in gel are almost similar with the plain drug gel with respect to the gel characteristics and ease of applications.



**5A.7.3 In-vitro and ex-vivo membrane permeation studies and determination of permeation flux for liposomal gel and transferosomal gel:-**

In-vitro and ex-vivo permeation studies were performed as per the ethical guidelines approved by institutional animal ethics committee. (IAEC approval no. PHD/13-14/23 date.14<sup>th</sup> December, 2013).The in-vitro permeation studies were carried out for all the experimental design batches whereas ex-vivo studies were performed for optimized batch. The permeation flux for experimental batches of liposomal gel, transferosomal gel and plain drug gel were determined.

❖ **In-vitro permeation studies of liposomes:** - Studies were carried out for all the experimental batches of liposomes and the cumulative drug release as well as the permeation flux was determined.

**Table 5A.24 Cumulative drug release (µg) in 6 hrs by experimental batches**

Time (hr)	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	M13	Plain drug gel
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1	323.1	364.5	<b>588.2</b>	376.2	396.9	396.2	380.4	316.1	382.1	337.2	398.7	347.9	365.2	314.9
2	475.5	469.0	<b>697.1</b>	524.7	582.7	531.3	585.3	566.8	467.4	454.8	530.7	468.3	587.1	468.7
3	644.8	653.3	<b>762.8</b>	675.4	638.4	690.1	667.4	639.0	616.3	613.5	659.1	626.5	639.6	615.1
4	876.1	762.9	<b>831.9</b>	863.2	881.2	856.2	846.3	826.8	787.2	732.6	996.3	758.1	813.4	746.8
5	998.4	875.4	<b>1072</b>	997.8	991.8	873.8	869.7	1171	819.3	864.2	1048	886.3	997.2	831.7
6	1186.5	1108	<b>1236.2</b>	1180	1101	1149	1185	1240	1190	1146	1148	1176	1135	1078
24	1741	1377	<b>1680</b>	1504	1568	1397	1635	1624	1521	1535	1601	1523	1587	1233

Permeation flux is the slope of percentage drug release v/s time. It is expressed as  $\mu\text{g}\cdot\text{cm}^{-2}/\text{hr}^{-1}$

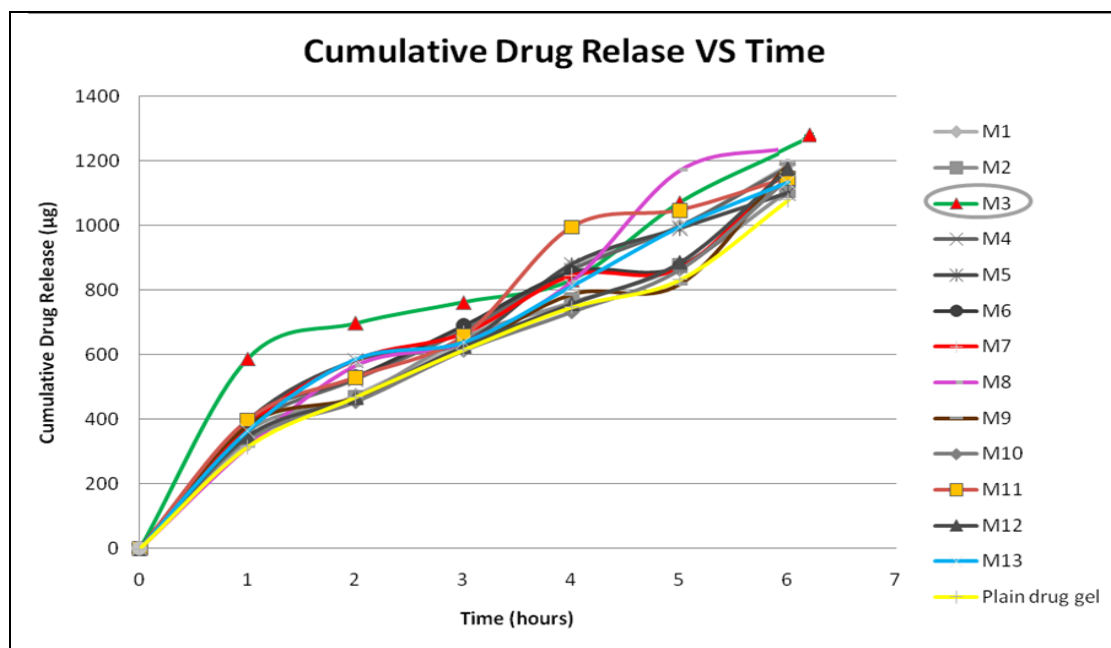


Figure 5A.26 Graph of in-vitro permeation studies of liposomal gel.

Table 5A.25 % Drug entrapment and in-vitro permeation flux ( $\mu\text{gcm}^{-2} \text{hr}^{-1}$ ) of liposomal gel batches

Formulation	Liposomes		Liposomal gel
	Size	% Drug entrapment	Permeation Flux ( $\mu\text{g.cm}^{-2} \text{hr}^{-1}$ )
M1	232	47±1.8	24.18
M2	119	32±1.6	21.06
<b>M3</b>	<b>263</b>	<b>49±2.6</b>	<b>26.08</b>
M4	228	44±1.9	23.11
M5	278	43±2.1	24.05
M6	216	37±1.1	22.34
M7	298	45±2.6	23.75
M8	212	47±1.7	24.33
M9	302	42±2.2	23.24
M10	285	38±1.8	22.31
M11	317	47±2.7	24.86
M12	204	42±1.6	22.91
M13	221	45±2.9	23.14

**Interpretation:-**

- Drug permeation studies were carried on by in-vitro studies. At the end of 6 hrs maximum drug release was found to be maximum for liposomal gel batch M3. At the end of 6 hrs, maximum transdermal flux of  $26.08 \mu\text{gcm}^{-2} \text{hr}^{-1}$  was achieved for batch M3 that is greater than the transdermal flux observed for aceclofenac plain gel. The drug release pattern was also found to be sustained release.
- The drug entrapment for batch M3 was also high i.e. 49%.
- The average size of liposomal gel batch M3 was found to be **263 nm** respectively with a zeta potential value of **-39.14 mv** indicating good stability

**❖ In-vitro permeation studies of transferosomes:-**

In-vitro Studies were carried out for all the experimental batches of transferosomes and the cumulative drug release as well as the permeation flux was determined.

**Table 5A.26 Cumulative drug release ( $\mu\text{g}$ ) in 6 hrs by experimental batches of transferosomal gel**

Formulation	Time(hrs)						
	1	2	3	4	5	6	24
F1	459.34	685.41	759.82	988.49	1018.12	1226.64	1876
F2	417.3	598.4	712.6	879.2	996.2	1278	1423
<b>F3</b>	<b>649.1</b>	<b>789.5</b>	<b>814.3</b>	<b>992.4</b>	<b>1034</b>	<b>1536.2</b>	<b>1897</b>
F4	435.6	610.1	746.8	904.1	1007	1253	1698
F5	448.1	673.4	752.1	976.2	1009	1201	1868
F6	486.4	624.1	743.4	975.3	984.1	1253	1416
F7	439.1	668.8	742.3	964.7	996.8	1198	1835
F8	446.8	675.4	746.0	976.2	1002	1217	1844
F9	431.8	599.1	729.5	897.3	994.0	1218	1684
F10	421.0	579.6	708.1	818.0	983.1	1197	1659
F11	467.8	699.8	768.2	1003	1069	1257	1889
F12	436.7	589.6	719.4	847.5	994.7	1201	1685
F13	438.8	613.4	742.1	909.8	1002	1249	1687
F14	471.1	701.1	770.8	1008	1074	1260	1896
F15	482.5	618.0	736.	972.1	978.1	1252	1403
<b>Plain drug gel</b>	314.9	468.7	615.1	746.8	831.7	1078	1233

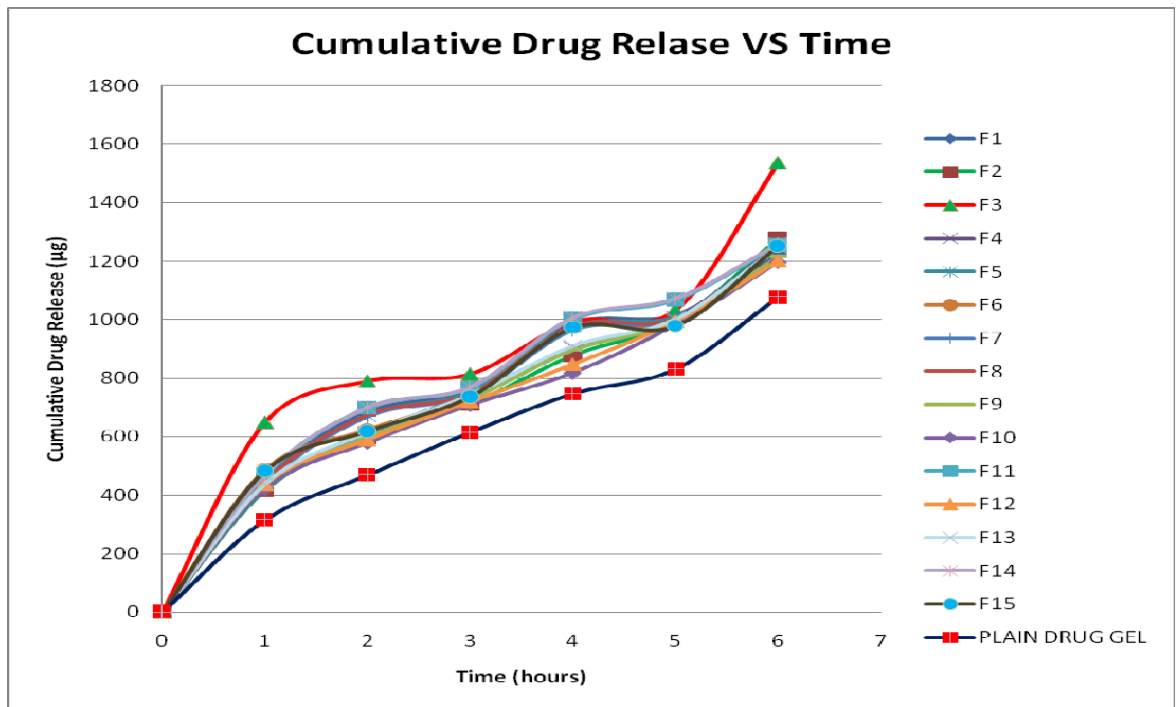


Figure 5A.27 Graph of in-vitro permeation studies of transferosomal gel.



Figure 5A.28 Permeation studies of liposomal and transferosomal gel of aceclofenac

**Table 5A.27 % Drug entrapment and in-vitro permeation flux ( $\mu\text{g}\cdot\text{cm}^{-2}\text{hr}^{-1}$ ) of transferosomal gel batches.**

Formulation	Transferosomes		Transferosomal gel
	Size	% Drug entrapment	Permeation Flux ( $\mu\text{g}\cdot\text{cm}^{-2}\text{hr}^{-1}$ )
F1	234	51±3.4	26.36
F2	218	36±1.2	23.17
<b>F3</b>	<b>368</b>	<b>57±3.7</b>	<b>28.11</b>
F4	387	48±2.4	25.49
F5	358	49±2.6	26.1
F6	298	40±1.5	24.22
F7	386	49±2.2	25.98
F8	401	50±3.1	26.18
F9	379	48±1.4	25.37
F10	354	43±1.2	24.77
F11	384	51±3.3	26.44
F12	378	46±1.9	24.87
F13	391	48±3.2	25.44
<b>F14</b>	<b>364</b>	<b>53±2.8</b>	<b>26.60</b>
F15	264	38±1.4	24.12

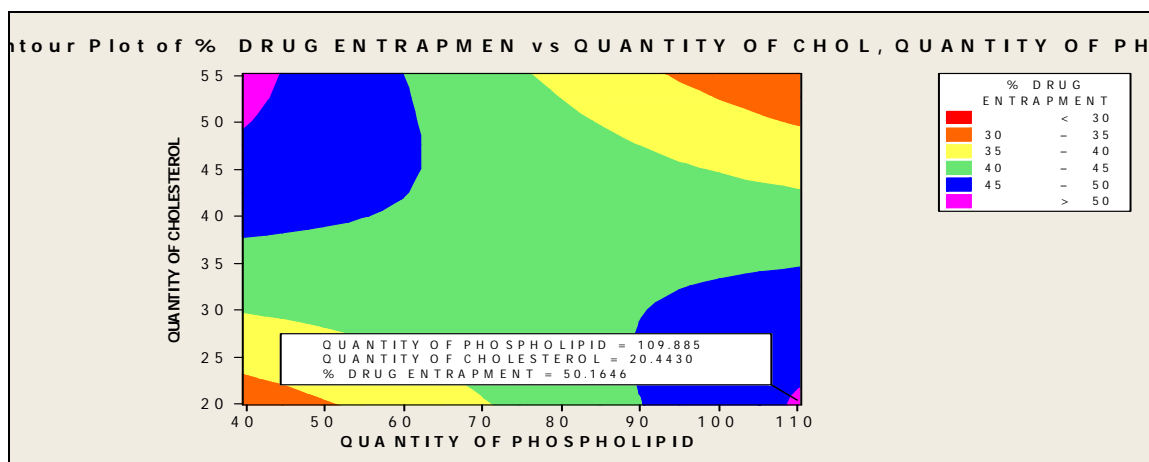
**Interpretation:** - The drug entrapment for transferosomal gel batch F3 and F14 were found to be high i.e. 57 % and 53% with permeation flux of  $28.11\ \mu\text{g}\cdot\text{cm}^{-2}\text{hr}^{-1}$  and  $26.60\ \mu\text{g}\cdot\text{cm}^{-2}\text{hr}^{-1}$  respectively.

The average size of transferosome formulations F3 and F14 were found to be 368 nm and 364 nm respectively, whereas the zeta potential values were also found to be -52.68 and -47.34 indicating good stability.

## 5A.8 Statistical analysis and optimization of formulation:-

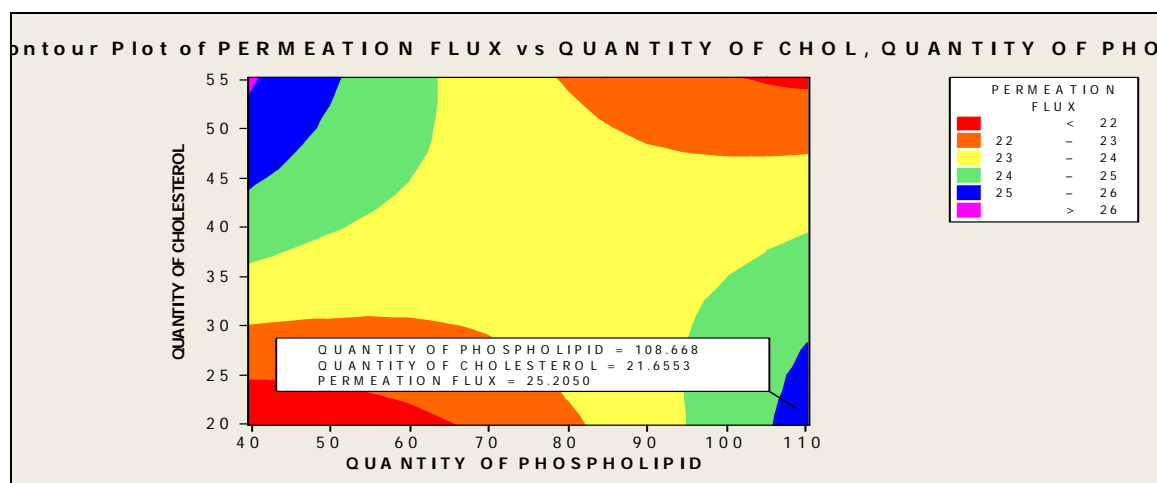
### 5A.8.1 Analysis of liposomal gel design batches by response surface methodology:

The liposomal batches were further analyzed by contour plot to screen the suitable quantities of excipients for maximum responses.



**Figure 5A.29 Contour Plot for %drug entrapment**

Interpretation:- From the contour plot of maximum % drug entrapment, the desired quantity of components in formulation will be phospholipid 109.88 mg and cholesterol 20.44 mg.



**Figure 5A.30 Contour plot for permeation flux**

Interpretation:- From the contour plot of in-vitro permeation flux, the desired quantity of components in formulation will be phospholipid 108.66 mg and cholesterol 21.65 mg

**5A.8.2 Optimization plot based on response surface methodology for liposomes:-**

The optimization plot provided the levels of factors in formulation which has the possibility to achieve the set target responses of size and entrapment efficiency. The desirability of 1.00 predicted the possibility of achieving responses of % drug entrapment and permeation flux same to set target values.

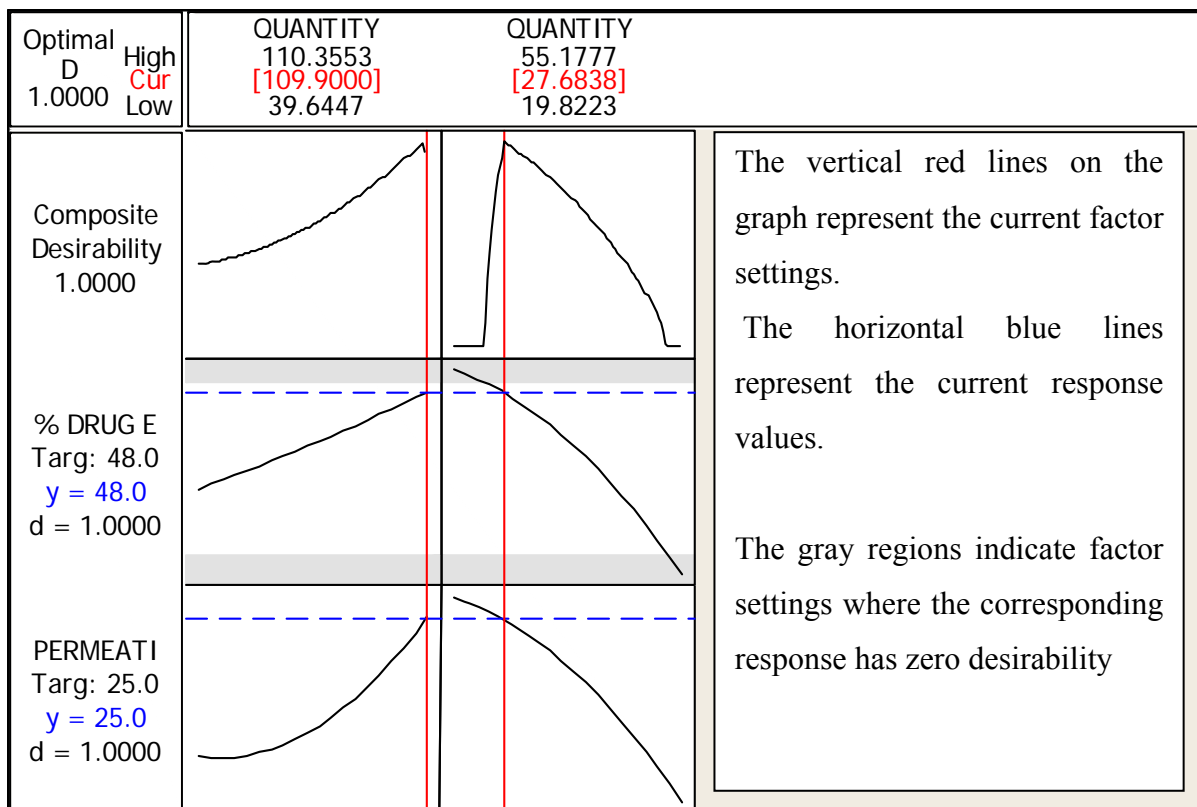


Figure 5A.31 Optimization plot of liposomes of aceclofenac

➤ From the optimization plot it was found that predicted formula for maximum drug entrapment and permeation flux is composed of phospholipid= 109.9 mg (0.14 mMol), and cholesterol= 27.68 mg (0.071 mMol) in formulation. The formulation was prepared using the obtained formula.

**5A.8.3 Analysis of transferosomal gel design batches by response surface methodology:-**

The transferosomal gel batches were further analyzed by contour plot to observe the suitable quantities of excipients for maximum responses.

## ❖ Contour plots based on response surface methodology:-

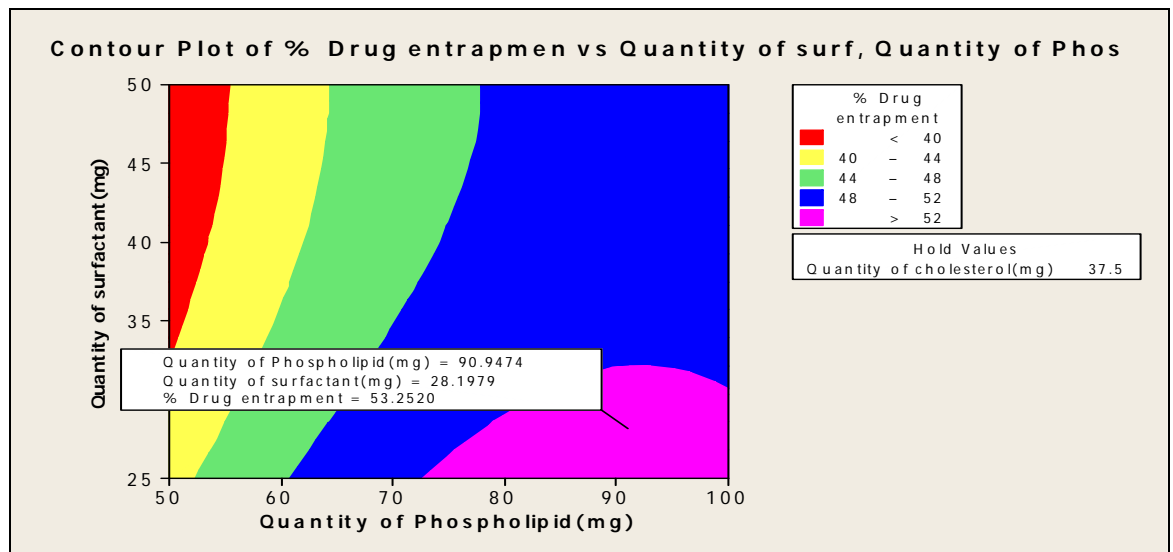


Figure 5A.32 Contour Plot of % Drug entrapment of transferosomes

Interpretation- From the contour plot of maximum % drug entrapment, the desired quantity of components in formulation will be phospholipid 90.94 mg and surfactant 28.19 mg.

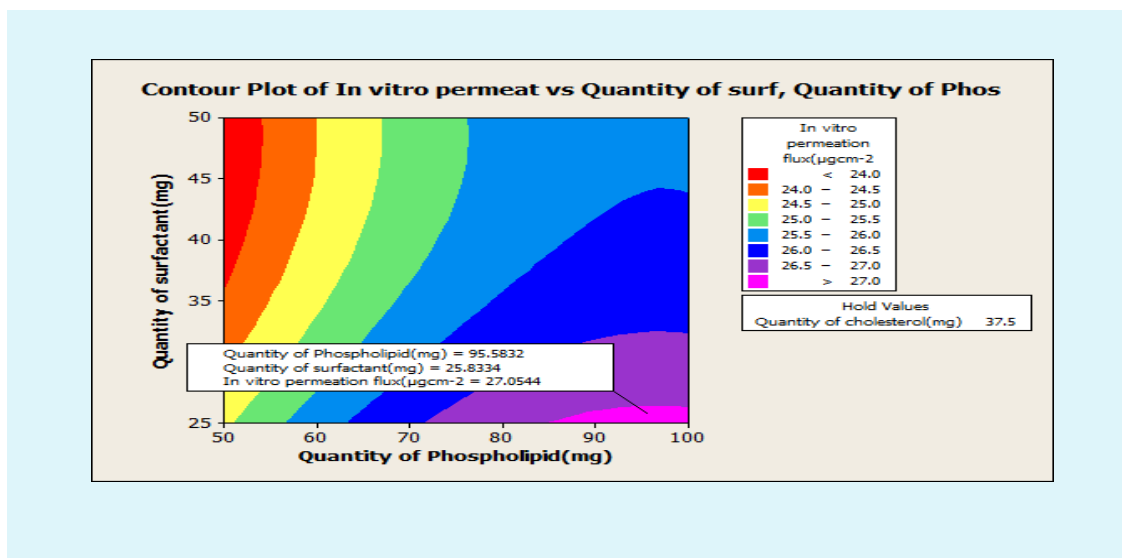


Figure 5A.33 Contour Plot of permeation flux of transferosomal gel

Interpretation- From the contour plot of in-vitro permeation flux, the desired quantity of components in formulation will be phospholipid 95.58 mg and surfactant 25.83 mg.

- ❖ The quantity of phospholipids and surfactants were found to be between 90 to 95 mg and 25 to 28 mg respectively for achieving maximum drug entrapment and in-vitro permeation flux.



#### 5A.8.4 Optimization plot based on response surface methodology for transferosomes:-

The optimization plot provided the levels of factors in transferosome formulation which has the possibility to achieve the set target responses of size and entrapment efficiency. The desirability of 0.94 predicts the possibility of achieving responses of % drug entrapment and permeation flux close to target values.

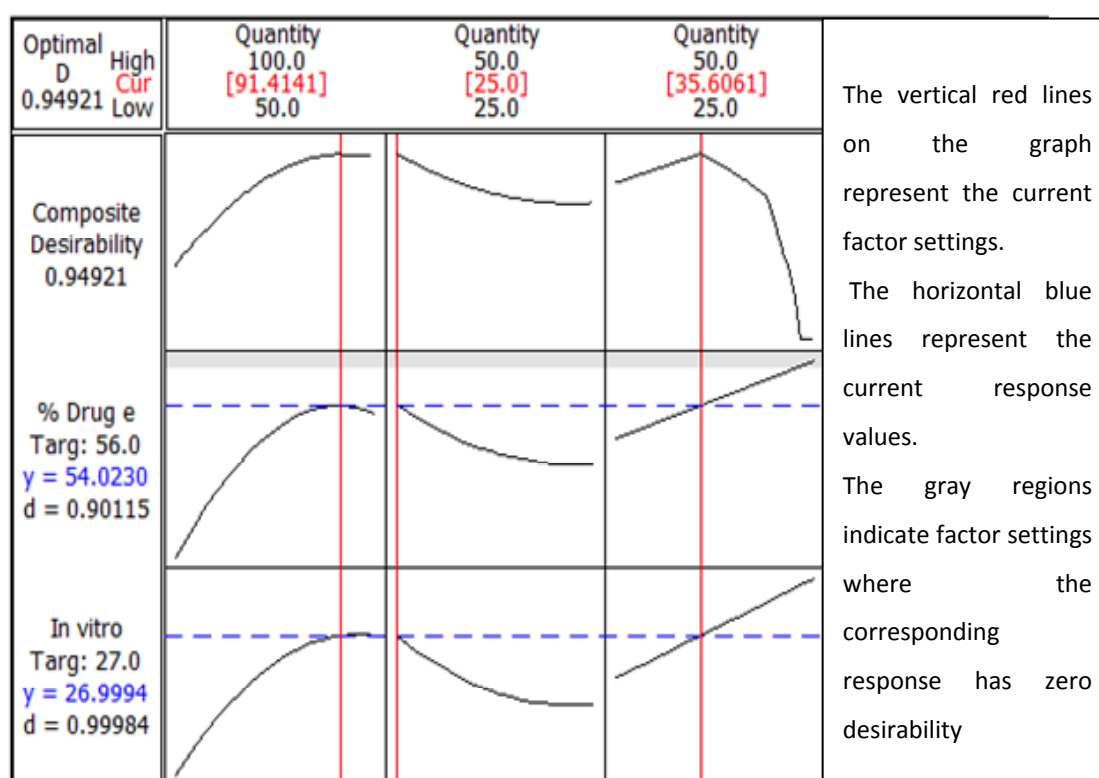


Figure 5A.34 Optimization plot of transferosome of aceclofenac

- From the optimization plot, it was found that predicted formula to achieve the set target values of drug entrapment and permeation flux is composed of phospholipid= 91.41 mg (0.12 mMol), surfactant= 25mg (0.06 mMol) and cholesterol= 35.60 mg (0.09 mMol) in formulation.

### 5A.8.5 Development of optimized formulation of transferosomal and liposomal gel:-

The novel transdermal gel has been developed containing drug loaded liposomes and transferosomes based on the optimized formula and process. The optimized batch was further evaluated to check the ability to achieve the target responses. The developed formulations were also compared with the conventional transdermal gel formulations.

### 5A.8.6 Evaluation data of optimized batch of transferosome and liposomes in gel based on response surface methodology:-

The optimized batches of liposomal gel and transferosomal gel batch was evaluated for responses of gel characteristics and ex-vivo permeation studies using excised skin of rat.

**Table 5A.28 Evaluation of optimized batch of transferosomal and liposomal gel formulation**

Optimized formula	Size (nm)	Zeta potential	%drug entrapment	Ex-vivo Permeation flux ( $\mu\text{gcm}^{-2} \text{hr}^{-1}$ )	Viscosity (cp)	pH	Spreadability (gm.cm/sec)	Gel strength (gm)
Transferosomal gel	351.9	-42.7	57.44%	28.69	4768	6.4	1.446	22.80
Liposomal gel	92.94	-26.0	51.02%	26.88	4872	6.0	1.3989	23.57
Plain drug gel	-	-	-	17.46	5938	6.0	1.3595	17.23

❖ The optimized formulation was evaluated and the evaluation results of optimized formula were matched with the desired target responses fixed for optimization. The optimized batch showed closeness to the target and thus found to exhibit good % drug entrapment and ex-vivo permeation flux as well as satisfactory handling characteristics and ease of application.

The transferosomal gel showed better % drug entrapment and ex-vivo permeation flux as compared to liposomal gel formula.

**5A.8.7 Size and morphology determination of optimized batch of drug carriers:-**

**Transfersosomal dispersion**

- The size and stability of transfersosomal dispersion was determined using Zeta Sizer (Malvern Instruments Ltd. Malvern, UK MAL10020)

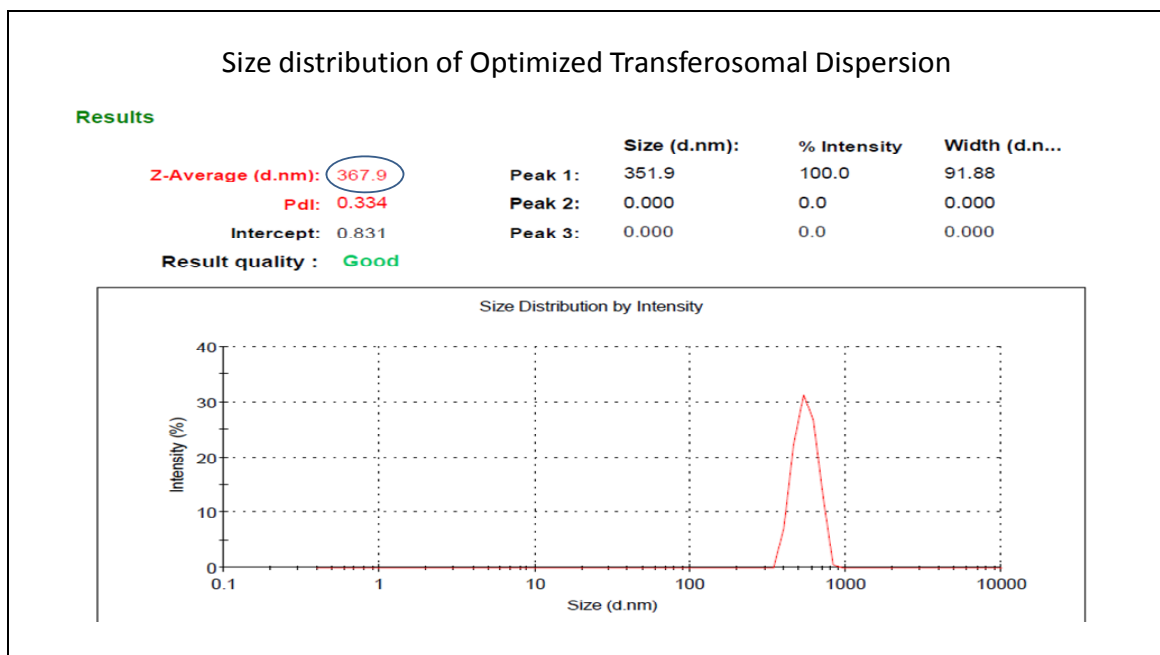
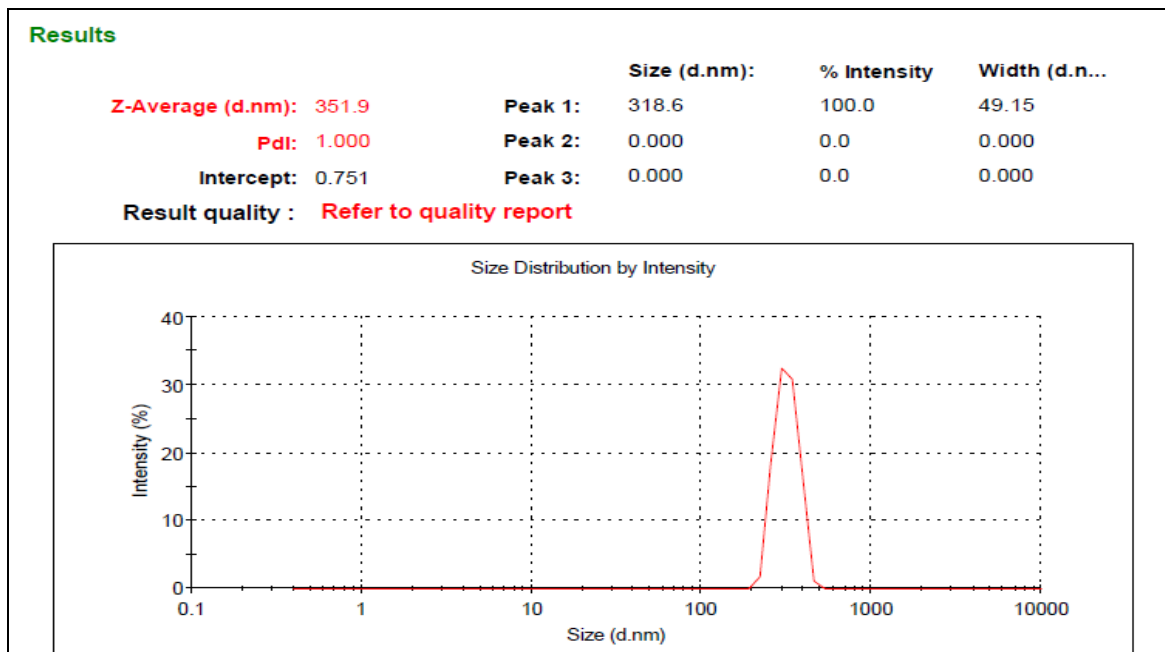


Figure 5A.35 Size of optimized batch of transfersomes

Table 5A.29 Properties of optimized batch of transferosomes

Formulation	Size	Zeta Potential	PDI
Transferosomal dispersion	351.9 nm	- 42.7	0.231

➤ **Surface morphology of transferosomes:** - Surface morphology of optimized formulation of transferosomes and liposomes was determined using transmission electron microscopy (TEM) (Model: Tecnai 20, Make: Philips, Holland) at SICART, Gujarat.

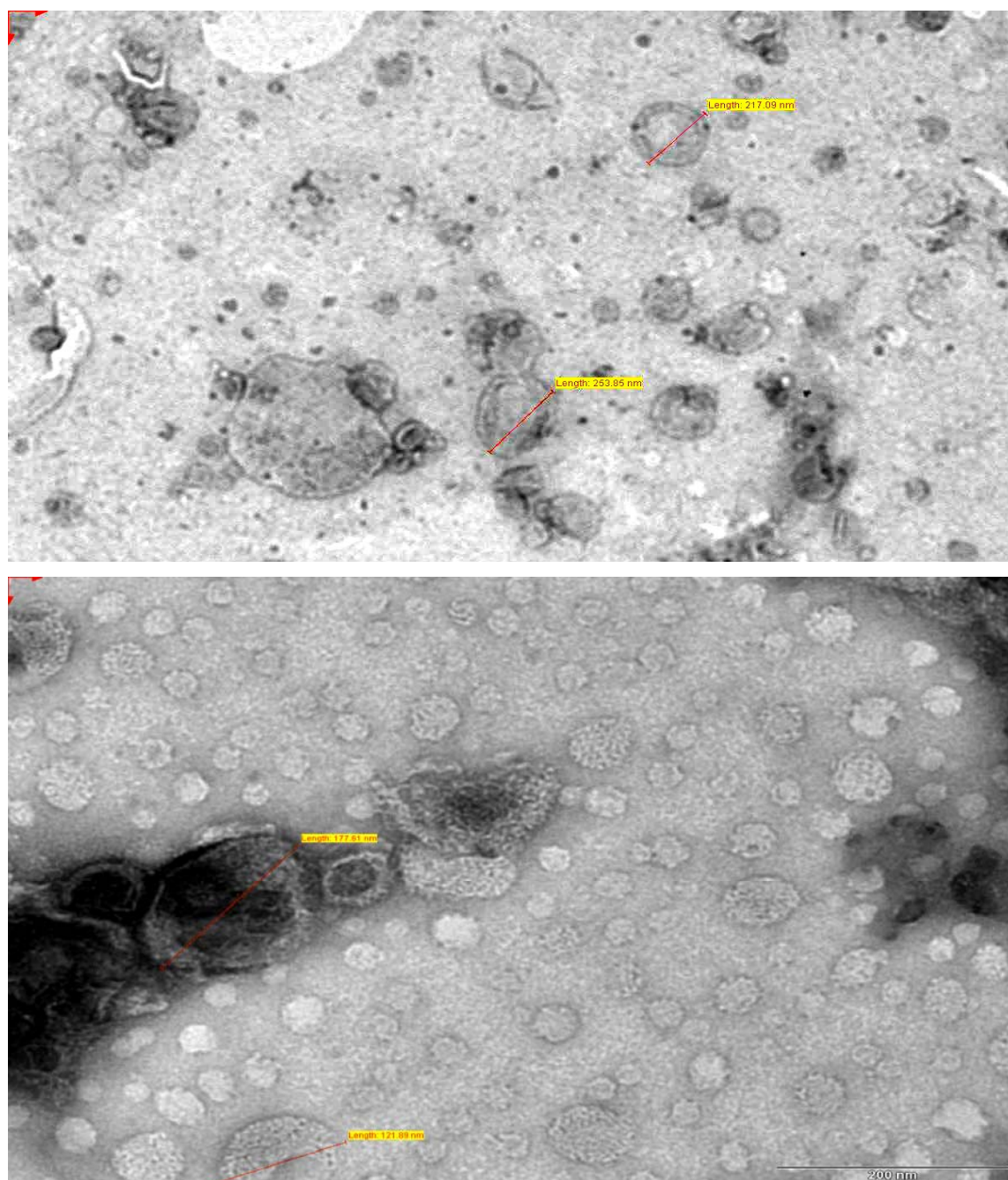
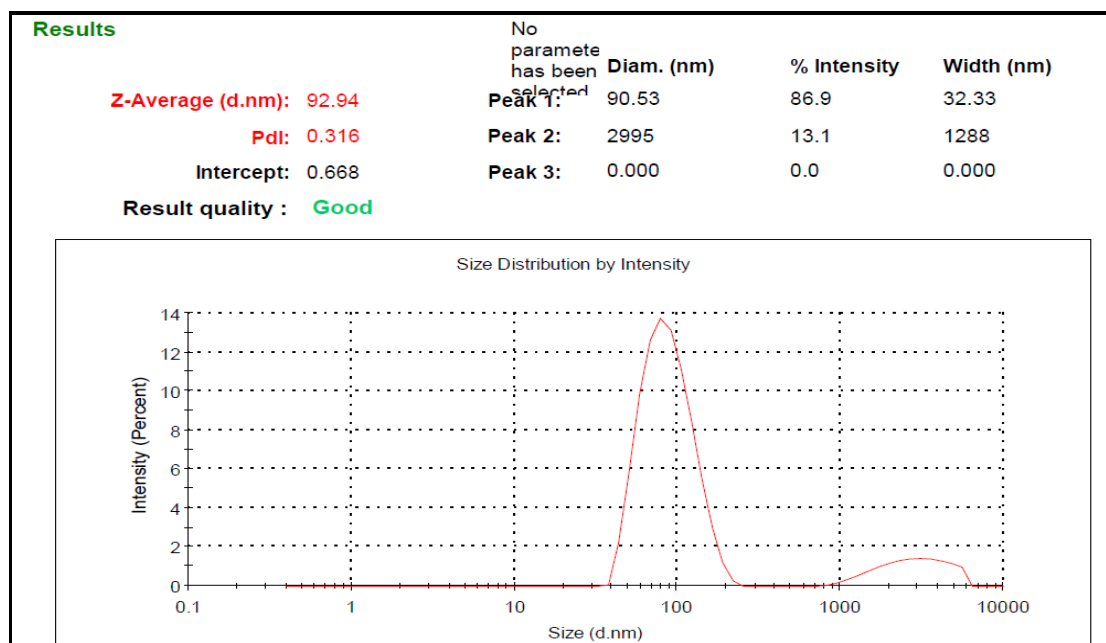
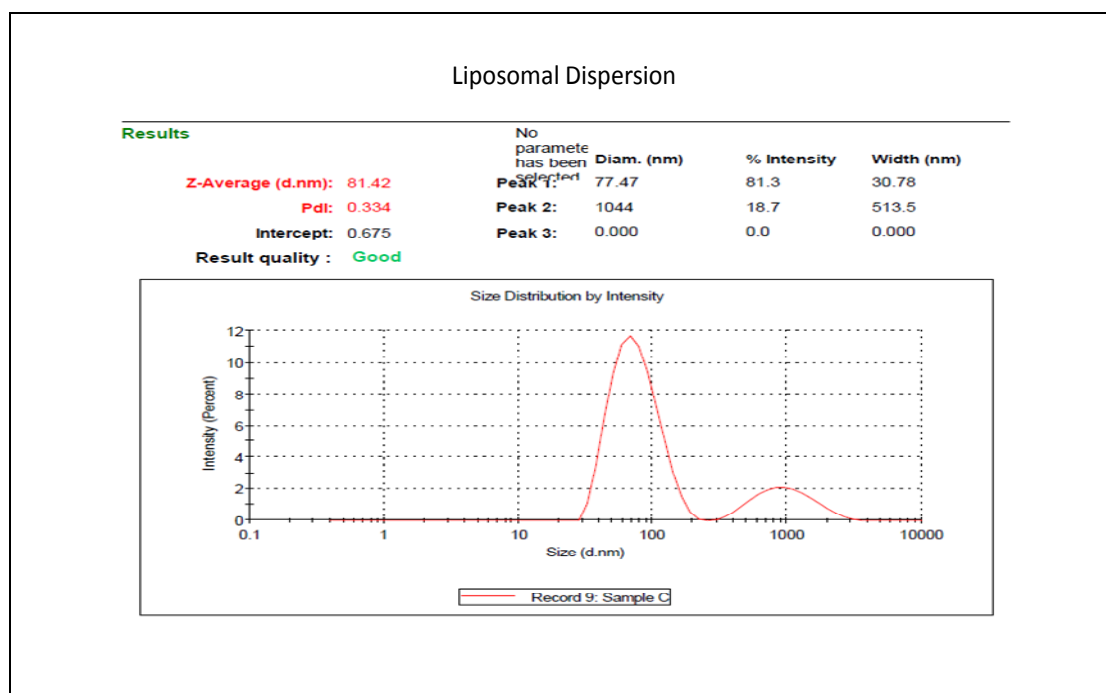


Figure 5A.36 TEM images of optimized batch of transferosomes

**5A.8.8 Size and morphology determination of optimized batch of drug carriers:-**

**Liposomal dispersion**

- The size and stability of liposomal dispersion was determined using Zeta Sizer (Malvern Instruments Ltd. Malvern, UK MAL100206)

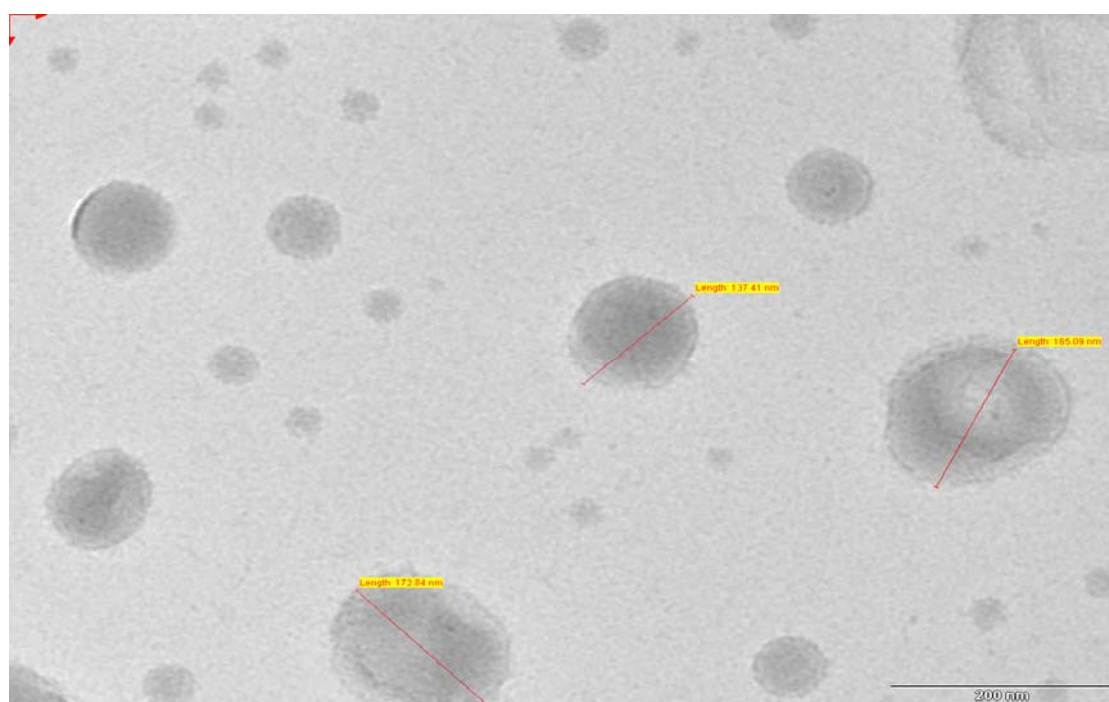
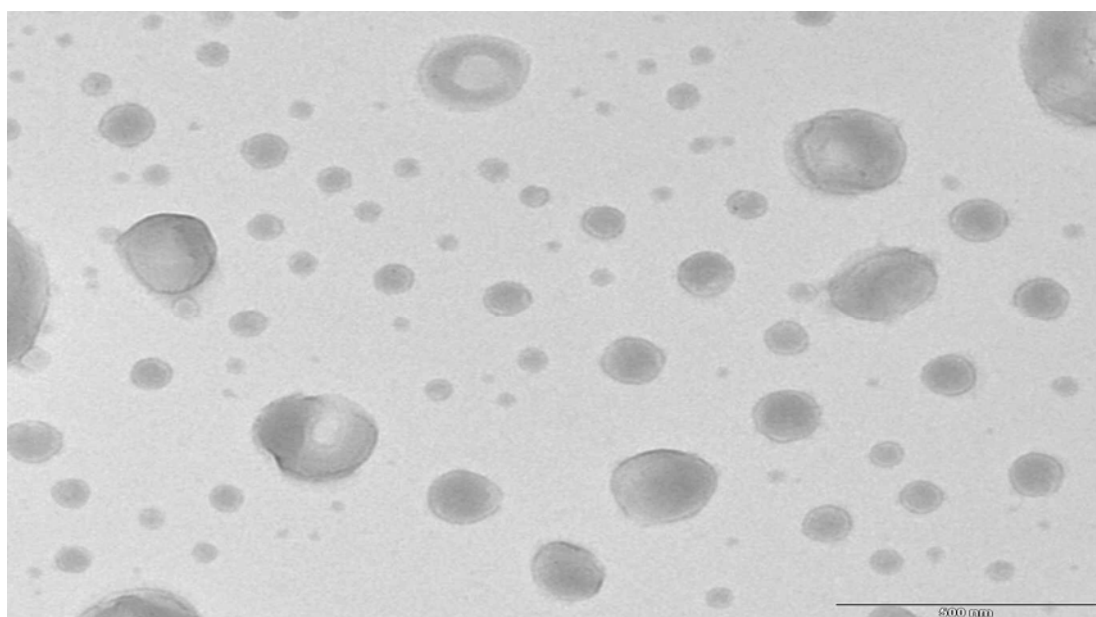


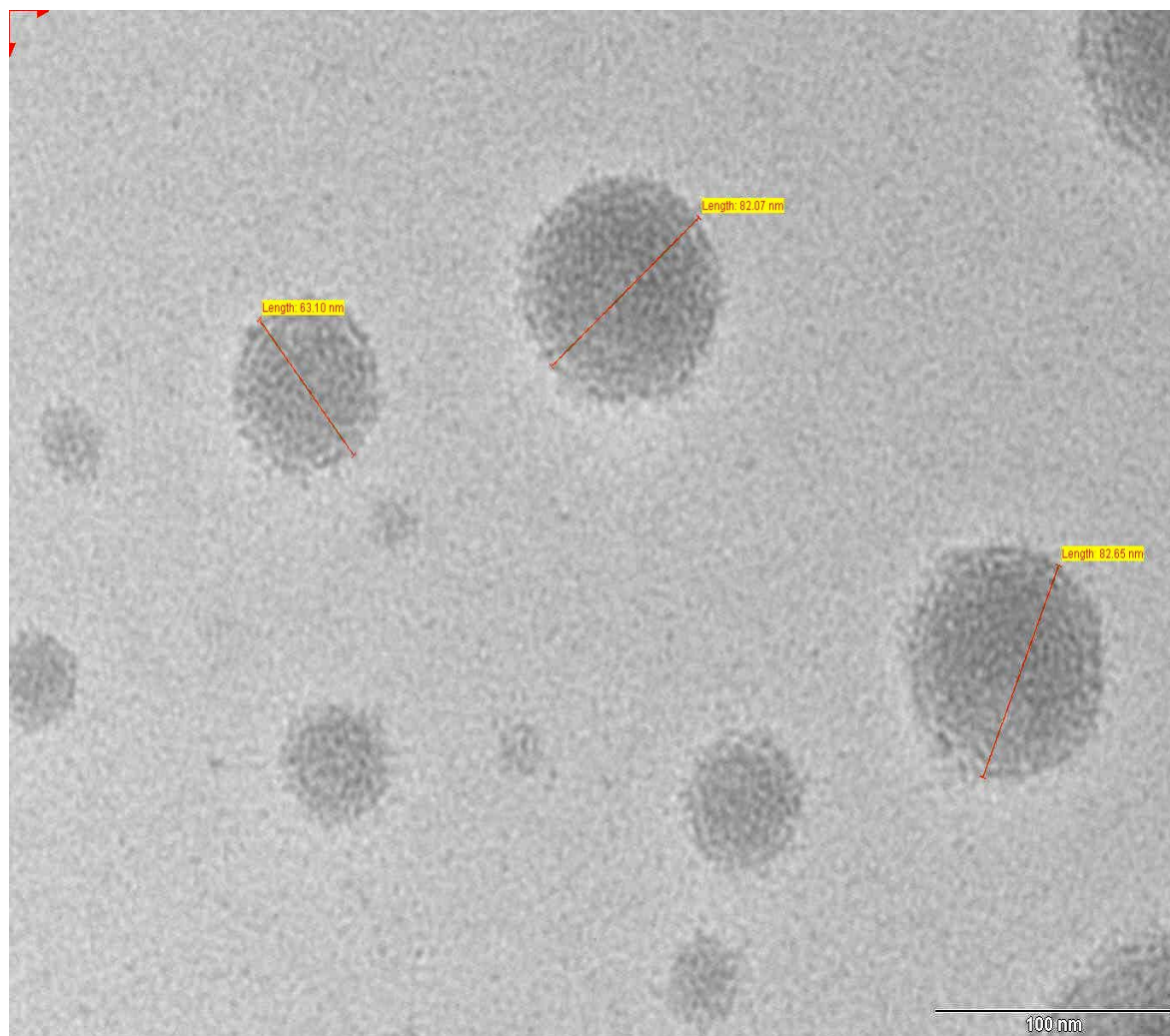
**Figure 5A.37 Size of optimized batch of liposomes**

Table 5A.30 Properties of optimized batch of liposomes

Formulation	Size	Zeta Potential	PDI
Liposomal dispersion	92.94 nm	-26.0	0.412

➤ **Surface morphology of liposomes:-** Surface morphology of optimized formulation of transferosomes and liposomes was determined using transmission electron microscopy (Model: Tecnai 20, Make: Philips, Holland) at SICART, Gujarat.





**Figure 5A.38 TEM image of optimized batch of liposomes**

- The TEM images shows that the both the vesicles liposomes and transferosomes are well identified, spherical vesicles.

#### **5A.8.9 Rheological studies of optimized carrier incorporated gel:-**

The rheological behavior of prepared liposomal gel, transferosomal gel was studied to ensure the ease of application and capability to withstand the stress of handling and storage. The properties were compared with the plain drug gel also to observe any change in gel characteristics that may occur due to presence of drug carriers in place of plain drug in gel. The rheological behavior of gel was determined by Brookfield viscometer using helipath with spindle no. 96.

Table 5A.31 Rheological properties of gel

RPM	Viscosity in centipoises					
	Liposomal gel		Transferosomal gel		Plain drug gel	
	↓	↓	↓	↓	↓	↓
2	30388	27989	32300	25400	34890	23478
3	20219	19432	22403	17555	22646	20112
5	15110	13420	16225	11234	18766	12789
10	10119	9100	10152	7865	12057	11075
20	5125	4999	5232	3898	7645	6346
30	4228	4228	4784	4784	5938	5938

**Interpretation-** Drug carrier incorporated gel showed pseudoplastic behavior. This indicated that upon application of minimum shear stress, they would thin out but once the shear stress is removed they would regain their normal thickness. This ensures the physical integrity of the formulated gels under various stress conditions like manufacturing, handling and packaging.

#### 5A.8.10 Determination of amount of aceclofenac permeated and absorbed in excised rat skin by after permeation studies:-

The percentage drug diffused into in acceptor compartment, percentage drug absorbed in skin and percentage drug retained on skin in donor compartment was determined by ex-vivo studies for optimized batch of liposomal gel, transferosomal gel and plain drug gel.

**5A.8.11 The ex-vivo release data for optimized formulations-**The evaluation outcomes of ex-vivo studies carried out for transferosomal gel, liposomal gel and plain drug gel are as follows:-



Table 5A.32 Comparison of ex-vivo release data

% Drug (after 6 hrs of studies)				
Optimized Formula	In acceptor compartment	Retained On Surface Skin	In skin	Loss
Transferosomal gel	82.85 %	10.02 %	4.28 %	2.85 %
Liposomal gel	74.16 %	12.89 %	9.63 %	3.32 %
Plain gel	59.04 %	26.88 %	3.76 %	10.32 %

**Interpretation-** It was observed that, maximum permeation (82.85%) of drug across rat skin takes place through transferosomal gel, followed by liposomal gel. The permeation through Plain gel is significantly less than both the drug carriers incorporated gel. The percentage of drug retained in skin was found to be maximum (9.63 %) for liposomal gel followed by transferosomal and plain drug gel. The reason may be the fusion of liposomes with rat skin releasing drug slowly in the skin layers.

### 5A.9 Residual solvent analysis for optimized batch of transferosomes and liposomes:-

As methanol and chloroform were used as solvent in the thin film hydration method during preparation of transferosomes and liposomes. The removal of complete organic solvent in the process was confirmed. Methanol and chloroform comes under solvent of Class II, their amount must be under limits of 3000 ppm and 60 ppm respectively in the formulation according to ICH Guideline Q3C\_R5. Therefore residual solvents were analyzed by Gas Chromatography (Auto System XL, Perkin Elmer) at SICART, Anand, Gujarat.

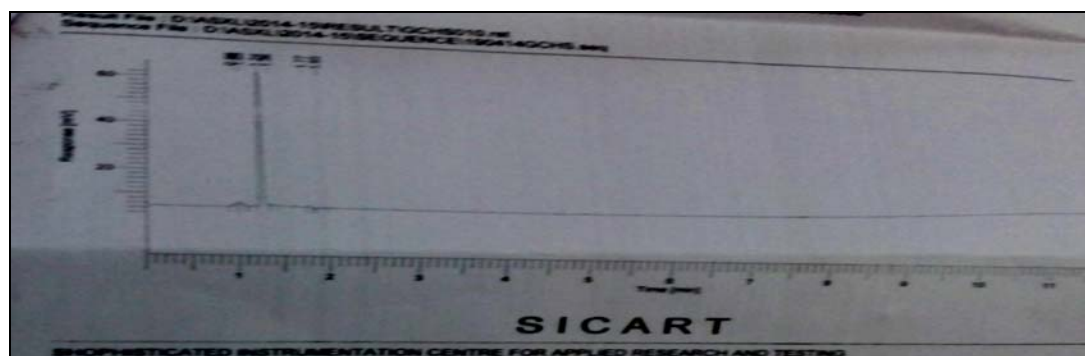


Figure 5A.39 GC of standard methanol

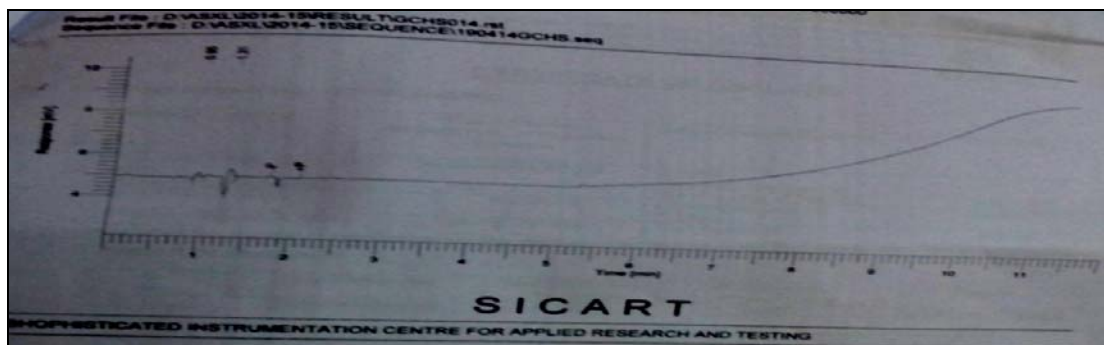


Figure 5A.40 GC of liposome sample for detection of methanol

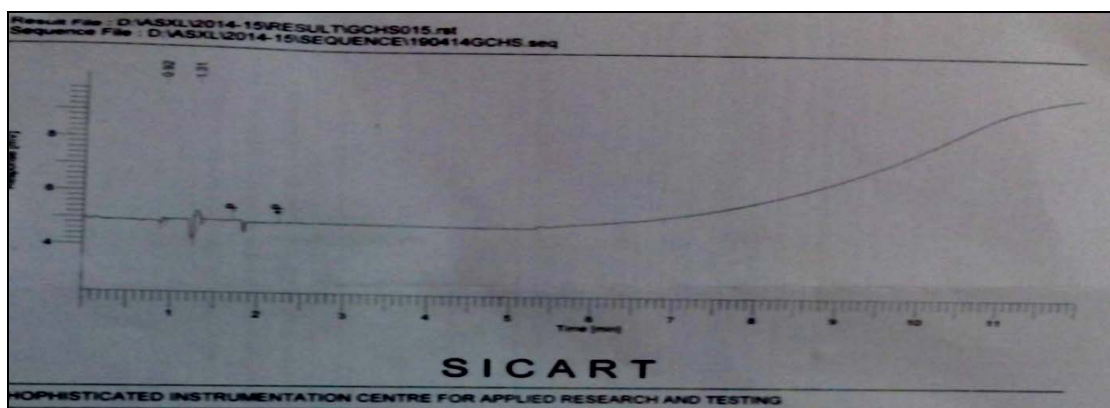


Figure 5A.41 GC of transferosome sample for detection of methanol

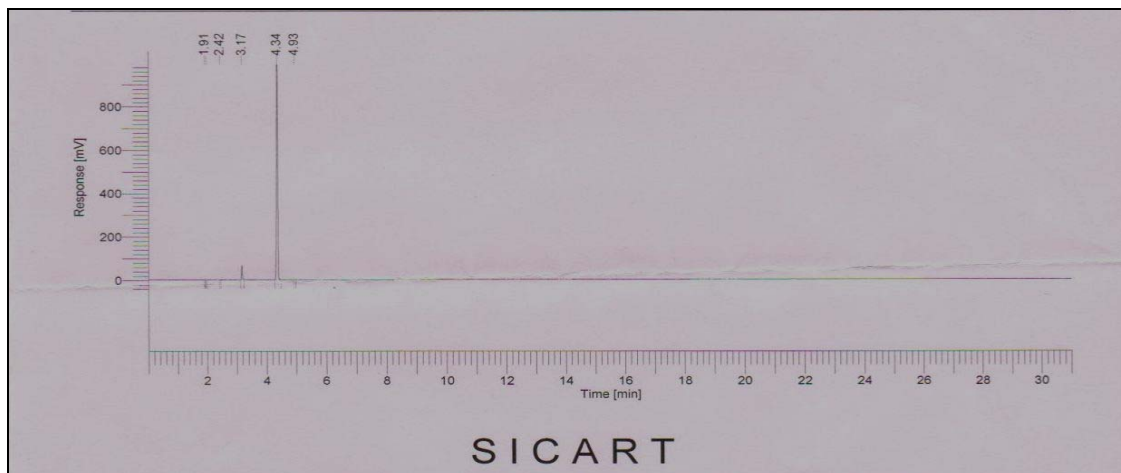
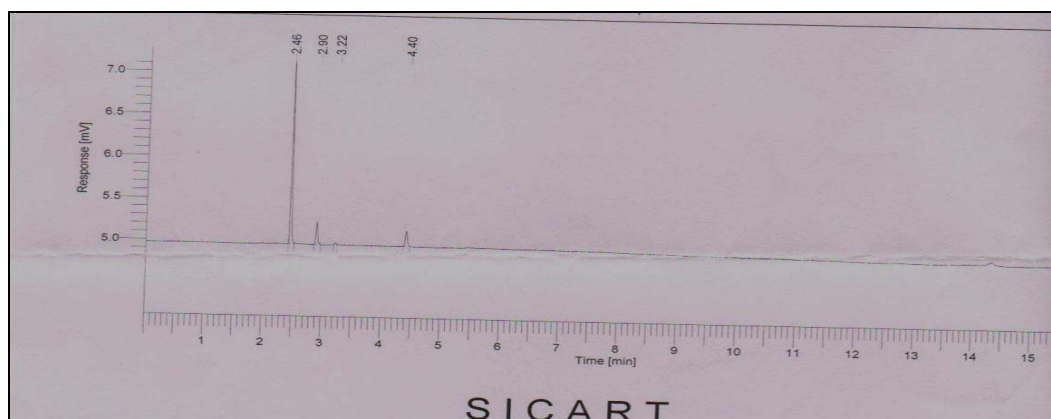


Figure 5A.42 GC of standard Chloroform



**Figure 5A.43 GC of transferosome sample for detection of chloroform**

**Interpretation:** - According to the reports of analysis of SICART, Anand, methanol and chloroform were not detected as residual solvents with respect to their standard solvents in both transferosomes and liposomes therefore the formulations were found to comply with the ICH guideline. (Q3C\_R5)

### **5A.10 Pharmacokinetics studies and comparative pharmacokinetic profiles of transferosomal gel, liposomal gel and plain gel of aceclofenac:-**

#### **5A.10.1 HPLC method details for analysis of aceclofenac in plasma and preparation of standard curve of drug in plasma:-**

Mobile phase:- 30 volume of water and 70 volume of acetonitrile

Injection volume:-10 $\mu$ l,

Flow rate:-1 ml/min,  $\lambda_{\max}$ :-273 nm

Column: - C<sub>18</sub> column (100 mm  $\times$  4.6 mm, 3.5  $\mu$ ).

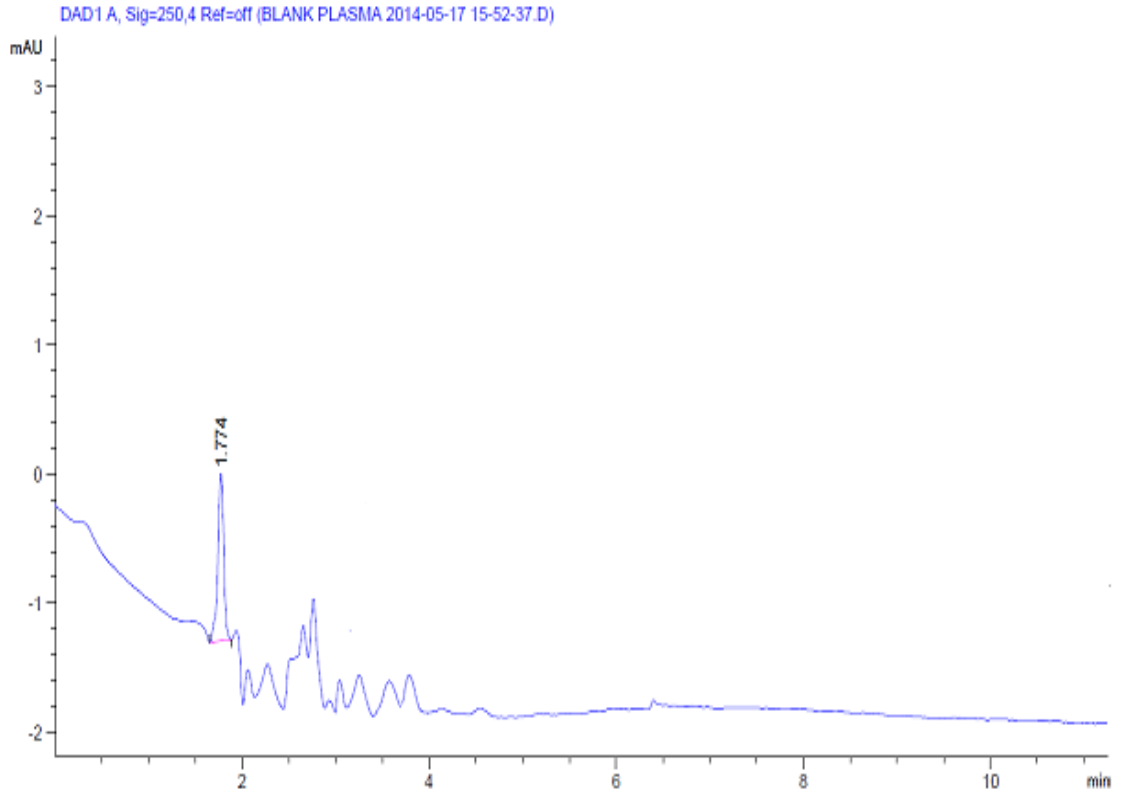


Figure 5A.44 HPLC chromatogram of blank Plasma

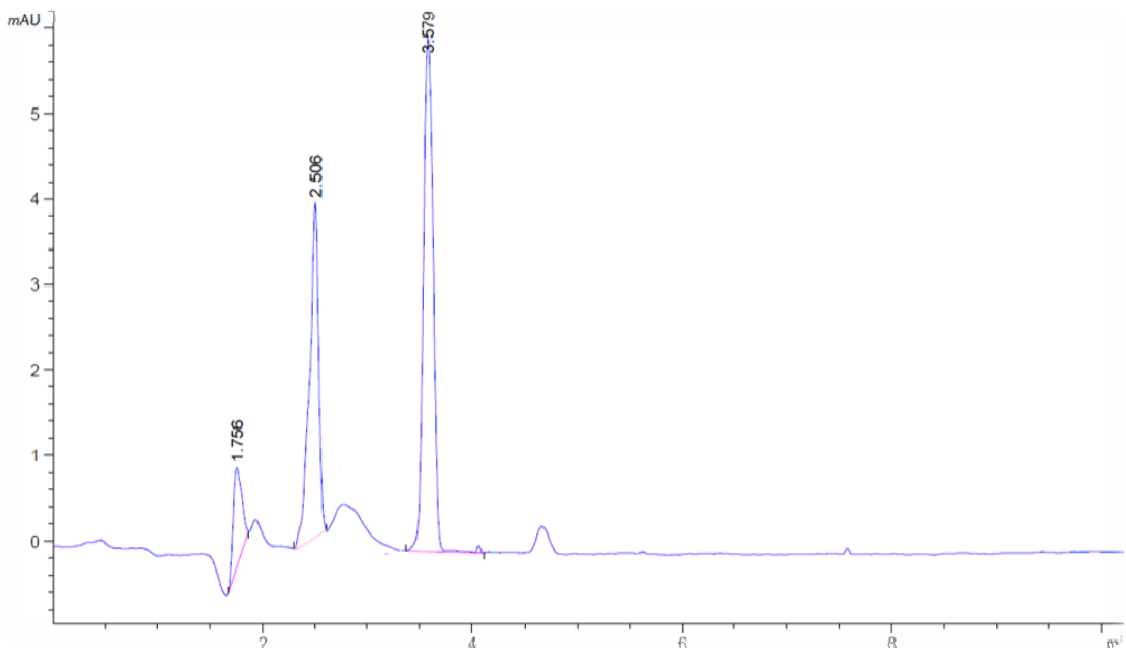


Figure 5A.45 HPLC chromatogram of plasma containing standard drug concentration

Table 5A.33 Standard curve of drug aceclofenac in plasma

Concentration	Peak area of analyte	Peak area of internal standard	Peak ratio of analyte / Int. standard
1 (2 µg/ml)	7.76911	27.2552	0.2850
2 (4 µg/ml)	11.6614	26.5002	0.4400
3 (6 µg/ml)	14.3273	26.6049	0.5385
4 (8 µg/ml)	22.6492	28.5956	0.7920
5 (10 µg/ml)	29.7864	27.8996	1.0676

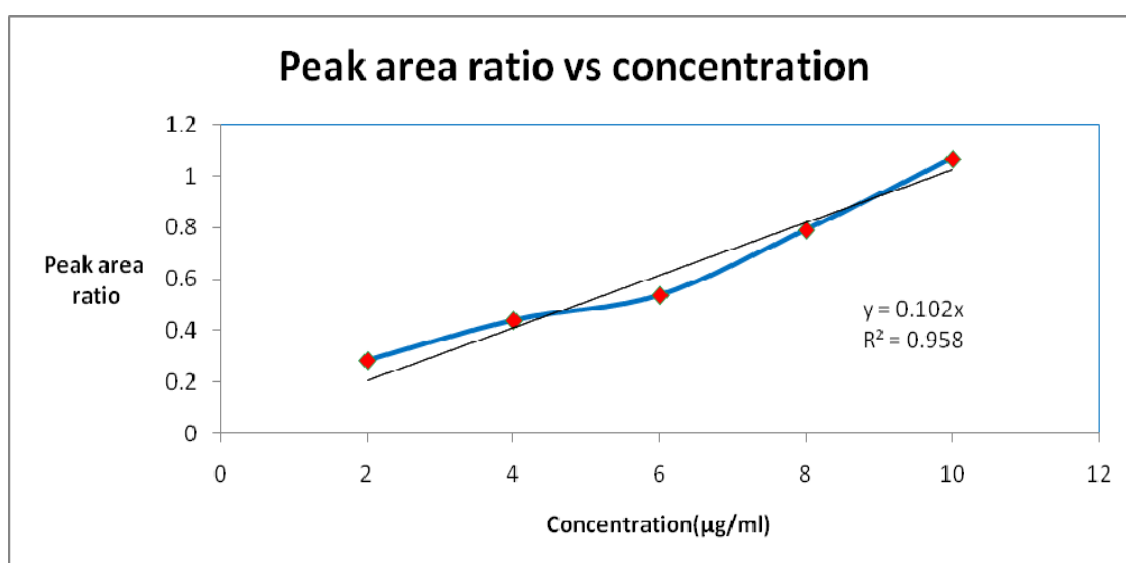


Figure5A.46 Standard curve of drug in plasma

**Interpretation-** Linearity was observed in the curve between peak area ratio and concentration of drug in plasma as  $R^2$  value was found to be 0.958.

#### 5A.10.2 Plasma profile of drug administered through drug carriers incorporated transdermal gel:-

Approval was taken from the Institutional Animal Ethics Committee to carry out pharmacokinetic studies. (Approval no- AEP. PhD/13-14/23)

**Table 5A.34 Plasma concentration profile of liposomal gel of aceclofenac**

<b>Time</b>	<b>Peak area of analyte</b>	<b>Peak area of internal standard</b>	<b>Peak ratio of analyte / Int. standard</b>	<b>Concentration (µg/ml)</b>
30 minutes	6.8643	27.2552	0.2518	2.468
1 hour	8.5432	26.5002	0.3223	3.160
2 hour	13.1198	26.6049	0.493	4.834
3 hour	19.7309	28.5956	0.6590	6.461
4 hour	19.906	27.8996	0.7135	6.996
6 hour	21.3219	29.8543	0.7142	7.002
8 hour	17.8621	28.3324	0.6304	6.180

**Table 5A.35 Plasma concentration profile of transferosomal gel of aceclofenac**

<b>Time</b>	<b>Peak area of analyte</b>	<b>Peak area of internal standard</b>	<b>Peak ratio of analyte / Int. standard</b>	<b>Concentration (µg/ml)</b>
30 minutes	8.6753	27.2552	0.3183	3.121
1 hour	11.1274	26.5002	0.4199	4.116
2 hour	16.9792	26.6049	0.6382	6.257
3 hour	23.7057	28.5956	0.8290	8.128
4 hour	24.6213	27.8996	0.8825	8.652
6 hour	27.0360	29.8543	0.9056	8.879
8 hour	21.4759	28.3324	0.7580	7.432

**Table 5A.36 Plasma concentration profile of plain gel of aceclofenac**

<b>Time</b>	<b>Peak area of analyte</b>	<b>Peak area of internal standard</b>	<b>Peak ratio of analyte / Int. standard</b>	<b>Concentration (µg/ml)</b>
30 minutes	5.4101	27.2552	0.1985	1.946
1 hour	7.4398	26.5002	0.2807	2.752
2 hour	12.1476	26.6049	0.4565	4.476
3 hour	15.0659	28.5956	0.5268	5.165
4 hour	16.2375	27.8996	0.0582	5.711
6 hour	18.2618	29.8543	0.6117	5.998
8 hour	12.4545	29.3324	0.4246	4.163

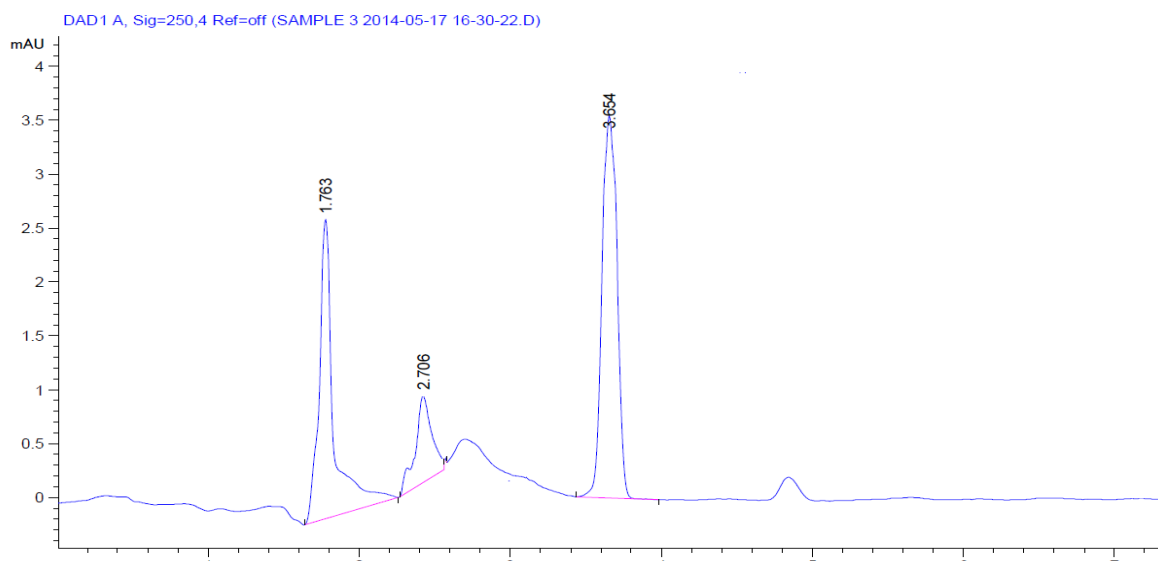
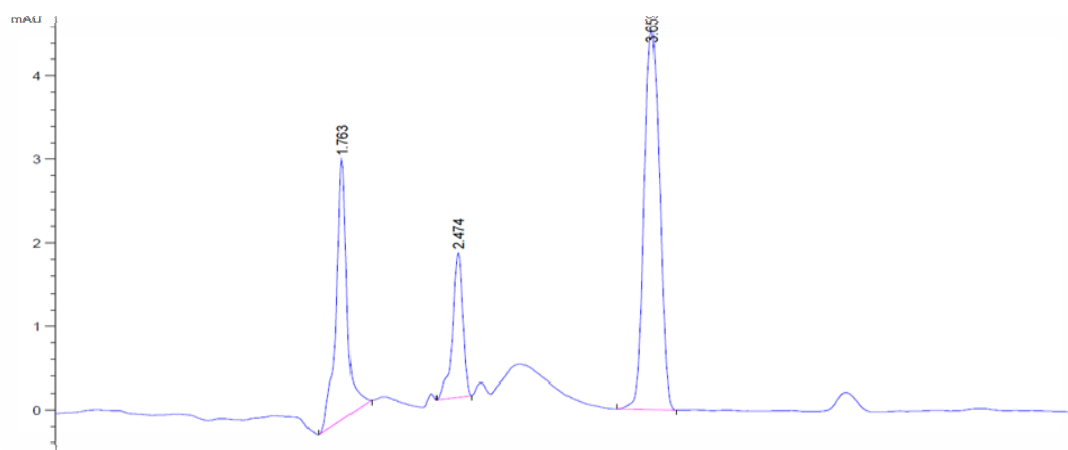
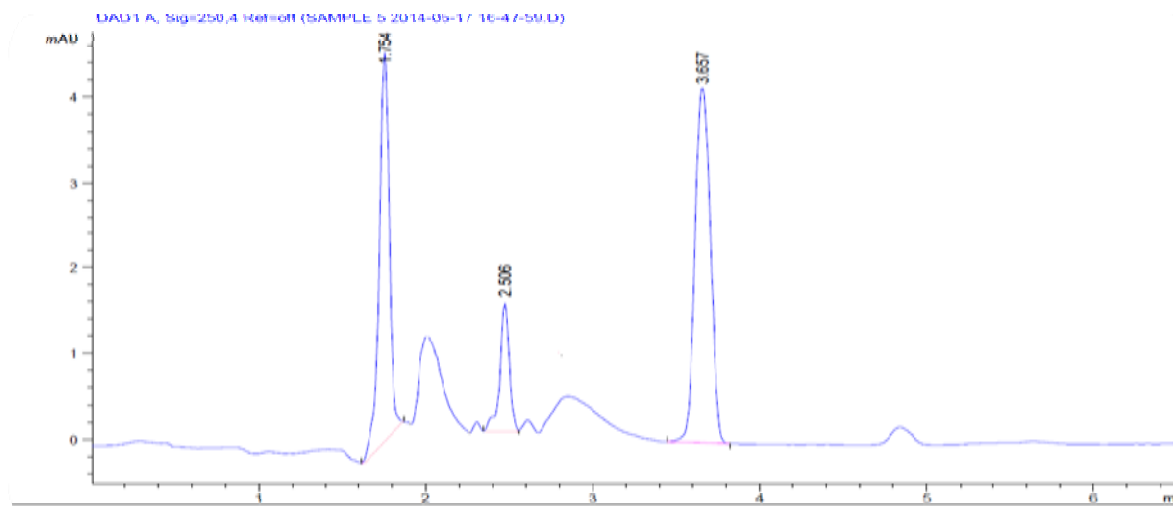


Figure 5A.47 Plasma concentration of drug after administration in rats.

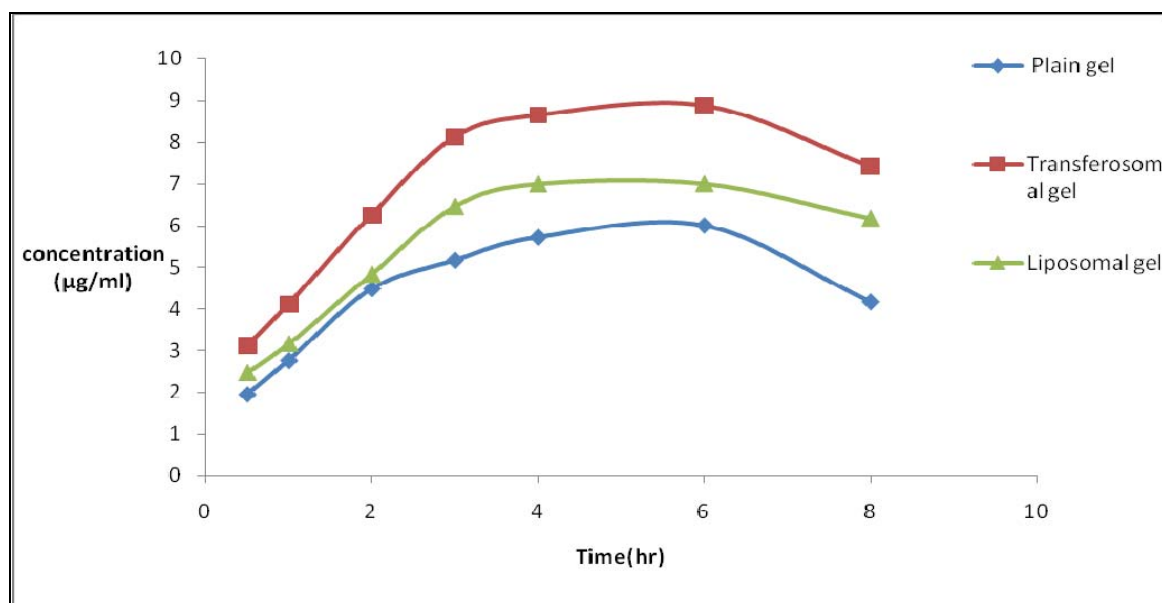


Figure.5A.48 Comparative plasma profile of drug carriers incorporated gel

Table 5A.37 Comparative pharmacokinetics profile of drug carriers incorporated gel

Formulation	$C_{max}$ (µg/ml)	$T_{max}$ (hr)	$AUC_{0-t}$ µg.hr/ml	$AUC_{t-\infty}$ µg.hr/ml	$AUC_{0-\infty}$ µg.hr/ml
Liposomal gel	7.002	6	44.957	8.917	53.874
Transfersosomal gel	8.879	6	56.419	10.724	67.143
Plain gel	5.998	6	36.916	6.007	42.923

**Interpretation** – The blank sample of plasma was found to be free of any other component. The aceclofenac was detectable in standard solution of drug in plasma by the HPLC method.

The concentration of drug in plasma after transdermal application of transfersosomal gel was detected and quantified. The blank sample of plasma was found to be free of any other component. The aceclofenac was detectable in standard solution of drug in Plasma by the used HPLC method.

The concentration of drug in plasma after transdermal application of optimized formulations of drug loaded liposomal gel, transfersosomal gel and plain gel was detected and quantified.



The liposomal gel showed  $C_{\max}$  of 7.002  $\mu\text{g/ml}$  and a  $T_{\max}$  of 6 hrs, whereas the transferosomal gel showed  $C_{\max}$  of 8.879  $\mu\text{g/ml}$  and a  $T_{\max}$  of 6 hrs. The plain gel of aceclofenac as compared to liposomal and transferosomal gel, showed lesser  $C_{\max}$  but the same  $T_{\max}$ . The bioavailability as measured by AUC was found to be highest for transferosomal gel followed by liposomal gel formulation. Both the liposomal gel and transferosomal gel were found to have better bioavailability as compared to plain gel of aceclofenac.

### 5A.11 Radioactive tagging experiment for skin permeation studies:-

The radiolabeling of aceclofenac transferosome formulation was carried out at Institute of Nuclear Medicine and Allied Sciences, DRDO, New Delhi by standard methods described as follows:-

- Aceclofenac transferosomal dispersion 1ml (2.5 mg/ml) was mixed with 0.1 ml  $\text{SnCl}_2$  (1 mg/ml in 10% glacial acetic acid). Then, 1 ml  $^{99\text{m}}\text{Tc-NaTcO}_4$  (2 mci/ml) was mixed with it.
- The pH of mixture was adjusted to 7 using  $\text{NaHCO}_3$  solution (1%)
- The mixture was incubated for 30 minutes at  $30^\circ\text{C}$  to produce labeled  $^{99\text{m}}\text{Tc}$ -aceclofenac transferosomes.

#### 5A.11.1 Determination of conditions for maximum labeling efficiency:-

The chemical form of  $^{99\text{m}}\text{Tc}$  available from the Moly generator is sodium pertechnetate ( $^{99\text{m}}\text{Tc-NaTcO}_4$ ) which is nonreactive. In  $^{99\text{m}}\text{Tc}$ -labeling, prior reduction of  $^{99\text{m}}\text{Tc-NaTcO}_4$  from the  $7^+$  state to a lower oxidation state is required using reducing agent  $\text{SnCl}_2$ .

The radiolabeling of compound was performed as per standard protocol of INMAS and radiolabeling efficiency was determined at various pH, temperature and incubation time by instant thin layer chromatography.

Table 5A.38 % Labeling efficiency at different pH in radioactive tagging studies

pH of Medium	% LE $\pm$ STD (n=3)
4.0	90.24 $\pm$ 0.82
6.0	92.46 $\pm$ 0.78
<b>7.0</b>	<b>94.40 <math>\pm</math> 0.45</b>
8.0	88.25 $\pm$ 1.45

Table 5A.39 % Labeling efficiency at different temperature in radioactive tagging studies

Temperature ( $^{\circ}$ C)	% LE $\pm$ STD (n=3)
25	89.12 $\pm$ 0.64
<b>30</b>	<b>94.76 <math>\pm</math> 0.52</b>
50	93.42 $\pm$ 0.56
60	94.12 $\pm$ 0.62

Table 5A.40 % Labeling efficiency at different incubation time in radioactive tagging studies

Incubation Time	% LE $\pm$ STD (n=3)
5	54.22 $\pm$ 0.58
10	78.27 $\pm$ 0.34
20	86.32 $\pm$ 0.47
<b>30</b>	<b>93.46 <math>\pm</math> 0.52</b>

- % labeling efficiency was found to be maximum at pH 7, temperature of 30 $^{\circ}$ C and incubation time of 30 minutes, so radiolabeling was further performed at these fixed parameters.

#### 5A.11.2 In-vitro Saline stability of radiolabeled drug:-

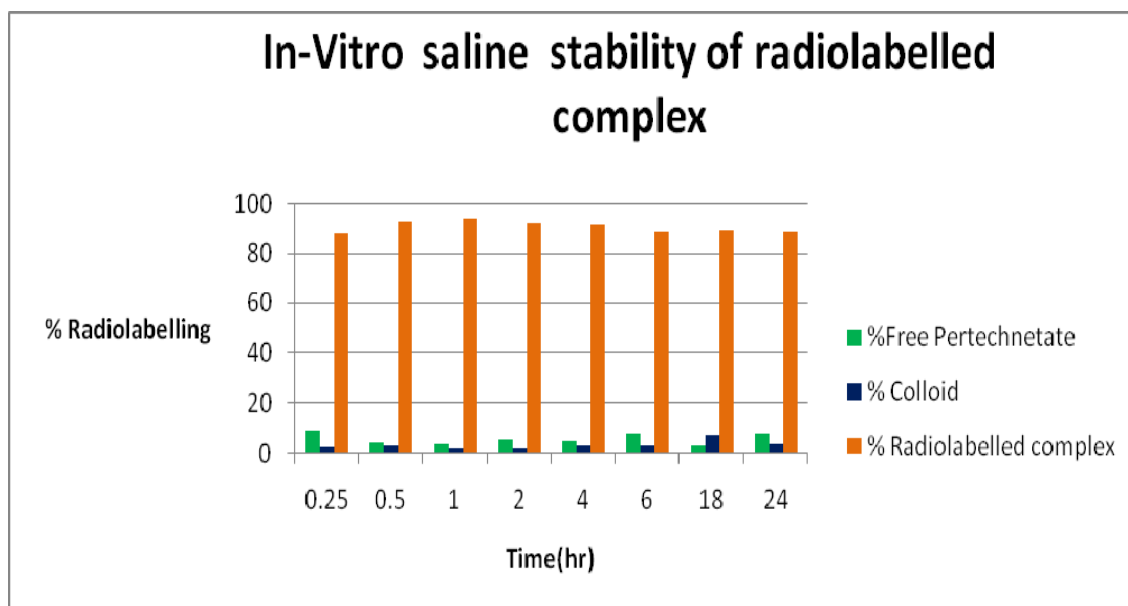
- ❖ In vitro saline stability of radiolabeled compound was determined by instant thin layer chromatography. Study was performed by mixing 100  $\mu$ l of  $^{99m}$ Tc-Compound with 900  $\mu$ l of saline, vortex it for proper mixing.
- ❖ Mixture was incubated at 37 $^{\circ}$ C and small aliquots were withdrawn at 0.25, 0.5, 1, 2, 4, 6, 18, 24 hrs. and radiochemical purity of  $^{99m}$ Tc-compound was evaluated by standard ITLC method using acetone as mobile phase.
- ❖ The developed strips were cut in 7:3 ratios and radioactivity in each part was measured to calculate the % labeled compound
- ❖ Any decrease in percentage of radiolabeled complex was taken as degradation.

Three components were detected:-

- Free  $^{99m}\text{Tc}$  as  $^{99m}\text{TcO}_4^-$  that has not been reduced by  $\text{Sn}^{2+}$ .
- Reduced  $^{99m}\text{Tc}$  bound to hydrolyzed  $\text{Sn}^{2+}$  ( $\text{Sn}(\text{OH})_2$ ).
- Bound  $^{99m}\text{Tc}$  with formulation, which is the desired group formed by binding of reduced  $^{99m}\text{Tc}$  to the formulation.

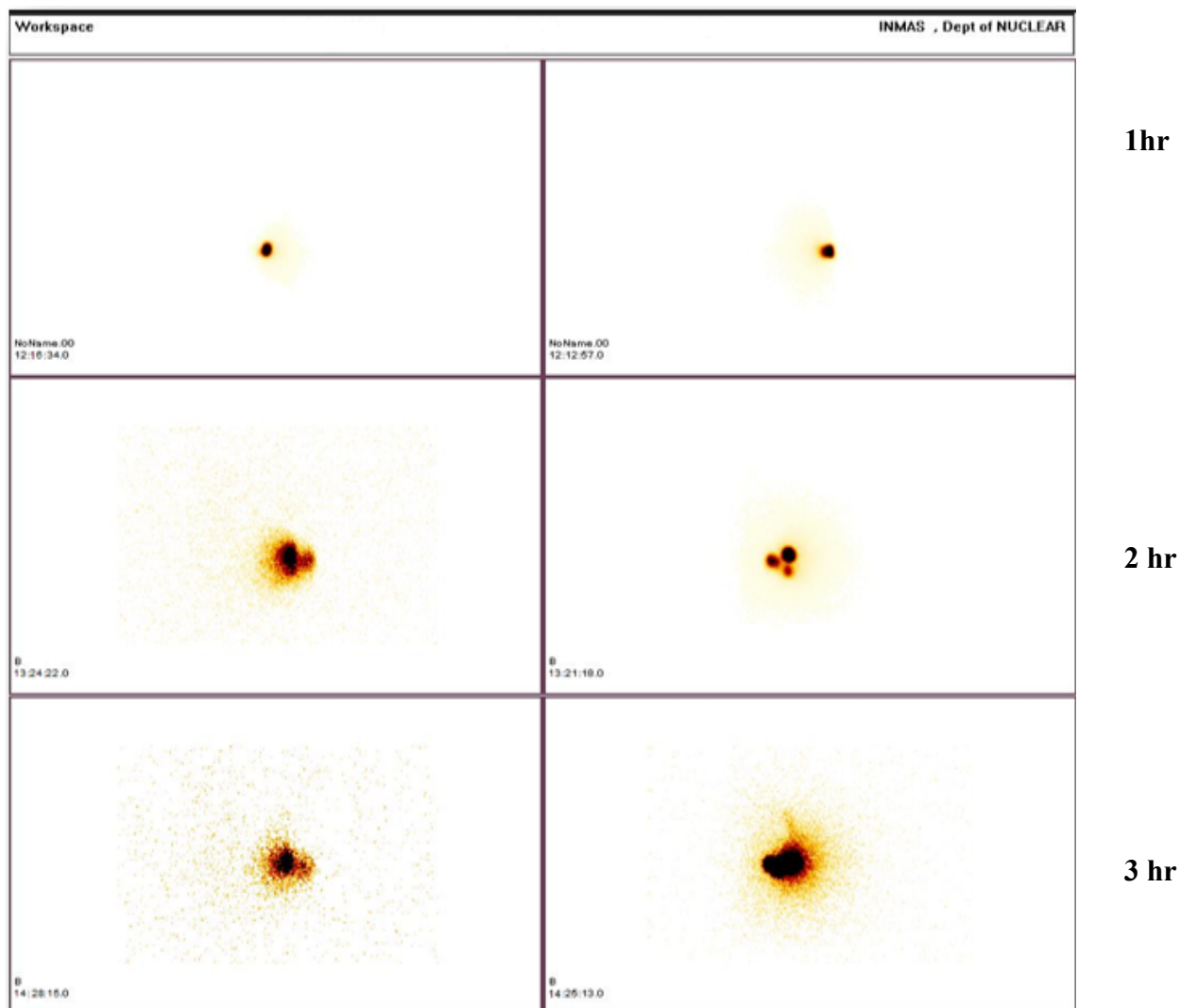
**Table 5A.41 In-vitro saline stability of radiolabelled complex**

Time (hr)	% Free Pertechnetate	% Colloid	% Radiolabeled complex
0.25	9.12	2.64	88.24
0.5	4.44	3.26	92.30
1	3.89	2.31	93.80
2	5.65	2.20	92.15
4	5.13	3.19	91.68
6	7.89	3.31	88.80
18	3.56	7.36	89.08
24	7.75	3.79	88.46



**Figure 5A.49 In-vitro saline stability of radiolabeled complex**

**Interpretation-** The radiolabeled drug was found to be stable in saline for 24 hrs as the fraction of radiolabeled complex in saline was found to be present as more than 90 % till 24 hours.

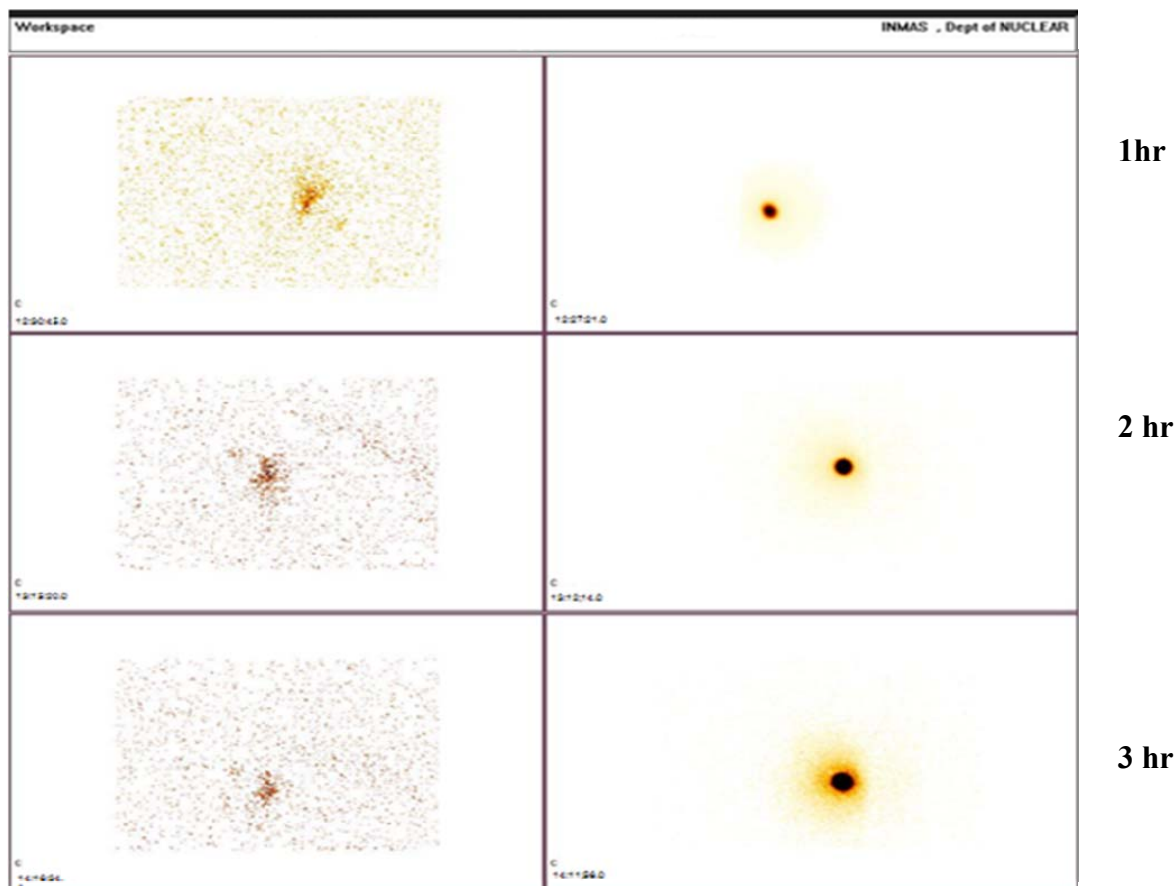
**5A.11.3 Drug permeation study of transferosomal gel through Newzealand rabbit skin :-**

**Figure 5A.50 Scintigraphy image analysis of drug permeation and retention in skin from transferosomal gel**

- The radioactivity was detected in skin .The drug was found to permeate at every hour and retained in skin after washing also. The drug retained in skin can be released in a sustained manner.

**5A.11.4 Drug permeation study of plain drug gel through Newzealand rabbit skin**

:-



**Figure 5A.51 Scintigraphy image analysis of drug permeation and retention in skin from plain drug gel**

- The radioactivity was detected in skin. The drug was found to permeate at every hour and retained in skin after washing also but the concentration of permeated drug was found to be very low as compared to transferosomal gel.
- The scintigraphic image analysis of drug permeation showed that more drug got permeated from transferosomal gel as compared to plain gel at every time point and so also, the retention of drug in skin was in case of transferosomal gel was more .

### 5A.11.5 Scintigraphy image analysis of drug permeation and retention in skin from transferosomal gel and plain drug gel:

Table 5A.42 Scintigraphy image analysis of aceclofenac transferosomal gel

Time(minutes)	Counts in region of interest		
	Test Counts (before washing)	Test Counts(after washing)	% drug in skin
60	6527	4480	68.637
120	6494	5004	77.055
180	6476	5118	79.130

Table 5A.43 Scintigraphy image analysis of plain aceclofenac gel

Time(minutes)	Counts in region of interest		
	Test Counts (before washing)	Test Counts (after washing)	% drug in skin
60	6511	3124	47.980
120	6134	2982	48.614
180	6123	3356	54.809

#### Finding from radioactive tagging studies:-

- Drug was detected in skin after removal of gel from skin.
- The drug concentration in skin was found to increase at every hour.
- The drug permeation through skin was found to be more from aceclofenac transferosomal gel as compared to aceclofenac plain gel.

### 5A.12 Anti-inflammatory studies of formulations by rat paw edema method:-

The paw edema was measured using plethysmometer and the % inhibition of edema was for each group using the following equation-

$$\% \text{ inhibition of edema} = 1 - [(a-x / b-y)] \times 100$$

a= mean paw volume of treated animal after carageenan injection

x= mean paw volume of treated animal before carageenan injection

b= mean paw volume of control animal after carageenan injection

y= mean paw volume of control animal before carageenan injection

### Anti inflammatory activity determination by rat paw edema method using plethysmometer:-

Table 5A.44 Applied transdermal formulations on rat paw

Rat group	Applied formulation
I	Control
II	Aceclofenac liposomal gel
III	Aceclofenac transferosomal gel
IV	Aceclofenac marketed gel

Table 5A.45 Rat paw volume measured after formulation application at different time intervals

Rat group	Paw volume in ml at different time intervals							
	time 0	1 hr	2 hrs	3 hrs	4 hrs	5 hrs	6 hrs	24 hrs
I	0.54	1.00	1.05	1.20	1.15	1.05	0.95	0.82
II	0.50	0.75	0.78	0.79	0.77	0.70	0.67	0.59
III	0.51	0.78	0.80	0.82	0.75	0.72	0.65	0.61
IV	0.53	0.92	0.91	0.93	0.90	0.81	0.79	0.67

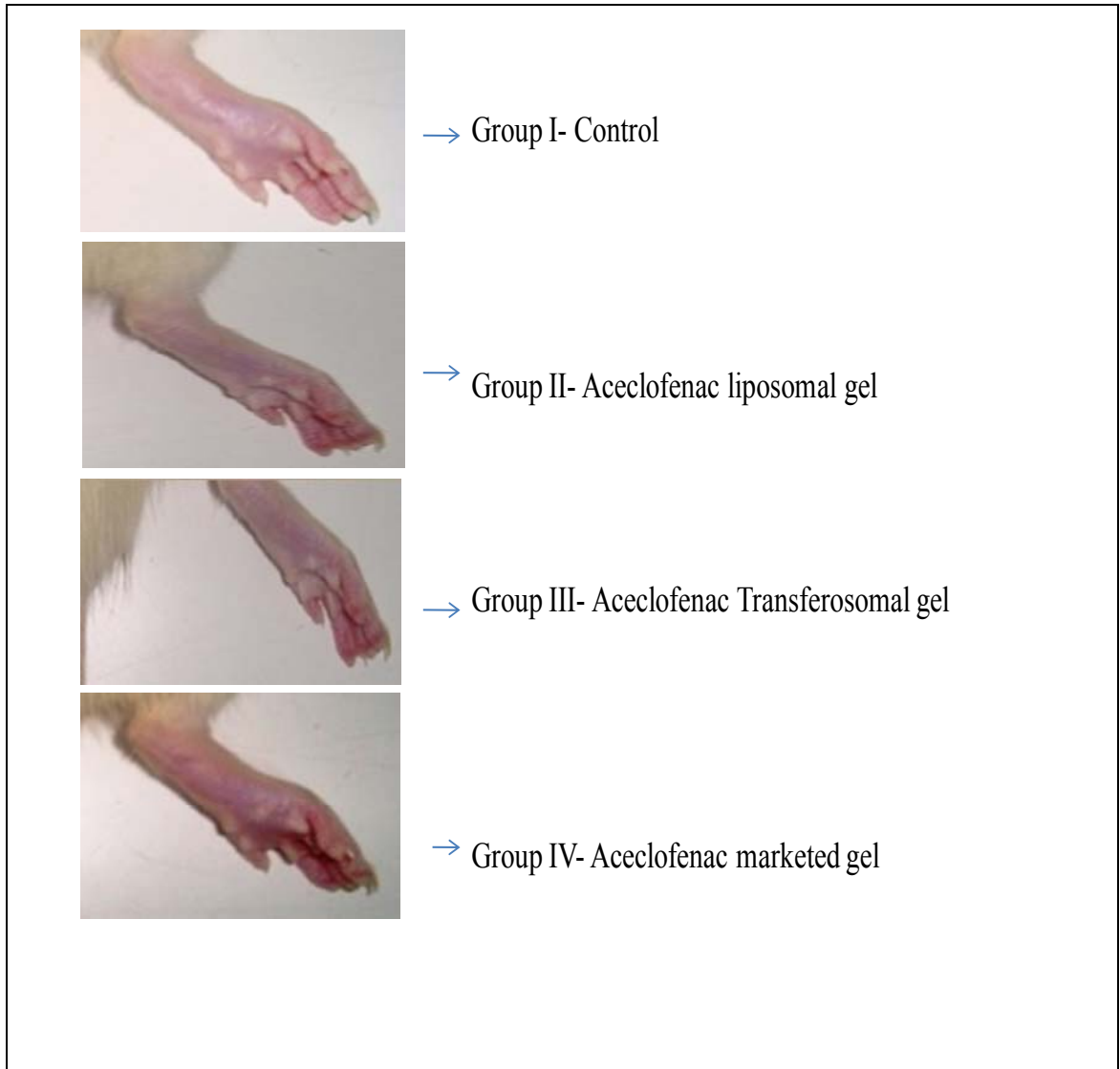


Figure 5A.52 Paw edema observed in rats after 3 hours.

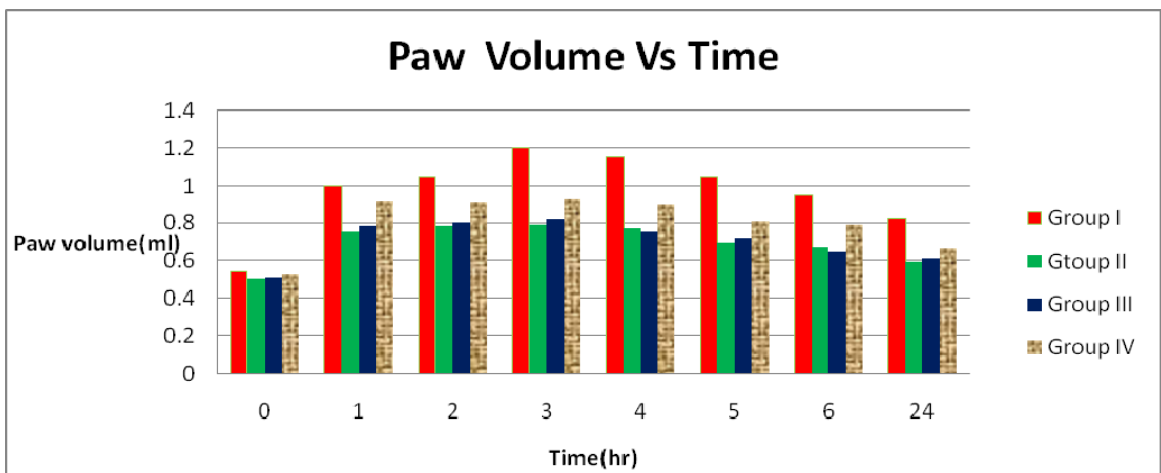


Figure 5A.53 Comparison of paw volume in rats.



Table 5A.46 % Anti-inflammatory activity of formulations in groups of rat

Group	% Anti-inflammatory activity						
	1 hr	2 hr	3 hr	4 hr	5 hr	6 hr	24 hr
II	45.83	44.28	50.00	52.00	59.37	59.00	65.62
III	40.0	42.85	50.66	57.33	57.50	62.50	63.75
IV	16.66	31.14	40.00	40.00	46.87	48.75	50.00

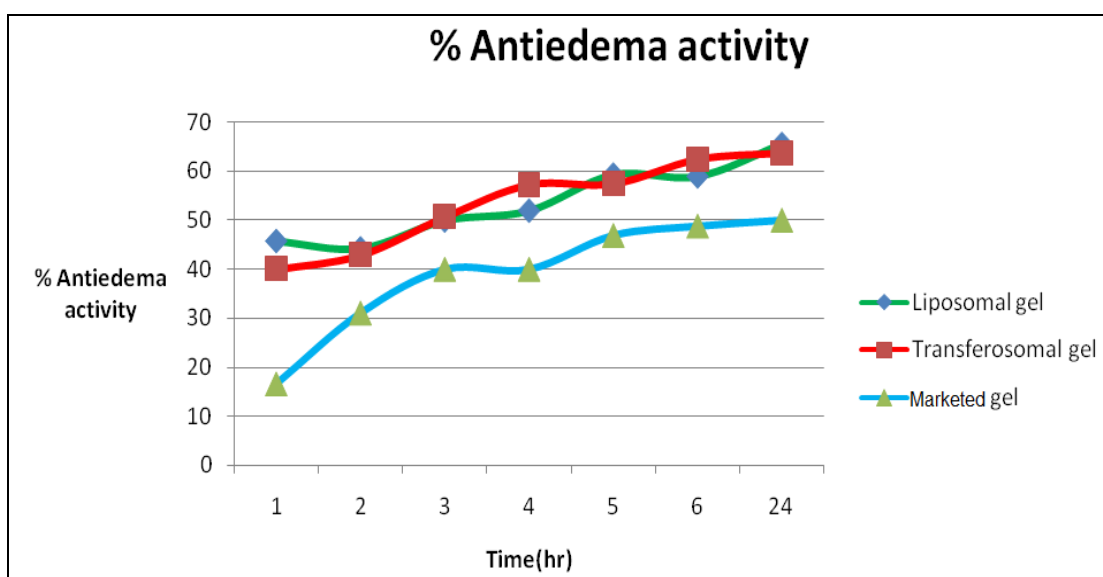


Figure 5A.54 Comparison of antiedema activity

**Interpretation:-**

- On determination of paw volume and thereafter % inhibition of edema, after application of liposomal gel, transferosomal gel and plain drug gel of aceclofenac on the right hind paws of group of rats, it was observed that, transferosomal gel showed slightly higher anti-inflammatory activity than liposomal gel and significantly higher activity than marketed gel of aceclofenac.

**5A.12.1 Statistical analysis of data to confirm improvement in Anti-inflammatory activity:-**

The anti-inflammatory activity of liposomal gel, transferosomal gel and marketed gel were compared by ANOVA analysis at 95% confidence interval to confirm statistically significant improvement due to drug carriers incorporated gel.

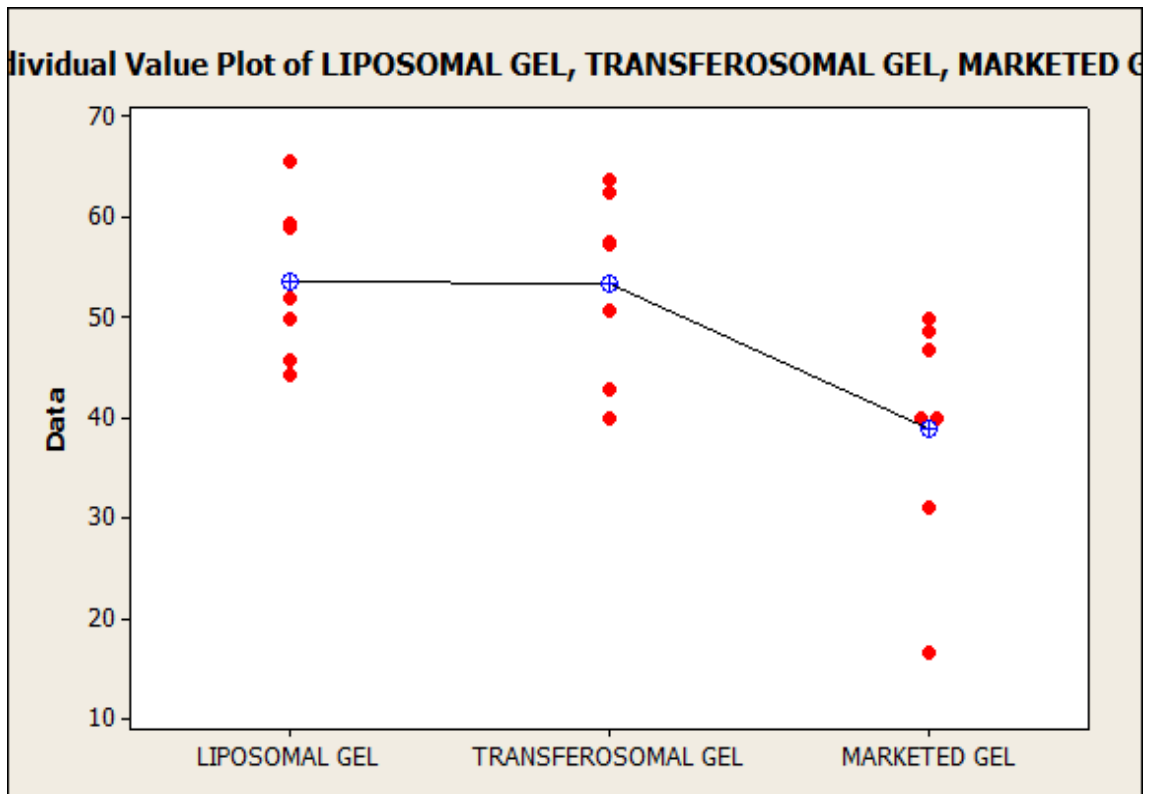
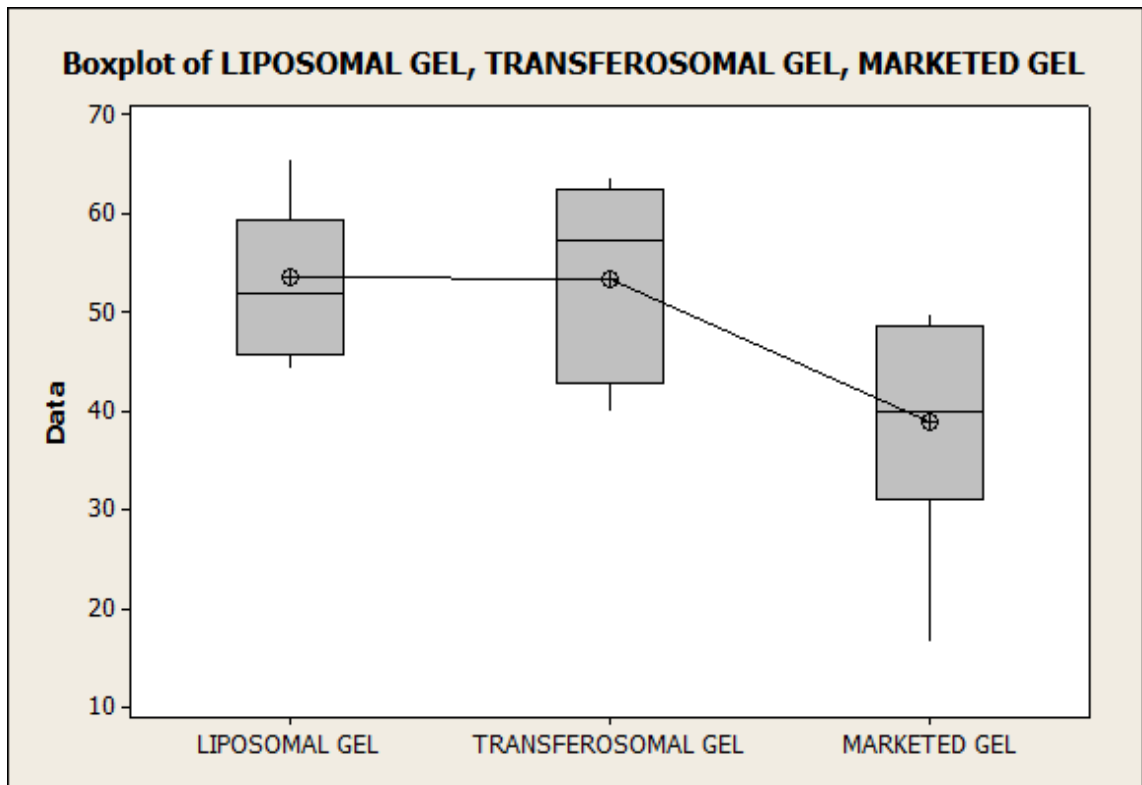


Figure 5A.55 Box lot and individual plots for statistical comparison of anti-inflammatory activity

❖ **Results of One-way ANOVA: 95% Confidence interval****Liposomal gel, Transferosomal gel, Marketed gel**

source	df	ss	ms	f	p
factor	2	989.6	494.8	5.15	<b>0.017</b>
error	18	1730.9	96.2		
total	20	2720.4			

- At 95% confidence interval, P value of less than 0.05 indicated that there is significant improvement in anti-inflammatory activity of aceclofenac in the form of transferosomal gel formulation as compared to marketed aceclofenac gel.

### 5A.13 Determination of analgesic activity by Eddy's hot plate method in rats:-

The time of latency was determined as the time period between the zero point, when the animal is placed on the hot plate surface, and the time when the animal jumps off to avoid thermal pain.



**Figure 5A.56** Observation of reaction time on Eddy's hot plate in analgesic activity studies

Table 5A.47 Analgesic activity of formulations in rats

Formulation	Reaction time in seconds at time intervals (minutes)					
	30	60	90	120	150	180
Control	3	4	4	5	4	4
Liposomal gel	7	11	11	12	12	11
Transferosomal gel	8	11	12	13	13	10
Marketed Gel	6	8	10	10	9	8

## ❖ Comparison of analgesic activity:-

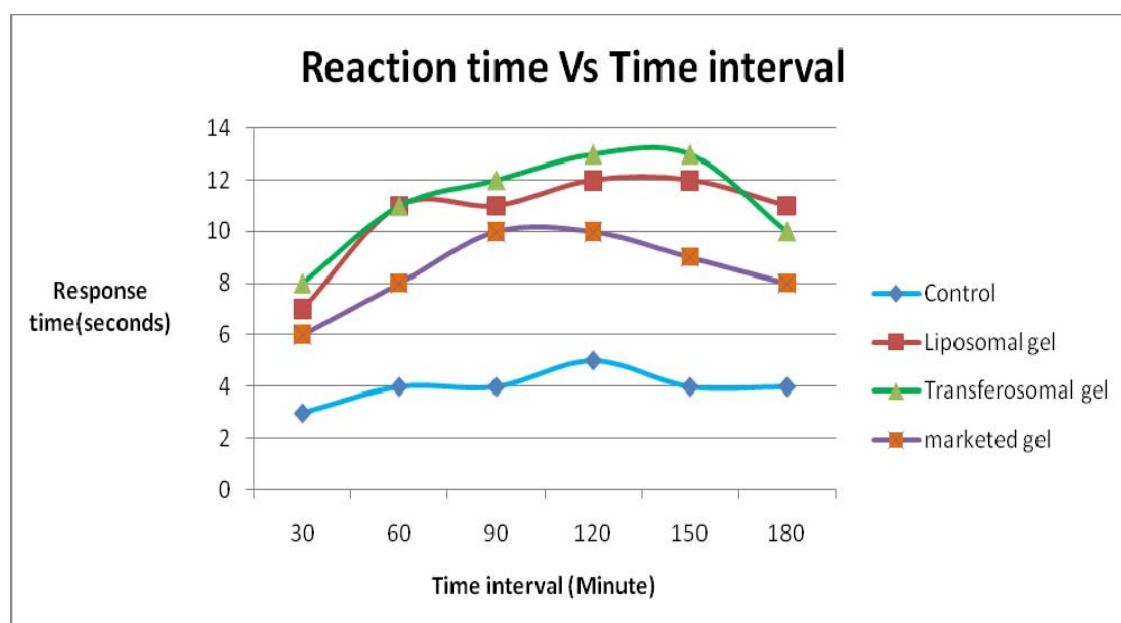


Figure 5A.57 Comparison of reaction in rats in analgesic activity studies

**Interpretations:-**

- The group of rats who received the application of liposomal and transferosomal gel of aceclofenac showed more tolerance to pain as compared to the group of rats who received the application of marketed gel.
- The transferosomal gel of aceclofenac showed maximum analgesic activity followed by liposomal gel.
- Both the carrier incorporated gels showed better activity than marketed aceclofenac gel.

**5A.13.1 Statistical analysis of data to confirm improvement in analgesic activity:-**

The analgesic activity of liposomal gel, transferosomal gel and marketed gel were compared by ANOVA analysis at 95% confidence interval to confirm statistically significant improvement due to drug carriers incorporated gel.

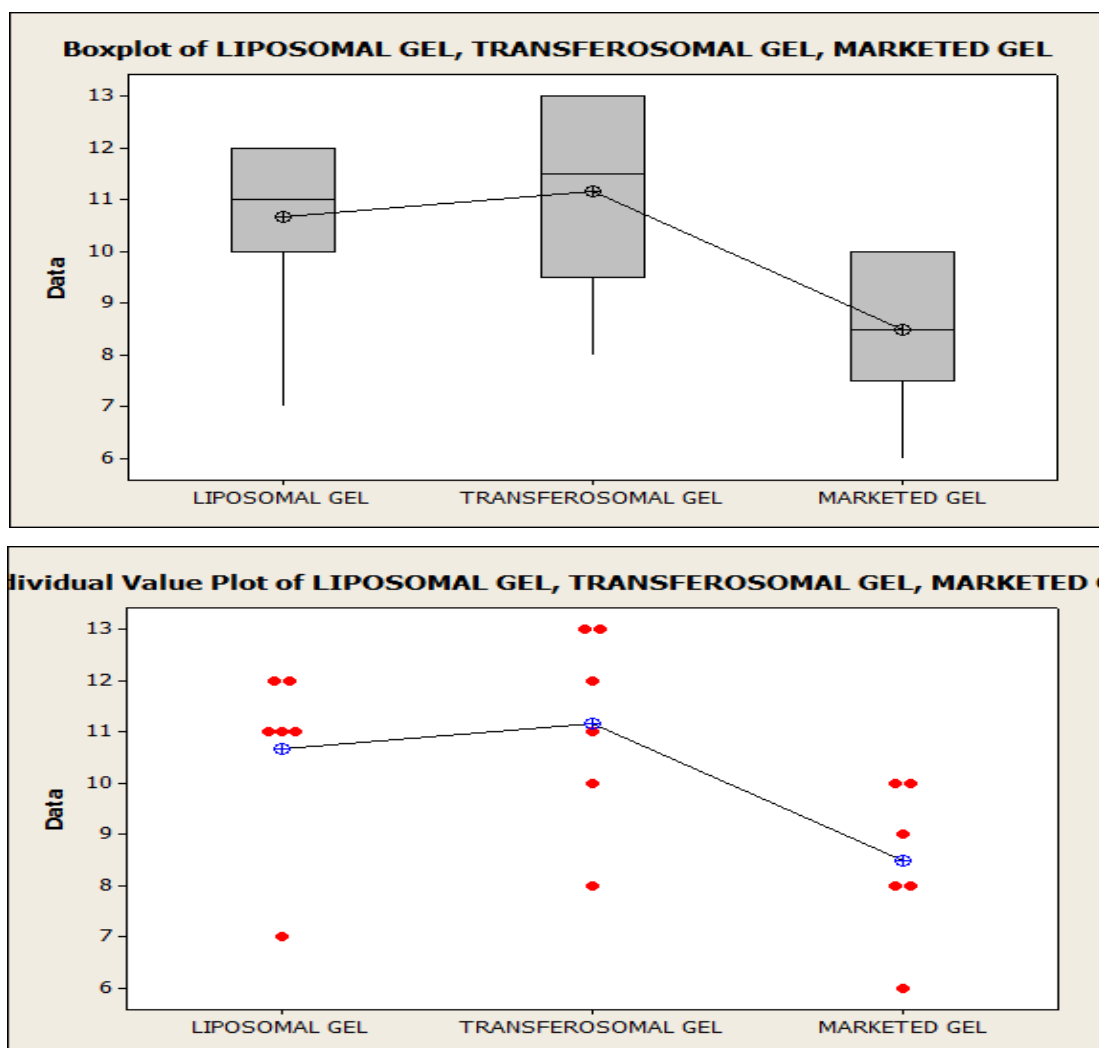


Figure 5A.58 Box lot and individual plots for statistical comparison of analgesic activity

❖ **One-way ANOVA: 95%CI**

Source	DF	SS	MS	F	P
Factor	2	24.11	12.06	3.79	<b>0.046</b>
Error	15	47.67	3.18		
Total	17	71.78			

➤ At 95% confidence interval, P value of less than 0.05 indicated that there is significant improvement in analgesic activity of aceclofenac in the form of transferosomal gel formulation as compared to plain drug gel.

### 5A.14 Stability Studies as per ICH guidelines:-

#### Conditions:-

- Long term = Temperature of  $5^{\circ}\text{C} \pm 3^{\circ}\text{C}$  for 12 months
- Accelerated condition = Temperature and humidity of  $25^{\circ}\text{C} \pm 2^{\circ}\text{C}/60\% \text{ RH} \pm 5\% \text{ RH}$  respectively for three months and six months.

#### 5A.14.1 Characterization of transferosome dispersion and transferosomal gel:-

Transferosomal dispersion was characterized by evaluation of size, zeta potential, entrapment efficiency and transferosomal gel was evaluated by permeation flux and gel characteristics

**Table 5A.48 Characteristics of optimized formulation of transferosome after stability studies**

Sampling time	Size in nm	Zeta Potential	PDI
Initial	351 nm	-42.7	0.334
3 months	358 nm	-24.0	0.231
6 months	383 nm	-15.9	0.374

**Table 5A.49 Characteristics of optimized formulation of transferosomal gel of aceclofenac after stability studies**

Sampling time	Ex-vivo Permeation flux ( $\mu\text{gcm}^{-2} \text{ hr}^{-1}$ )	Viscosity (cp)	pH	Spreadability (gm.cm/sec)
Initial	28.69	4768	6.4	1.446
3 months	23.02	4113	6.2	1.423
6 months	19.91	4003	6	1.411

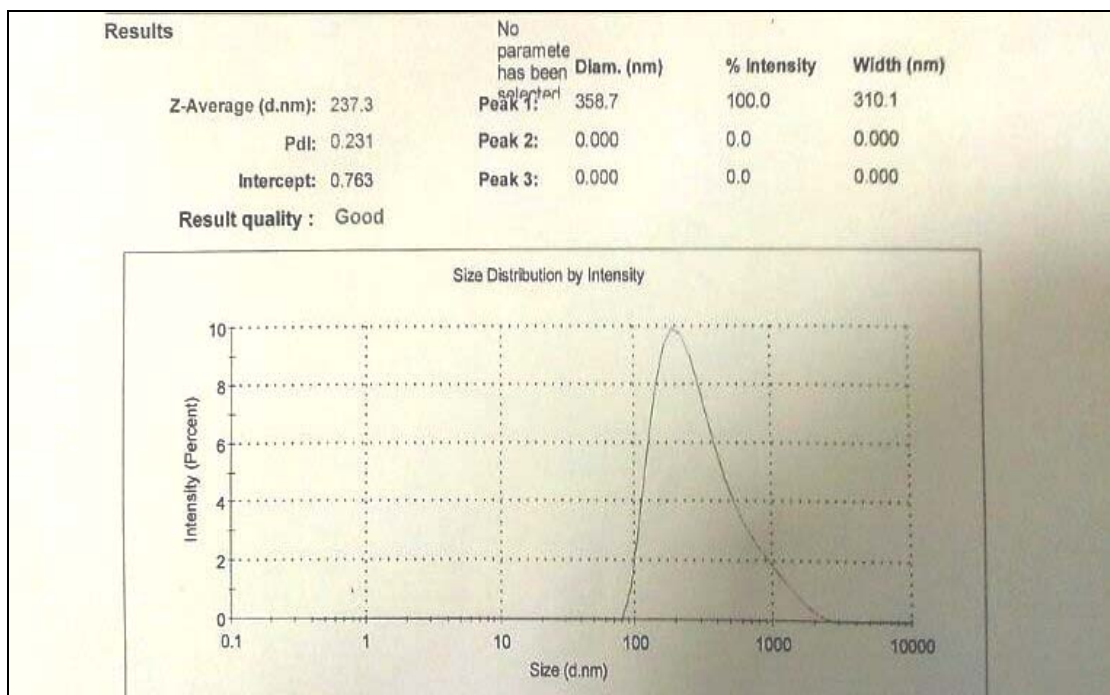


Figure 5A.59 Size of aceclofenac transferosome after storing for 3 months at temperature and humidity conditions of  $25^{\circ}\text{C} \pm 2^{\circ}\text{C}/60\% \text{RH} \pm 5\% \text{RH}$ .

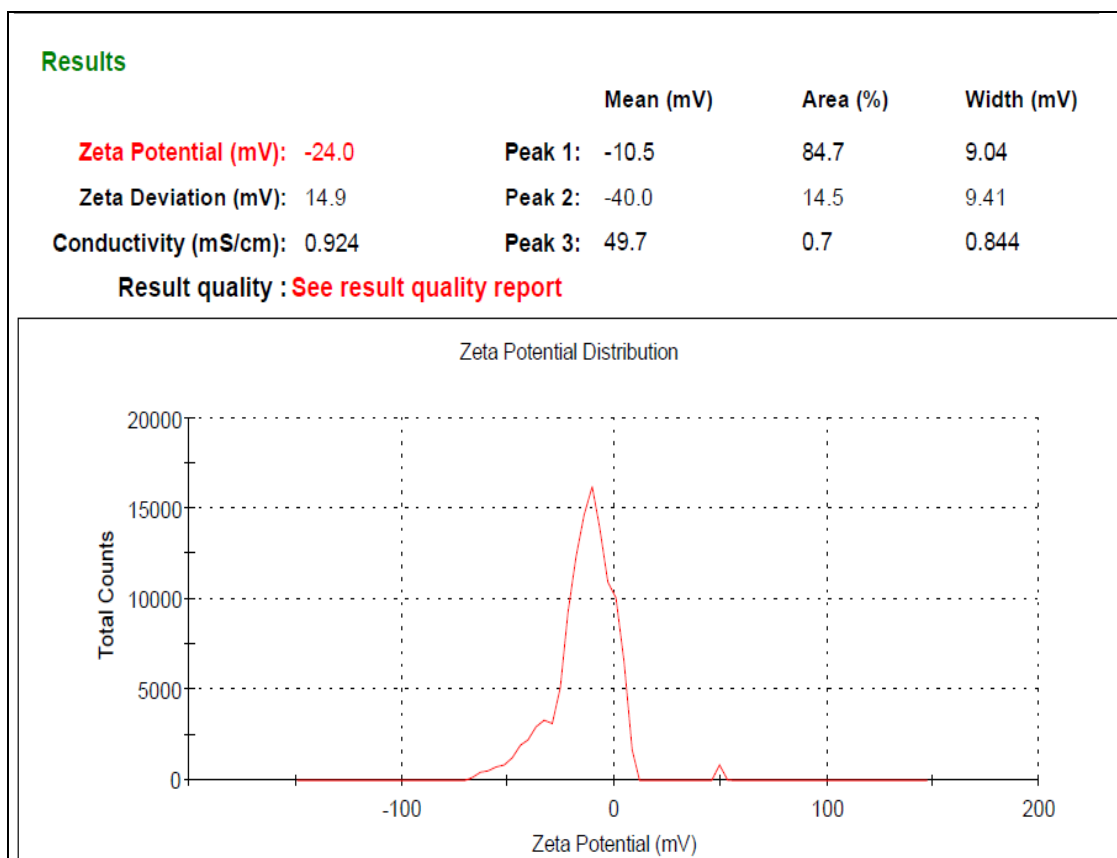


Figure 5A.60 Zeta potential of aceclofenac transferosome after 3 months at temperature of  $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$  and humidity of  $60\% \text{RH} \pm 5\% \text{RH}$

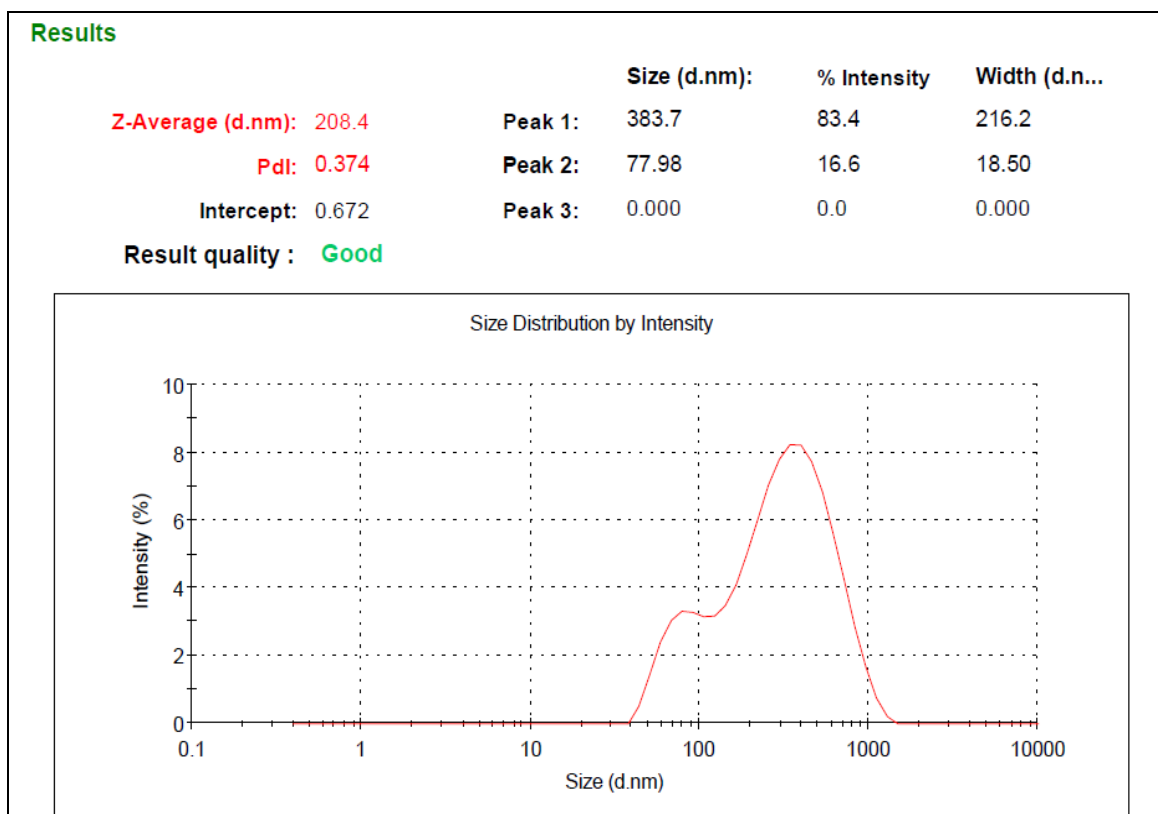


Figure 5A.61 Size of aceclofenac transferosome after storing for 6 months at conditions of temperature and humidity of  $25^{\circ}\text{C} \pm 2^{\circ}\text{C}/60\% \text{RH} \pm 5\% \text{RH}$ .

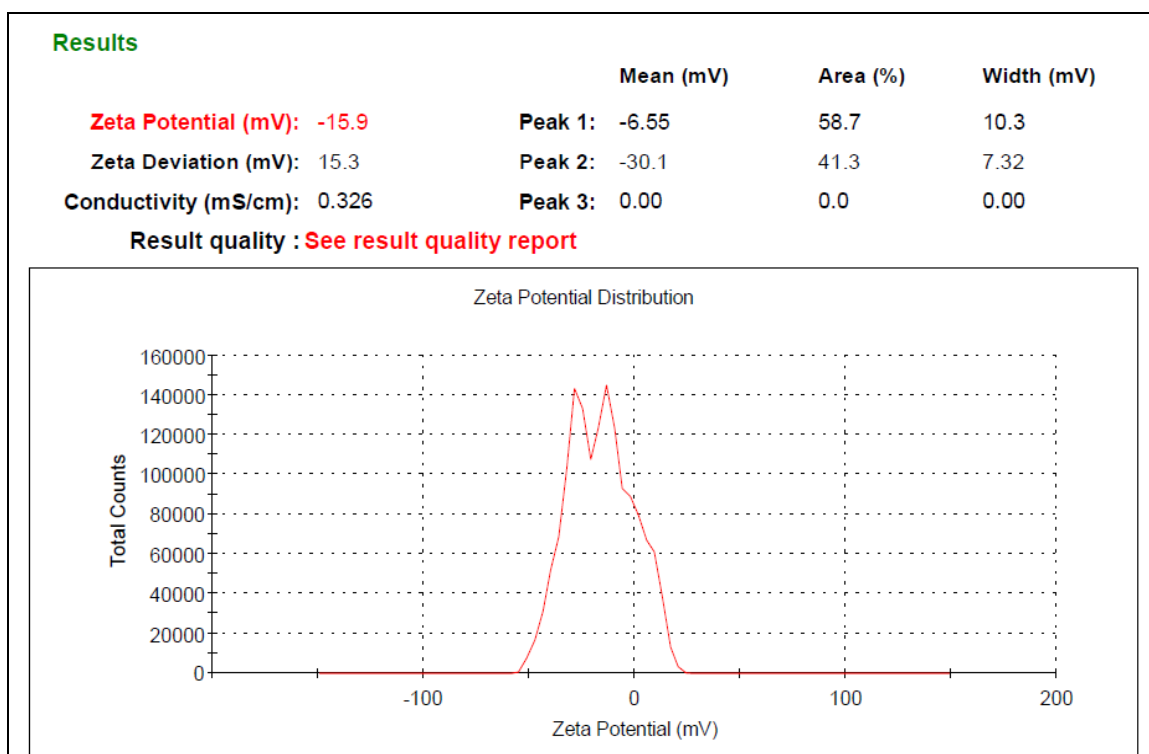


Figure 5A.62 Zeta potential of aceclofenac transferosome after storing for 6 months at temperature and humidity conditions of  $25^{\circ}\text{C} \pm 2^{\circ}\text{C}/60\% \text{RH} \pm 5\% \text{RH}$



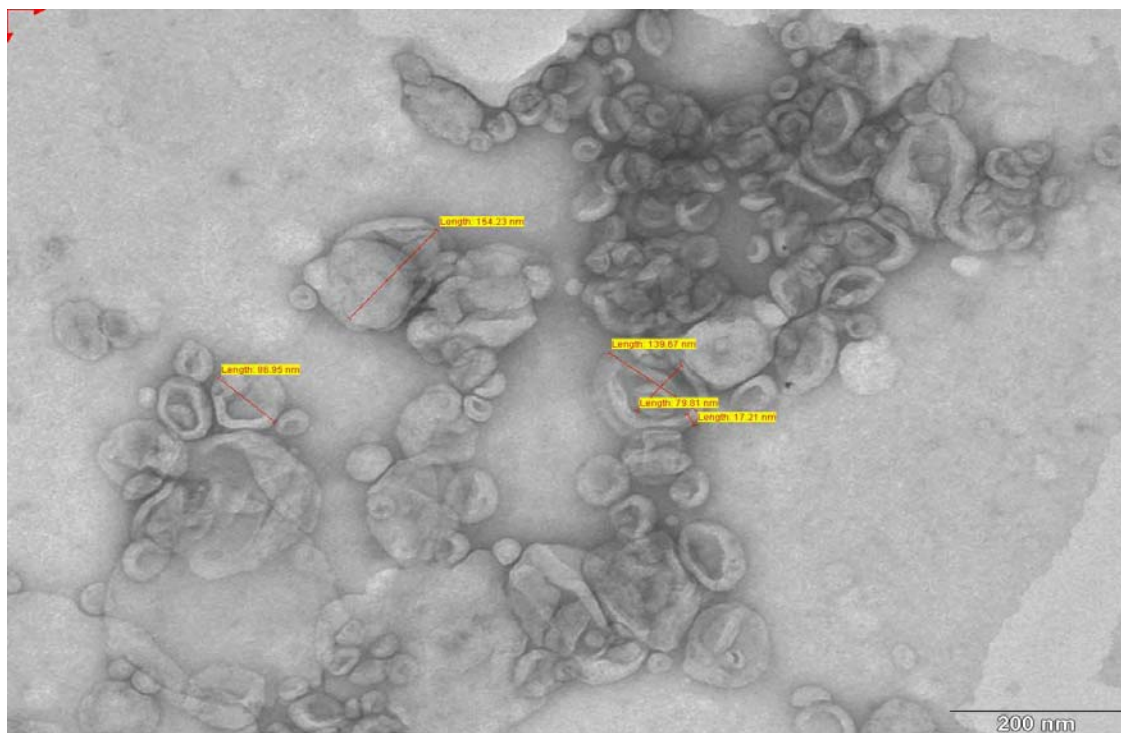


Figure 5A.63 TEM images of aceclofenac transferosome after storing for 3 months at temperature and humidity of  $25^{\circ}\text{C} \pm 2^{\circ}\text{C}/60\% \text{RH} \pm 5\% \text{RH}$

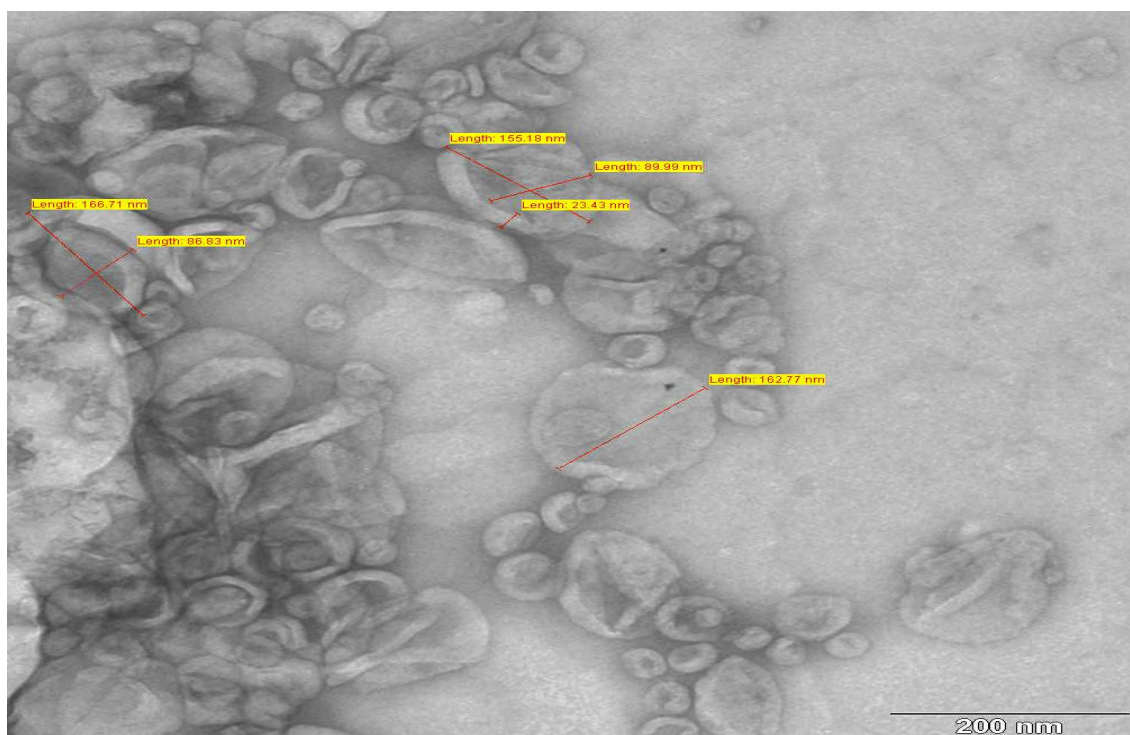


Figure 5A.64 TEM images of aceclofenac transferosome after storing for 6 months at conditions of temperature and humidity of  $25^{\circ}\text{C} \pm 2^{\circ}\text{C}/60\% \text{RH} \pm 5\% \text{RH}$

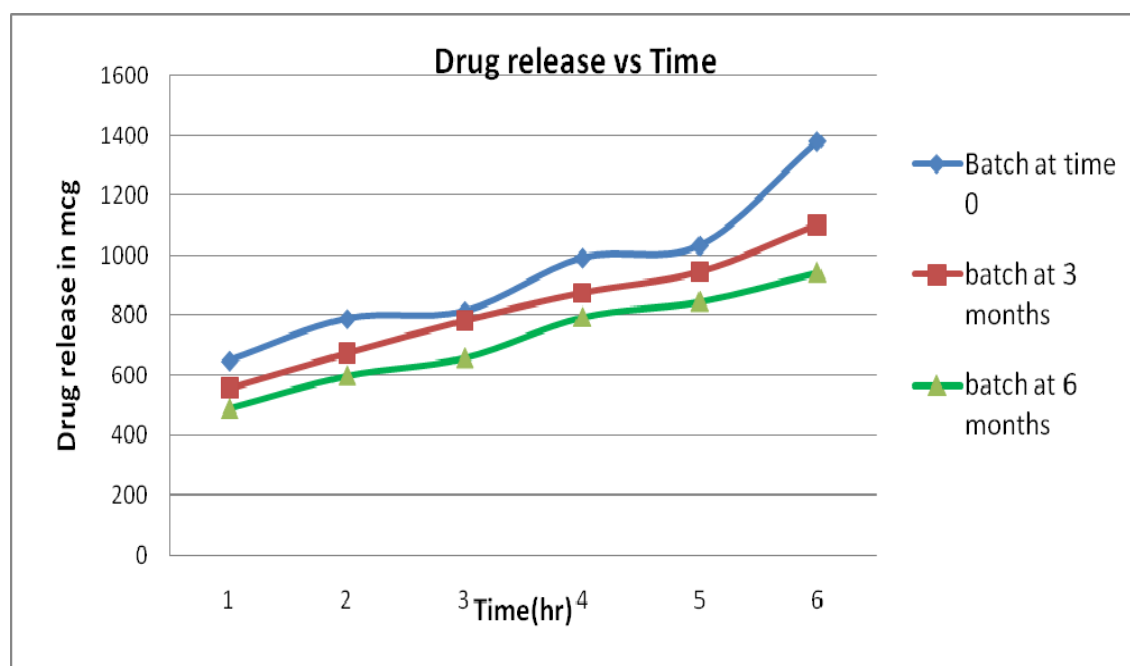
#### 5A.14.2 Cumulative drug release ( $\mu\text{g}$ ) in 24 hrs by transferosomal gel of aceclofenac for stability batches:-

The optimized transferosomal gel batch was subjected to stability studies for 3 months

and 6 months at temperature and humidity conditions of  $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$  and  $60\% \pm 5\%$  respectively and then drug release studies was performed. The purpose was to confirm the stability of the batch by finding out any significant change in the drug release after storing for the mentioned time period.

**Table 5A.50 Cumulative drug release ( $\mu\text{g}$ ) by stability studies batches transerosomal gel of aceclofenac**

Time (Hr)	Cumulative drug release ( $\mu\text{g}$ )		
	Initial batch	Batch at 3 months	Batch at 6 months
0	0	0	0
1	649.1	556.7	489.4
2	789.5	674.3	598.7
3	814.3	783.1	658.2
4	992.4	875.9	794.3
5	1034	946.2	846.3
6	1382.40	1103.6	943.6
24	1523	1387.4	1227.9



**Figure 5A.65 Drug release studies from batches at time- 0, 3 months and 6 months**

#### 5A.14.3 Statistical analysis for confirmation of stability of formulation:-

**One-way ANOVA analysis for drug permeation data of batches at 95% confidence interval:** – 6 months, 0 month, 3 months at temperature and humidity conditions of  $25^{\circ}\text{C} \pm 2^{\circ}\text{C}/60\% \text{RH} \pm 5\% \text{RH}$

Source	DF	SS	MS	F	P
Factor	2	189105	94553	1.18	<b>0.331</b>
Error	18	1444857	80270		
Total	20	1633962			

➤ The p value larger than the  $\alpha$ -level selected (0.05), so, the means are not significantly different.

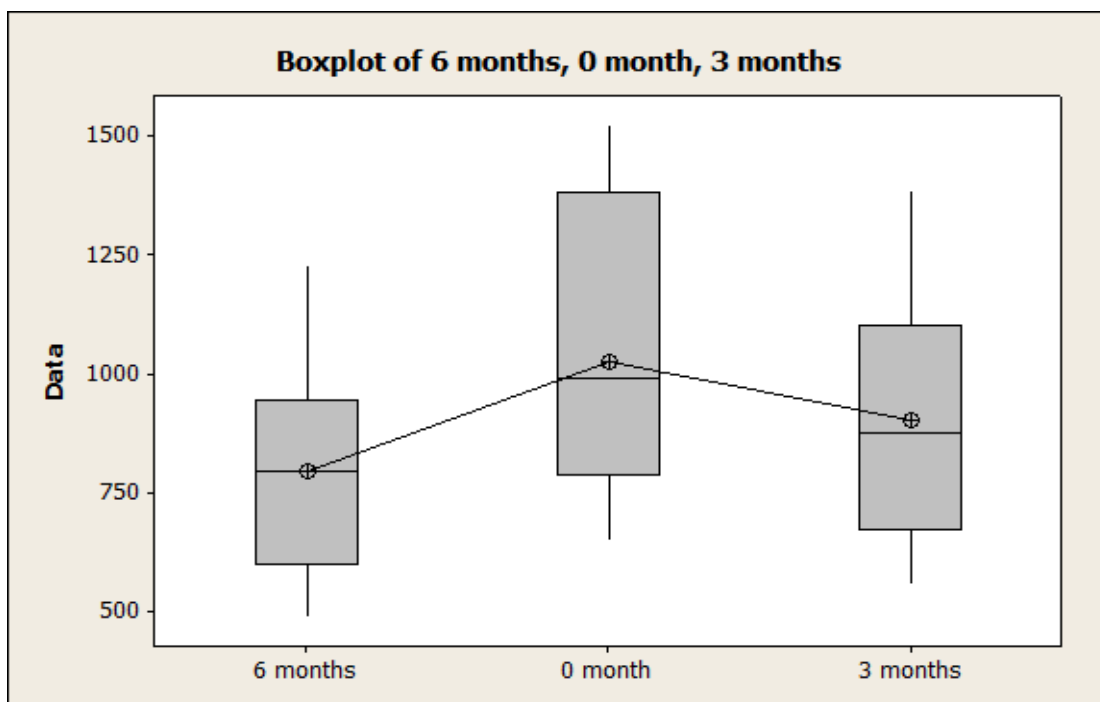
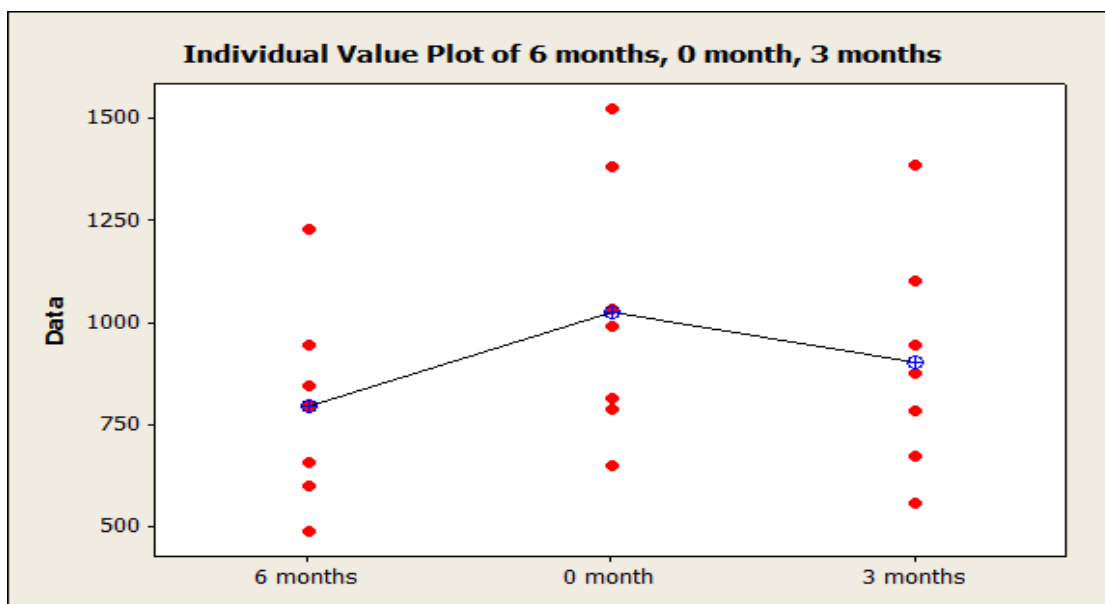


Figure 5A.66 ANOVA Analysis of stability batches of aceclofenac transferosomal gel.

- The drug permeation was found to be highest for batch at 0 month followed by 3 months and 6 months but statistically the difference was found to be not significant.

### ❖ Findings of stability studies:-

- On storage of aceclofenac transferosomal dispersion for 3 months at  $25^{\circ}\text{C} \pm 2^{\circ}\text{C}/60\% \text{RH} \pm 5\% \text{RH}$ , a slight increase in size and decrease in zeta potential in vesicles was observed, whereas on storage for 6 months at the same condition, slightly more increase in size and decrease in zeta potential was observed.
- The drug entrapment efficiency was also found to slightly decrease after storage at temperature and humidity conditions of  $25^{\circ}\text{C} \pm 2^{\circ}\text{C}/60\% \text{RH} \pm 5\% \text{RH}$  for 3 months and 6 months. The ex-vivo permeation flux was also found to decrease slightly, whereas the gel characteristics were found to be satisfactory.
- It was found that statistically, there is no any significant difference among the drug permeation profile from the batches kept at 0 month, 3 months and 6 months.

## 5B. Transferosome based transdermal gel of indomethacin:-

### 5B.1 Preformulation studies:-

#### 5B.1.1 FTIR studies-

The FTIR of drug sample was carried out by FTIR Shimadzu Corporation- 8400S DRS. The Sampling technique involved KBr pellet with resolution of 6 and scanning for 12-24 times. IR scan of drug indomethacin was taken after keeping at temperature and humidity conditions of  $40^{\circ}\text{C} \pm 2^{\circ}\text{C}$  and  $75\% \text{RH} \pm 5\% \text{RH}$  for 3 months. The IR spectrum of the plain drug was compared with reference IR Spectrum.

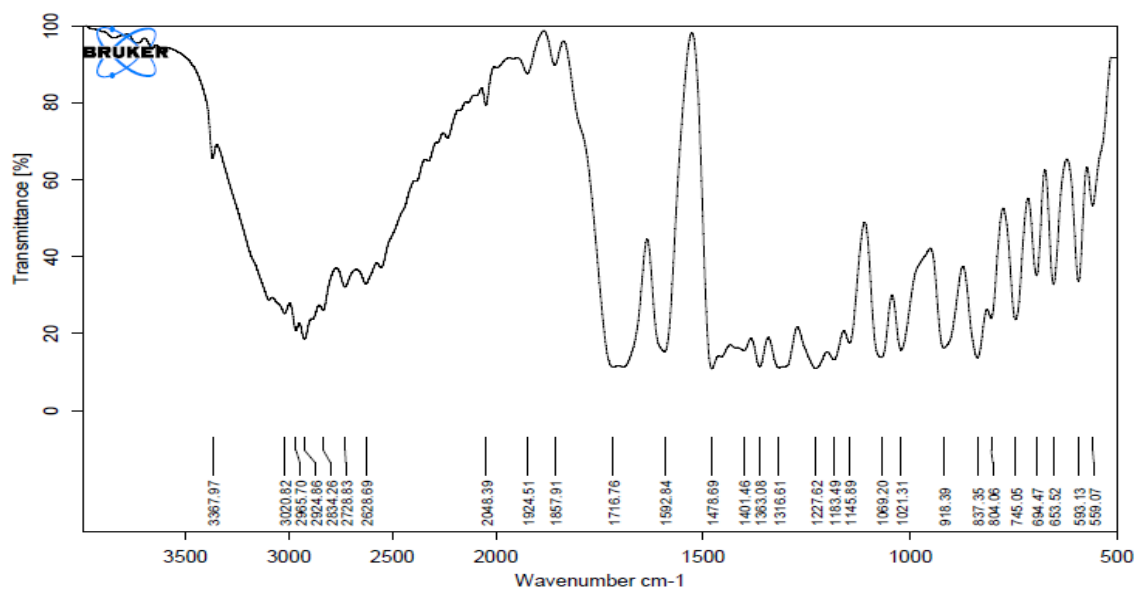


Figure 5B.1 FTIR of pure drug indomethacin

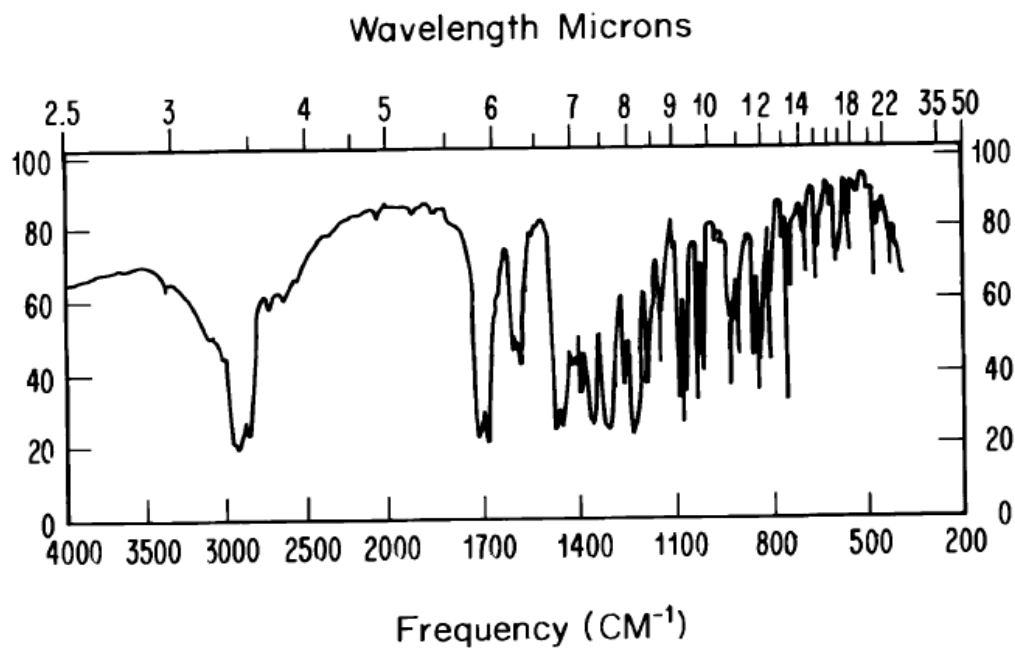


Figure 5B.2 Standard FTIR of pure drug Indomethacin

Source:-Analytical Profile of drug substances, Vol.13, pp:235

#### FTIR Interpretation:

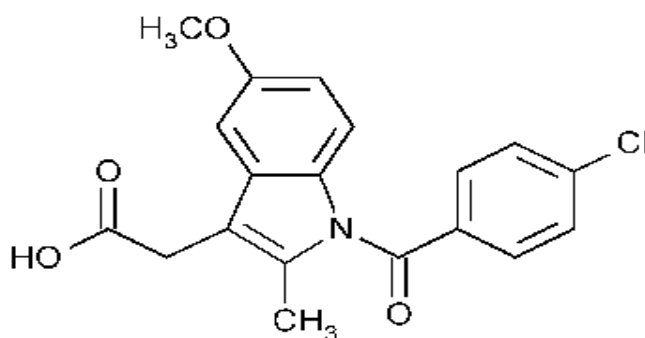


Figure 5B.3 Structure of indomethacin

Table 5B.1 Interpretation of FTIR spectra of indomethacin

Functional groups	Absorption (cm <sup>-1</sup> )	Range (cm <sup>-1</sup> )
Aromatic C-H stretching	3020.12	3400-2500
Aromatic C=C stretch	1601	1600
C=O stretching vibrations	1716.76	1715,1695
C-Cl stretching	653.52	750

**Interpretation:-** The FTIR spectra of sample fairly match with that of reference spectra particularly in fingerprint region thereby confirming the identity of indomethacin.

### 5B.1.2 UV Scan for determining $\lambda_{\max}$ of indomethacin in methanol :-

The indomethacin drug concentration of 10  $\mu\text{g/ml}$  in methanol was scanned between wavelengths of 200 nm to 400 nm using UV-Visible spectrophotometer Shimadzu Corporation, Japan, UV-1800.

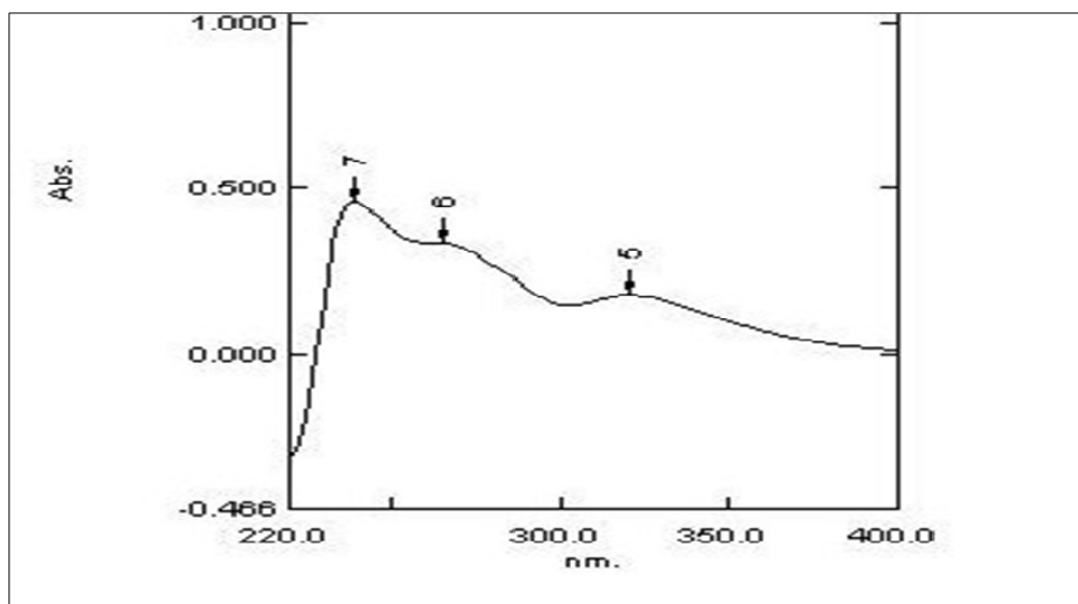


Figure 5B.4 UV scan of indomethacin in methanol

- From the UV scan of indomethacin, maximum absorbance was observed at wavelength of 239 nm in media methanol at different concentrations.

### 5B.1.3 Standard calibration curve of indomethacin in methanol:-

Standard plot was prepared using solutions of concentration range 2 to 16  $\mu\text{g/ml}$ .

Methanol was used as blank solution and absorbance was measured for each solution at  $\lambda_{\max}$  of 239 nm using Shimadzu Corporation, Japan (UV-1800).

Table 5B.2 Standard curve data of indomethacin in methanol

S. no	Conc.( $\mu\text{g/ml}$ )	Absorbance
1.	2	0.133 $\pm$ 0.001
2.	4	0.275 $\pm$ 0.005
3.	6	0.515 $\pm$ 0.005
4.	8	0.712 $\pm$ 0.01
5.	10	0.845 $\pm$ 0.01
6.	12	0.997 $\pm$ 0.005
7.	14	1.118 $\pm$ 0.01
8.	16	1.143 $\pm$ 0.01

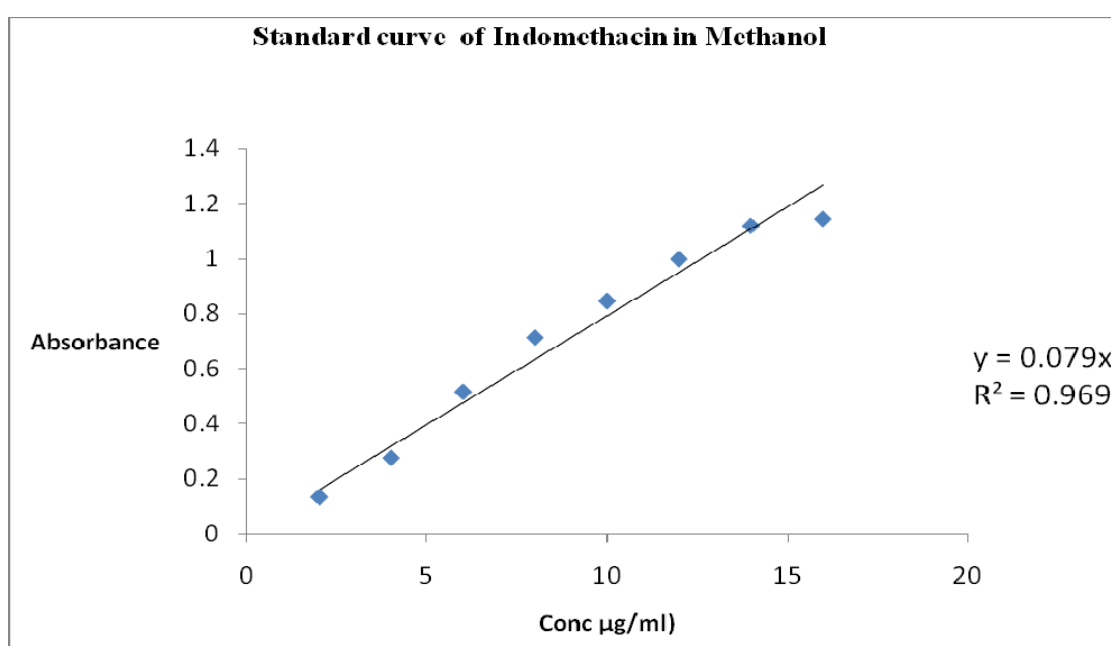


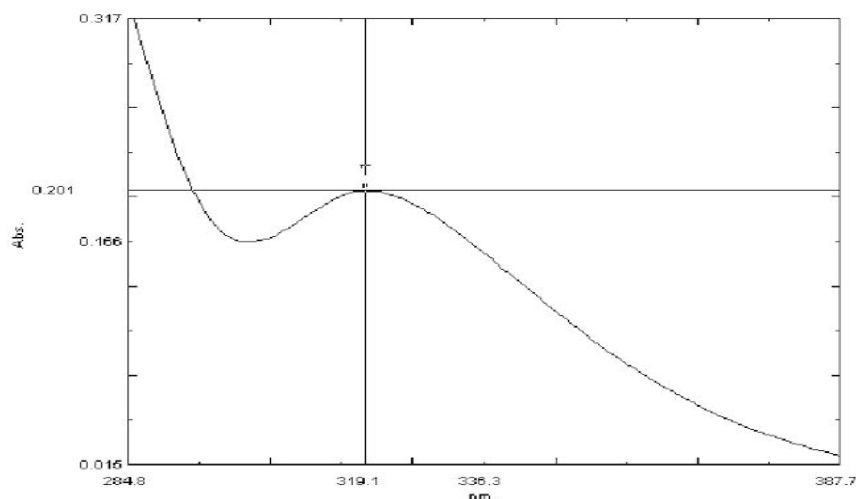
Figure 5B.5 Calibration curve of indomethacin in methanol

## 5B.2 Method of analysis of indomethacin in phosphate buffer pH 7.4:-

### 5B.2.1 UV Scan for determining $\lambda_{\text{max}}$ of indomethacin in phosphate buffer pH 7.4:-

The indomethacin drug concentration of 10  $\mu\text{g/ml}$  in phosphate buffer pH 7.4 was scanned between 200 nm to 400 nm using UV-visible spectrophotometer Shimadzu Corporation, Japan UV 1800.





**Figure 5B.6 UV scan of indomethacin in phosphate buffer pH 7.4**

Interpretation:- From the UV scan of indomethacin, maximum absorbance was observed at 319.1 nm in media Phosphate buffer pH 7.4 at different concentration. The reported  $\lambda_{\max}$  is 320 nm, hence it can be considered an evidence for identification of indomethacin.

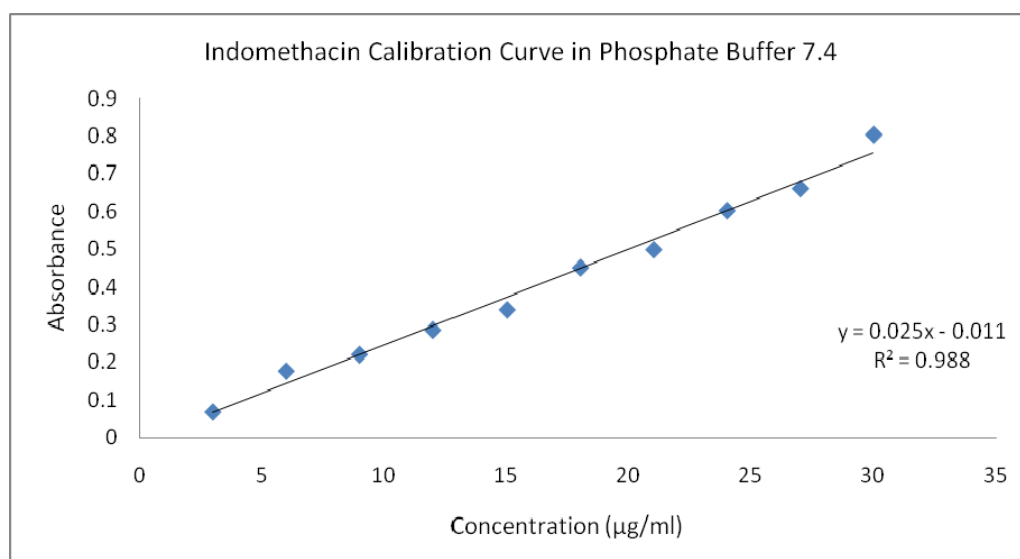
#### **5B.2.2 Standard calibration curve of indomethacin in phosphate buffer pH 7.4 :**

Standard plot was prepared using a concentration range 3 to 30  $\mu\text{g/ml}$ .

Phosphate buffer saline pH 7.4 was used as blank solution and absorbance was measured for each solution at  $\lambda_{\max}$  of 320 nm using Shimadzu Corporation, Japan (UV-1800).

**Table 5B.3 calibration curve of indomethacin in phosphate buffer pH 7.4**

<b>Concentration (<math>\mu\text{g/ml}</math>)</b>	<b>Absorbance</b>
3	0.0683 $\pm$ 0.001
6	0.1767 $\pm$ 0.005
9	0.2202 $\pm$ 0.01
12	0.2853 $\pm$ 0.01
15	0.3388 $\pm$ 0.01
18	0.4505 $\pm$ 0.005
21	0.4977 $\pm$ 0.01
24	0.6015 $\pm$ 0.01
27	0.659 $\pm$ 0.01
30	0.802 $\pm$ 0.01



**Figure 5B.7** Calibration curve of indomethacin in phosphate buffer pH 7.4

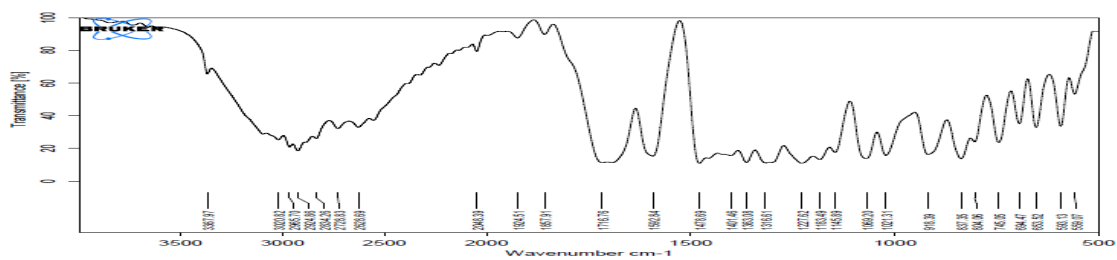
**Table 5B.4** Results of linearity and range

Solvent system	Range (µg/ml)	Coefficient of linearity found from graph
Phosphate buffer pH 7.4	3-30	0.988

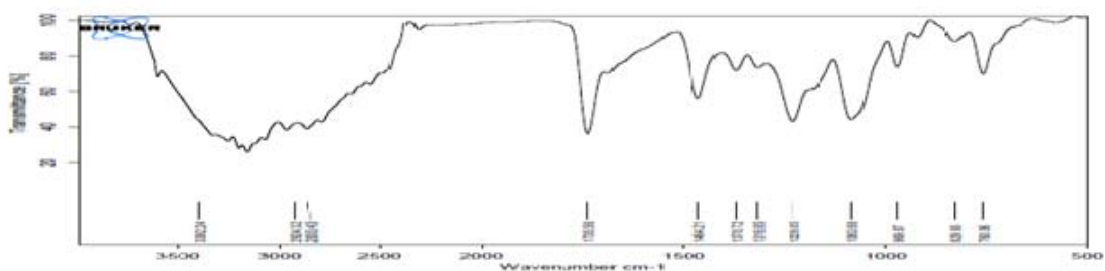
➤ The calibration curve of indomethacin was found to be linear as per the  $R^2$  value. The values of coefficient of linearity were found to be nearer to 1 for all solutions which concludes the linearity of the range selected.

### 5B.3 Drug-excipient compatibility studies:-

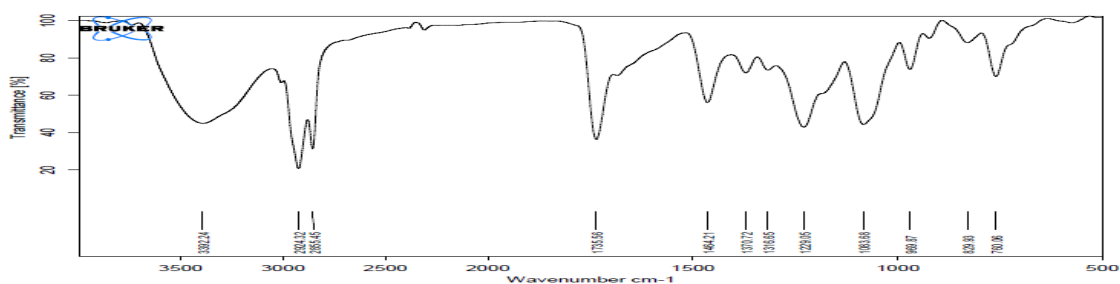
The screened phospholipid, 1,2-disteroyl-sn- glycerol-3-phospho-ethanolamine, Na salt and surfactant span 60 were subjected to compatibility studies with drug indomethacin to find out any interaction with drug. The interaction of cholesterol and carbopol gel base with drug, phospholipid and surfactant mixture was also studied.



A. IR Scan of indomethacin



B. IR scan of mixture of indomethacin, phospholipids, cholesterol and surfactant in ratio of 1:1:1:1.



C. IR scan of mixture of indomethacin, phospholipids, cholesterol, surfactant and gel base carbopol 934 in ratio of 1:1:1:1:1.

Figure 5B.8 FTIR Spectra of Physical mixture of drug and excipients

Table 5B.5 Results of FTIR spectra

Compounds	FTIR Interpretation
Indomethacin	Identity confirmed
Indomethacin + phospholipids and cholesterol in ratio of 1:1:1.	No interaction
IR scan of mixture of indomethacin, phospholipids, cholesterol, surfactant and gel base carbopol 934 in ratio of 1:1:1:1:1.	No interaction

➤ Indomethacin retains its characteristic peaks in physical mixture with excipients so, it can be concluded that there is no interaction between drug and excipients.

### 5B.4 Preparation of experimental design batches of transferosomes based on Box-Behnken design:-

The experimental design batches were prepared using the screened factors and their levels with the help of statistical software minitab 16. The responses of size, % drug entrapment and % drug release were determined and analyzed.

The quantity of phospholipid was varied as 50 mg to 100 mg (0.067-0.133 mMol), the quantity of surfactant varied as 25 mg to 50 mg (0.06-0.12 mMol) and quantity of cholesterol was varied as 25 mg to 50 mg (0.064-0.129 mMol) for the preparation of factorial batches. Quantity of indomethacin was taken as 100 mg (0.279 mMol).

**Table 5B.6 Experimental design batches of transferosomes of indomethacin based on Box- Behnken design.**

Formulation	Quantity of Phospholipid (mg)	Quantity of Surfactant(mg)	Quantity of Cholesterol(mg)	Avg. Size(nm)	% Drug entrapment
K1	75	50	25	250±20	38±1.4
K2	75	25	25	350±20	47±2.8
K3	50	50	37.5	300±55	52±3.9
<b>K4</b>	<b>100</b>	<b>37.5</b>	<b>50</b>	<b>380±60</b>	<b>58±1.9</b>
<b>K5</b>	<b>100</b>	<b>25</b>	<b>37.5</b>	<b>450±35</b>	<b>61±2.1</b>
K6	75	25	50	400±20	57±4.3
K7	50	37.5	25	350±40	49±3.7
K8	75	37.5	37.5	200±50	37±3.6
K9	75	37.5	37.5	200±40	39±2.4
K0	100	50	37.5	400±30	54±6.1
K11	50	25	37.5	380±20	53±2.3
K12	50	37.5	50	320±25	47±7.1
K13	75	37.5	37.5	220±15	36±6.9
K14	75	50	50	230±30	37±1.9
K15	100	37.5	25	360±20	50±2.6

**Interpretation:** - Highest drug entrapment was observed in the batch containing 100 mg of phospholipid, 25 mg of surfactant and 37.5 mg of cholesterol but the size of vesicles was higher than other formulations.

**5B.5 Evaluation of experimental design batches of transferosomes of indomethacin:-**

The experimental design batches were evaluated for in-vitro drug release characteristics using modified Franz diffusion cell using dialysis membrane. The in-vitro drug release studies were performed for all the fifteen batches of transferosomal gel and also for the plain drug gel for comparison.

**Table 5B.7 Cumulative drug release (µg) by experimental batches of transferosomal gel of indomethacin.**

Formulations	Time (hrs)							
	0	1	2	3	4	5	6	24
K1	0	389.12	564.33	698.28	898.37	989.54	1174.77	1349
K2	0	387.2	513.1	672.4	779.6	914.1	1268	1399
K3	0	454.2	612.8	784.5	917.3	1088	1234.11	1462
<b>K4</b>	<b>0</b>	<b>482.9</b>	<b>680.3</b>	<b>816.5</b>	<b>974.2</b>	<b>1103</b>	<b>1427</b>	<b>1517</b>
<b>K5</b>	<b>0</b>	<b>546.3</b>	<b>713.8</b>	<b>863.4</b>	<b>992.9</b>	<b>1176.2</b>	<b>1460</b>	<b>1568</b>
K6	0	497.1	610.8	811.2	1003.4	1064.1	1346	1485
K7	0	473.2	679.3	835.1	919.5	997	1255	1375
K8	0	476.3	684.1	794.8	998.6	1006	1281	1454
K9	0	411.8	597.2	713.8	889.1	1008.4	1263	1413
K10	0	453	578.3	728.6	863.2	982.5	1231	1416
K11	0	478.8	687.3	786.5	1012	1109	1276	1426
K12	0	475.3	619.7	749.4	937.2	1014	1310	1468
K13	0	518.5	646.1	756.2	919.6	988.2	1202	1387
K14	0	491.4	641.3	788.3	948.6	1064	1110	1416
K15	0	437.4	598	715	962.1	1078	1201	1396
Plain drug gel	0	374.6	479.2	658.9	816.4	998.2	1196	1403

Table 5B.8 % Drug entrapment and permeation flux ( $\mu\text{gcm}^{-2} \text{hr}^{-1}$ ) of transferosomal gel batches

Formulation	% Drug entrapment	In -vitro permeation flux ( $\mu\text{gcm}^{-2} \text{hr}^{-1}$ )
K1	38±1.4	24.15
K2	47±2.8	26.11
K3	52±3.9	24.50
<b>K4</b>	<b>54±1.9</b>	<b>28.14</b>
<b>K5</b>	<b>57±2.1</b>	<b>28.96</b>
K6	57±4.3	26.64
K7	49±3.7	21.26
K8	37±3.6	22.82
K9	39±2.4	25.80
K10	54±6.1	23.10
F11	53±2.3	24.61
K12	47±7.1	25.04
K13	36±6.9	19.12
K14	37±1.9	18.58
K15	50±2.6	14.79
Plain drug gel	-----	21.69

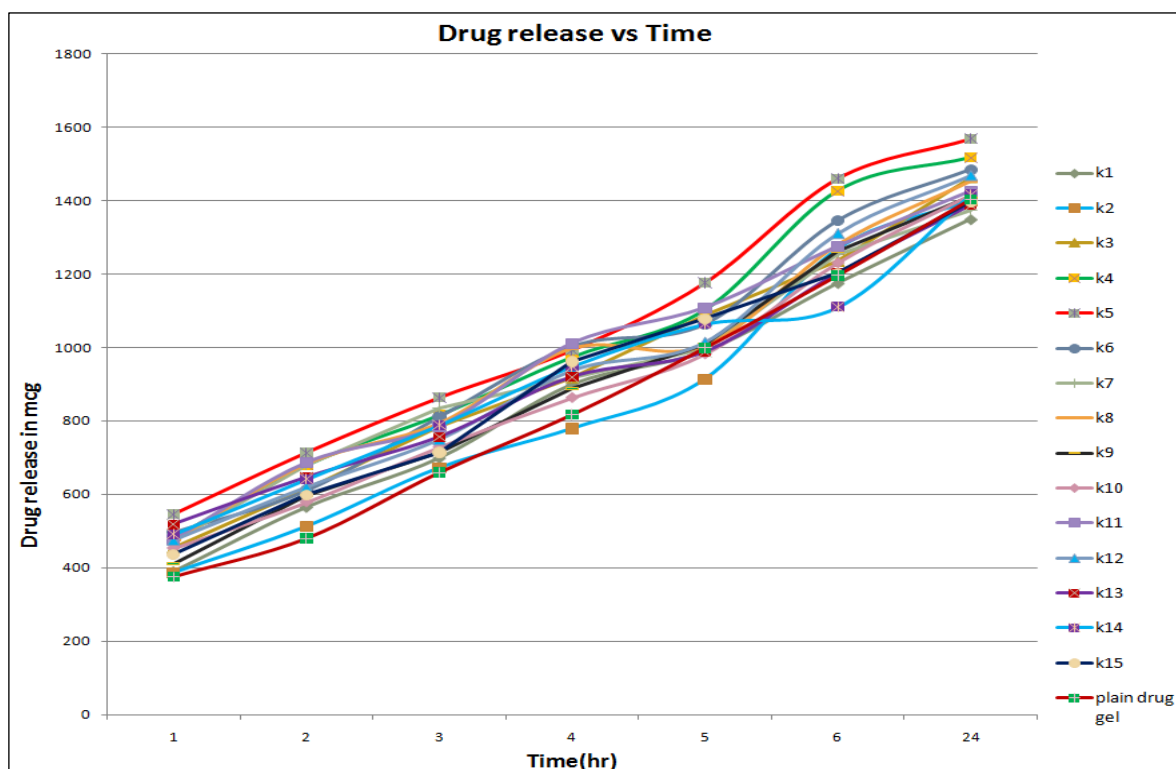


Figure 5B.9 Graph of in-vitro permeation studies of transferosomal gel of indomethacin

**Interpretation-**The drug entrapment for K4 and K5 were found to be high i.e. 54 % and 57% with permeation flux of  $28.14 \mu\text{g}\cdot\text{cm}^{-2} \text{hr}^{-1}$  and  $28.96 \mu\text{g}\cdot\text{cm}^{-2}\text{hr}^{-1}$  respectively. The average size of transferosome formulations F4 and F5 were 380 nm and 450 nm respectively, whereas the zeta potential values were also found to be -25.4 and -28.5 indicating moderate stability.

### **5B.6 Analysis of experimental design batches based on response surface methodology and formulation optimization:-**

The batches of transferosome prepared using experimental design were analyzed by statistical tools of response surface methodology to investigate the probable combinations of factors and their levels to achieve the closest target responses. The purpose was to find out an optimized formula of transferosome which can provide maximum drug entrapment and permeation through skin.

## ❖ Analysis of batches by contour plot :-

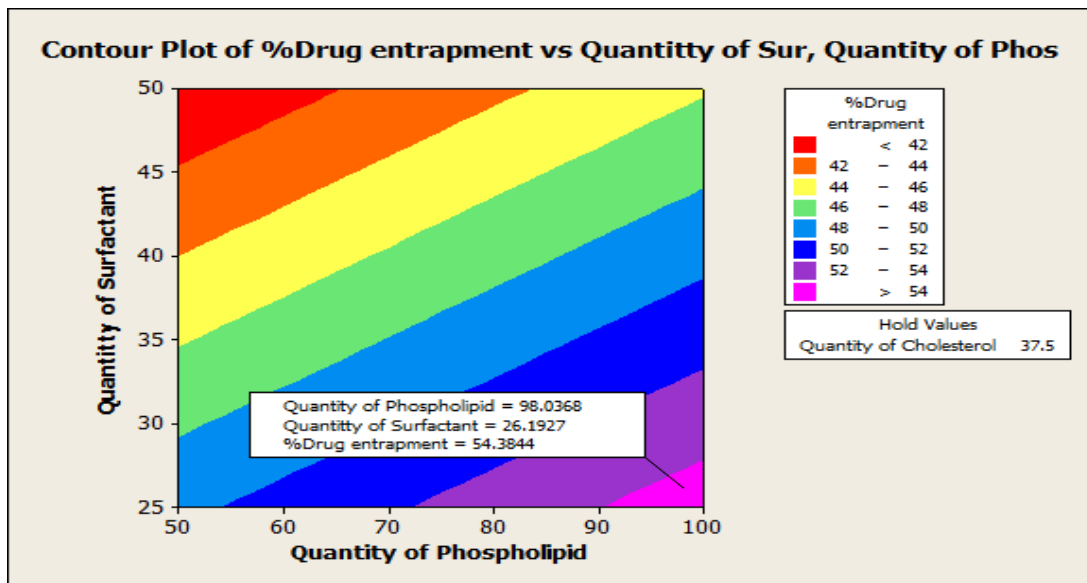


Figure 5B.10 Contour Plot for %drug entrapment of transferosome of indomethacin

Interpretation- From the contour plot of maximum % drug entrapment, the desired quantity of components in formulation will be phospholipid 98.03 mg and surfactant 26.19 mg

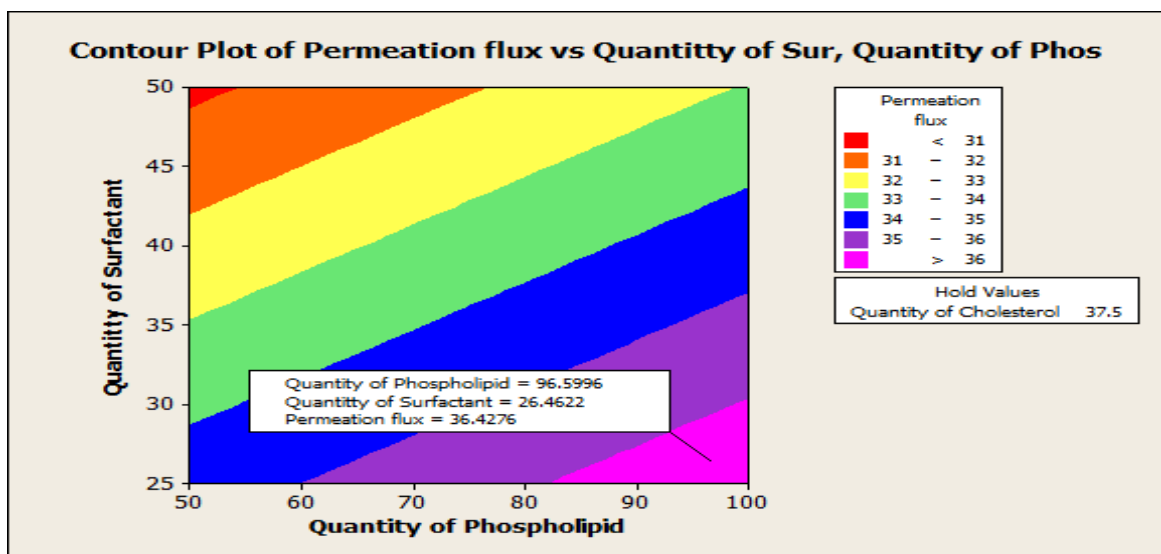


Figure 5B.11 Contour Plot for permeation flux of transferosomal gel of indomethacin

Interpretation- From the contour plot of maximum permeation flux, the desired quantity of components in formulation will be phospholipid 96.59 mg and surfactant 26.46 mg

- The contour plots showed the design space with some probable levels of factors which can provide maximum desired responses of drug entrapment and permeation flux.



❖ Probability Plots:-

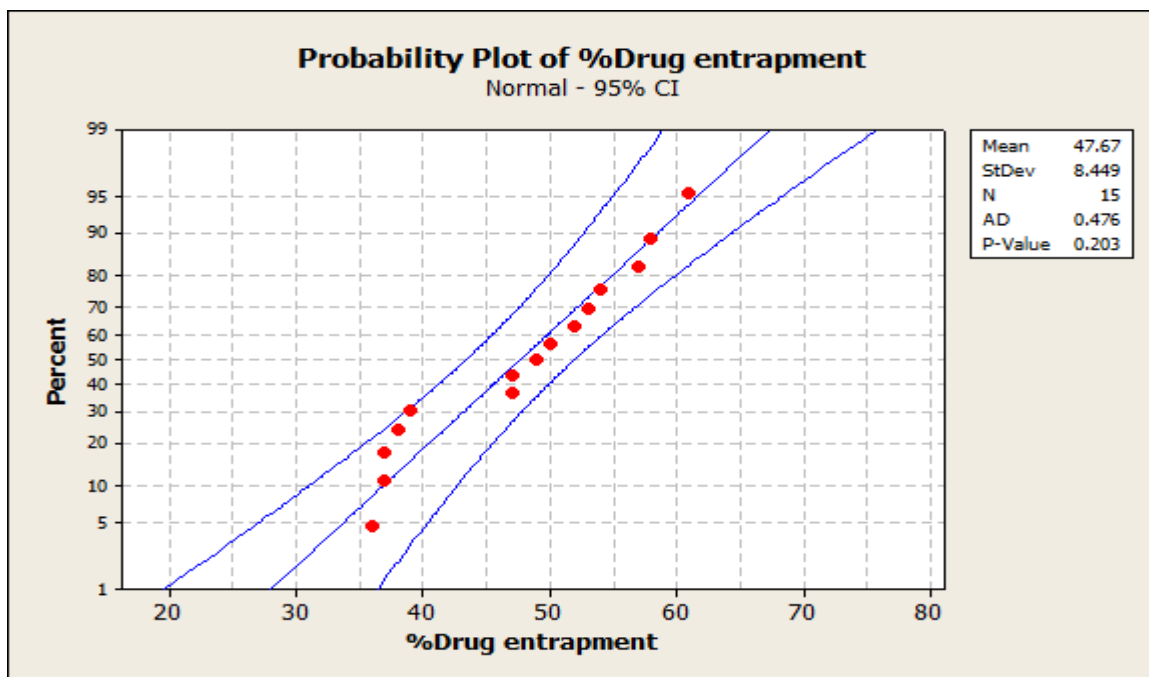


Figure 5B.12 Probability Plot for % drug entrapment

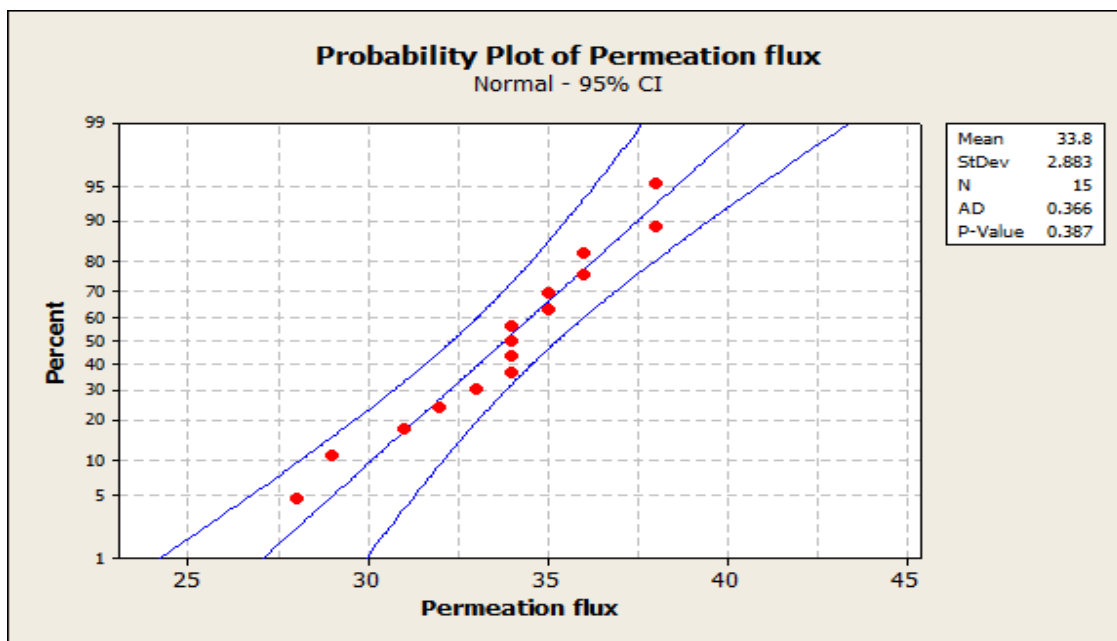


Figure 5B.13 Probability plot for permeation flux

- The probability plot showed that the drug entrapment and permeation flux data of all the experimental batches were normally distributed at 95% confidence interval.

### 5B.6.1 Optimization plot for determining the formula which can provide maximum desired responses:-

The desired target responses of % drug entrapment and skin permeation flux were set in the statistical software to obtain the best combination of levels of the factors to achieve the target responses. The target responses were the values of responses close to the best responses of % drug entrapment and permeation flux obtained from the experimental design batches. The desirability value mentioned in the optimization plot gave the prediction of possibility of achieve the target responses by preparing the transferosome formulation using the given combination of quantity of phospholipid, surfactant and cholesterol.

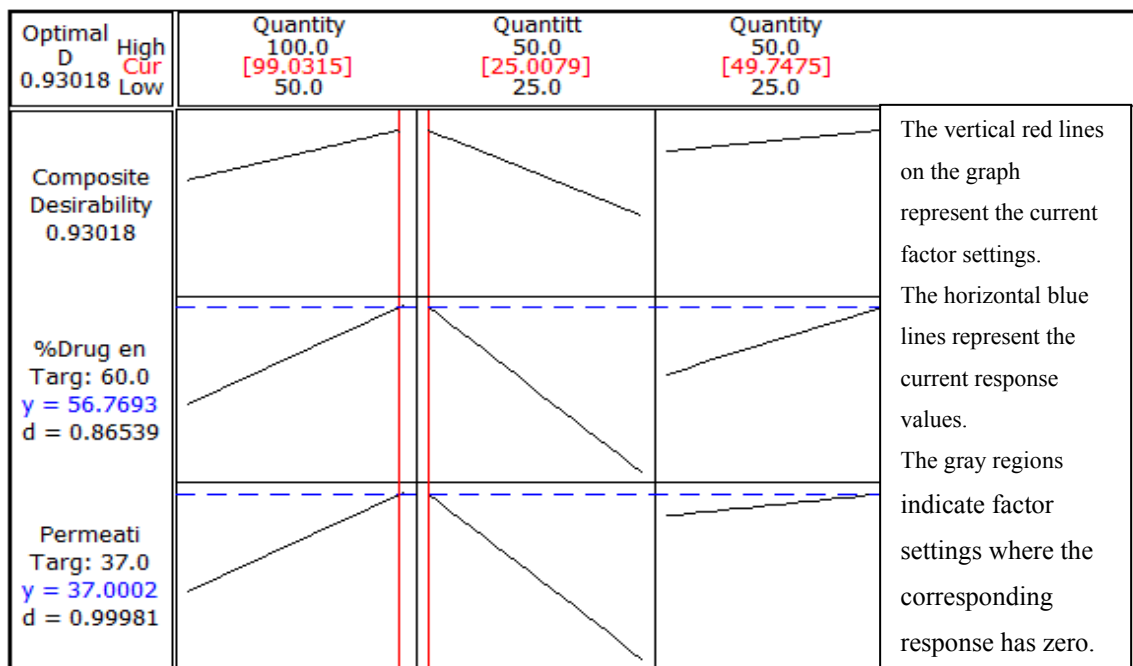
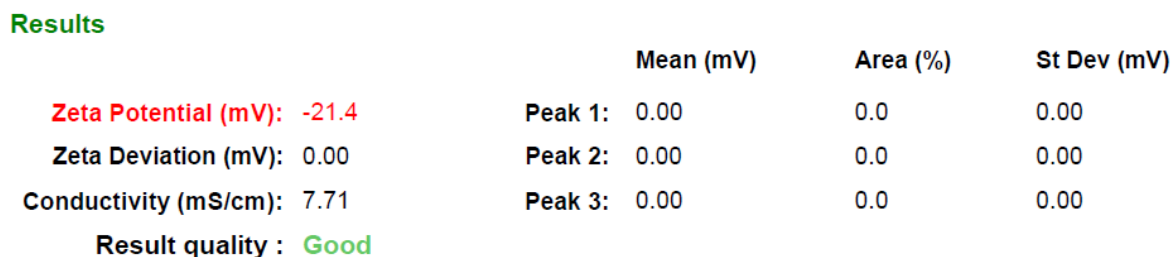
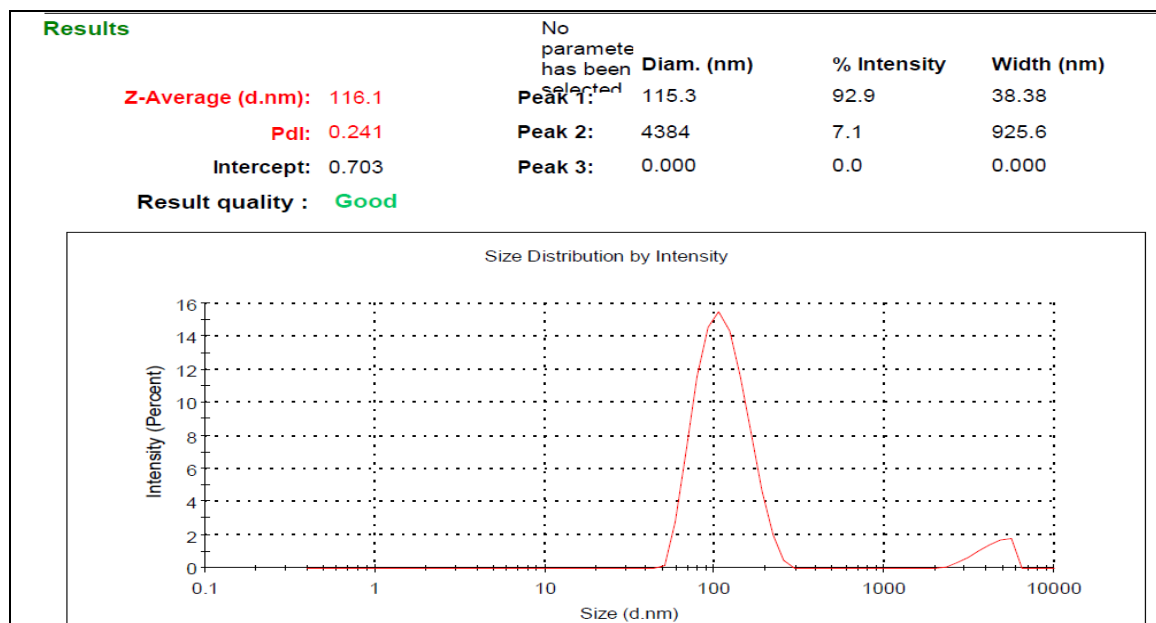


Figure 5B.14 Optimization plot for transferosome composition of indomethacin

**Optimization Plot:-** From the optimization plot it was found that predicted formula for maximum drug entrapment and permeation flux is phospholipid 99.03 mg, surfactant-25 mg and cholesterol 49.74 mg in formulation.

➤ The transferosomes of indomethacin were prepared as per the predicted formula for optimization and evaluated further for size, shape, zeta potential, % drug entrapment, ex-vivo permeation flux using excised rat skin, viscosity, pH, spreadability, gel strength and rheological properties.

**5B.6.2 Size and Zeta potential of optimized transferosome formulation as determined by dynamic light scattering (Zeta Sizer) :-**



**Figure 5B.15 Size and zeta potential of optimized batch of transferosomes of indomethacin**

- The optimized batch of transferosome shows size of 116 nm and a zeta potential of -21.4 indicating lesser size and uniform vesicle size distribution.

**5B.6.3 Rheological studies of optimized carrier incorporated gel :-**

The rheological properties of the transferosomal gel was determined and also compared with the plain drug gel to ensure the capability to withstand the stress due to handling and transportation and also to confirm the ease of application.

Table 5B.9 Rheological behavior of gel (Brookfield helipath: spindle no. 96)

RPM	Viscosity in centipoises			
	Transferosomal gel		Plain gel	
	↓	↑	↓	↑
2	31800	26338	33987	24860
3	27308	18669	23784	21976
5	17598	12552	19442	14931
10	11009	7895	13632	12014
20	6873	4978	8879	7114
30	5673	5673	5668	5668

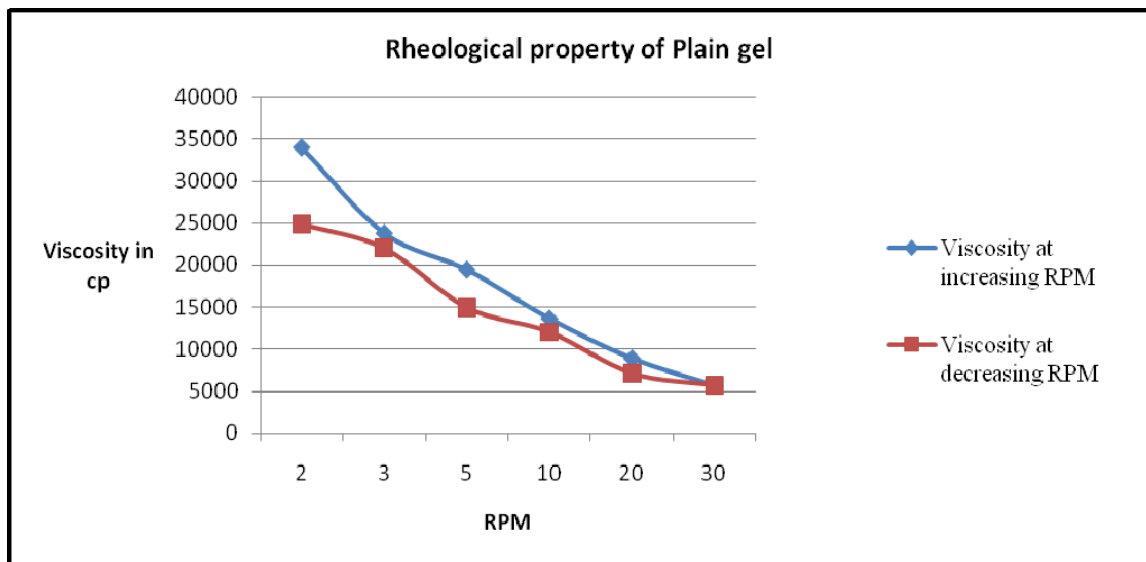


Figure 5B.16 Rheological behavior of plain drug gel

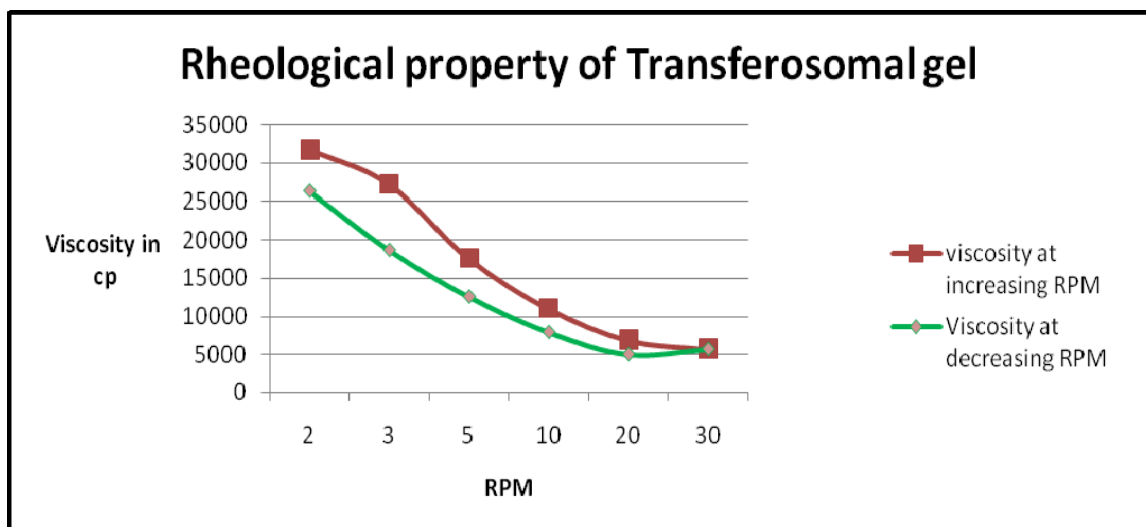


Figure 5B.17 Rheological behavior of transferosomal gel

**Interpretation:-** Drug carrier incorporated gel showed pseudoplastic behavior. This indicated that upon application of minimum shear stress, they would thin out but once the shear stress is removed they would regain their normal thickness. This ensures the physical integrity of the formulated gels under various stress conditions like manufacturing, handling and packaging.

**5B.6.4 Evaluation data of optimized batch of transferosome and transferosome in gel based on response surface methodology :-**

**Table 5B.10 Evaluation results of optimized batch of transferosomal gel of indomethacin**

Optimized formula	Size (nm)	Zeta potential (mv)	% drug entrapment	Ex-vivo Permeation flux ( $\mu\text{gcm}^{-2}\text{hr}^{-1}$ )	Viscosity (cp)	pH	Spreadability (gm.cm/s)	Gel strength (gm)
Transferosomal gel	116.1	-21.4	52.32%	26.11	5673	6.2	1.398	21.76
Plain drug gel	-	-	-	21.69	5668	6.0	1.363	24.23

- The optimized batch of transferosome shows size of 116 nm and a zeta potential of -21.4 indicating lesser size and uniform vesicle size distribution.
- The optimized batch showed closeness to the target and thus exhibit a good % drug entrapment and ex-vivo permeation flux as well as good handling characteristics.
- The transferosomal gel showed better % drug entrapment and ex-vivo permeation flux as compared to plain drug gel.

**5B.6.5 Transmission electron microscopy images of optimized formulation:-**

Transmission Electron Microscopy (Tecnai 20, Make: Philips, Holland) was performed at SICART, Gujarat. For analysis 2 drops of sample was placed on carbon support film, dried and then 1% solution of urenyl acetate was used for negative staining.

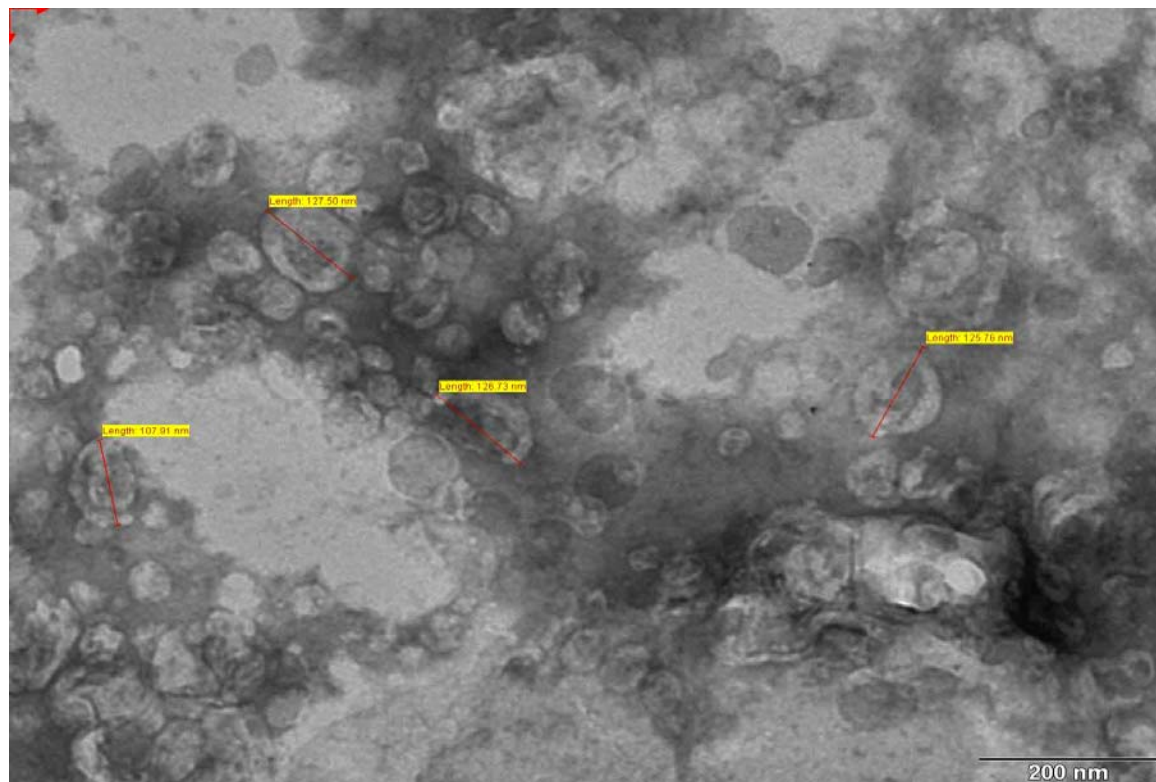


Figure 5B.18 TEM images o transferosomes of indomethacin

**5B.6.6 The Ex-vivo release data of optimized transferosomal gel formulation after 6 hrs of release studies:-****5B.6.7**

Table 5B.11 The Ex-vivo release data after 6 hrs of release studies

Formulation	% Drug			
	In acceptor compartment	Retained On Surface Skin	In skin	Loss
Transferosomal gel	78.40%	14.02%	4.33%	3.25%
Plain gel	70.15 %	17.56%	6.24%	6.05%

**Interpretation-** It was observed that, better permeation (78.40 %) of drug across rat skin takes place through transferosomal gel. The permeation through plain indomethacin gel is significantly less than the drug carriers incorporated gel.

### 5B.7 Analgesic activity by hot plate method in rats:-

The time of latency was determined as the time period between the zero point, when the animal is placed on the hot plate surface, and the time when the animal jumps off to avoid thermal pain.



**Figure 5B.19 Analgesic activity determination of transerosomal gel of indomethacin by Eddy's hot plate method**

**Table 5B.12 Analgesic activity of indomethacin transdermal formulations on rats**

Formulation	Reaction time in seconds at time intervals (minutes)					
	30	60	90	120	150	180
Control	4	3	5	4	3	4
Transerosomal gel	9	10	11	14	14	13
Plain drug Gel	8	9	11	11	7	6

- **Comparison of analgesic activity:-**

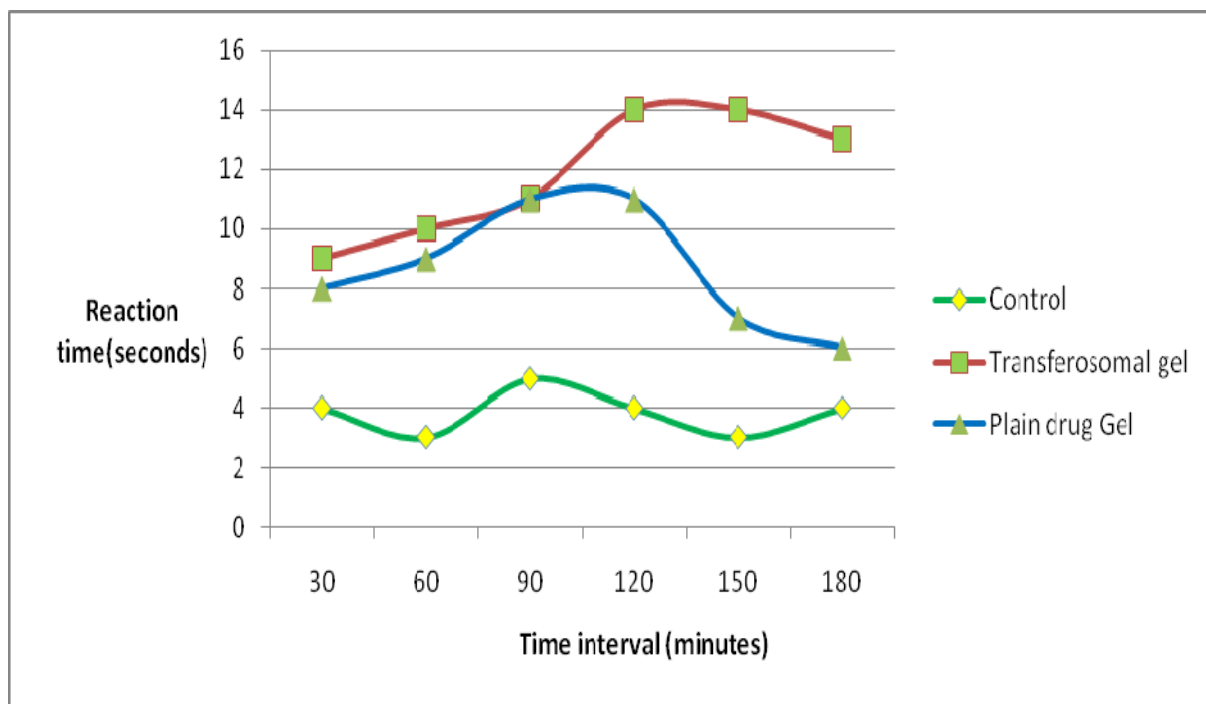


Figure 5B.20 Comparison of analgesic activity

- The group of rats who received the application of transfersosomal gel of indomethacin showed more tolerance to pain as compared to the group of rats who received the application of plain drug gel of indomethacin. Therefore, it can be interpreted that transfersosomal gel of indomethacin showed better analgesic activity than plain drug gel.



- ❖ Confirmation of improvement in analgesic activity by statistical analysis by two sample T test:-

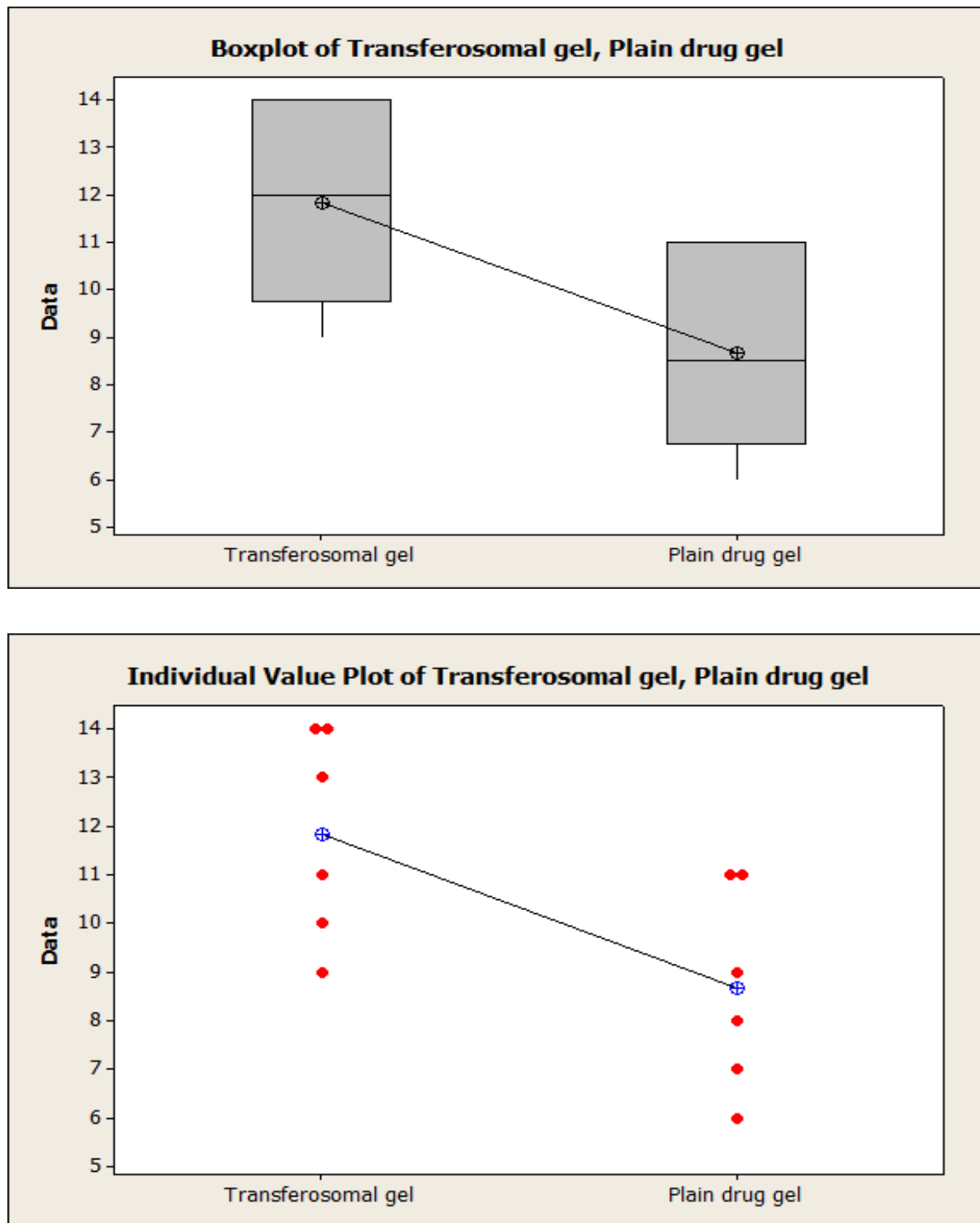


Figure 5B.21 Box plot and individual plot for statistical comparison

❖ **Two-Sample T-Test at 95% confidence interval:-**

	n	mean	st. dev
mean			
Transferosomal gel	6	11.83	2.14
Plain drug gel	6	8.67	2.07

difference =  $\mu$  (transferosomal gel) -  $\mu$  (plain drug gel)

Estimate for difference: 3.17, 95% CI for difference: (0.42, 5.91)

**p- Value = 0.028**

At 95% confidence interval, p value of less than 0.05 indicated that there is significant improvement in anti-inflammatory activity of indomethacin in the form of transferosomal gel formulation as compared to plain drug gel.

### **5B.8 Anti-inflammatory studies of formulations by rat paw edema method:**

The anti-inflammatory activity was carried out by carrageenan induced paw edema method to compare the activity of transferosomal gel and plain drug gel of indomethacin using plethysmometer.

The % inhibition of edema was calculated for each group using the following equation:-

$$\% \text{ inhibition of edema} = 1 - [(a - x) / (b - y)] \times 100$$

a= mean paw volume of treated animal after carageenan injection

x= mean paw volume of treated animal before carageenan injection

b= mean paw volume of control animal after carageenan injection

y= mean paw volume of control animal before carageenan injection

Table 5B.13 Rat paw volume measurement data

Group	Paw volume in ml at time intervals							
	0 hr	1 hr	2 hrs	3 hrs	4 hrs	5 hrs	6 hrs	24 hrs
I	0.59	0.98	1.12	1.18	1.19	1.19	1.14	0.87
II	0.58	0.84	0.86	0.82	0.82	0.76	0.74	0.66
III	0.58	0.86	0.97	0.93	0.89	0.84	0.82	0.71

Table 5B.14 Anti-inflammatory activity determination by rat paw edema method using plethysmometer

Rat Group	Applied Formulation
I	Control
II	Indomethacin transferosomal gel
III	Indomethacin plain gel

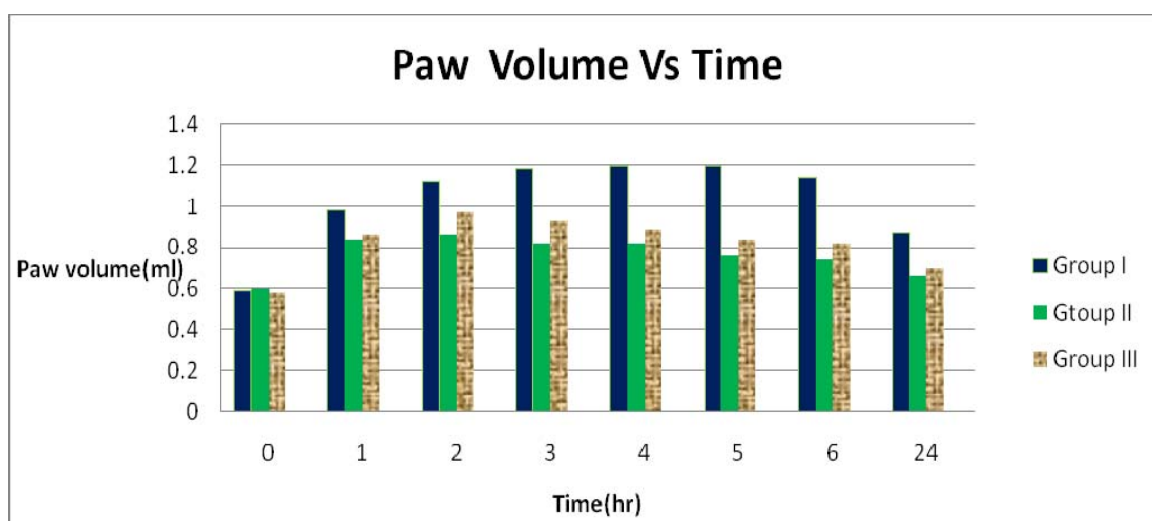


Figure 5B.22 Comparison of anti-inflammatory activity

Table 5B.15 Anti-inflammatory activity of formulations in groups of rat

Group	% Anti-inflammatory activity						
	1 hr	2 hr	3 hr	4 hr	5 hr	6 hr	24 hr
I							
II	33%	48%	50%	61.6%	70%	71.9%	71.4%
III	28%	26%	40.8%	44.6%	53.6%	51.8%	51.1%

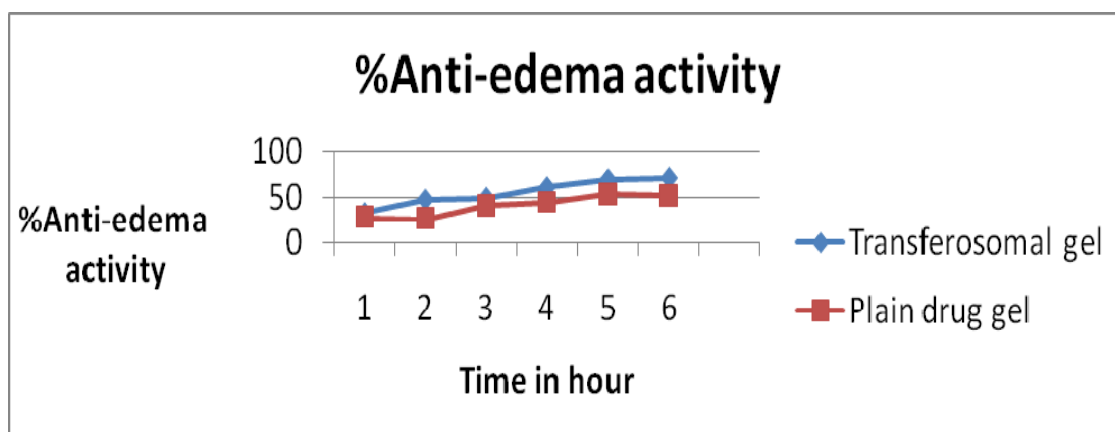
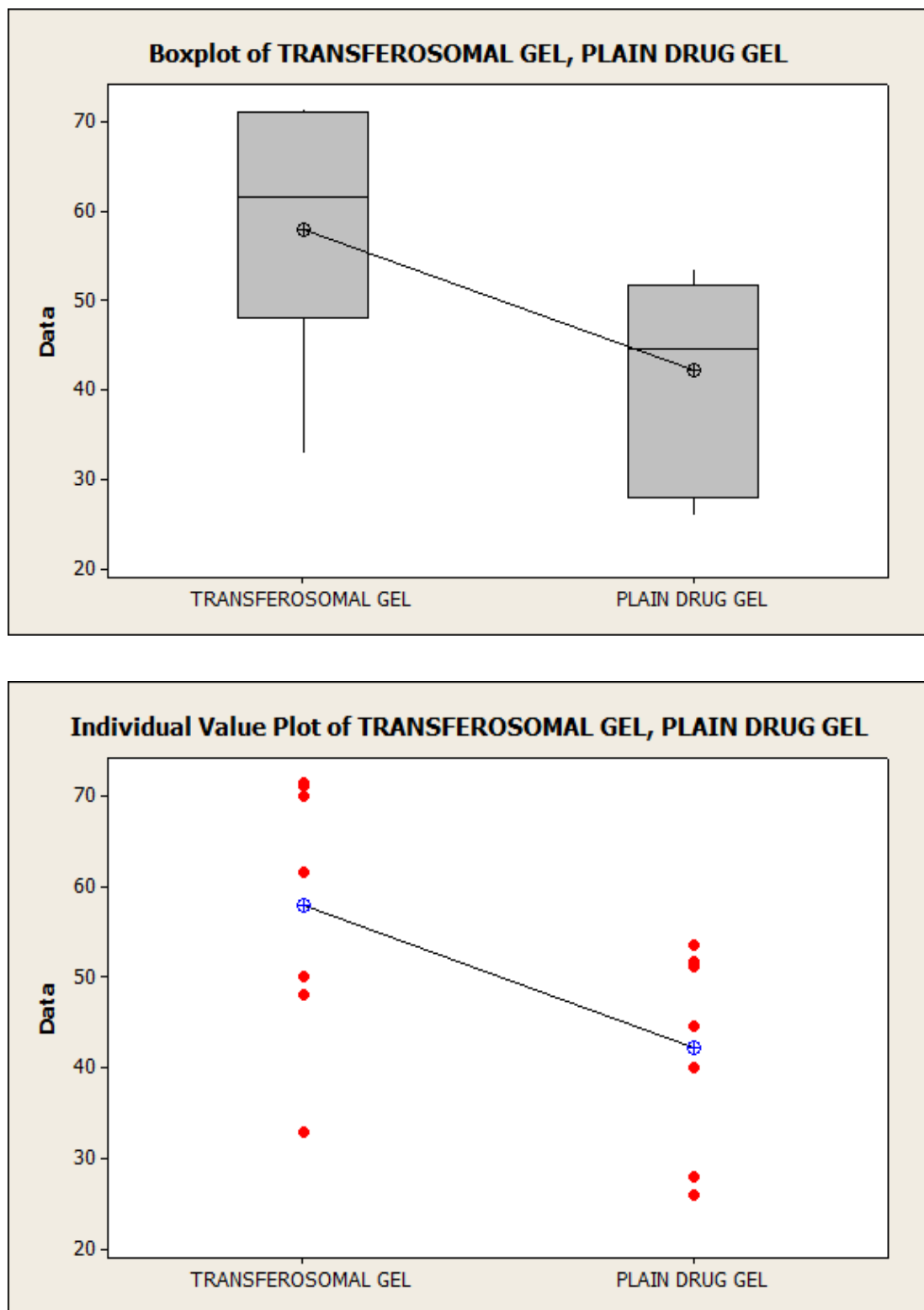


Figure 5B.23 Comparison of anti-inflammatory activity

**Interpretation:** - The paw volume in rats after topical application of transfersosomal gel was found to be lower than paw volume after application of plain drug gel, so the transfersosomal gel has better anti edema activity than plain drug gel.

**5B.8.1 Statistical confirmation of betterment in Anti-inflammatory activity by two sample T test:-**



**Figure 5B.24 Box plot and individual plots for statistical comparison of anti-inflammatory activity**

❖ **Two-sample t for transferosomal gel vs. plain drug gel:-**

	N	Mean	St. Dev
Transferosomal gel	7	57.9	14.7
Plain drug gel	7	42.2	11.4

Difference =  $\mu$  (Transferosomal gel) –  $\mu$  (Plain drug gel)

Estimate for difference: 15.70

95% CI for difference: (0.25, 31.15)

**P-Value = 0.047**

- At 95% confidence interval, P value of less than 0.05 indicated that there is significant improvement in anti-inflammatory activity of indomethacin in the form of transferosomal gel formulation as compared to plain drug gel.

### **5B.9 Stability studies of transferosomes and transferosomal gel of indomethacin:-**

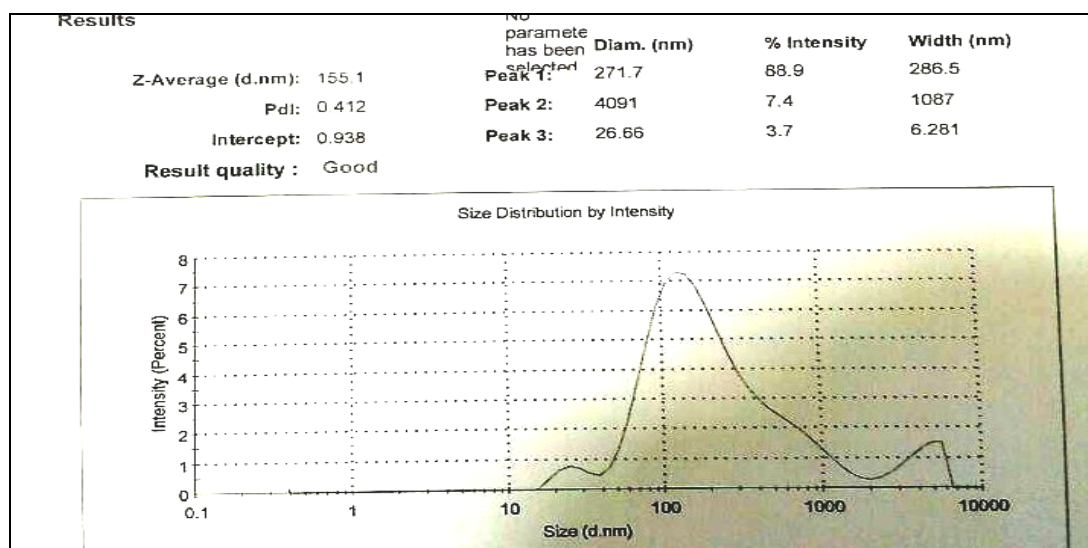
Conditions- The transferosomes and transferosomal gel of Indomethacin were subjected to accelerated stability testing.

- Long term = Temperature of  $5^{\circ}\text{C} \pm 3^{\circ}\text{C}$  for 12 months
- **Accelerated condition=  $25^{\circ}\text{C} \pm 2^{\circ}\text{C}/60\% \text{ RH} \pm 5\% \text{ RH}$  for six months**

- After keeping for specified time period at accelerated stability conditions, the formulations were evaluated for size, zeta potential and entrapment efficiency for transferosomes and permeation flux and gel characteristics for transferosomal gel.

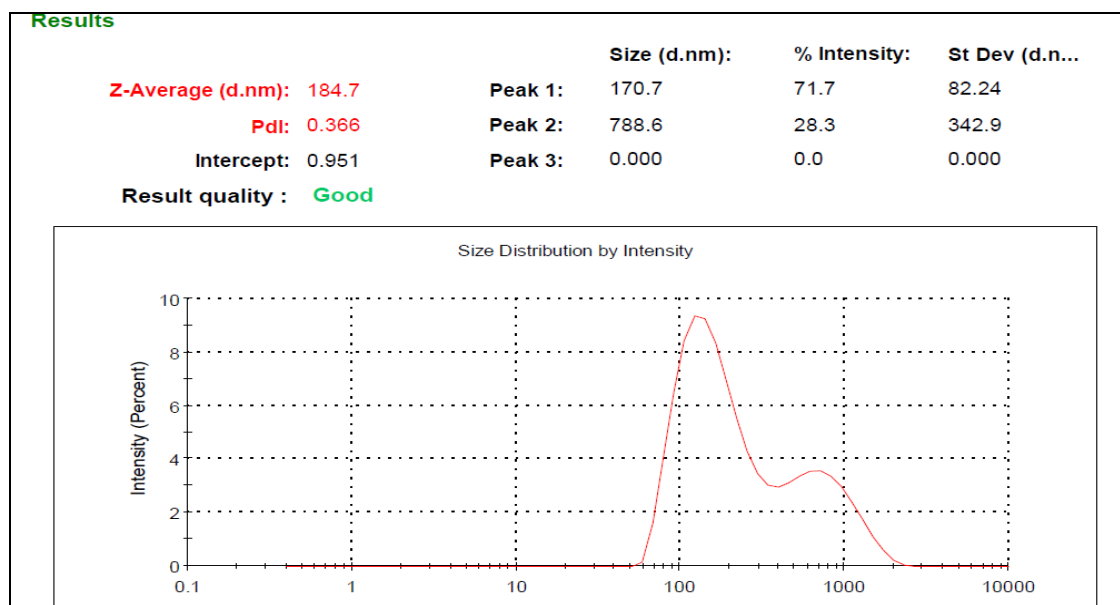
**5B.9.1 Size determination of indomethacin transferosome after storing for 3 months at temperature and humidity conditions of  $25^{\circ}\text{C} \pm 2^{\circ}\text{C}/60\% \text{RH} \pm 5\% \text{RH}$**

:-



**Figure 5B.25 Size of transferosomes of indomethacin after 3 months at  $25^{\circ}\text{C} \pm 2^{\circ}\text{C}/60\% \text{RH} \pm 5\% \text{RH}$**

**5B.9.2 Size of indomethacin transferosome after storing for 6 months at temperature and humidity conditions of  $25^{\circ}\text{C} \pm 2^{\circ}\text{C}/60\% \text{RH} \pm 5\% \text{RH}$ :-**



**Figure 5B.26 Size of transferosomes of indomethacin after storing for 6 months at temperature and humidity conditions of  $25^{\circ}\text{C} \pm 2^{\circ}\text{C}/60\% \text{RH} \pm 5\% \text{RH}$**

**Table 5B.16 Properties of transferosomal dispersion of indomethacin after stability testing**

Sampling time	Size in nm	Zeta Potential	PDI	% Drug entrapment
Initial	116.1	-21.4	0.241	52.33%
3 months	155.1	-34.0	0.412	49.12%
6 months	184.7	-52.9	0.366	43.23%

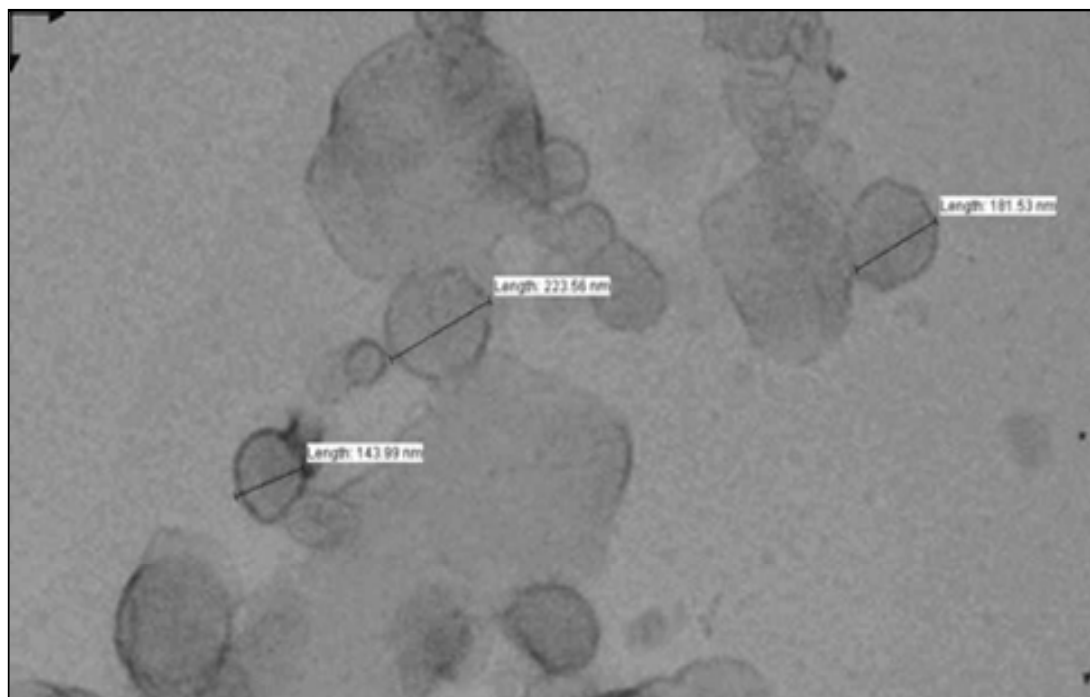
**5B.9.3 Transmission electron microscopy images of indomethacin transferosomal dispersion after 3 months and 6 months of stability testing:-**

The transmission electron microscopy was performed for the formulation kept for 3 months at  $25^{\circ}\text{C} \pm 2^{\circ}\text{C}/60\% \text{RH} \pm 5\% \text{RH}$  to observe any change in the size and shape of the transferosomes.

**Figure 5B.27 TEM images of transferosomes of indomethacin after storing for 3 months at temperature and humidity conditions of  $25^{\circ}\text{C} \pm 2^{\circ}\text{C}/60\%$**



## Indomethacin Transferosomal dispersion after 6 months



Size range- 150nm-200 nm

**Figure 5B.28** TEM images of transferosomes of indomethacin after storing for 6 months at temperature and humidity conditions of  $25^{\circ}\text{C} \pm 2^{\circ}\text{C}/60\% \text{RH} \pm 5\% \text{RH}$

**Interpretation:** Transferosomes were found to maintain their size of approximately 200 nm and no any major change in the surface morphology was observed.

#### 5B.9.4 Comparison of batches of transferosomal gel of indomethacin kept at temperature and humidity conditions of $25^{\circ}\text{C} \pm 2^{\circ}\text{C}/60\% \text{RH} \pm 5\% \text{RH}$ for time 0, 3 months and 6 months:-

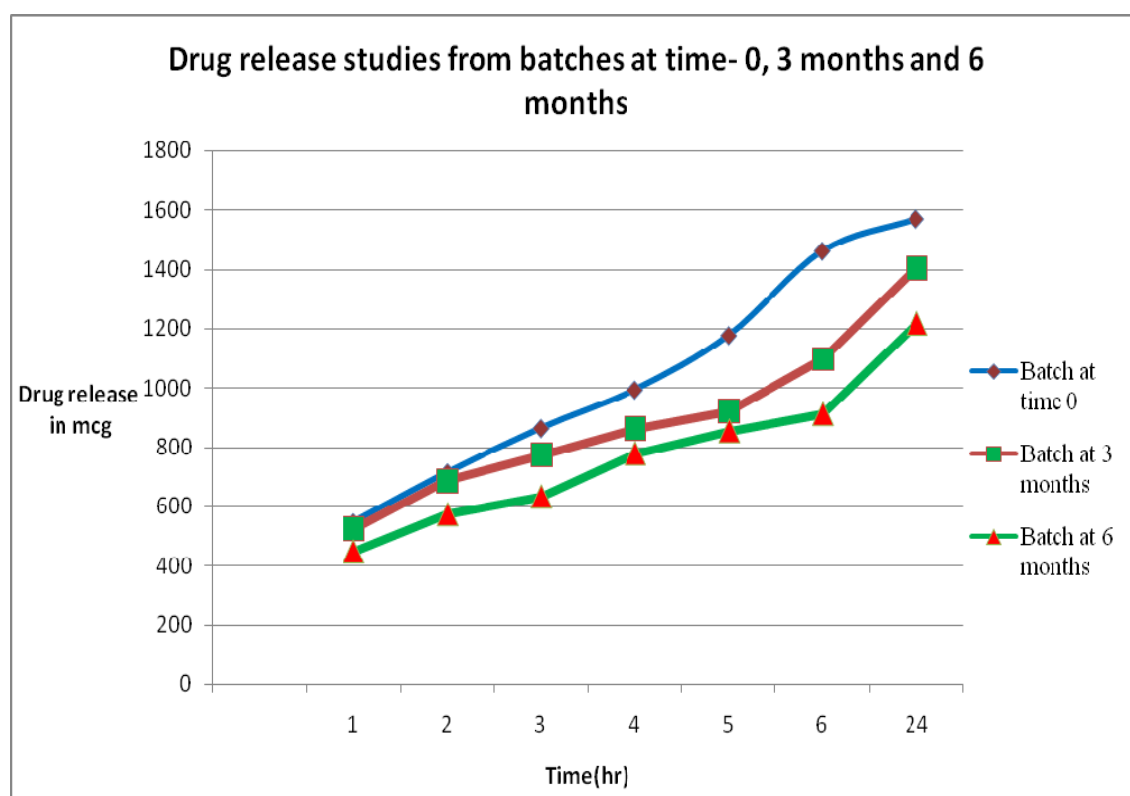
The batches kept at conditions of stability testing were analyzed for permeation flux and gel characteristics also.

**Table 5B.17** Comparison of batches of transferosomal gel of indomethacin kept at  $25^{\circ}\text{C} \pm 2^{\circ}\text{C}/60\% \text{RH} \pm 5\% \text{RH}$  for time-0, 3 months and 6 months

Sampling time	Permeation flux ( $\mu\text{gcm}^{-2} \text{hr}^{-1}$ )	Viscosity (cp)	pH	Spreadability (gm.cm/sec)
Initial	26.11	5673	6.2	1.398
3 months	22.32	4987	6.0	1.346
6 months	18.76	4146	6.0	1.319

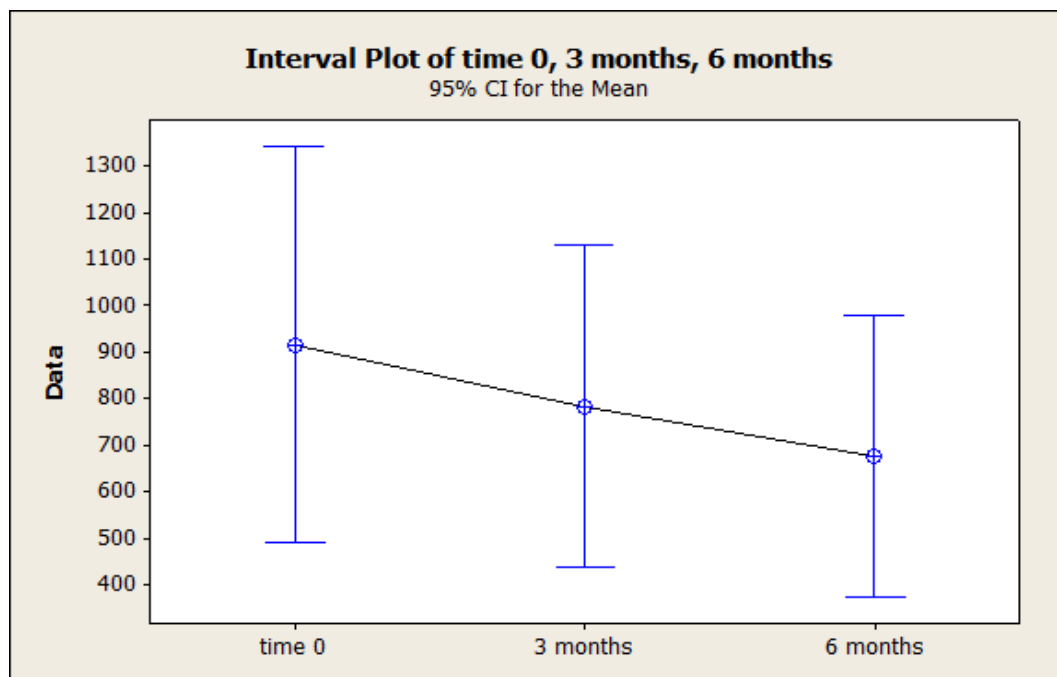
**Table 5B.18 Cumulative drug release ( $\mu\text{g}$ ) for 24 hours by transferosomal gel of indomethacin for stability batches**

Time(Hr)	Initial batch	Batch at 3 months	Batch at 6 months
0	0	0	0
1	546.3	523.5	447.2
2	713.8	686.1	573.2
3	863.4	773.4	632.7
4	992.9	863.1	778.5
5	1176.2	921.8	853.9
6	1460	1097.6	911.4
24	1568	1405.2	1216.5

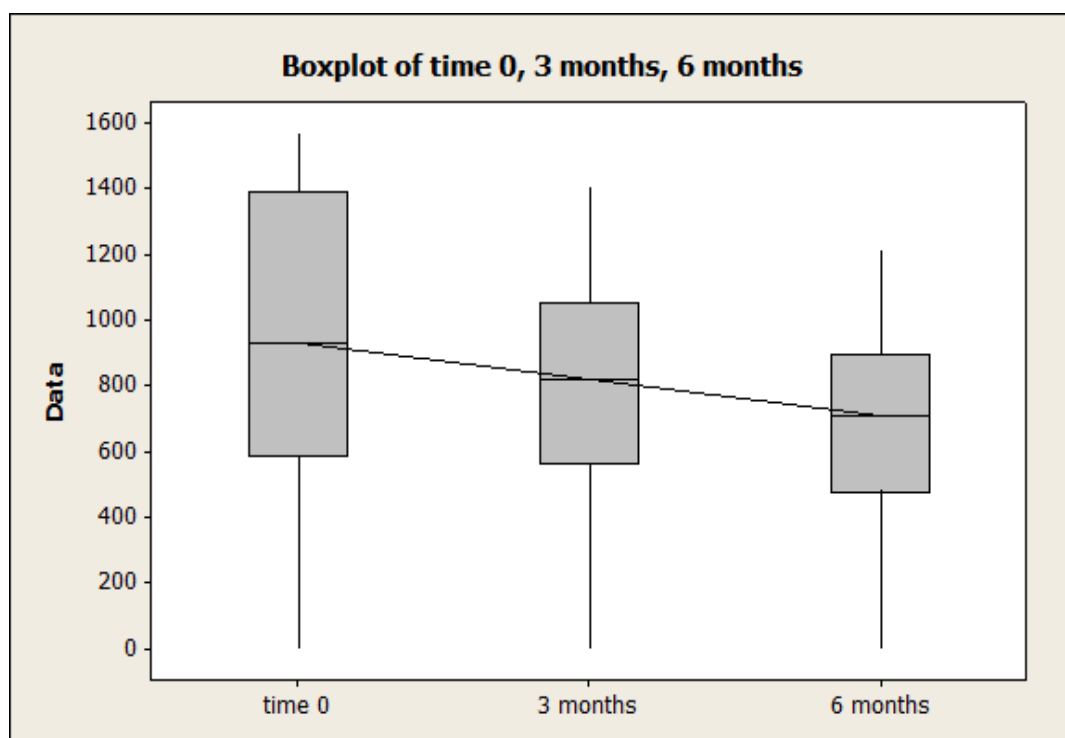


**Figure 5B.29 Comparison of drug release characteristics of batches of Transferosomal gel of Indomethacin kept at  $25^{\circ}\text{C} \pm 2^{\circ}\text{C}/60\% \text{RH} \pm 5\% \text{RH}$  for time-0,3 months and 6 months**

**5B.9.5 Statistical analysis of evaluation outcomes batches kept at accelerated stability conditions:-**



**Figure 5B.30 Individual plot and interval plots for statistical comparison of batches of transerosomal gel of indomethacin kept at  $25^{\circ}\text{C} \pm 2^{\circ}\text{C}/60\% \text{RH} \pm 5\% \text{RH}$  for time-0,3 months and 6 months**



**Figure 5B.31 Box plot for statistical comparison of batches of transerosomal gel of indomethacin kept at  $25^{\circ}\text{C} \pm 2^{\circ}\text{C}/60\% \text{RH} \pm 5\% \text{RH}$  for time-0, 3 months and 6 months**

❖ **One-way ANOVA: For drug permeation data of batches at 6 months, 0 month, 3 months at 25°C ± 2°C/60% RH ± 5% RH**

Source	DF	SS	MS	F	P
Factor	2	228111	114056	0.61	<b>0.553</b>
Error	21	3927202	187010		
Total	23	4155313			

The p value was found to be larger than the  $\alpha$ -level selected (0.05), so, the means of skin permeation of drug from the transferosomal gel formulation were not significantly different when kept at accelerated stability studies conditions.

#### **5B.9.6 Findings of stability testing studies:-**

- On storage of indomethacin transferosomal dispersion for 3 months at 25°C ± 2°C/60% RH ± 5% RH, a slight increase in size and decrease in zeta potential in vesicles was observed, whereas on storage for 6 months at the same condition, slightly more increase in size and decrease in zeta potential was observed.
- The drug entrapment efficiency was also found to be slightly decreased after storage at 25°C ± 2°C/60% RH ± 5% RH for 3 months and 6 months.
- The ex-vivo permeation flux was also found to decrease slightly, whereas the gel characteristics were found to be satisfactory.
- It was found that statistically, there is no any significant difference among the drug permeation profile from the batches kept at 0 month, 3 months and 6 months.

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## CHAPTER-VI

### Conclusion

The drug loaded carrier systems of nonsteroidal anti inflammatory drug aceclofenac and indomethacin were prepared and further incorporated in transdermal gel formulation. The formulations were prepared by experimental design using screened factors and their levels and by optimized process parameters.

The excipients were found to be compatible with the drug based on the results of drug excipient compatibility studies. The transferosomes and liposomes batches were prepared based on experimental design using minitab software 16. The process of rotary vacuum evaporation at 50° C and 90 rpm, for 20 minutes followed by probe sonication for 5 cycles and 2 cycles each of 2 minutes at amplitude of 60% using 13 mm standard probe produced transparent liposomes and transferosomes dispersion respectively with reproducibility and uniformity in vesicle size. For liposome preparation, 1, 2-disteroyl-sn-glycero-3-phospho-ethanolamine sodium salt was found to be better among the phospholipids, and for transferosome preparation, span 60 was screened as most suitable surfactant along with the mentioned phospholipid on the basis of size, drug entrapment efficiency and drug release of formulation.

The size of both drug carriers was found to be in the nanometric range with size uniformity and zeta potential values indicating stability of drug carrier suspension. The formulation of liposome and transferosomes were optimized based on drug entrapment efficiency and in-vitro drug permeation. The optimized formula of liposome contained phospholipid 109.9 mg (0.14 mM) and cholesterol 27.68 mg (0.071 mM) along with 100 mg (0.28 mM) aceclofenac, whereas the optimized formula of transferosomes contained phospholipid 91.41 mg (0.12 mM), surfactant 25 mg (0.06 mM) and cholesterol 35.60 mg (0.09 mM) in formulation with 100 mg (0.28 mM) aceclofenac. The % drug entrapment of optimized formulation of liposomes was 51.02 % and that of transferosomes was found to be 57.44 % which were very close to the target responses fixed in response surface methodology. Also, permeation flux of optimized liposome and transferosome transdermal gel formulations were 26.88 and 28.69  $\mu\text{gcm}^{-2} \text{hr}^{-1}$  respectively, very close to the target responses fixed in response

surface methodology that shows that optimized formulation followed the prediction of possibility to meet the target responses.

The ex-vivo studies through rat skin showed that better permeation of aceclofenac occurs from optimized liposomal and transferosomal gel as compared to marketed gel. The transdermal drug permeation was found to be highest for transferosomal gel, whereas drug was found to be slightly retained in skin during permeation from liposomal gel formulation. The reason may be fusion of phospholipids during diffusion through skin. Both the gel formulation showed sustained release of drug for more than 6 hrs.

The pharmacokinetic studies showed that,  $C_{max}$  for liposomal gel (7.002  $\mu\text{g/ml}$ ) and transferosomal gel (8.879  $\mu\text{g/ml}$ ) were significantly more than that for the conventional drug gel (5.998  $\mu\text{g/ml}$ ) however, the  $T_{max}$  was 6 hrs for all the three. The bioavailability as indicated by AUC was found to be highest for transferosomal gel (67.14  $\mu\text{g.hr/ml}$ ) followed by liposomal gel (53.87  $\mu\text{g.hr/ml}$ ). The AUC of both the drug carriers based gel were better as compared to conventional marketed gel of aceclofenac (42.92  $\mu\text{g.hr/ml}$ ). Both the transferosomal and liposomal gel were subjected to anti-inflammatory studies by rat paw edema method using plethysmometer and showed better anti-inflammatory action than marketed gel of aceclofenac and can be correlated with the pharmacokinetic parameters measured. The analgesic activity was also measured by Eddy's hot plate method and it was found that transferosomal gel and liposomal has better analgesic action than marketed gel.

The radio labeling experiment was carried out to understand the kinetics and dynamics of drug permeation and release from transferosomal gel and it demonstrated that percutaneous permeation of drug is rapid followed by depot formation in skin, thus the drug released in a sustained manner from the transferosomal gel.

As the results of transferosomal gel of aceclofenac was found to be more promising as compared to liposomal gel, the transferosomal gel was prepared for another drug indomethacin and tested. It was observed that transferosomal gel of aceclofenac and indomethacin, both were found to have better permeation characteristics as compared to their plain drug gel for arthritis, whereas the permeation flux of aceclofenac transferosomal gel was also found to be slightly higher than indomethacin transferosomal gel.

The formulation of indomethacin transferosome was optimized based on Size, drug entrapment efficiency and in vitro drug permeation. The optimized formula contained phospholipid 99.03 mg (0.13 mMol), surfactant 25 mg (0.05 mMol) and cholesterol 49.74 mg (0.12 mMol) in formulation with drug to lipid ratio of 0.27 to 0.13 mMol. The % drug entrapment of optimized formulation was 52.32 % which were very close to the target responses fixed in response surface methodology. Also, permeation flux of optimized transferosomal gel formulation was  $26.11 \mu\text{gcm}^{-2} \text{hr}^{-1}$ , very close to the target responses fixed in response surface methodology that shows that optimized formulation follow the prediction of possibility to meet the target responses. The ex-vivo studies through rat skin showed that better permeation of indomethacin from optimized transferosomal gel as compared to plain drug gel.

The gel characteristics and the analgesic activity and anti inflammatory activity of transferosomal gel were also found to be better than plain drug gel for both the drugs aceclofenac and indomethacin.

The rheological properties of transferosomal gel formulations indicated good spreadability, gel strength and extrudability for ease of application and a potential to withstand stress conditions of handling and packaging. The pH of the gel formulation was found to be compatible with the skin and the formulation was also found to be free of any toxic organic solvent.

The comparative characteristics of aceclofenac liposomal & transferosomal gel and indomethacin transferosomal gel along with their plain drug gel formulations has been summarized in the table no-6.1

**Table 6.1. Comparative characteristics of formulations**

Formulation	Vesicle Size (nm)	Zeta Potential (mv)	% Drug entrapment	Permeation flux ( $\mu\text{g.cm}^{-2} \text{hr}^{-1}$ )	Spreadability (gm.cm/sec)	pH	Analgesic activity (reaction time in seconds)	% Anti inflammatory activity
Aceclofenac liposomal gel	92.94	-26.0	51.02%	26.88	1.3989	6.0	11	59.00
Aceclofenac transferosomal gel	351.9	-42.7	57.44%	28.69	1.446	6.4	10	62.50
Marketed gel	--	--	--	17.46	1.3595	6.0	8	48.75
Indomethacin transferosomal gel	116.1	-21.4	52.32%	26.11	1.363	6.2	13	71.9
Plain drug gel	--	--	--	21.69	1.363	6.0	6	51.8

The transferosomal gel of NSAID aceclofenac showed maximum skin permeation, better bioavailability, analgesic action and anti-inflammatory action followed by liposomal gel. Both the transferosomal and liposomal gel of aceclofenac showed better efficacy than marketed gel.

The transferosomal gel of indomethacin also showed better skin permeation, analgesic action and anti-inflammatory action as compared to plain drug gel.

The transferosomal gel of both NSAIDs aceclofenac and indomethacin were found to be compatible with skin and having ease of handling and application.

Optimized transferosomal gel formulations of aceclofenac and indomethacin were stable as no statistically significant difference at 95% CI was observed among the size, zeta potential and drug permeation profile for the batches stored at accelerated stability testing for 6 months.

The NSAIDs aceclofenac and indomethacin in transferosomal gel formulation was found to have better bioavailability, anti-inflammatory & analgesic activity as compared to existing formulations of the mentioned drugs. The statistical analysis at 95% CI also proves stability and significant improvement in efficacy of formulation.

The work done has successfully produced nanocarriers based novel drug delivery system for drugs utilized for long term treatment of chronic joint pain and disability due to arthritis. The drug delivery system developed can target the inflamed tissue and avoid the accumulation of drugs in other tissues, thereby minimizing the side effects of drugs. The prepared formulation of drug carriers incorporated gel can be a novel approach for treatment of arthritis through transdermal route through which the drug can permeate through skin and also show sustained release characteristics. The localized action of drug through novel drug carriers based transdermal gel for prolonged period at the site of pain can provide more relief to arthritis patients and can replace the oral therapy thereby avoiding the gastrointestinal side effects of drug.



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## List of Publications

1. Pandey P, Pancholi SS, 2013, Nanocarriers: a novel treatment approach for arthritis, International Journal of Pharmaceutical Sciences & Research, 4(11), pp.4165-4174.
2. Pandey P, Pancholi SS, 2016, Effect of process parameters on design of vesicular systems carrying Aceclofenac, European Journal of Biomedical and Pharmaceutical Sciences, 3(6),pp.529-532.
3. Pandey P, Pancholi SS, 2016, Nanocarrier based transdermal formulation of NSAID: optimization of drug loading and analysis of permeation characteristics, Journal of Innovations in Pharmaceuticals and Biological Sciences, 3(3), pp.01-12.

### Patent Filed:-

1. Pandey P, Pancholi SS (2014) *Novel topical composition of non steroidal anti-inflammatory drugs*, IPO 1340/MUM/2014.

# APPENDIX-A


**BABARIA INSTITUTE OF PHARMACY**

**BITS EDU CAMPUS, VARNAMA, VADODARA, GUJARAT-391240**

**CERTIFICATE**

This is to certify that the experimental protocol titled “Novel carrier systems for targeted drug delivery in the treatment of Arthritis” and bearing the proposal number **Phd/13-14/23** has been approved by the IAEC vide its meeting held on **14<sup>th</sup> December 2013.**

Name of Chairman, IAEC:  
S.S Pancholi

  
14/12/13

Signature with date

Name of CPCSEA nominee, IAEC: Dr.  
Dr. B. Suresh

  
14.12.13

Signature with date

Name of the Principal Investigator:  
Ms. Prachi Pandey



# APPENDIX-B

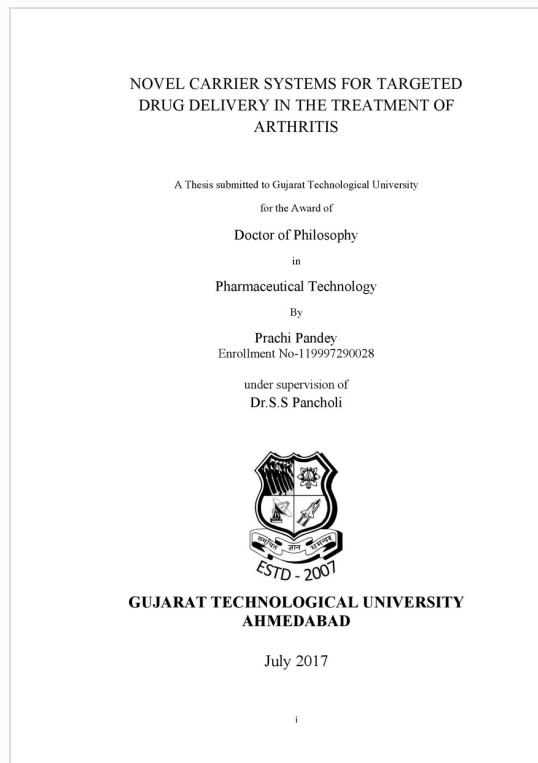


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