"Studies on adapalene loaded oil-in-water Janus emulsion for the management of *Acne vulgaris*"

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By

Yusuf Nawaz Khan

(Reg. No. 11505342)

Under the Guidance of

Dr. S. Tamilvanan

(Professor)

School of Pharmaceutical Sciences

Lovely Faculty of Applied Medical Sciences

Lovely Professional University

PUNJAB - 144411

INDIA

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DEDICATED TO MY PARENTS , MY MENTOR AND GOD

WITHOUT WHOM THIS WORK

COULD HAVE NOT BEEN POSSIBLE

TOPIC APPROVAL PERFORMA

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SR.NO.	NAME OF STUDENT	REGISTRATION NO	BATCH	SECTION	CONTACT NUMBER
1	Yusuf Nawaz Khan	11505342	2015	Y1507	7837380164

SPECIALIZATION AREA : Pharmaceutics **Supervisor Signature:** _____

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DAA Nominee Name: Dr. Sazal Patyar	UID: 17050	Recommended (Y/N): Yes

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This is to submit that this written submission in my thesis entitled **"Studies on adapalene loaded oil-in-water Janus emulsion for the management of *Acne vulgaris*"** represents original ideas in my own words and where other ideas and words have been included; I have adequately cited and referenced the original sources. I also declare that i have struck to all the principles of academic honesty and integrity and have not misrepresented or fabricated or falsified any idea/data/fact/source in my submission. I understand that any violation of the above will be cause for disciplinary action by the school and can also evoke penal action from the sources which thus have not been properly cited or from whom proper permission have not been taken when required.

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Forwarded Through

Dr. S. Tamilvanan
Coordinator
Domain of Pharmaceutics
School of Pharmaceutical sciences
LFAMS, LPU

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Place:

Date:

Dr. S. Tamilvanan

Professor

Coordinator

Domain of Pharmaceutics

School of Pharmaceutical Sciences

LFAMS, LPU

Dr. Monica Gulati

Senior Dean

School of Pharmaceutical Sciences

LFAMS, LPU

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Place: Yusuf Nawaz Khan

Date:

Abbreviations

Cm	:	Centimetre
COA	:	Certificate of analysis
Conc.	:	Concentration
DDW	:	Double distilled water
DHEAS	:	Dehydroepiandrosterone
DMSO	:	Dimethyl sulfoxide
EE	:	Entrapment efficiency
et al.	:	and others
FDA	:	Food and Drug Administration
Fig.	:	Figure
FSH	:	Follicle stimulating hormone
FT-IR	:	Fourier transform infrared
g	:	Gram
hr	:	hours
HIPR	:	High internal phase ratio
i.e.	:	That is
IgG	:	Immunoglobulin G
IR	:	Infra-red
IUPAC	:	International union of pure and applied chemistry
L	:	Litre
LH	:	Luteinizing hormone
LOD	:	Limit of detection
LOQ	:	Limit of quantification
Mg	:	Milligram
min.	:	Minute
mL	:	Milliliter
mm	:	millimetre
NLCs	:	Nanostructured lipid carriers
No.	:	Number
P. acne	:	*Propionibacterium acne*

PBS	:	Phosphate-buffered saline
pKa	:	Dissociation constant
RAR	:	Retinoic acid receptor
RBC	:	Red blood cell
Rf	:	Retention factor
ROS	:	Reactive oxygen species
rpm	:	Rotations per minute
RXR	:	Retinoids X receptor
S	:	Seconds
S.D.	:	Standard deviation
SLM	:	Solid lipid microparticle
SLN	:	Solid lipid nanoparticle
THF	:	Tetrahydrofuran
TLC	:	Thin layer chromatography
TEM	:	Transmission electron microscopy
UC	:	Ultracentrifugation
USP	:	United states pharmacopeia
UV	:	Ultra violet
VO	:	Vegetable oil
Wt.	:	Weight
%	:	Percentage
°C	:	Degree celsius
µm	:	Micrometre
Å	:	Angstrom
α	:	Alpha
λmax	:	Wavelength maxima

Abstract

The objectives of current research work were to see the solubility of adapalene in different oil and oil combination, to prepare topical emulsion formulation containing dispersed oil droplets on micro sized particles, to see the possibility of producing the dispersed oil droplet with double-head-faced structure (Janus particles), to determine the drug – entrapment efficiency as well as drug localization at oil water interface, to find out the actual structure of the dispersed oil droplets of emulsion through transmission electron microscopy, to calculate the *in vitro* drug release in phosphate buffer pH 6.0: THF (60:40) from Janus emulsion over the time period of 20 minutes, to find out the permeation capability of drug and drug loaded Janus emulsion through artificial cellophane membrane using a franz - diffusion cell apparatus, to calculate the *in vitro* antiinflammatory activity of pure adapalene powder and adapalene - loaded Janus emulsion via protein denaturation assay and to estimate the toxic nature of the developed Janus emulsion containing with or without drug via RBC break down assay.

Castor and silicone oils combination (1 : 2 ratio) was act as oil phase to disperse adapalene and tween 80 aqueous medium containing tween 80 was considered as continuous medium to make oil-in-water Janus emulsion. The produced Janus emulsion showed a mean particle diameter value [$d_{(0.5)}$] of 180.324 μm. About 74.74 ± 0.49 % of drug entrapment efficiency value was observed and 15.29 ± 0.17 % of pure drug was accumulated at oil-water-interface of Janus emulsion. However TEM analysis indicated the formation of drug nano-crystal inside the dispersed oil droplets of the Janus emulsion. This unexpected nano-crystal formation within the dispersed oil droplets might probably influenced the performance of Janus emulsion in an *in vitro* antiinflammatory study. A very similar antiinflammatory activity was observed between drug-loaded emulsion and drug solution in THF. Finally the RBC break down assay indicated that the adapalene-loaded Janus emulsion possessed the percentage haemolysis value that was relatively lower than the percentage haemolysis value shown by adapalene solution made with THF. Further research work is needed to confirm the advantage of Janus emulsion system to deliver adapalene for the treatment of inflammatory condition produced at acne.

Keywords: Adapalene, Janus emulsion, silicone oil, castor oil, *Acne vulgaris*.

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CHAPTER 1
INTRODUCTION

1.1 *Acne vulgaris*

Skin is perhaps the most vulnerable part of our body. It is a well-known fact that day to day exposure of human skin to the external environment leads to a number of problems such as acne, pimples, pigmentation and sunburn marks (Kapoor and Saraf, 2010). Acne, as a family of skin disorders, is one of the most prevalent dermatologic diseases in the world. Acne usually affects almost everybody during the life (Azimi et al., 2012). It is a common skin disorder encountered in the age group of 15 - 25 years owing to increased production of sebum followed by the attack of *P. acnes* (Khan et al., 2012).

The term acne is derived from Greek word- „acme" which means - prime of life. Although generally considered to be a benign, self-limiting condition, acne may cause severe psychological problems or disfiguring scars that can persist for a lifetime. (Tahir et al, 2010).

Acne vulgaris may be defined as any disorder of the skin whose initial pathology is the microscopic microcomedo. The microcomedo may evolve into visible open comedones ("blackheads") or closed comedones ("whiteheads"). Subsequently, inflammatory papules, pustules, and nodules may develop (Feldman et al., 2004).

1.2 Pathophysiology

There are at least 4 factors which are responsible for the acne.

a. Increased activity of sebum production.
b. Changed keratinisation in the follicular duct.
c. Increased microbial colonization.
d. Inflammatory and immunological reaction.

a. Increased activity of sebum production: In the very beginning of puberty, the sebaceous gland becomes more and more differentiated, increases in size, and changes its sebum composition. The sebaceous gland is under the control of androgens (testosterone) or by the adrenals (DHEAS). The proliferation and differentiation of the sebaceous glands mostly depend on the amount of cellular uptake of androgens, the activity of 5-α-reductase, and the receptor affinity (Gollnick et al., 1991).

b. Changed keratinisation in the follicular duct: Disturbed keratinization pattern in the infra infundibulum of the follicular duct can also be seen. Acne lesions can develop, due to the changes in the sebaceous gland or sebaceous follicular canal. During puberty, increase in the infra infundibulum, in the follicular canal can be observed. It starts comedo formation

which furthermore causes corneocytes to become denser and increase in their lipid content. Immunohistochemical studies revealed that filaggrin, a marker of terminal keratinization expressed in the very inner layer of the follicular wall, is increased in the infra infundibulum in seborrhoeic and even more in acne patients compared to normal.

c. Increased microbial colonization: Hyper seborrhoea and hyperkeratinization of the in infundibulum are followed by a hypercolonization with microbes. The most responsible microbe in acne is *Propionibacterium acnes*. Beside *P. acnes, P. granulosum, Staphylococcus* and *Micrococcus* species, aerobic *coryniform* bacteria, and *Malasezzia furfur* can be found in sebum rich areas of the skin. The prevalence of propionibacteria are highest in open comedones, compared to closed comedones and normal follicles (Leeming et al., 1985). In particular, *Propionibacterium acnes* is in two ways involved in the pathogenesis of acne: (a) it is capable of producing coenzymes like lipase which can split triglycerides to free fatty acids, which cause comedo formation and, (b) chemoattractive substances released by bacteria within the comedo can start a host defense reaction.

d. Inflammatory and immunological reactions: In closed comedones formation, follicular wall thinning can be found after some time. A chemoattractive substance produced by the bacteria can easily penetrate the follicular canal and by reacting with sebum and other components which are already present in the follicular canal, they can start defense reaction by accumulating lymphocytes and neutrophils. Interestingly, lower levels of linoleic acid can be found in comedones than in normal follicles (Wertz et al., 1985). The locally decreased linoleic acid in acne comedones can contribute in part to the production of inflammation through decreasing the phagocytosis by neutrophils and of ROS generation. Mostly the bacteria seen in the follicular canal even in the severe cases, but it can also cause activation of complement system and systemic response, for example, in conglobate type of acne, increase in the IgG – antibody can be seen in serum (Strauss and Pochi, 1965, Imamura et al., 1969, Puhvel et al., 1966).

1.3 Classification of *Acne vulgaris*

In 1990, the American Academy of Dermatology developed a classification scheme for primary *Acne vulgaris*. Acne lesions may be divided into inflammatory and non-inflammatory lesions. Non-inflammatory lesions consist of open and closed comedones. In general, acne manifested only by non-inflammatory lesions can rarely be characterized as severe, unless the number, size, and extent of such lesions are so overwhelming as to warrant such a designation. Inflammatory acne is traditionally characterized by the presence of one or more of the following types of lesions: papules, pustules, and nodules (cysts). Papules are

inflammatory lesions less than 5 mm in diameter. Pustules are similar in size to papules but have a visible central core of purulent material. Nodules are inflammatory lesions with a diameter of 5 mm or greater. These nodules may become suppurative or hemorrhagic. Nodular lesions, particularly when suppurative, have commonly been referred to as cysts because of their resemblance to inflamed epidermal cysts.

This grading scale delineates three levels of acne: mild, moderate, and severe. Mild acne is characterized by the presence of few to several papules and pustules, but no nodules. Patients with moderate acne have several to many papules and pustules, along with a few to several nodules. With severe acne, patients have numerous or extensive papules and pustules, as well as many nodules (Pochi et al., 1991).

Severity	Papules/ pustules	Nodules
Mild	Few to several	None
Moderate	Several to many	Few to several
Severe	Numerous and/or extensive	Many

Acne also is classified by type of lesion - comedonal, papulopustular, and nodulocystic. Pustules and cysts are considered inflammatory acne (Feldman et al., 2004).

Fig. 1: Different stages and types of acne

(Source: http://theacneproject.com/types-of-acne-pimples-07, accessed on 20th march, 2017)

1.4 Treatment for acne

Effective control of acne needs careful selection of anti-acne agents according to the symptoms and individual patient needs.

1.4.1 Topical treatment

It is commonly used for the mild to moderate cases of acne as such, in combination or as maintenance treatment with other treatments.

1.4.1.1 Benzoyl peroxide

It is available in different formulations as lotions, gels and creams, at different concentrations (2.5 - 10%) and its stability depend upon the vehicle. Gels are preferred over lotion and cream because of there more stability, activity and their less irritancy due to the presence of water base. It shows activity due to its oxidizing capacity (Packman et al., 1996). The drug has an anti-inflammatory, keratolytic and comedolytic activities, and is indicated in mild-to-moderate acne vulgaris. The main limitation of benzoyl peroxide is concentration dependent cutaneous irritation or dryness and bleaching of clothes, hair, and bed linen. It can induce irritant dermatitis with symptoms of burning, erythema, peeling, and dryness (Mills et al., 1986, Bojar et al., 1995).

1.4.1.2 Topical retinoids

Topical retinoid are being used as the first-line therapy, alone or in combination, for mild-to-moderate inflammatory acne and is also a preferred agent for maintenance therapy. Their biological effects are mediated through nuclear hormone receptors (retinoic acid receptor RAR and retinoids X receptor RXR with three subtypes α, β, and γ) and cytosolic binding proteins. The most studied topical retinoids for acne treatment worldwide are tretinoin and adapalene (Jain, 2004). Adapalene was generally better tolerated than all other retinoid with which it was compared (Percy, 2003, Shalita et al., 1996). The main adverse effects with topical retinoid are primary irritant dermatitis, which can present as erythema, scaling, burning sensation and can vary depending on skin type, sensitivity, and formulations.

1.4.1.3 Topical antibiotics

Topical antibiotics such as erythromycin and clindamycin are the most popular in the management of acne and available in a variety of vehicles and packaging. Clindamycin and erythromycin were both effective against inflammatory acne in topical form in a combination of 1 – 4% with or without the addition of zinc (Shahlita et al., 1984). Side effects though minor includes erythema, peeling, itching, dryness, and burning, pseudomembranous colitis which is rare but has been reported with clindamycin. A most important side effect of topical antibiotics is the development of bacterial resistance and cross-resistance; therefore, it should not be used as monotherapy (Parry and Rha, 1986).

1.4.2 Systemic therapy

1.4.2.1 **Systemic antibiotics**

Oral antibiotics are indicated in mainly moderate-to-severe inflammatory acne. Tetracyclines and derivatives still remain the first choice. Macrolides, co-trimoxazole and trimethoprim are other alternatives for acne. They have side effects like - gastrointestinal upset, vaginal candidiasis and resistance. Doxycycline can be associated with photosensitivity. Minocycline may produce pigment deposition in the skin, mucous membrane, and teeth. There is a significant association between antibiotic used in acne and the incidence of upper respiratory tract infection (Meynadier and Alirezai, 1998, Margolis et al., 2005).

1.4.2.2 Hormonal therapy

It may be needed in female patients with severe seborrhoea, clinically apparent androgenic alopecia and with proven ovarian or adrenal hyperandrogenism. The main approach of hormonal therapy in acne is to prevent the effects of androgens on the sebaceous gland and probably follicular keratinocytes as well. Oral contraceptives and androgen receptor blockers like spironolactone and cyproterone acetate can be used to treat acne. Anti-acne effect of oral contraceptive governed by decreasing level of circulatory androgens through inhibition of luteinizing hormones (LH) and follicle-stimulating hormone (FSH). The currently FDA approved agents include norgestimate with ethinyl estradiol, and norethindrone acetate with ethinyl estradiol (Huber and Walch, 2006). Spironolactone act by causing hyperkalemia in higher dose (Muhlemann et al., 1986). Cyproternoe acetate higher dose is more effective and it is given in combination with ethinyl estradiol for acne treatment (Fugère et al., 1990).

1.4.2.3 Oral isotretinoin

It is a retinoid that affects all four pathogenic factors implicated in the etiology of acne. Side effects include those of musculoskeletal, mucocutaneous, and ophthalmic systems, as well as a headache, and central nervous system effects. Most of the side effects are temporary and resolve after the drug is discontinued. Oral isotretinoin is a potent teratogen so it should be avoided by the pregnant women (Rathi, 2011).

CHAPTER 2
TERMINOLOGY

Janus emulsion these are the highly structured fluids with emulsion drops composed by two non-mixable oil components.

Acne vulgaris it is a chronic disease which affects the pilosebaceous units on the skin and manifests when hair follicles become clogged with dead skin cells and oil.

Retinoid is a class of drugs consists of vitamin A and its natural as well as synthetic derivatives.

Diffusion is a process in which particle moves from higher concentration to lower concentration through a membrane.

Inflammation it is the body response to damage to its tissues and cells by pathogens, noxious stimuli such as chemicals, or physical injury.

Haemolysis is the rupturing of the RBCs and releasing its content to the plasma.

Chromatography is a technique by which any mixture of the substance is divided into its components with the help of mobile phase and stationary phase.

CHAPTER 3

REVIEW OF LITERATURE

3.1 Drug Profile:

3.1.1 Adapalene

Table 3.1

3.1.2 Properties

Table 3.2

3.1.3 Marketed formulation

Table 3.3

Brand Name	**Dosage form**
Differin	Cream and Gel (0.1% and 0.3%)
Adife Aqs	Gel 0.1 %
Adaferin	Gel 0.1 %

3.2 Previous work done on Adapalene

Harde et al (2015) prepared adapalene loaded solid lipid nanoparticles by hot homogenization technique using glyceryl monostearate and pluronic F68 followed by carbopol gelation. It promising drug carrier for follicular delivery of adapalene also reduces the skin irritation.

Ramezanli et al (2017) developed polymeric nanocarriers based on tyrosine-derived nanospheres (TyroSpheres) for adapalene delivery. Their result shows that TyroSpheres are promising carrier system to deliver adapalene (hydrophobic drug) to hair follicles and upper epidermis while minimizing skin irritation of the encapsulated drug.

Pereira et al (2016) developed different formulations of adapalene and dapsone like lipid-core polymeric nano-capsule suspensions containing adapalene and dapsone (AD-LCNC) and a carbopol 940® hydrogel containing adapalene and dapsone (AD-LCNC HG). Also, nano-emulsion of adapalene and dapsone (AD-NE) was prepared and incorporated in a carbopol 940® hydrogel (AD-NE HG). The *in vitro* skin permeation studies showed a higher amount of adapalene in epidermis and dermis for ADNE HG. The AD-LCNC HG presented a higher amount of dapsone in both skin layers. The assay showed a significant difference between AD-LCNC HG and AD-NE HG, and no drug was found in the receptor medium.

Dubey et al (2015) prepared niosomal gel containing adapalene by lipid film hydration technique using non-ionic surfactants (span 60) and cholesterol at different concentrations. They reported that niosomal gel of adapalene offer a good topical drug delivery system and provide a sustained action.

Bhalekar et al (2015) prepared solid lipid nanoparticles loaded adapalene to reduce the limitations of the drug like poor penetration, limited localization, and associated incompatibility of photosensitization and skin irritation. Hence, it was concluded that the studied adapalene loaded solid lipid nanoparticles formulation with skin localizing ability may be a promising carrier for topical delivery of adapalene.

Jain et al (2015) fabricated and characterized a topically applicable gel loaded with nanostructured lipid carriers (NLCs) of adapalene and vitamin C. Concluded that NLCs has a great potential as a novel carrier for the dermal delivery of adapalene and incorporation of vitamin C has a synergistic effect.

Kumar and banga (2015) prepared adapalene-encapsulated liposomes and concluded that adapalene was efficiently encapsulated in liposomes and led to enhanced delivery in the target site.

Guo et al (2014) developed acid-responsive nanocarrier for adapalene to increase its efficacy at the acne site (acidic pH) and found it to be a very effective drug delivery system for the treatment of acne.

Jain et al (2014) developed adapalene loaded solid lipid nanoparticles for effective topical delivery and concluded that SLNs are a novel carrier and have a potential for topical delivery of adapalene.

Lauterbach and Goymann (2014) formulated adapalene in novel solid lipid microparticle (SLM) dispersion and evaluated its efficacy. An improved potency and efficacy were reported.

Rolland et al (1993) developed a carrier system for using 50:50 poly (DL-lactic-co-glycolic acid) microspheres and carried out *in vitro* and *in vivo* studies. The results showed that the polymeric microspheres incorporated with adapalene represented a promising therapeutic approach for the treatment of pathologies associated with pilosebaceous units.

3.3 Previous work done on Janus emulsion

Ge et al., (2016) prepared water–oil Janus emulsions by using a double-bore capillary microfluidic device. Ethoxylated trimethylolpropane triacrylate monomer is used as inner oil phase and liquid paraffin is used as outer oil phase while tween 80 was used as surfactant. They have seen the effect of changing the surfactant amount and by changing the flow ratio of water phase to the oil phase and concluded that adjustment of tween 80 amount will show more effect on the surface are of the required phase.

Leonardi et al., (2015) prepared the Janus emulsion by using the silicone oil and vegetable oil, by using tween 80 as a stabilizer. They have found that the dispersions tended to contain both single and flocculated drops irrespective of the emulsification intensity. They concluded that Janus drop free energy was less in comparison to the engulfed and separated drops.

Jeong et al., (2015) prepared liquid crystal Janus droplets by using solvent induced phase separation and microfluidics technique. They have demonstrated that concentration of surfactant and volume ratio of the compartments affects the prepared droplets morphology. The difference in the densities and solubility of the components produce gravity-induced alignment, tumbling, and directional self-propelled motion which show remarkable optical properties and dynamical behaviors of these particles.

Ge et al., (2015) prepared anisotropic particles by using Janus emulsion droplet method. They have used ethoxylated trimethylolpropane tri acrylate as polymer, fluorocarbon oil as non-polymer and Pluronic F127 as a stabilizer. They obtained the emulsion droplets in batch scale simply by one-step mixing. They concluded that control of topology from crescent to moon shape can be achieved simply by adjusting the emulsion composition and size control is seen by controlling the emulsification energy. By further UV-induced polymerization process, they have removed solid anisotropic particles by removal of immiscible oil.

Kovach et al., (2014) prepared the Janus emulsion by adding phospholipids in the olive oil/water/silicone oil. They concluded that droplet size of the prepared emulsion can be decreased by adding phosphatidylcholine or by decreasing the viscosity of the silicone oil. Furthermore, they explained that Tween molecules are not tightly packed on the interface between olive oil and water, but might be in a tilted state, that’s why phosphatidylcholine tend to form a more ordered structure.

Ge et al., (2014) prepared Janus emulsion by using tripropyleneglycol diacrylate, silicone oil and tween 80 as an emulsifier by using on step traditional high energy mixing processing which overcame the volume restraints suffered by microfluidic methods. They showed that the volume ratio of two oils within an individual droplet changes correspondingly to the total composition of the emulsion. Increasing the speed of stirring results in a significant reduction in the droplet size, i.e. a five times increase in the stirring speed produces a droplet size reduction from hundreds to a few microns.

Ge et al., (2014) prepared Janus emulsion using sunflower oil, silicone oil and tween 80 through one-step high energy mixing and investigate the droplets by image observation. They reported that location of the contact plane is the dominant factor determining the volume ratio of two oil lobes. Composition change in the emulsion results in the corresponding tune of the location of contact plane and subsequently, the volume ratio of two oils within Janus droplets.

Tu and Lee, (2014) prepared multiple emulsions and they have used Janus particles as emulsifiers. Janus emulsion is prepared and other oil phase includes toluene by using styrene and acrylic acid. They have demonstrated that highly stable multiple emulsions can be generated with Janus particles via one-step emulsification, enabling the one-step encapsulation of hydrophilic species in multiple emulsions. Furthermore, destabilization of the emulsion causes the release of encapsulated product which can be achieved by simply increasing the pH of the environment.

Hasinovic et al., (2011) prepared Janus emulsion via vegetable oil and silicone oil using vibration mixer. They discussed the basis for the unique structure in relation to pair-wise interactions between the components with especial emphasis on the surfactant concentration in the aqueous phase. The vegetable oil/ water interfacial tension is less than the silicone oil/ water interfacial tension (with tween 80) which causes the vegetable oil to spreading on the water during the emulsification, and it results in the Janus emulsion formation.

Hasinovic and Friberg, (2011) prepared a high internal phase ratio (HIPR) aqueous Janus emulsions using silicone oil and a vegetable oil (VO), were prepared by the application of vibration mixer. They have reported that by lowering the fraction of the aqueous phase, Janus emulsion gets converted into the triple emulsion forming a drop inside the vegetable oil to give a double Janus emulsion and triple Janus emulsion.

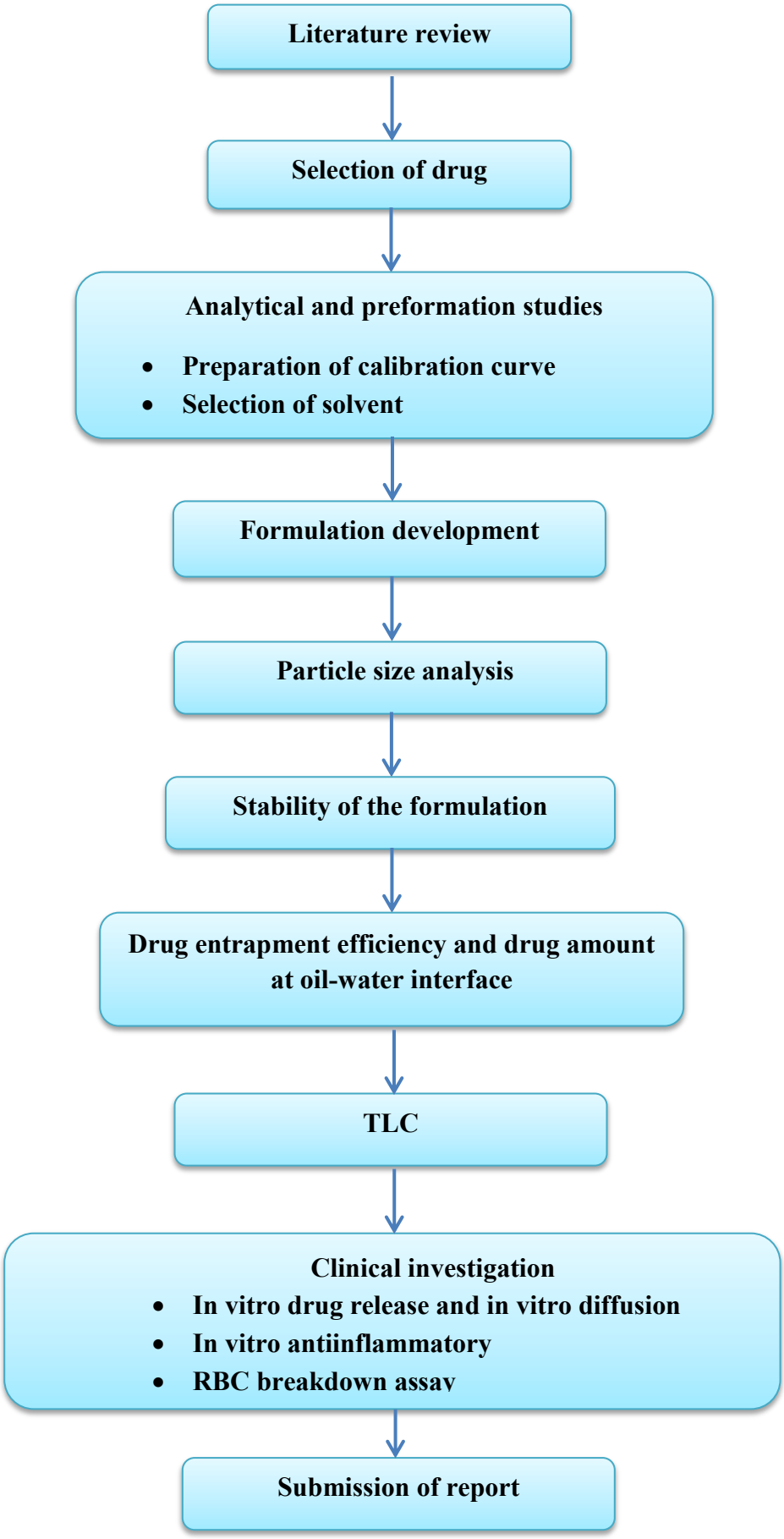
CHAPTER 4

RATIONALE AND SCOPE OF THE STUDY

4.1 Rationale

Adapalene is a retinoid and it is highly environmental sensitive (light and moisture) drug molecule, therefore, it requires the protection of its molecular structure during formulation development stage. Since adapalene itself having very less solubility in aqueous medium, it is not possible to make aqueous based formulation with adequate adapalene concentration into it. This drug has been recommended and regularly being used nowadays for the management of inflammatory conditions produced at *Acne vulgaris* skin diseases conditions. To improve the water solubility of the drug, there are a numerous number of formulation approaches. Among the gamut of approaches available to increase the water solubility of the drugs, the oil in water type emulsion is particularly received considerable attention in recent years. The current research work was undertaken to incorporate the adapalene within the oil in water emulsion system. Few of the physicochemical characterisation of the developed emulsion system were studied in the current research work which includes particle size analysis by the microscopic method and Malvern mastersizer instrument, drug entrapment efficiency and drug availability at the oil-water interface of the emulsion etc. An *in vitro* antiinflammatory study and an *in vitro* drug diffusion study via cellophane membrane were also performed for the adapalene-loaded emulsion.

4.2 Plan of work



CHAPTER 5

AIM AND OBJECTIVES OF THE STUDY

5.1 Aim

The aim of the current investigation was, therefore to design, develop and perform few *in vitro* test of adapalene - loaded oil-in-water topical Janus emulsion.

5.2 Objectives

1. To see the solubility of adapalene in different oil and oil combination.
2. To prepare topical emulsion formulation containing dispersed oil droplets on micro-sized particles.
3. To see the possibility of producing the dispersed oil droplet with double head face structure (Janus particles).
4. To determine the drug – entrapment efficiency as well as drug localization at the oil-water interface.
5. To calculate the *in vitro* drug release in phosphate buffer pH 6.0: THF (60:40) from Janus emulsion over the time period of 20 minutes.
6. To find out the permeation capability of drug and drug loaded Janus emulsion through artificial cellophane membrane using a Fraz-diffusion cell apparatus.
7. To calculate the *in vitro* antiinflammatory activity of pure adapalene powder and adapalene-loaded Janus emulsion via protein denaturation assay.
8. To estimate the toxic nature of the developed Janus emulsion containing with or without drug via RBC break down assay.

CHAPTER 6

MATERIALS AND RESERCH METHODOLOGY

6.1 Materials and Equipments Used

6.1.1 Materials Used

Table 6.1 List of various materials used in current project

Chemical Name	Supplier
Adapalene	Taj Pharmaceutical Ltd, India
Castor oil	Khurana Oils, Ludhiana, India
Coconut oil	Khurana Oils, Ludhiana, India
Diclofenac Sodium	Kwality Pharma, Amritsar, India
DMSO	Loba Chemie, Mumbai , India
EDTA	Loba Chemie, Mumbai, India
Egg Albumin	Loba Chemie, Mumbai, India
Methanol	Loba Chemie, Mumbai, India
Olive oil	Khurana Oils, Ludhiana, India
Potassium chloride	Loba Chemie, Mumbai, India
Potassium di hydrogen orthophosphate	Loba Chemie, Mumbai, India
Silica gel G	Loba Chemie, Mumbai, India
Silicone oil	Loba Chemie, Mumbai, India
Sodium hydroxide	Loba Chemie, Mumbai, India
THF	Loba Chemie, Mumbai, India
Tween 20	Loba Chemie, Mumbai, India
Tween 80	Loba Chemie, Mumbai, India

6.1.2 Equipments Used

Table 6.2 List of various equipment used in current project

Equipments	Suppliers
Centrifuge	Remi Electrotechnik Ltd. Vasai, India
Double beam UV- Visible spectrophotometer	Shimadzu 1800, Japan
Fourier transform Infrared Spectrophotometer	Shimadzu FTIR-8400 S Japan
Franz diffusion cell	Pefit glasswares Pvt. Ltd., India
Hot Air Oven	Navyug, Punjab, India
Incubator	Navyug, India
Magnetic Stirrer	Remi Motors Pvt. Ltd. Vasai, India
Mechanical water bath shaker	Remi Motors Pvt. Ltd. Vasai, India
Melting Point Apparatus	Popular Traders, Ambala Cantt. , Ind
Microscope	Kyowa Getner, Japan
pH Meter	Labtronics, Punjab , India
Refrigerator	Kelvinator ,USA
Rota evaporater	IKA HB 10
Transmission Electron Microscope	FEI Tecnai, Netherlands
Ultrasonicator	Athena Technology, India
UV visualizer	Perfit, India
Weighing Balance	Shimadzu, Japan

6.2 Research methodology

6.2.1 Pre-formulation Studies

6.2.1.1 Physical appearance and characterisation

The sample of adapalene was identified and characterised as per requirements of COA (certificate of analysis) issued by the manufacturer and (USP 30 NF 25, 2007).

6.2.1.2 Melting Point Analysis

Melting point of adapalene was determined using capillary tube method. In this method the double-end opened capillary tube was taken and the one end was sealed using direct flame. Then the drug was filled in the capillary tube before inserting it into the melting point apparatus. The transformation of solid state drug particle into liquid state was noted and the corresponding temperature at which this solid to liquid transformation occurred was also noted down.

6.2.1.3. Identification of the drug by FT-IR spectrum

The most well-known method of preparing the pallet by mixing the drug molecule and KBr in the ratio of 1:40 was used to find the FT-IR spectra of adapalene. The functional groups present in the drug molecule structure were scanned between the wave number values of 600 to 6000 cm^{-1} using a FT-IR spectrophotometer.

6.2.1.4 Solubility of adapalene in various oil, organic solvents and oil combination

The solubility of adapalene was determined in presence of various oils, organic solvents and oil combination such as castor oil, coconut oil, DMSO, methanol, olive oil, silicone oil and water. Excess amount of adapalene was added into the selected oils, solvents or oil combinations and all the samples were shaken for four hours using a mechanical water bath shaker. All the samples were filtered and the filtrates after suitable dilution with THF were measured at 320 nm spectrophotometrically.

6.2.2 Analytical method development

6.2.2.1 Selection and optimisation of solvent

The selection of solvent has a profound influence on the quality and shape of the peak. For UV method development, the major solvents used are: DMSO, Methanol, THF etc. Under this step, different solvents were optimized. Out of which THF satisfied all the conditions relative to peak quality and non-interference at specified wavelength.

6.2.2.2 Determination of wavelength maxima (λmax)

Adapalene 15 µg/mL solution was scanned in UV spectrophotometer in range of 200 - 800 nm. THF is used as blank. Wavelength corresponding to maximum absorbance of adapalene in THF was observed at 320 nm.

6.2.2.3 Preparation of standard calibration curve of adapalene in THF

The standard calibration curve of adapalene was obtained by measuring the absorbance of adapalene solution in concentration range (3-15 µg/mL) prepared from stock solutions in THF at 320 nm in triplicate. The calibration curve of adapalene was then plotted with absorbance on y-axis and adapalene concentration on x-axis.

6.2.2.4 Validation of calibration curve

6.2.2.4.1 Linearity

Linearity can be determined by taking the drug concentration between 3 – 15 µg/mL. It is determined by taking aliquots from stock solution of adapalene 1 mg/mL. Linearity was observed in range of 3 - 15 µg/mL by scanning 200 - 800 nm using THF as blank. The calibration curve was plotted on graph of concentration vs. absorbance to check the linearity [(ICH Q2 (R1)]

Acceptance Criteria:

Correlation Coefficient ≥ 0.99

6.2.2.4.2 Precision

The precision is determined by assay of five determinations at 100% test concentration (15 ppm) and relative standard deviation (% RSD) was calculated. The results were reported in terms of % relative standard deviation [ICH Q2 (R1)].

Acceptance Criteria:

The Relative Standard Deviation should not be more than 2%.

6.2.2.4.3 Accuracy

The accuracy of an analytical method was determined using the samples in which known amount of analyte is added. The accuracy is calculated from the results by calculating the percentage of analyte recovered using the assay at three different levels i.e. 50 %, 100 % and 150 % [ICH Q2 (R1)]. The method of accuracy was determined by measuring the reference standard recovery in triplicate at three levels, 50 %, 100 % and 150 % of the method concentration (200 µg/mL). A standard stock solution 1 mg/mL of adapalene was prepared. In volumetric flasks of 10 mL, aliquots of 0.15, 0.3 and 0.45 mL of this solution (which would yield concentrations of 15, 30 and 45 µg/mL respectively) were combined with 1.5 mL of the 200µg/mL sample solution. The volume was made upto 10 mL using THF. Thus the final concentrations were 45.0, 60.0 and 75 µg/mL, which correspond to 50, 100 and 150% of the target concentration, respectively.

The sample was prepared in triplicate and analyzed by using UV-Vis spectrophotometer at 320 nm.

Acceptance Criteria:

Mean recovery = 98 to 102%.

6.2.2.4 Robustness

Adapalene (3-15 µg/mL) solution was analyzed six times at two different temperatures (18°C and 24°C) to determine robustness of the method. The result is indicated as %RDS.

6.2.2.5 Limit of detection

"It is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated under the stated experimental conditions". Limit of detection is calculated using following equation.

$$\mathbf{LOD = 3.3 \times \sigma / S} \qquad \textbf{..... (1)}$$

Where,

σ is Standard deviation of the response and

S is Slope of the calibration curve.

6.2.2.6 Limit of quantification

"It is the lowest concentration of analyte in a sample that can be determined with the acceptable precision and accuracy under stated experimental conditions." Limit of quantification is calculated using following equation.

$$LOQ = 10 \times \sigma / S \quad \text{..... (2)}$$

Where,

σ is Standard deviation of the response and

S is Slope of the calibration curve

6.2.3 Formulation Development

6.2.3.1 Ingredients used in the preparation of Janus emulsion

The various ingredients used in the preparation of Janus emulsion were described briefly below.

6.2.3.1.1 Silicone oil

Silicone oil is prepared from tetrachlorosilane and silicon metal obtained from the reduction of sand at high temperature.

In the most commonly used silicone oil, all the organic groups are methyl, called methylsilicone oil. Silicone oils are the polymers of dimethyl siloxane. According to the need, various organic groups can be attached in place of dimethyl group, like – hydrogen, ethyl, phenyl, chlorophenyl etc. and it will effect there properties and there application

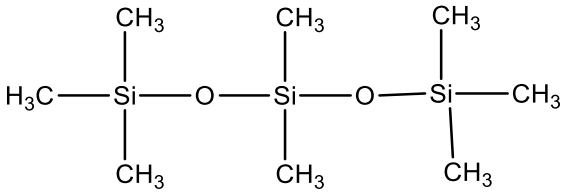


Fig 6.1: Chemical structure of methylsilicone oil

Silicone oil is generally colourless, odourless, non-toxic, non-volatile liquid. It is insoluble in water, methanol, slightly soluble in ethanol, acetone and mutually dissolved in benzene, diethyl ether and carbon tetrachloride. It has a small vapour pressure, high flash point and ignition point, low freezing point.

Clinically there are two types of silicone oil which are used in pharmaceutical industry.

(a) Lighter than water: for example polymethyl siloxane

(b) Heavier than water: for example polytrifluropropyl methyl siloxane.

Silicone oil has excellent heat resistance, electrical insulation, weather resistance, hydrophobicity, physiologically inert and smaller surface tension. In addition, it also has a low viscosity-temperature coefficient, high compression resistance. Its viscosity was increased by the number of silicone chain sections n in the molecule, from the easily flowing liquid to a thick semi-solid. Has excellent electrical insulating properties and heat resistance.

6.2.3.1.2 Castor oil

It is a vegetable oil obtained by pressing the seeds of the castor oil plant (*Ricinus communis* L.). They yield viscous, pale yellow nonvolatile and nondrying castor oil. It has density less than water. Castor oil is known to consist of up to 90% ricinoleic, 4% linoleic, 3% oleic, 1% stearic. Castor oil is valuable due to the high content of ricinoleic acid, which is used in a variety of applications in the chemical industry. The hydroxyl functionality of ricinoleic acid makes the castor oil a natural polyol providing oxidative stability to the oil, and a relatively high shelf life compared to other oils by preventing peroxide formation.

It is used as powerful laxative, if given orally. Castor oil is often administered orally to induce diarrhea in rats.

In modern-day medicine, castor oil is also used as a drug delivery vehicle. An example is **Kolliphor EL** or formerly known as **Cremophor EL**. The product is a polyexthoxylated castor oil, a mixture that is prepared when ethylene oxide is made to react with of castor oil. This product is often used as an excipient or additive in drugs and is also used to form stable emulsions of nonpolar materials in various aqueous systems. (Patel et al., 2016)

6.2.3.1.3 Coconut oil

It is edible oil obtained from the coconut palm (*Cocos nucifera*). It has various applications in food, medicine and industry. It contains 92% of saturated fatty acids. Coconut oil is colourless at or above 30˚ C. It is white when in its solid form. It solidifies below 25˚C. It form a homogenous mixture with water, when heated, otherwise it is insoluble in water.

It has many beneficial effects in heart diseases as it contains 50% lauric acid, used in various heart problems like high cholesterol levels and high blood pressure. It is frequently used in creams, tanning lotions, and body lotions. Coconut oil is also beneficial in weight loss. It has high anti-oxidant activity which is due to the phenolic content present in it.

6.2.3.1.4 Tween 80

Tween-80 is light yellow to amber oily viscose liquid, non-toxic. It is easily soluble in water, soluble in ethanol, vegetable oil, ethyl acetate, methanol, and insoluble in mineral oil. It has HLB value 15, which shows that it can be used as emulsifier in water in oil emulsion preparation.

6.2.3.2 Formulas used in the preparation of Janus emulsion

The ingredients used to prepare both blank-and adapalene-loaded Janus emulsion are shown in **Table 6.2 – 6.3** with formulation notations F_1, F_2, $F_{3.....}$ F_{14}.

Fig 6.2 : Steps involved to prepare Janus emulsion in small scale

6.2.4 Evaluation of the emulsion

6.2.4.1 Stability of emulsion

The stability of the Janus emulsion was carried for the four weeks at different temperatures. The Janus emulsion stability was carried out at 37 ˚C (room temperature) and 25 ˚C (refrigerator). The stability of the Janus emulsion was estimated microscopically with the appearance of Janus particles for 4 weeks.

6.2.4.2. Entrapment Efficiency and drug amount on oil water interface

The EE of the emulsions were determined by measuring the concentration of adapalene in the aqueous layer obtained by ultracentrifugation (UC) (Wang et al., 2006). Centrifugation was carried out using a HITACHI ultracentrifugation apparatus, operated at 12,000 rpm at 25° C for 15 min. Polyallomer tubes were used and their bottoms were pricked after centrifugation with a syringe needle to collect the aqueous phase. Concentrations of adapalene in both the aqueous layer and the whole emulsion were determined by UV spectrophotometer after diluting it 100 times with THF.

The EE was calculated according to the following equation (Groves et al., 1985; Ferezou et

al., 1994):

$$\% \textbf{ drug entrapment efficiency} = \frac{(\textbf{C total} * \textbf{V total}) - (\textbf{C water} * \textbf{V water})}{(\textbf{C total} * \textbf{V total})} \textbf{X 100.....(3)}$$

Where,

- C_{total} is total drug concentration
- V_{total} is total volume of emulsion
- C_{water} is drug concentration in water phase
- V_{water} is total volume of water phase

$$\text{Drug amount on oil water interface} = \frac{\text{Drug amount in whole emulsion} - \text{drug amount in water phase}}{\text{initial amount of the drug added}} \text{X } 100. \textbf{(4)}$$

6.2.4.3 Particle size determination

6.2.4.3.1 Particle size determination by using microscope

1. Microscope was cleaned properly and was placed in proper place where light was suitable for projection.

2. Then the eye-piece was fixed in microscope with micrometer.

3. The eye-piece micrometer was calibrated with a standard stage micrometer.

4. Then sample was mounted on the plain side.

5. Then the size of particles was measured with the help of eye-piece micrometer.

6. 100 particles were counted accurately and then picture was captured through Asus Zenfone 5.

6.2.4.3.2 Determination of particle size by Malvern mastersizer

Mastersizer equipment was developed by Malvern, instrumentation limited; London, UK was used for determining the mean particle size of the emulsion. This equipment works on the basis of Frauenhofer or Mie scattering theory. About 100-200 µl of emulsion was mixed with 150 mL of dispersion water (Hydro S). A laser beam of He-Ne light source was used to detect the particle size ranging from 0.02-2000 microns.

6.2.4.4 Thin layer chromatography (TLC)

Mobile phase: THF and methanol in ratio of 18 : 82

Stationary phase: Silica gel G

Visualising aid: UV rays

Procedure:

To perform the TLC, the mobile phase containing THF and methanol in the ratio of 18 : 82 was kept in kept in a TLC chamber and closed with lid for a period of 40 to 50 minutes to make the chamber saturated with mobile phase vapours. Slurry (5 - 10% w/v) of silica gel G was made in distilled water and poured onto a glass plate/slide to make the stationary phase. The prepared plate was activated by keeping it in a hot air oven for a period of 30 minutes at 110° C. A solution of adapalene in THF and equivalent amount of adapalene-loaded Janus emulsion were applied using a micropipette directly on the TLC plate, leaving 2 cm from the border. The plate is developed with the prepared mobile phase mixture in a TLC chamber for half an hour until the solvent rise up to 3/4th of the plate. After development, the plate was dried at room temperature to make the plate ready for visualization. The identification of spot was done with fluorescent indicator UV-254. The Rf value was calculated with help of the following equation, and the experiment was repeated thrice under the identical experimental conditions.

$$\textbf{Retention factor (Rf)} = \frac{\textbf{Distance travelled by sample}}{\textbf{Distance travelled by solvent}} \textbf{.....(5)}$$

6.2.4.5. Transmission Electron Microscopy (TEM)

6.2.4.5.1. Preparation of Staining Solution:

1. Add 0.1 g of sodium phosphotungstate (PTA) powder to 10 mL of distilled water in a shell vial. Check the PTA solution pH with the pH meter and adjust the pH to 7.2–7.4 with the 0.1N NaOH.

2. Add 500 µg bacitracin powder to the phosphotungustic acid solution.

6.2.4.5.2 Staining Procedure :

1. Pick up a carbon – coated grid with forceps and push the forceps locking ring down so that the grid is held firmLy. Lay forceps down on petridish lid with tips extending over the edge with the grid held coated-side up. The low thermal stability of the carbon-coated pioloform film on copper grids (piloform grids) in combination with PTA for contrast in the TEM is utilized.

2. The prepared Adpalene-loaded coarse emulsion was diluted at 5 times with deionized water. A drop of the diluted emulsion was applied to a carbon-coated copper grid using Pasteur pipette.

3. After 3-5 min, remove diluted emulsion by touching ragged torn edge of filter paper to the edge of the forceps jaws (where they contact the grid) until the grid surface is nearly dry. Never let the grid surface totally dry out because it will produce a coating of culture or body fluids and yield an excessively dirty grid.

4. Add one drop of PTA/bacitracin solution to the grid.

5. After 1 min, dry the grid as before with the ragged torn edge of filter paper except this time; dry the grid quickly and completely. Immediately touch the sample-side of the grid to a clean piece of filter paper in the bottom of a petridish. Slide a fresh piece of filter paper down between the forceps jaws to push the dried grid out of the forceps tips and onto a clean, dry part of the filter paper in the petridish.

6. Let the grid dry for 15 min in the petridish (covered) and then examine with a TEM.

6.2.4.6. *In vitro* drug release

1. The adapalene-loaded emulsion was prepared freshly.
2. 3 beakers of 10 mL capacity were taken and washed properly with double distilled water (DDW).
3. All three beakers were kept in hot air oven at 60 ± 5 ˚C for drying the beakers completely.
4. 8 mL of phosphate buffer pH 6.0: THF (60:40) was added to each beaker.
5. 1.6, 0.8 and 0.2 mLs of emulsion were added to the first, second and third beaker, respectively.
6. 0.5 mL of sample was withdrawn from each beaker at a time interval of 1, 5, 10, 15 and 20 minutes and no replenishment was made at each of the sampling times.
7. All the withdrawn samples (0.5 mL) were diluted separately upto 10 mL using phosphate buffer pH 6.0: THF (60:40).

6.2.4.7. *In vitro* permeation study

1. The adapalene-loaded Janus emulsion was prepared freshly.
2. 2 beakers of 100 mL capacity were taken and washed properly.

3. 50 mL of phosphate buffer pH 6.0: THF (60:40) was added to each beaker.
4. Cellophane membranes were put in previous contact with phosphate-buffered pH 6.0: THF (60:40) solution, 30 min before placing the samples.
5. 1 mL of emulsion (containing 3.1 mg of adapalene) was added to the donor compartment having cellophane membrane at one end.
6. In another assembly, standard drug solution was prepared and its 1 mL is added in donor.
7. During the experiment, the acceptor compartment was continuously homogenized at 100 rpm.
8. 5 mL of sample was withdrawn from acceptor compartment at a time interval of 30, 60, 90 and 120 minutes and replenishment was made after each sampling.
9. All the withdrawn samples were diluted separately using phosphate buffer pH 6.0: THF (60:40) and analysed by using UV spectrophotometer at 320 nm.
10. Permeability coefficient is calculated by using the following formula

$$Papp = \frac{\Delta Q}{\Delta t \times Co \times A \times 60}\ (\mathrm{Cm/sec}) \qquad \textbf{……. (6)}$$

Where,

- Co = Initial concentration of drug in solution
- 60 = Represents the conversion of the minutes into the seconds.
- A = Area of cellophane membrane
- $\Delta Q/\Delta t$ = steady state of drug permeation

11. Steady state flow rate is calculated by using the following formula

$$\mathrm{Jass} = \mathrm{Co} \times \mathrm{Papp} \qquad \textbf{……(7)}$$

Where,

- Jass = Steady state flow rate
- Papp = Permeability coefficient

6.2.4.8 *In vitro* antiinflammatory activity

1. In brief, the adapalene (40 mg) - loaded coarse emulsion (13 mL) was prepared freshly.
2. From this, 1 mL of emulsion (equivalent to 3.1 mg of adapalene) was taken and diluted with 100 mL phosphate buffer solution pH 7.4.

3. From this stock solution, 2 mL (equivalent to 62 µg of adapalene) was mixed with 0.2 mg of egg albumin powder and 2.8 mL of phosphate buffer solution of pH 7.4. Hence the final concentration was 12.4 µg/mL.
4. Step (2) was repeated by using 2, 3, 4 and 5 mL of emulsion (equivalent to 6.2, 9.3, 12.4 and 15.5 mg of adapalene respectively).
5. The step (3) was repeated by taking 2 mL from each of the solutions obtained from step (4) therefore, the obtained final concentration were 248, 372, 496 and 620 µg , respectively. Similarly the final solution concentration corresponding to step (4) were 49.6, 74.4, 99.2 and 124 µg/mL.
6. A control solution was also prepared using emulsion without drug and phosphate buffer solution of pH 7.4 and step (2) and (3) were followed.
7. All the samples were incubated at 37±2˚C in an incubator for 15 minutes.
8. After incubation, all the samples were heated for 70˚C for 5 min in a water bath.
9. After cooling to room temperature, the absorbance of all the solutions were measured blank.
10. The reference standard stock solutions were prepared by dissolving 40 mg of adapalene in 13 mL of phosphate buffer solution of pH 7.4. Steps (2) and (3) were performed to get the final concentration of 49.6, 74.4, 99.2 and 124 µg/mL and the absorbance values for these solutions were also measured at 660 nm.
11. The percentage inhibition of protein denaturation was calculated by using the following formula :

$$\%\ inhibition = 100(\frac{Vt}{Vc} - 1) \qquad \textbf{.....(8)}$$

Where,

V_t = absorbance of the sample

Vc = absorbance of the control

6.2.4.9 RBC breakdown assay

1. Freshly collected blood from a healthy male volunteer was mixed with anticoagulant solution (74.8 mm sodium citrate/ 38.07 mm citric acid/ 124.3 mm glucose 1:1:19 v/v or 2% EDTA solution) and centrifuged at 650 g for 10 min.
2. The supernatant was discarded and the erythrocytes were re-suspended in

phosphate buffered saline (PBS, pH = 7.4). Next, the erythrocytes were washed three times with an isotonic buffer (PBS), and upper phase with a buffy coat containing precipitated debris and serum proteins was carefully removed at each wash step.

3. After last washing, the packed cells were suspended in a buffer to a haematocrit of 50%.
4. All the erythrocytes suspensions used in the experiments were prepared daily.
5. To determine the haemolytic effect, 100 µL of each samples (pure drug, Janus emulsion containing equivalent amount of drug) containing 230 µg of adapalene was diluted with 10 mL of buffer and 1 mL from this diluted samples (containing 23 µg of adapalene) was added to 20 µL of erythrocytes suspension (50% haematocrit) and adjusted to a 10 mL volume with PBS.
6. The samples were stirred and incubated for 30 min at 37 ˚C. Debris and intact erythrocytes were removed by centrifugation at 650 *g* for 10 min.
7. The haemoglobin released into the supernatant was detected spectrophotometrically at 490 nm against the corresponding blank sample.
8. The haemolytic effect, measured as the percentage of haemolysis (H), was determined on the basis of released haemoglobin, according to the following formula.
9. The procedure from (5) to (8) was repeated with emulsion prepared based on castor oil only (without silicone oil) and also without adapalene.

$$H\ (\%) = \frac{As - Ac1}{Ac2 - Ac1}\ X\ 100 \qquad \text{.....(9)}$$

Where,

H = Haemolysis

As_1 = Absorbance of adapalene-loaded Janus emulsion

As_2 = Absorbance of pure adapalene solution

As_3 = Absorbance of castor oil based emulsion without adapalene

Ac_1 = Absorbance due to mechanical haemolysis (RBC 20 µL + 4 mL PBS)

Ac_2 = 100% haemolysis (RBC 20 µL + 4 mL DDW)

CHAPTER 7

RESULT AND DISCUSSION

7.1 Result

7.1.1 Pre-formulation Studies

7.1.1.1 Physical appearance and characterisation

Table 7.1 shows the physical appearance and characteristics of adapalene and it confirms that the colour, odour, and physical state at room temp condition observed for the sample obtained were the same as that of the specification mentioned in the COA.

Table 7.1. Physical appearance and characterisation of adapalene in room temperature.

Parameters	Specifications as per COA	Observation
Physical State	Solid	Solid
Colour	White	White
Odour	Odourless	Odourless

7.1.1.2 Melting point analysis

Table 7.2 shows the melting point of adapalene, which complies with the melting point mentioned in the COA.

Table 7.2. Melting point of adapalene

Parameter	Specified as per COA	Observation
Melting Point	319 – 322 ˚C	320 – 326 ˚C

7.1.1.3 Identification of the drug by FT-IR spectrum

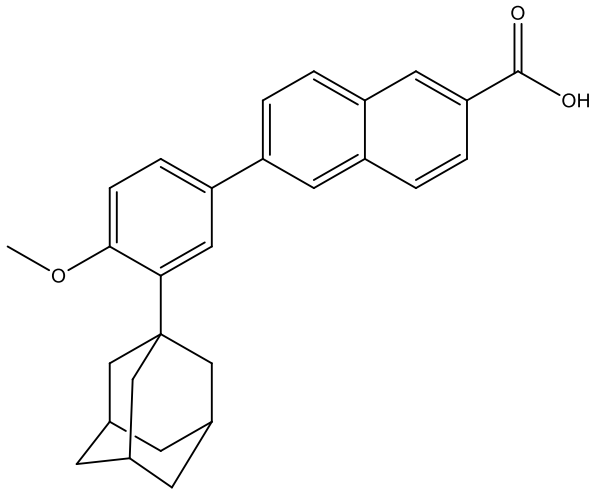


Fig 7.1 Structure of adapalene

Fig 7.1 and **Fig 7.2** depict the structure and FT-IR spectrum of adapalene respectively.

Table 7.3 shows the various peaks of functional groups present in the adapalene which complies with the reported values.

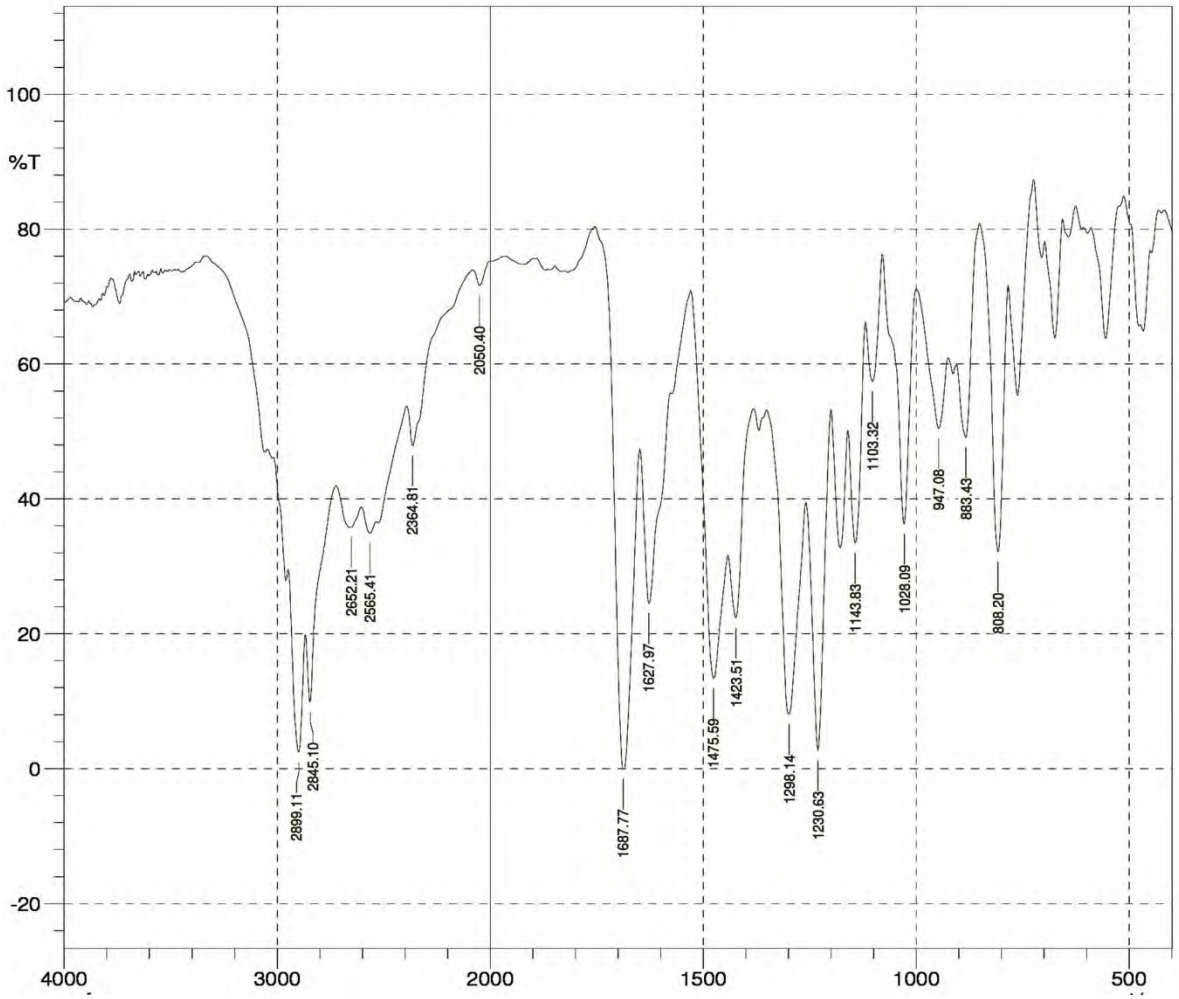


Fig 7.2. FT-IR spectrum of adapalene on Shimadzu 1800S FT-IR

Table 7.3. Typical wavenumbers for functional group of adapalene in IR spectrum

Functional group	Reported value (cm^{-1})	Observed value (cm^{-1})
O-H stretching	3400 – 2400	2849
C-H stretching	3000 – 2850	2899
C=O stretching	1820 – 1660	1687
C=C (alkene) stretching	1660 – 1600	1627
C-H bending	1450 – 1375	1423
C-O stretching	1320 – 1210	1298
=C-H out of plane bending	900 – 600	883

7.1.1.4 Solubility of adapalene in various oil, organic solvents and oil combination

Table 7.4 shows the solubility of adapalene in various oils, organic solvents and oil-combinations. **Fig 7.3** depicts the solubility graph of adapalene in oils, organic solvents and oil-combinations.

Table 7.4 Solubility of adapalene in various oils, organic solvents and oil combination

Solvent	Absorbance	Solubility (mg/mL)
DMSO	0.035	0.58
Methanol	0.045	0.75
THF	0.632	10.98
Castor oil	0.546	9.33
Coconut oil	0.902	15.43
Olive oil	0.475	8.12
Silicone oil	0.311	5.31
Castor oil + Silicone oil (1:3)	0.674	11.52
Castor oil + Silicone oil (1:4)	0.741	12.67
Coconut oil + Silicone oil + (1:3)	0.996	17.04
Coconut oil + Silicone oil (1:4)	1.033	17.67
Olive oil + Silicone oil (1:3)	0.512	8.75
Olive oil + Silicone oil (1:4)	0.584	9.98

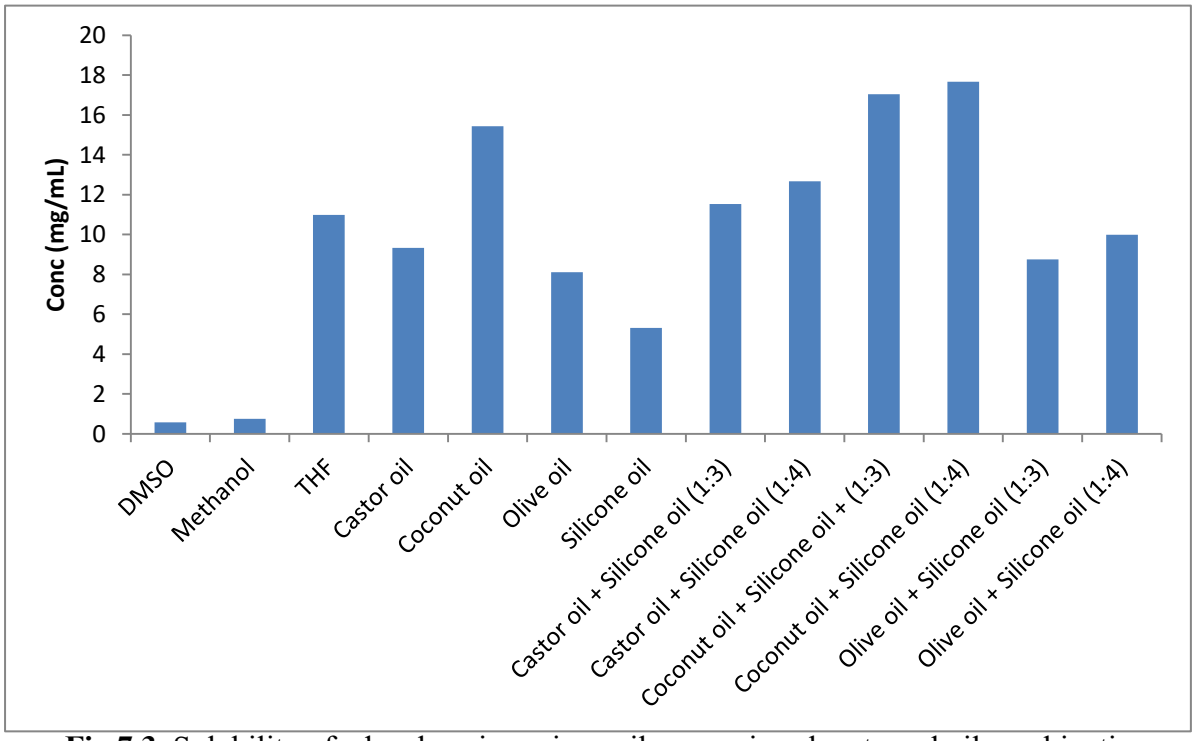


Fig 7.3. Solubility of adapalene in various oils, organic solvents and oil combination

7.1.2. Analytical method development

7.1.2.1 Selection and optimisation of solvent

Fig 7.4 and 7.5 show the scan of adapalene in between 200 – 400 nm using THF and DMSO respectively.

The overall experimental work depicted that THF satisfied all the conditions relative to peak quality and non-interference at specified wavelength.

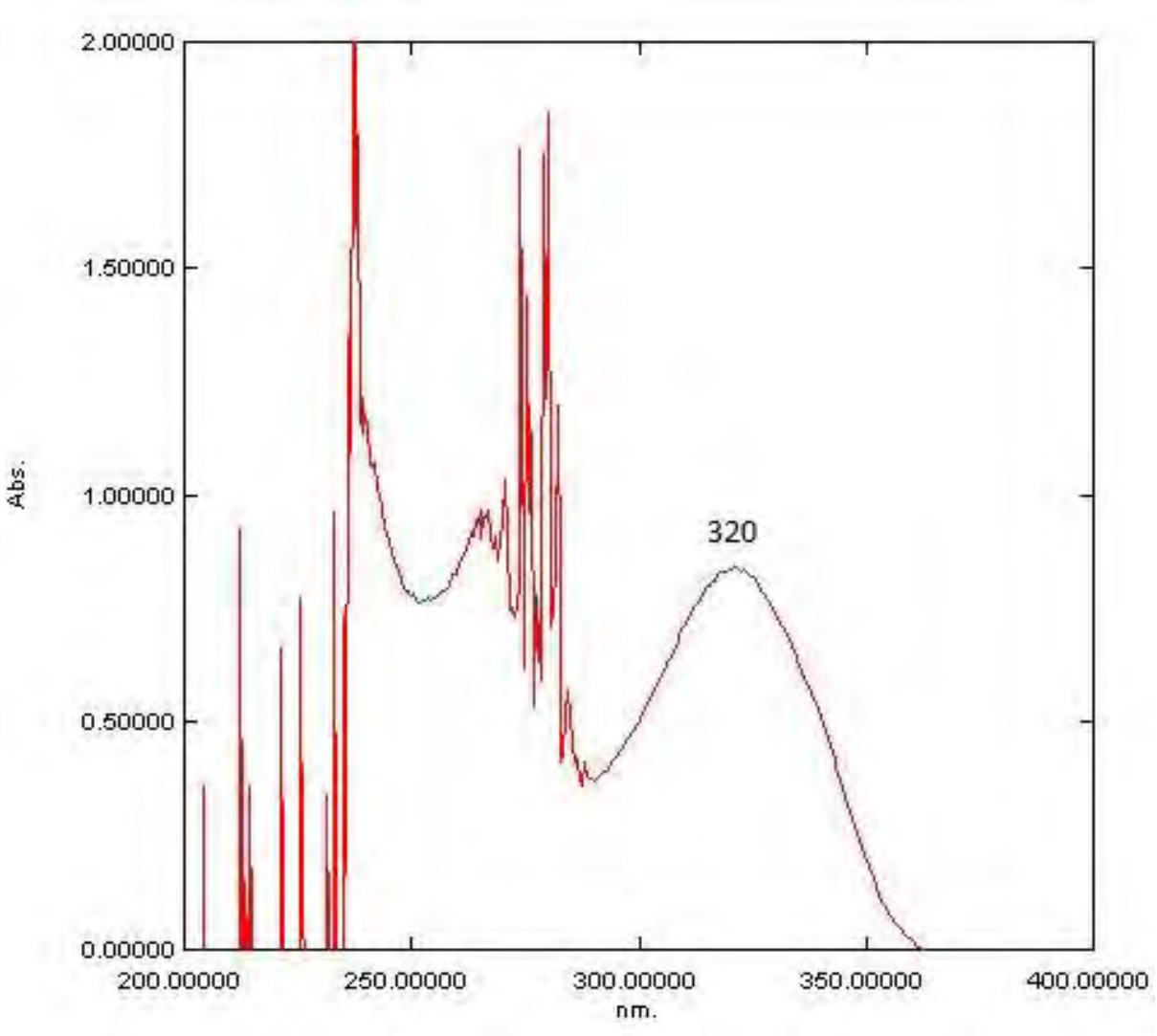


Fig 7.4. Scan of adapalene in between 200 – 400 nm using THF.

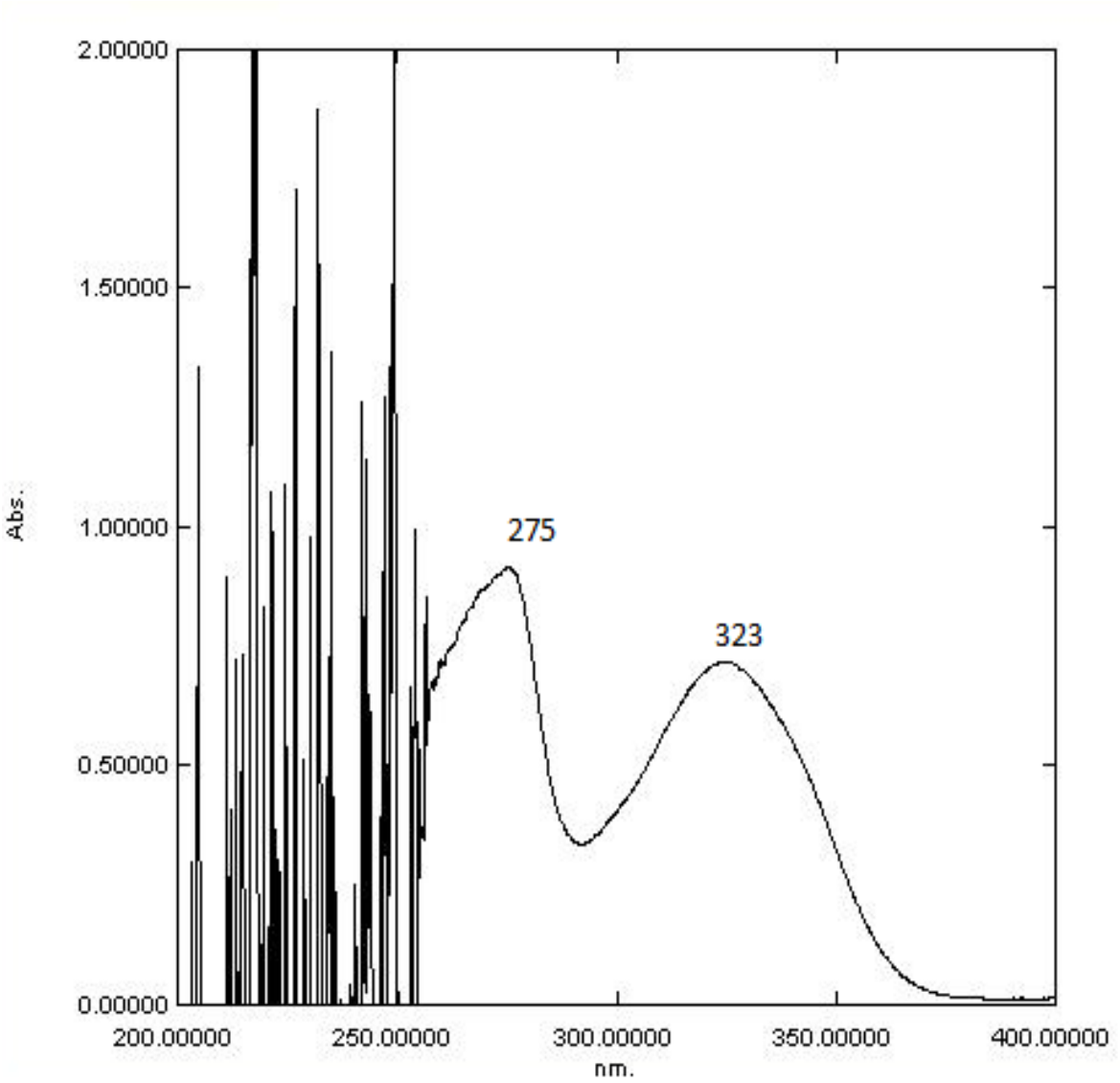


Fig 7.5. Scan of adapalene in between 200 – 400 nm using DMSO.

7.1.2.2 Determination of wavelength maxima (λmax)

Fig 7.4 shows the maximum wavelength of the THF at 320 nm when it is scanned in between 200 – 400 nm.

7.1.2.3 Preparation of standard calibration curve of adapalene in THF

Table 7.5 shows the absorbance values obtained in triplicate with 3, 6, 9, 12 and 15 µg/mL drug solution at 320 nm. **Fig 7.6** depicts the calibration curve of adapalene along with regression equation showing the slope value of the adapalene in THF.

7.1.2.4 Validation of calibration curve

7.1.2.4.1 Linearity

Table 7.5 shows the shows the absorbance values obtained in triplicate with 3, 6, 9, 12 and 15 µg/mL drug solution at 320 nm.

Fig 7.6 depicts the calibration curve of adapalene and correlation coefficient values obtained with concentration versus absorbance was found to be 0.999 confirming the linearity of the analytical method development.

Fig 7.6. Calibration curve of adapalene in THF at λ_{max} = 320 nm

7.1.2.4.2 Precision

Table 7.6 shows the percentage relative standard deviation calculated from absorption values obtained with five different determinations containing the same drug concentration. Interday and intraday precision study was also done by taking different concentrations which is shown in the **Table 7.7 - 7.9**. The calculated percentage relative standard deviation value was less than 2 indicate that the precession of the developed analytical method is within the acceptance criteria reported by ICH Q2 (R1) guidelines.

Table 7.6 Precision result showing repeatability in adapalene concentration of 3 – 15 µg/mL at 320 nm.

Concentration (µg/mL)	Absorbance	Mean	SD
15	0.877		
15	0.874		
15	0.875	0.875	0.002739
15	0.878		
15	0.871		

Table 7.7 Intraday precision of adapalene concentration 3 – 15 µg/mL at 320 nm.

Concentration	Absorbance 1	Absorbance 2	Absorbance 3	Mean	SD

(µg/mL)					
3	0.176	0.173	0.175	0.175	0.001617
6	0.350	0.351	0.350	0.350	0.000503
9	0.527	0.525	0.526	0.526	0.000808
12	0.702	0.703	0.705	0.703	0.001617
15	0.877	0.871	0.875	0.874	0.003055

Table 7.8 Intraday precision by using only one concentration (15 µg/mL) of adapalene

Concentration (µg/mL)	Absorbance 1	Absorbance 2	Absorbance 3	Mean	SD	Average %RSD
15	0.875	0.868	0.874	0.872	0.003786	
15	0.872	0.871	0.869	0.871	0.001528	0.33
15	0.876	0.871	0.877	0.875	0.003215	

Table 7.9 Interday precision of adapalene concentration 3 – 15 µg/mL at 320 nm.

Concentration (µg/mL)	Absorbance 1	Absorbance 2	Absorbance 3	Mean	SD
3	0.172	0.170	0.174	0.172	0.002
6	0.348	0.352	0.357	0.352	0.004509
9	0.526	0.521	0.528	0.525	0.003606
12	0.699	0.704	0.702	0.702	0.002517
15	0.875	0.868	0.874	0.872	0.003786

Table 7.10 Interday precision by using only one concentration (15 µg/mL) of adapalene

Concentration (µg/mL)	Absorbance 1	Absorbance 2	Absorbance 3	Mean	SD	%RSD
15	0.871	0.875	0.872	0.873	0.002082	0.24
15	0.874	0.873	0.868	0.872	0.003215	0.37
15	0.872	0.874	0.87	0.872	0.002	0.23

7.1.2.5.3 Accuracy

Table 7.11 and **Table 7.12** shows the percentage mean recovery 100.36 % which complies with the acceptance limit.

Table 7.11 Result showing accuracy data

Conc (µg/mL)	Absorbance 1	Absorbance 2	Absorbance 3	Mean	SD	Conc found (µg/mL)
45	0.264	0.263	0.261	0.263	0.00153	44.81
60	0.358	0.36	0.361	0.360	0.00153	61.42
75	0.434	0.437	0.435	0.435	0.00153	74.37

Table 7.12 Result showing mean percentage recovery

Sample (µg/mL)	Reference Standard Concentration Added (µg/mL)	Total Concentration of solution (µg/mL)	Concentration of drug found (µg/mL)	Recovery (%)	Mean Recovery (%)
30	15	45	44.81	99.57	100.36
	30	60	61.42	102.36	

45	75	74.37	99.16	

7.1.2.5.4 Robustness: Table 7.13 and 7.14 shows the percentage relative standard deviation calculated from absorption values containing the concentration between 3- 15 µg/mL and same drug concentration at 18˚C and 24˚C. The calculated percentage relative standard deviation value was less than 2 indicate that the precession of the developed analytical method is within the acceptance criteria reported by ICH Q2 (R1) guidelines.

Table 7.13 Results showing robustness at 18˚C and 24˚C

Concentration (µg/mL)	Absorbance at 18˚C	Absorbance at 24˚C	Mean	SD
3	0.175	0.176	0.176	0.00070
6	0.351	0.354	0.353	0.00212
9	0.528	0.525	0.527	0.00212
12	0.701	0.704	0.703	0.00212
15	0.876	0.879	0.878	0.00212

Table 7.14 Robustness by using only one concentration (15 µg/mL) at 18˚C and 24˚C

Concentration (µg/mL)	Absorbance at 18˚C	Absorbance at 24˚C	Mean	SD	Average %RSD
15	0.876	0.879	0.877	0.002121	
15	0.875	0.878	0.877	0.001414	0.19
15	0.876	0.878	0.877	0.001414	

7.1.2.5.5 Ruggedness:

Table 7.15 and 7.16 shows the percentage relative standard deviation calculated from absorption values containing the concentration between 3- 15 µg/mL and same drug concentration by 2 different analyst. The calculated percentage relative standard deviation value was less than 2 indicate that the precession of the developed analytical method is within the acceptance criteria reported by ICH Q2 (R1) guidelines.

Table 7.15 Results showing ruggedness by two analyst

Concentration (µg/mL)	Analyst 1 absorbance	Analyst 2 absorbance	Mean	%RSD
3	0.178	0.177	0.178	0.40
6	0.351	0.352	0.352	0.20
9	0.528	0.527	0.527	0.05
12	0.704	0.703	0.704	0.10
15	0.875	0.876	0.876	0.08

Table 7.16 Results showing ruggedness at one concentration (15 µg/mL) by two analyst

Concentration (µg/mL)	Analyst 1 Absorbance	Analyst 2 Absorbance	Mean	SD	%RSD	Average %RSD
15	0.875	0.876	0.875	0.000707	0.08	
15	0.877	0.873	0.875	0.002828	0.32	0.27
15	0.877	0.872	0.875	0.003536	0.40	

7.1.2.5.6 Limit of detection (LOD) and limit of quantification (LOQ):

LOD and LOQ were found to be 0.235 and 0.713 respectively as given in **Table 7.17**. These results demonstrate that the method is sensitive and can detect the drug in the above mentioned concentration range.

Table 7.17 Observed values of LOD and LOQ

Standard Deviation of response (**σ**)	0.0004
Slope (**S**)	0.0584
Ratio (**σ /S**)	0.071
LOD	0.235
LOQ	0.713

Table 7.18 Characteristics of adapalene in THF

S. No.	Parameter Result	
1.	Absorption Maxima	320 nm
2.	Linearity range	3-15 µg/mL
3.	Correlation coefficient	0.999
4.	Regression equation	y = 0.0584 x + 0.001
5.	Slope	0.0584
6.	Intercept	0.001
7.	Accuracy	100.36 %
8.	Precision, Intraday and Interday	0.313, 0.173 and 0.299
9.	LOD µg/mL	0.0235
10.	LOQ µg/mL	0.713

7.1.3 Formulation development

7.1.3.1 Preparation of blank Janus emulsion

Fig 7.7 Janus emulsion without adapalene (**F_1 – F_{13}**) and with adapalene (**F_{14}**).

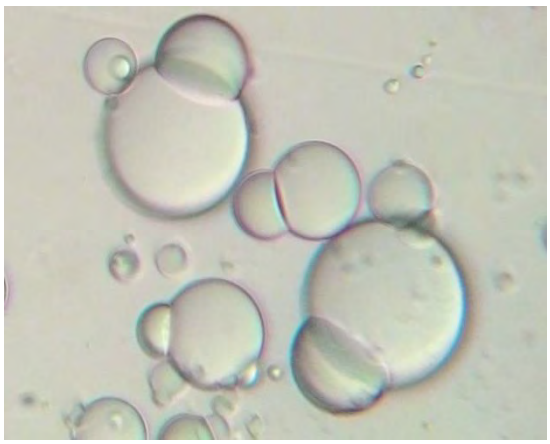
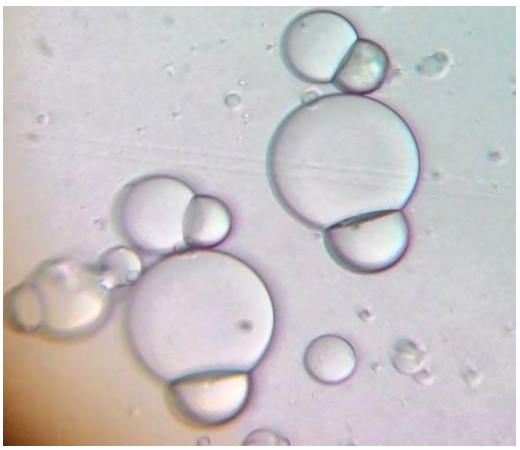
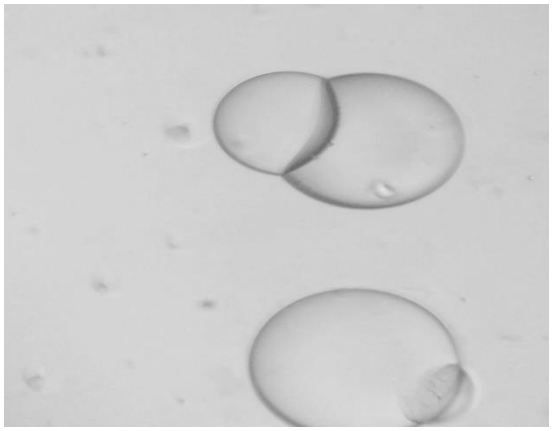
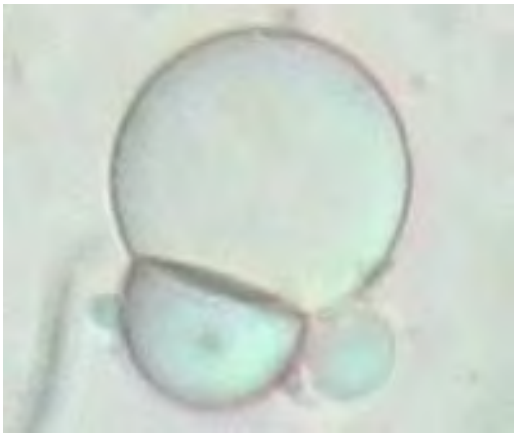
7.1.3.2 Selection of best formulation by using compound microscope

Formulations were prepared according to **Table 6.1** and their optimization was done by using the microscopy, shown in the **Fig 7.8.**

Fig 7.8. Photographic pictures of various Janus formulations without adapalene (**F_1 - F_{13}**) and with adapalene (**F_{14}**)

7.1.3.3 Selection of best formulation by comparing the photographic pictures taken using compound microscope

For checking the stability of prepared Janus emulsion, fresh emulsion is prepared and is kept at 37 ˚C and 25 ˚C. They are observed after each week through microscope as shown in the **Fig 7.9**.

At 25˚C (0 week) 	**At 37˚C (0 week)** 
At 25˚C (1 week) 	**At 37˚C (1 week)** 

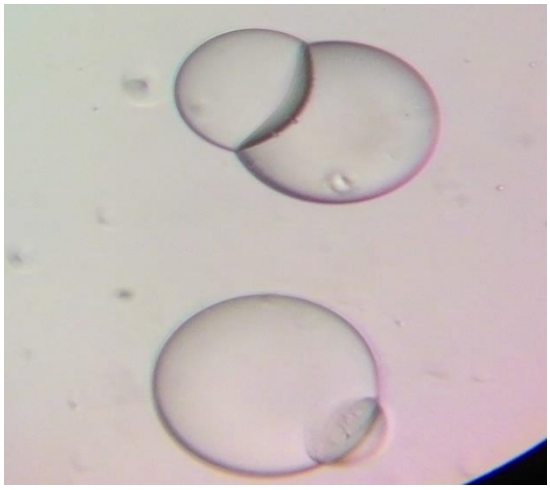
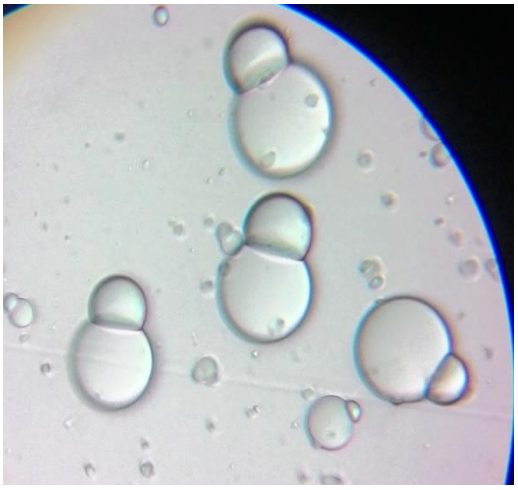
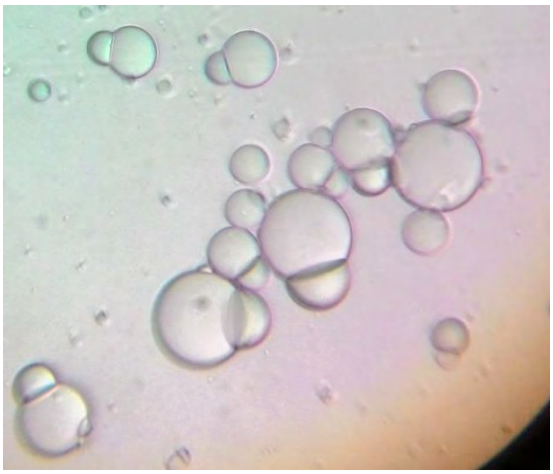
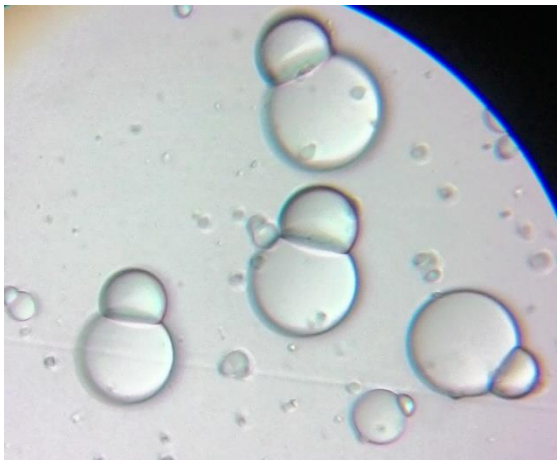
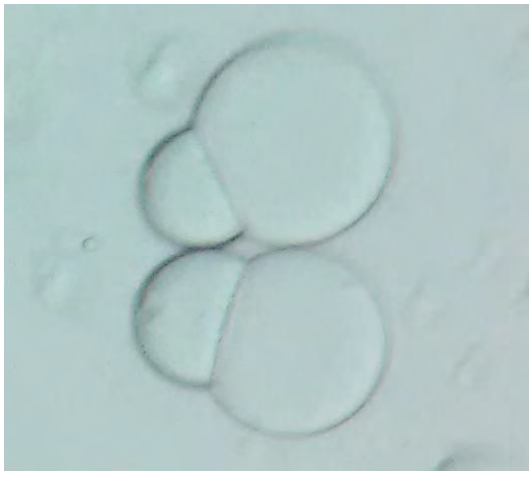
At 25˚C (2 week)	**At 37˚C (2 week)**
	
At 25˚C (3 week)	**At 37˚C (3 week)**
	
At 25˚C (4 week)	**At 37˚C (4 week)**
	

Fig 7.9 Microscopy of Janus emulsion at 25˚ and 37˚ C for 4 weeks.

7.1.3.4 Determination of drug amount at oil water interface & drug entrapment efficiency percentage.

Equation **3** and **4** were used to calculate the drug entrapment efficiency percentage and drug amount in oil water interface. **Table 7.19** shows the % entrapment efficiency and percentage drug amount on oil water interface values and it shows that Janus emulsion is having 74.74% entrapment efficiency and almost 15% of drug is present in the oil-water interface.

7.1.3.5 Particle size determination

7.1.3.5.1 By using microscope

By using the data present in **Table 7.20**, particles size was determined as **14.8 µ.**

7.1.3.5.2 By using Malvern mastersizer

Fig 7.10 - 7.11 depicts the particle size of Janus emulsion without drug and with drug was determined by using Malvern mastersizer and it shows that after loading the drug there are no significant changes in the particle size.

Fig 7.10 Particle size of Janus emulsion without adapalene

Fig 7.11 Particle size of Janus emulsion with adapalene

7.1.3.6 Qualitative TLC

Fig 7.12 depicts the TLC of adapalene-loaded Janus emulsion as test and adapalene solution as reference. **Table 7.21** confirms the Rf value for both is 0.96 which confirm the drug loading in the emulsion.

Table 7.21 Rf value calculation of adapalene-loaded Janus emulsion and adapalene solution by using methanol : THF (18:24)

7.1.3.7 TEM

Fig 7.13 a - c shows the Transmission electron microscopy pictures of adapalene-loaded Janus emulsion**.**

Fig 7.14 Amount of adapalene released from oil-in-water Janus emulsion in phosphate buffer pH 6.0 and THF (60:40) over 20 min dissolution time period as a function of increasing dissolution rate.

7.1.3.9 *In vitro* diffusion

Table 7.25 shows the adapalene permeation from adapalene-loaded Janus emulsion and pure adapalene in the **Table 7.26**. The diffusion comparison of both adapalene-loaded Janus emulsion and pure adapalene is depicted in **Fig 7.15. Table 7.27 and 7.28** shows the steady state drug permeation and flow rate which was calculated by using the equation **(5)** and **(6)**.

Fig 7.15 *In vitro* diffusion of adapalene-loaded emulsion and standard drug solution in phosphate buffer pH6.0 : THF (60:40)

7.1.3.10 *In vitro* antiinflammatory

Table 7.29 and Table 7.30 shows the *in vitro* antiinflammatory activity of adapalene-loaded Janus emulsion as test and powder adapalene as reference respectively. **Fig 7.16** depicted the difference in the antiinflammatory activity shown by the adapalene-loaded Janus emulsion and standard drug solution.

Table 7.29 *In vitro* antiinflammatory activity showing the percentage inhibition value for the albumin denaturation phenomena obtained with adapalene-loaded Janus emulsion (test).

Table 7.30 *In vitro* antiinflammatory activity showing the percentage inhibition value for the albumin denaturation phenomena obtained with Adapalene solution (reference).

Conc (µg/mL)	**Adapalene solution (reference)**			**% Inhibition**
	Absorbance 1	**Absorbance 2**	**Absorbance 3**	
24.8	0.183	0.197	0.188	23.99 ± 6.17
49.6	0.289	0.291	0.295	64.30 ± 6.14
74.4	0.545	0.588	0.561	154.13 ± 4.68
99.2	0.795	0.789	0.810	177.43 ± 3.02
124.0	0.921	0.954	0.945	204.25 ± 4.88

Fig 7.16 *In vitro* antiinflammatory activities showing the percentage inhibition value for the albumin denaturation phenomenon obtained with adapalene-loaded Janus emulsion and pure adapalene solution.

7.1.3.11 RBC break down assay

Table 7.31 shows the effect of adapalene-loaded Janus emulsion, pure adapalene solution and solution without adapalene on RBC's breakdown. % Haemolysis was calculated by using the equation 6. **Fig 7.17** depicts the % haemolysis shown by adapalene-loaded Janus emulsion, pure adapalene solution and castor oil based emulsion without adapalene.

Table 7.31 RBC breakdown showing the % haemolysis value obtained with adapalene-loaded Janus emulsion, pure adapalene solution and solution without adapalene.

7.2 Discussion

It was around more than 50 years that colloidal drug delivery system is being designed. The main use of this kind of dispersed is to increase the solubility of hydrophobic drug molecules and thus enhance their GI epithelial transport mechanism for achieving desired bioavailability. Among the gamut of so far designed dispersed system, such as liposome, nanospheres, nanocapsules, niosomes, transferosomes, ethosomes, cubosomes, etc., the oil-in-water type dispersion system called 'emulsion' receives a considerable interest to make the successful commercial pharmaceutical formulation for hydrophobic drug moiety.

Basically emulsions are heterogeneous dispersion of oil-in-water or water-in-oil. The stabilization of these two immiscible liquids is achieved by the incorporation of the third substance called emulsifiers, emulgators, emulgents or emulsifying agent into the dispersion system. Furthermore in order to obtain the emulsion dispersion system stable over the desired period of time, it become necessary to keep the dispersed oil or water droplets in a particle diameter range that should be well below sub-micrometre level or even much below one micrometre level. In this context, different size reduction machineries, like homogenizers, micro-fluidizers and ultrasonicator are routinely used for making the emulsion dispersion system.

Apart from incorporating the hydrophobic drug molecules into the emulsion dispersion system, it could be of interest to find the way to entrap also the hydrophilic drug molecules into the dispersion system along with the hydrophobic drug moieties. In this direction, the emulsion dispersion scientists focus to find the way how to incorporate both the hydrophilic and hydrophobic drug molecules together in a single emulsion dispersion system. One of the way which was found during the year 2000 is to develop positively-charged submicron emulsion prepared based on medium chain triglyceride (MCT) stearylamine, Lipoid-E80, glycerine, poloxamer-188, vitamin E and doubled distilled water (Teixeira et al., 2000). This particular emulsion dispersion system, when stearylamine molecule was incorporated, it shows an unexpected bi - compartmental structure, termed as 'handbag'. The handbag structure formation is believed due to the association of lipoid-E80, stearylamine and poloxamer-188 at the oil water interface to prevent the random collision of dispersed oil droplets, coalescence and ostwald ripening. While performing this activity by covering the dispersed oil droplets, with mono or multi – emulsifiers film, the presence of cationic lipidic stearylamine promotes the elevation of polyoxyethylene structure of the hydrophilic poloxamer-188 towards the water dispersion medium of oil-in-water emulsion system. The elevation of hydrophilic head structure towards water phase in one hand/side, whereas the complimentary emulsifiers film formed around the dispersion oil droplets tries to keep the dispersed droplet intact, by preventing the droplet interactions. These two competing driving forces, exerted by stearylamine and poloxamer-188 ultimately lead to the formation of unexpected handbag structure or bi - compartmental structure within the emulsion dispersion system.

In the present study, the emulsion dispersion system is being developed based on two different non-volatile oils, one-single emulsifier molecule and doubled distilled water. The presently developed emulsion dispersion system is also never utilized the so called particle size reduction machinery but simple magnetic stirrer is enough to produce a stable emulsion dispersion system. The unique feature of this presently developed emulsion dispersion system is the presence of the double-head-faced structure similar to the one, which was shown after – incorporation of cationic lipidic stearylamine emulsifier moiety into the emulsion. In the literature, the presence of double-head-faced structure is simply called as 'Janus particles' honouring the roman God 'Janus'. Again from the literature, it is classified that the observed double-head-faced structure, within the emulsion dispersion system, is capable of entrapping both hydrophilic and hydrophobic drug molecules together.

However in the present study, the hydrophobic anti-acne drug, adapalene is being selected as the candidate drug molecule to be incorporated into the Janus emulsion dispersion system. Since the *Acne vulgaris* is multi-model syndrome and both hydrophilic and hydrophobic drug candidates are used separately to control / contain the progression of acne, the presently developed Janus emulsion dispersion system could pave the way of incorporating the anti-acne drug molecules, possessing the diverse solubility.

After receiving the drug as a gift sample from Taj Pharmaceutical Ltd., the external appearance of the drug was accessed visually. The physical shape, colour and odour were found respectively to be solid, white and colourless **(Table 7.1),** as per the certificate of analysis (COA) provided by the company. Similarly, the melting point of the drug was also determined by capillary rise method and it was found to be in the range of 320 – 326 ˚ C (**Table 7.2**). The observed melting point range for the adapalene powder was in agreement with the melting point ranges, specified in the COA. The authenticity of the adapalene drug sample received was also confirmed by FT-IR analysis after checking the wavenumber values for the different functional groups present in the adapalene with that of the wavenumber values of the different functional groups reported (**Table 7.3 and Fig 7.2**) in the literature.

After performing the different authentication test's (physical assessment, melting point determination and wavenumber comparison), the solubility of the adapalene was determined in various oils, organic solvents and oil combinations (**Table 7.4 and Fig 7.3**). In case of organic solvents tested, the THF showed higher drug solubility (10.98 mg/mL). Among the oil combination tested, the coconut and silicone oils combination at two different ratios (1 : 3 and 1 : 4) showed the higher drug solubility (17.04 and 17.67 mg/mL, respectively). The next oil combination, which exhibits the higher drug solubility (11.57 and 12.67 mg/mL) was castor and silicone oils combination at the same ratios was tested. Based on the photo micrographic observation as shown in the **Fig 8.1 (a) and (b)** which indicates that the formation of dispersed oil droplets with two-head-faced structure was comparably less in number for coconut and silicone oils based Janus emulsion with that of Janus emulsion prepared based on castor and silicone oil combination.

7.18 Janus emulsion prepared using (a) coconut and silicone oils combination, (b) castor and silicone oils combination.

Between the THF and DMSO solvent, for making the calibration curve for adapalene, the THF was chosen based on the peak obtained for drug solution scanned from 200 – 400 nm (**Fig 7.4 and 7.5**). Therefore the linearity, precision, accuracy, robustness, rigidness, LOD and LOQ were also checked using the adapalene solution in THF (**Table 7.5 – 7.18**).

The Janus emulsion dispersion system was prepared by following the composition shown in the **Table 6.2 and 6.3**. After thoroughly analysing the formation of dispersed oil droplets with double-head-faced structure against each one of the formula tested (**Fig 7.7**), the Janus emulsion prepared using the $\mathbf{F_{14}}$, resulted to produce, a prominent double–head-faced structure. Therefore the formulation $\mathbf{F_1}$ was carried forward to perform the emulsion characterisation studies, drug amount determination at oil-water interface, drug entrapment efficiency, topological analysis by using TEM, *in vitro* drug release, *in vitro* diffusion, *in vitro* antiinflammatory and RBC break down assay.

The drug stability inside the Janus emulsion and in THF solution was checked through qualitative TLC analysis (**Fig 7.12 and Table 7.21**). Since the R_f value obtained for adapalene-loaded Janus emulsion and adapalene-THF solution was virtually the same, this result ruled out the possibility of occurrence of either drug degradation or drug interactions with excipients within the Janus emulsion. Immediately after preparing the Janus emulsion, the particle distribution of the dispersed oil droplets was checked by using microscopy (**Table 7.20**). This preliminary microscopy analysis revealed 14.8 μm as the log number mean diameter value for Janus emulsion. This method was performed by selecting randomly around 100 dispersed oil droplets of the Janus emulsion randomly. Another way to see the mean diameter of the Janus emulsion particles was by analysing with Malvern mastersizer. The Janus emulsion with or without adapalene showed a mean diameter values of the dispersed oil droplets $d_{(0.5)}$ 171.65 μm and 180.53 μm, respectively **(Fig 7.10 and 7.11)**. An almost similar particle diameter values observed with the Janus emulsion prepared with or without adapalene was the indication of the stable emulsion formation. Furthermore, the %

drug amount present at the oil-water interface of the Janus emulsion and drug entrapment efficiency value were found to be 15.29 ± 0.17 % and 74.74 ± 0.49 % respectively. The remaining 85% of the drug might be solubilized in the selected castor oil and silicone oil combinations (**Table 7.19**). Following unexpected observation were seen when adapalene-loaded Janus emulsion was subjected to TEM analysis **(Fig 7.13 a, 7.13 b and 7.13 c).**

- Either complete loss or presence of very few double-head-faced structures.
- Appearance of drug crystals within the dispersed oil droplets in Janus emulsion
- Presence of some dark shadow within a single dispersed oil droplet of the Janus emulsion.

The scarcity in doubled-head-faced structures after subjecting the Janus particles in TEM might be due to the destruction of doubled-head-faced structure during the negative staining in sample preparation step. This result is in contradictory with the result obtained for positively charged submicron emulsion following cryo-TEM analysis as shown by Teixeira et al., (2000). **Fig 7.19** shows the structure taken directly from the publication of Teixeira et al., (2000) where the 'handbag' structure after cryo-TEM analysis was clearly visible.

Fig 7.19 Positively charged submicron emulsion after cryo-TEM analysis (Teixeira et al., 2000)

The presence of drug crystals within the dispersed oil droplets of the Janus emulsion following negative staining TEM analysis was somewhat surprising although drug nano crystal formation is not the new case for the emulsion dispersion delivery system (McClements et al., 2014). The gradual step which might be involved to initiate and induce the adapalene drug particles to undergo drug nano crystals formation, after subjecting the emulsion into negative staining TEM analysis requires further elaborative mechanistic investigation.

The formation of dark shadow within a single dispersed oil droplet of the Janus emulsion after subjecting the emulsion into negative staining TEM analysis might plausibly be collaborated within the sudden loss of double-head-faced structure of the dispersed oil-droplets. Since castor and silicone oils were selected to produce the Janus emulsion, both of them were not soluble with each other and thus tend to form a doubled-head-faced structure in presence of a single emulsifier molecule (Tween 80). The application of negative staining followed by the vacuum pressure created during the TEM analysis, eventually tried to

compress these two selected oil droplets, to come closer together and to lose its previously developed double-head-faced structure. But the joining point of these two oils in the single oil droplets appeared as probably a dark shadow after negative staining TEM analysis.

The adapalene-loaded Janus emulsion was found to be stable for at least up to 3 weeks, after their storage at two different temperature condition **(Fig 7.8)**. At 4 weeks of storage, at both of the tested temperature condition, the Janus emulsion were stable but the presence of Janus type dispersed oil droplets were found to be less in number, in comparison to 0, 1, 2 and 3 weeks storage time. From this result, one may conclude that adapalene-loaded Janus emulsion could be stable for up to 3 weeks at both of the studied two different storage temperature conditions.

Since emulsion dispersion system is not considered as an affective system to produce controlled drug release characteristics, it's advantages are in general to increase the drug solubility and to protect the drug structure following different storage conditions. Upon mixing with dissolution medium, the emulsion system is expected to release the entrapped drug molecule very rapidly within initial first few seconds or minutes. This general concept of drug delivery system is also applicable for the currently developed adapalene-loaded Janus emulsion. Within 5 minutes of dissolution, about 23% of the drug release was occurred from the Janus emulsion. The released drug might able to reduce the papules and pustules produced in the acne conditions.

At a concentration range of 23 µg of adapalene, the free drug induced 61.46 ± 2.21% haemolysis while the adapalene-loaded Janus emulsion showed only the RBC destruction value of 36.51 ± 1.25% **(Fig 7.17)** following incubation for 30 minutes. The emulsion prepared based only on castor oil showed the haemolysis % value 41.30 ± 2.56%. According to the Student's *t* – test, no significant difference (p vale at 0.05) was noticed in the % haemolysis values shown by Janus emulsion containing adapalene and emulsion prepared based only on castor oil. However there was a significant difference between the % haemolysis values obtained by the free drug and emulsion samples. This result dictates that there was a reduction in the haemolysis effect of the drug when the adapalene was incorporated in the adapalene-loaded Janus emulsions.

From **Fig 7.16** and **Table 7.29 - 7.30** shows the % inhibition value for the protein (albumin) denaturation phenomenon obtained with adapalene-loaded Janus emulsions (test) and adapalene solution (reference). When looking at the value of the % inhibition, it can be

concluded that both the test and reference products produced the % inhibition values for the protein denaturation phenomenon that was concentration-dependent (24.8, 49.6, 74.4, 99.2 and 124 µg/mL). Both the test and reference showed the IC_{50} value at or around the same concentration level (~ 30 and 30.5 µg/mL). Furthermore, all of the studied concentration ranges, there was a similarity in % inhibition values obtained for both and reference samples. It indicates that upon topical application of adapalene-loaded emulsion onto the inflamed skin surfaces (produced due to acne), a similar antiinflammatory effect was likely to occur like to that of adapalene-THF solution. Since THF is an organic solvent, the adapalene solution prepared using the THF might not be of clinical significance. Therefore adapalene-loaded Janus emulsion could be of clinical interest for managing the inflammations produced at acne skin disorder conditions.

Table 7.25 to **7.28** and **Fig 7.15** show the *in vitro* diffusional profile of adapalene solution and adapalene-loaded Janus emulsion. Both of these formulations were shown linear relationships in the time verses drug amount permitted plot as the correlation values were in the range from 0.986 to 0.997. Whereas adapalene solution possessed the percentage permeation value of 48.16 ± 0.67 %, only 16.25 ± 1.69 % permeation value was observed for adapalene-loaded Janus emulsion over the tested time period of 120 minutes. Again the steady state flux (J_{ss}) value was also higher for adapalene solution than the J_{ss} value observed with the adapalene-loaded Janus emulsion. As expected, the adapalene solution showed a higher apparent permeability coefficient value (P_{app}) value of 5×10^{-5} cm/sec, whereas the adapalene-loaded Janus emulsion demonstrated the P_{app} value of 1.9×10^{-5} cm/sec. The observed noticeable reduction in the drug permeation value for emulsion formulation in the *in vitro* diffusion experiment can be attributed to the fact that the adapalene was entrapped at oil - water interface of the emulsion or simply dissolved in a vesicular type oil droplet protected by a monolayer emulsifier film.

CHAPTER 8

CONCLUSION AND FUTURE SCOPE

8.1 Conclusion

The current research work was undertaken in order to improve the solubility problem of adapalene. The castor oil and silicone oil (1 : 2 ratios) hence the drug was selected to act as oil phase of the emulsion hence, the drug was dispersed. Upon mixing the drug dispersed oil phase into the tween 80 containing water phase by a simple magnetic stirrer, it is observed that there was a formation of handbag structure of dispersed oil droplets within the aqueous continuous medium. When viewing through TEM, it was again observed that there was a drug nano-crystal formation within the oil droplets of the emulsion system. About 75% of drug entrapment efficiency was observed while 15% of the drug was accumulated at oil-water interface of the emulsion. However, *in vitro* protein denaturation assay indicates that a similar antiinflammatory effect was observed between the adapalene solution and adapalene-loaded Janus emulsion. On the other hand, a concentration dependent *in vitro* release characteristic was observed when membrane free dissolution study was conducted for adapalene-loaded Janus emulsion. The *in vitro* permeation study via cellophane membrane indicated a reduction in P_{app} value and J_{ss} value for adapalene-loaded Janus emulsion in comparison to the P_{app} and J_{ss} values observed for adapalene solution. Although the *in vitro* antiinflammatory effect observed with emulsion formulation was similar to the adapalene solution, it could be of clinical interest by seeing results of *in vitro* permeation study results that indicated a delay in adapalene permeation via cellophane membrane in comparison with the adapalene solution counterpart.

8.2 Future prospective

The presence of handbag structure in the dispersed oil droplet structure is one of the newly added academic knowledge for colloidal dispersion system. However, the drug selected in the current investigation showed a nano-crystal formation within the dispersed oil droplets of the emulsion. The propensity of drug nano-crystal formation within the dispersed oil droplets of the emulsion system should be of further research focus to investigate the parameters involved to control the drug nano-crystal formation inside the emulsion system. Furthermore, the relationship needs to be studied in between the drug nano-crystal formation and its consequence to influence the antiinflammatory potential of adapalene, when incorporated into the oil - water Janus emulsion system.

CHAPTER 9

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